Natural Resources for Human Health
eISSN: 2583-1194

Original Research

Received 10 May 2026
Revised 15 June 2026
Accepted 28 June 2026

Natural Resources for Human Health 2026, Vol. 6 (5): 644–665

KEYWORDS:
Escherichia coli, Urinary tract infection, Biofilm, Multidrug resistance, Virulence genes

Integrated Phenotypic and Molecular Characterization of Biofilm Formation, Virulence Determinants, and Multidrug Resistance in Clinical Escherichia Coli

Hiba Mohssin Ali[1]*, Sarah Abdulsalam wahwah[2], Baneen A. MohammedAli[3], Hadeer Mohammed Ali[3], Aya Ibrahim Safar4, Sura Mouaid Abbas[1]

[1]Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq
[2]Department of Medical Laboratory Techniques, Imam Ja'afar Al-Sadiq University (IJSU), Al-Muthanna Branch, Iraq
[3]Department of Microbiology, College of Medicine, Mustansiriyah University, Baghdad, Iraq
[4]Department of Microbiology, College of Science, Al-Mustansiriyah University, Baghdad, Iraq
*Corresponding author: Hiba Mohssin Ali. Email: Hiba.M.Ali@uomustansiriyah.edu.iq

ABSTRACT:

Background: Escherichia coli is responsible for the majority of UTI cases, with an increasing number of multidrug resistance (MDR) and biofilm-forming E. coli isolates being isolated from such cases. In this study, we assessed the MDR profile, biofilm formation ability, and occurrence of specific biofilm-associated and MDR-related genes in isolates.

Methods: One hundred isolates of non-duplicate UP E. coli EC were tested for their antibiotic susceptibility using the Kirby-Bauer disc diffusion technique according to CLSI guidelines. Biofilm formation was evaluated using the MTP and CRA tests. The presence of fimH, csgA, luxS, bcsA, acrB, and blaCTX-M genes was determined by conventional PCR. Statistical correlations among biofilm formation, distribution of genes, and MDR were established.

Results: Nitrofurantoin had the highest sensitivity (92%), while the highest levels of resistance were seen with amoxicillin-clavulanate (95%), cefixime (83%), tetracycline (79%), ceftriaxone (78%), ceftazidime (78%), cefepime (77%), and trimethoprim-sulfamethoxazole (71%). MDR isolates were found in 95% of the isolates. Production of biofilm was seen in 98% of the isolates, with 18% as strong, 24% as moderate, and 56% as weak producers. CRA method yielded 81.6% sensitivity and 100% specificity than MTP test. Common gene targets included fimH (93%), acrB (90%), csgA (77%), luxS (72%), blaCTX-M (69%), and bcsA (58%). Significance was found in association of biofilm genes to biofilm phenotype while acrB and blaCTX-M were independent predictors of MDR.

Conclusions: MDR was highly prevalent in isolates. The presence of biofilm formation and virulence factors was also high. Combination of both phenotypic and molecular approaches offers useful epidemiologic information which can lead to better antibiotic stewardship.

Natural Resources for Human Health
eISSN: 2583-1194

INTRODUCTION

Urinary tract infections (UTIs) are one of the most frequently occurring bacterial infections globally and present a considerable public health issue due to their high prevalence, tendency to recur, and fast development of resistance to antimicrobial [1]. UTIs can affect people of all age groups, but the disease is more commonly found in women due to specific anatomic and physiological features allowing easier colonization by bacteria of the urinary tract. Over half of all females will have at least one episode of UTI in their lifetime, and around 20-30% will suffer from recurrent infections [1, 2]. Besides being burdensome, UTIs contribute to the rising cost of healthcare due to excessive use of antibiotics, hospitalization, and productivity losses. The increasing occurrence of MDR uropathogens has further complicated empirical therapy and underscored the need for enhanced surveillance and alternative therapy approaches [1- 3].

In the case of causative agents responsible for UTIs, *Escherichia coli* stand out as the primary culprit and are responsible for 70-90% of cases of UTIs in the community. *E. coli*, as opposed to normal *E. coli*, has several virulence factors that facilitate the process of attachment, invasion, immune evasion, intracellular survival, and repeat infection [3, 4].

Formation of biofilm is one of the significant strategies that are responsible for the persistent nature of *E. coli*. Biofilms are bacterial colonies which have an ordered structure and are surrounded by a protective EPS matrix of polysaccharides, proteins and extracellular DNA produced by the bacteria [3, 5]. Biofilm-forming bacteria are more resistant to antibiotic agents, are resistant to the immune responses of the host organism and survive longer than planktonic bacteria in the presence of environmental stresses. Biofilm development is thus an important component of chronic UTIs, catheter-related infections, and treatment failure [5, 6].

The growing number of antimicrobial-resistant isolates has emerged as a formidable hurdle in the successful treatment of this condition. Resistance against β-lactam, fluoroquinolone, trimethoprim-sulfamethoxazole and other widely used drugs has led to the emergence of multiple drug resistant isolates. In terms of resistance mechanisms, one of the most significant is the synthesis of extended spectrum β-lactamases (ESBLs), particularly CTX-M enzymes carried by $bla_{CTX\text{-}M}$ [5]. Another notable form of resistance is related to the use of multidrug efflux systems such as the AcrAB-TolC, where the *acrB* gene actively expels antimicrobial agents from inside the cell, thus lowering their levels in the bacterial cytoplasm and also playing a part in persistence and biofilm formation [7, 8].

Some virulence factors genes play direct roles in biofilm formation. *FimH* is responsible for the primary adhesion of bacteria to the uroepithelial cell via type 1 fimbriae, while *csgA* plays a critical role in bacterial aggregations by encoding the protein making up curli fimbriae [3, 9]. *luxS* is involved in quorum sensing via autoinducer-2 signals, while *bcsA* is responsible for cellulose biosynthesis, which forms part of the biofilm matrix Coordinated expression of these genes helps in the formation of stable biofilms and ensures bacterial persistence in the urinary tract [3, 9-11].

While past research has been conducted on antimicrobial resistance, biofilm formation, and virulence factors individually, there is limited research involving the combination of phenotypic antimicrobial susceptibility testing, quantification of biofilm production, molecular identification of biofilm-associated genes and resistance-related genes, co-occurrence pattern of these genes, hierarchical clustering, and multivariable statistical analyses using a single *E. coli* population. An extensive knowledge of such relationships would help in advancing empirical therapy and finding out well-adapted MDR *E. coli* isolates. The current research was intended to study antimicrobial susceptibility profiles, assess biofilm production capacity, detect the prevalence of chosen biofilm-related genes (*fimH, csgA, luxS, bcsA*), along with *acrB* and $bla_{CTX\text{-}M}$, examine gene co-occurrence, and reveal predictors of MDR.

MATERIALS AND METHODS

Study design and setting

This cross-sectional study was carried out in the Microbiology Laboratory of Medical City Teaching Hospital, Baghdad, Iraq, which is a tertiary care hospital. This study was carried out within the period of August 1, 2025, to June 2026. The major aim of this study was to study the antimicrobial susceptibility pattern, MDR, biofilm formation ability, and the relationship between antimicrobial resistance and biofilm formation in *E. coli* isolated from urine specimens of UTI suspects.

Collection of Urine Samples and Isolation of *E. coli*

A total of 389 mid-stream clean catch urine samples were obtained from in- and out-patients presenting with clinical suspicion of UTI. Urine samples were obtained in sterile tubes and immediately taken to the laboratory for microbiological processing as per the established microbiological procedures.

The culture procedure involved the use of MacConkey agar and 5% sheep blood agar, followed by an aerobic incubation at 37°C for 18–24 hours. Significant bacteriuria was determined as per CLSI. Only those urine cultures which yielded a pure isolate of *E. coli* with significant counts were considered. Contaminated cultures, mixed cultures, duplicate cultures and those cultures that failed to meet the criteria for significant bacteriuria were excluded.

Identification and confirmation of *E. coli* isolates

Bacterial isolates were first characterized using traditional microbiological techniques. Presumptive identification of *E. coli* was conducted through colony identification on MacConkey agar, Gram staining properties, and common biochemical tests such as indole test, citrate utilization, urease test, and triple sugar iron (TSI) test [12].

The presumptive identification was later confirmed using VITEK 2 Compact automated identification system (bioMérieux, France) for bacteria identification as per the manufacturer's guidelines. The quality control strain used for bacterial identification and antibiotic susceptibility testing is *Escherichia coli* ATCC 25922. For the definitive identification of bacterial isolates, molecular characterization was done through the amplification of the 16S rDNA using polymerase chain reaction (PCR) technique [13, 14].

Antimicrobial susceptibility testing

Susceptibility testing of the isolates of *E. coli* was conducted by means of Kirby-Bauer disk diffusion test in accordance with CLSI guidelines (CLSI M100, 35th edition, 2025). Specifically, the bacterial suspensions were prepared from fresh overnight cultures and diluted to match the turbidity equal to that of a 0.5 McFarland standard. The standardized inoculum was spread evenly on Mueller-Hinton agar plates [15, 16].

Antimicrobial susceptibility testing was done by a set of 17 antimicrobials belonging to different antimicrobial classes, namely, β-lactams, fluoroquinolones, aminoglycosides, tetracyclines, sulfonamides, nitrofurans, and carbapenems. These antimicrobial agents include amoxicillin, amoxicillin-clavulanate, ceftriaxone, cefixime, ceftazidime, cefepime, cefoxitin, ciprofloxacin, levofloxacin, nalidixic acid, gentamicin, amikacin, tetracycline, trimethoprim-sulfamethoxazole, nitrofurantoin, imipenem, and chloramphenicol. After being cultured under appropriate conditions, the inhibition zone diameters were recorded in mm and analyzed based on the CLSI clinical breakpoints for 2025. Thus, isolates were classified into groups of susceptible, intermediate, and resistant. The *Escherichia coli* ATCC 25922 were selected as the quality control strain in order to validate the antimicrobial susceptibility testing procedures.

The MDR phenotype was determined according to the international definition proposed by Magiorakos *et al.* (2012). An isolate was classified as multidrug-resistant when it demonstrated acquired non-susceptibility to at least one antimicrobial agent in three or more antimicrobial categories [17].

Biofilm formation assay

Two different phenotypic techniques have been used to test the biofilm-forming capacity of the *Escherichia coli* isolates, namely the quantitative microtiter plate technique and qualitative Congo red agar (CRA) technique.

Quantitative measurement of biofilm formation by microtiter plate technique

The biofilm formation was measured quantitatively by using crystal violet microtiter plate technique as described before with some modifications. Overnight cultures of *E. coli* isolates were made using freshly grown bacterial colonies, and the bacterial suspensions were standardized to turbidity comparable to that of 0.5 McFarland standard. These suspensions of bacteria were inoculated in sterile 96-well flat-bottom polystyrene microtiter plates containing TSB. The microplates that had been inoculated with the suspension were left statically at 37°C for 24-48 h to allow biofilm formation. The planktonic cells were then decanted, and each well was washed three times with PBS solution. The attached biofilm biomass was fixed and stained with 0.1% crystal violet solution for 15-20 min. Excess crystal violet was rinsed off, and the stained crystals were dissolved with ethanol-acetone

solution. The OD of each well was read at 570 nm using the microplate reader. All tests were done in triplicates, and the OD was averaged for each isolate [18, 19]. The biofilm production capabilities of the isolates were determined based on ODc value. ODc value was computed as per the formula developed by Stepanović *et al.* (2007) [20]. This is as follows:

$$ODc = \text{mean OD of negative control} + (3 \times \text{standard deviation of negative control})$$

Whereas ODc is the optical density cut-off value, the Mean OD of the negative control is the mean of the optical density values of the negative control, and SD stands for the standard deviation of the negative control.Based on the calculated ODc value, the isolates were classified into four categories as follows:

- Non-biofilm producers: $OD \leq ODc$
- Weak biofilm producers: $ODc < OD \leq 2 \times ODc$
- Moderate biofilm producers: $2 \times ODc < OD \leq 4 \times ODc$
- Strong biofilm producers: $OD > 4 \times ODc$

Qualitative detection of biofilm formation using Congo red agar method

Biofilm production among *E. coli* isolates was screened by Congo red agar (CRA). In the CRA, bacteria were cultured on brain heart infusion agar with Congo red (0.08%) and sucrose (5%). Cultures were grown in aerobic conditions at 37°C for 24-48 hours. Biofilm production by isolates was assessed based on colony appearance. Isolates producing black, dry, and rough colonies were regarded as positive biofilm producers while those producing red, smooth, and non-crystalline colonies were considered negative for biofilm production [21].

Molecular Detection of Biofilm-Associated and Antimicrobial Resistance Genes

Genomic DNA Extraction

The genomic DNA was purified from the cultured *E. coli* isolates using the Presto™ Mini gDNA Bacteria Kit (Geneaid Biotech Ltd., Taiwan). Initially, the pure culture bacteria were selected and then genomic DNA was isolated from the cultured bacteria using the above kit according to the standard procedure. Then the quantity and quality of the isolated genomic DNA was determined using a NanoDrop™ Spectrophotometer (Thermo Fisher Scientific, Wilmington, DE, USA) by measuring the A260/A280 absorbance ratio [22].

PCR Amplification of Biofilm-Associated and Antimicrobial Resistance Genes

Conventional PCR was conducted for the molecular identification of bacteria through amplification of the 16S rDNA gene, and detection of specific biofilm (*fimH, csgA, bcsA*, and *luxS*) and antibiotic resistance-associated (*acrB* and $bla_{CTX\text{-}M}$) genes. Target-specific primers were employed for amplification of respective genes. Primer sequences, amplicon sizes, and references are provided in **Table 1**.

The conventional PCR mixture was set up at a total volume of 25 µL that included 12.5 µL GoTaq® Green Master Mix (2x) (Promega Corporation, Madison, WI, USA), 1 µL template DNA, 1 µL of each forward and reverse primer (10 pmol/µL), and 9.5 µL nuclease-free water. PCR amplification was done using a T100™ Thermal Cycler (Bio-Rad Laboratories, Hercules, CA, USA). PCR cycling conditions included initial denaturation at 95°C for 5 min and 35 cycles of denaturation at 95°C for 30 s, annealing at 30 s at specific gene temperatures (55°C for 16S rDNA, *csgA*, and *luxS*; 58°C for *bcsA* and *acrB*; 60°C for *fimH* and $bla_{CTX\text{-}M}$), extension at 72°C for 60 s, and final extension at 72°C for 5 min.

PCR products were electrophoresed using 1.5% agarose gel containing 1x Tris-Borate–EDTA (TBE) buffer. Staining of the gel was achieved using GelRed® Nucleic Acid stain (Biotium, Fremont, CA, USA), while 100 bp DNA Ladder (Bio-Rad Laboratories, Hercules, CA, USA) was used as the molecular weight standard. Electrophoresis was carried out at 100 V for 60 min. Visualization of DNA bands were achieved using Gel Doc™ EZ Imaging System (Bio-Rad Laboratories, USA). Positive amplification was indicated by the appearance of DNA bands that matched the expected amplicon sizes [2, 4].

Table 1. Primers used for molecular identification and detection of biofilm-associated and antimicrobial resistance genes in *E. coli* isolates.

Target gene	Target function	Primer sequence (5'–3')	Annealing temperature (°C)	Amplicon size (bp)	Reference
16S rDNA	Molecular identification and confirmation of *E. coli* isolates	**F:** GGAAGAAGCTTGCTTCTTTGCTGAC **R:** AGCCCGGGGATTTCACATCTGACTTA	55	544	[23]
fimH	Type 1 fimbrial adhesin involved in adhesion and biofilm formation	**F:** TGCAGAACGGATAAGCCGTGG **R:** GCAGTCACCTGCCCTCCGGTA	60	506	[24]
csgA	Curli fimbriae involved in biofilm matrix formation	**F:** GCAATCGTATTCTCCGGTAG **R:** GATGAGCGGTCGCGTTGTTA	55	418	[24]
bcsA	Cellulose biosynthesis involved in biofilm formation	**F:** GCTTCTCGGCGCTAATGTTG **R:** GAGGTATAGCCACGACGGTG	58	826	[25]
luxS	Quorum sensing (AI-2 signaling)	**F:** TGCCACACTGGTAGACGTTC **R:** TGATTGGTACGCCAGATGAG	55	116	[26]
acrB	Multidrug efflux pump	**F:** GGTCGATTCCGTTCTCCGTTA **R:** CTACCTGGAAGTAAACGTCATTGGT	58	107	[27]
bla$_{CTX-M}$	Extended-spectrum β-lactamase (ESBL)	**F:**ATGTGCAGYACCAGTAARGTKATGGC **R:**TGGGTRAARTARGTSACCAGAAYSAGCGG	60	592	[28]

F: Forward primer, **R:** Reverse primer, **bp:** Base pairs, **AI-2:** Autoinducer-2, **ESBL:** Extended-spectrum β-lactamase.

Statistical analysis

Statistical analyses were carried out with the software IBM SPSS Statistics version 26. Antimicrobial resistance profile, MDR, biofilm production, and gene distribution among *E. coli* isolates were expressed by descriptive statistics. Categorical associations were assessed with Chi-square test and Fisher's exact test where appropriate. Cohen's kappa index was determined to compare the microtiter plate technique with the Congo red agar method for assessing biofilm production. Binary logistic regression was

done to assess the relation between biofilm production and MDR. Odds ratios (OR) with 95% confidence intervals (CI) were calculated, and $p<0.05$ was considered as statistically significant.

Ethical considerations

The present study was carried out in accordance with the ethics guidelines of biomedical research. Ethical clearance was obtained from the Scientific Research Ethics Committee of the College of Science, Mustansiriyah University (Ethical Clearance No.: BCSMU/9025/000087M; Date: August 1, 2025). This study was based on bacterial isolates from clinical urine samples only. All bacterial isolates were anonymous, and there was no collection or use of any personal data or patient ID number. Since the study involved only the analysis of bacterial isolates, without any intervention or information about patients, confidentiality and privacy of clinical samples were ensured.

RESULTS

Antimicrobial susceptibility profile of *Escherichia coli* isolates

Antimicrobial susceptibility testing was performed on 100 isolates against a panel of commonly prescribed antibiotics. The findings revealed substantial variation in susceptibility patterns among the tested antimicrobial agents. Nitrofurantoin showed the highest susceptibility rate 92% (92/100) followed by chloramphenicol (82%), amikacin (76%), cefoxitin (68%), and imipenem (61%).

On the other hand, the highest rate of resistance was recorded against amoxicillin-clavulanate (95%), cefixime (83%), amoxicillin (81%), tetracycline (79%), ceftriaxone (78%), ceftazidime (78%), cefepime (77%), and trimethoprim-sulfamethoxazole (71%). Fluoroquinolones also had relatively high resistance rate with 58% of the isolates being resistant to ciprofloxacin and 54% to levofloxacin. This means that there are high levels of resistance among the isolates against β-lactams and oral drugs. The detailed antimicrobial susceptibility distribution is presented in **Table 2** and graphically illustrated in **Figure 1**.

Table 2. Antimicrobial susceptibility profile of *E. coli* isolates against tested antimicrobial agents (n = 100)

Antibiotic	Sensitive n (%)	Resistant n (%)
Chloramphenicol	82 (82%)	18 (18%)
Trimethoprim–Sulfamethoxazole	29 (29%)	71 (71%)
Nitrofurantoin	92 (92%)	8 (8%)
Tetracycline	21 (21%)	79 (79%)
Ciprofloxacin	42 (42%)	58 (58%)
Nalidixic acid	34 (34%)	66 (66%)
Levofloxacin	46 (46%)	54 (54%)
Gentamicin	60 (60%)	40 (40%)
Amikacin	76 (76%)	24 (24%)
Imipenem	61 (61%)	39 (39%)
Ceftriaxone	22 (22%)	78 (78%)
Cefixime	17 (17%)	83 (83%)
Ceftazidime	22 (22%)	78 (78%)
Cefoxitin	68 (68%)	32 (32%)
Cefepime	23 (23%)	77 (77%)
Amoxicillin–clavulanate	5 (5%)	95 (95%)
Amoxicillin	19 (19%)	81 (81%)

n: number of isolates; percentages represent the proportion of sensitive or resistant isolates for each antibiotic relative to the total number of isolates.

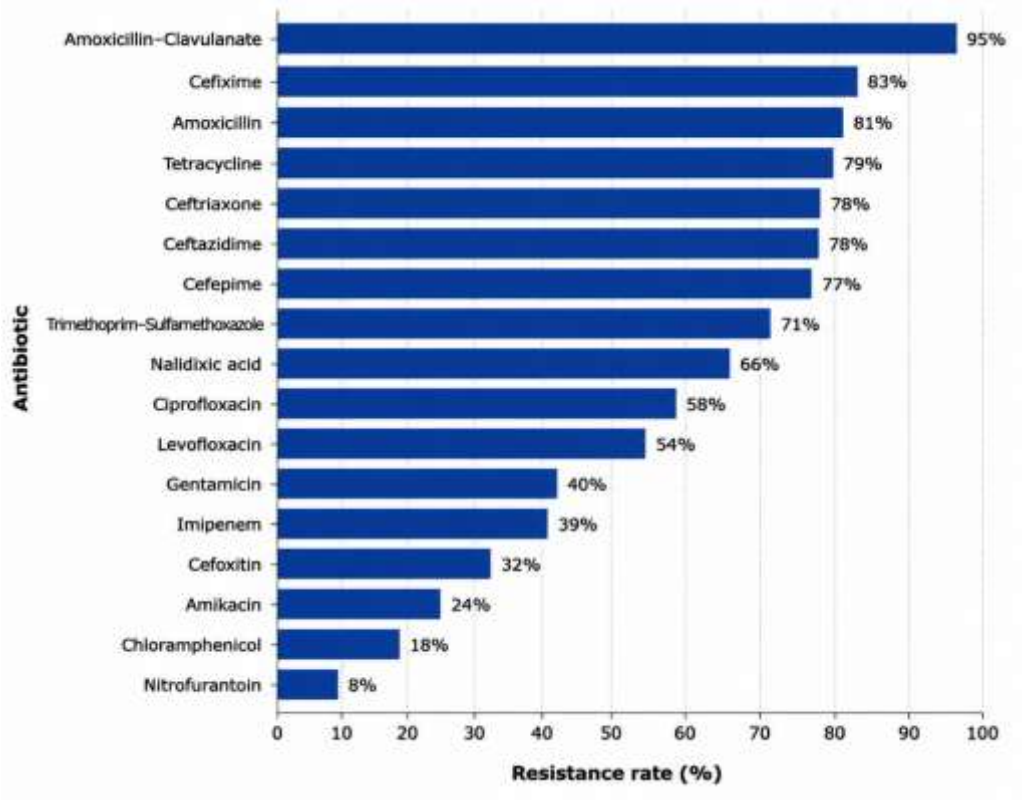

Figure 1. Antimicrobial resistance rates of *E. coli* isolates against tested antimicrobial agents (n = 100).

Regarding MDR, 95 (95%) out of 100 *E. coli* isolates were found to be MDR whereas 5 (5%) isolates were found to be non-MDR. The proportion of MDR and non-MDR isolates has been shown in **Table 3**.

Table 3. Distribution of multidrug-resistant and non-multidrug-resistant *E. coli* isolates (n = 100)

Category	Definition	No. of isolates	Percentage
MDR	Resistant to ≥1 agent in ≥3 antimicrobial categories	95	95%
Non-MDR	Did not fulfill MDR criteria	5	5%

MDR: multidrug resistance; defined according to Magiorakos *et al.* criteria as resistance to ≥3 antimicrobial classes; values represent number and percentage of isolates.

Biofilm formation ability among *E. coli* isolates

The biofilm production of the isolates was assessed through two phenotypic techniques which include the quantitative microtiter plate assay and the qualitative CRA method. From the OD values obtained from the microtiter plate technique, 98 (98%) out of 100 isolates were found to be biofilm producers while 2 (2%) were not. From the 98 biofilm producers, 56 (56%) were found to be weak, 24 (24%) moderate and 18 (18%) strong biofilm producers. Conversely, the Congo Red Agar (CRA) method revealed that 80 (80%) isolates were biofilm producers while 20 (20%) were not. The frequency of biofilm production by both techniques is illustrated in **Table 4** and **Figure 2**.

Table 4. Distribution of biofilm-forming ability among *E. coli* isolates detected by microtiter plate and Congo red agar assays (n = 100)

Method	Category	Number (%)
Congo red agar	Positive	80 (80%)
	Negative	20 (20%)
Microtiter plate	Strong producer	18 (18%)
	Moderate producer	24 (24%)
	Weak producer	56 (56%)
	Non-producer	2 (2%)

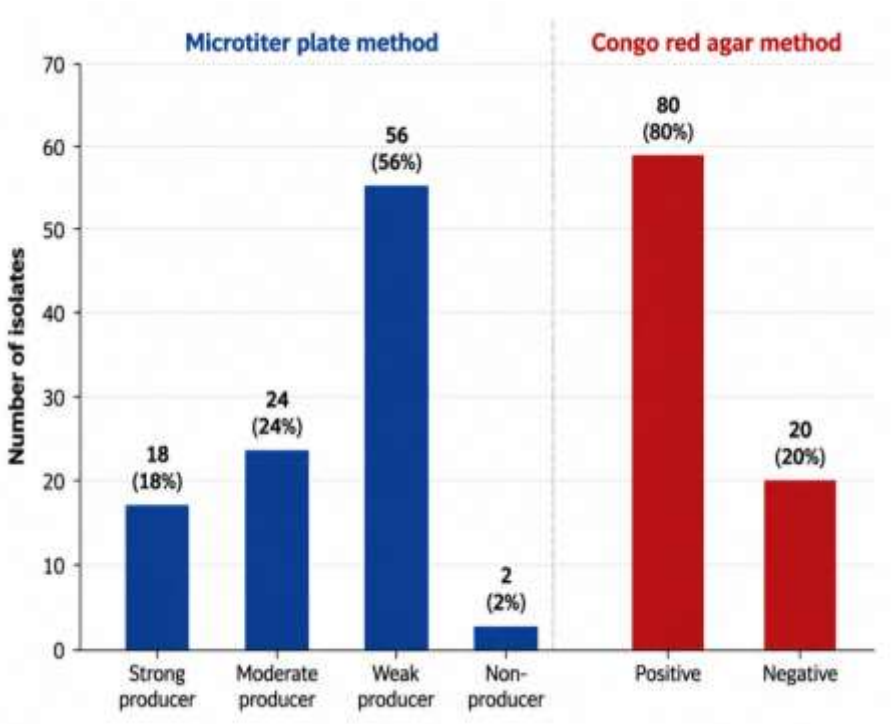

Figure 2. Biofilm formation detaction by microtiter plate and Congo red agar methods (n = 100)

Comparison between microtiter plate assay and Congo red agar method for biofilm detection

From the microtiter plate biofilm production test, 98 (98%) isolates were classified as biofilm producers while 2 (2%) were non-biofilm producers. On the other hand, from the CRA) biofilm prodution test, 80 (80%) isolates were biofilm producers while 20 (20%) were non-biofilm producers. The percentage of concordance between the two methods was 82% (82/100 isolates) with Cohen's Kappa (ϰ) coefficient being 0.15. There were 18 isolates that were discordant with all of them being positive through the microtiter plate assay and negative through the CRA. For the weak biofilm producers, CRA test showed 45/56 (80.4%) to be positive while 11/56 (19.6%) were negative. For the moderate biofilm producers, there were 22/24 (91.7%) positive and 2/24 (8.3%) CRA negative. Finally for the strong biofilm producers, 13/18 (72.2%) were positive and 5/18 (27.8%) negative by the CRA test. The two non-biofilm-producing isolates were negative through the CRA. With regard to the microtiter plate method as reference, the sensitivity of the CRA method was 81.6% while its specificity was 100%. The comparison of biofilm detection results of the two methods is shown in **Table 5** and **Figure 3**.

Table 5. Comparison of biofilm detection results between microtiter plate assay and Congo red agar method among *E. coli* isolates (n = 100)

Microtiter plate classification	CRA positive n (%)	CRA negative n (%)	Total
Strong producer	13 (72.2)	5 (27.8)	18
Moderate producer	22 (91.7)	2 (8.3)	24
Weak producer	45 (80.4)	11 (19.6)	56
Non-producer	0 (0.0)	2 (100)	2
Total	80	20	100

Agreement between the two methods: 82%, Cohen's kappa coefficient (ϰ): 0.15, CRA sensitivity: 81.6%, CRA specificity: 100%

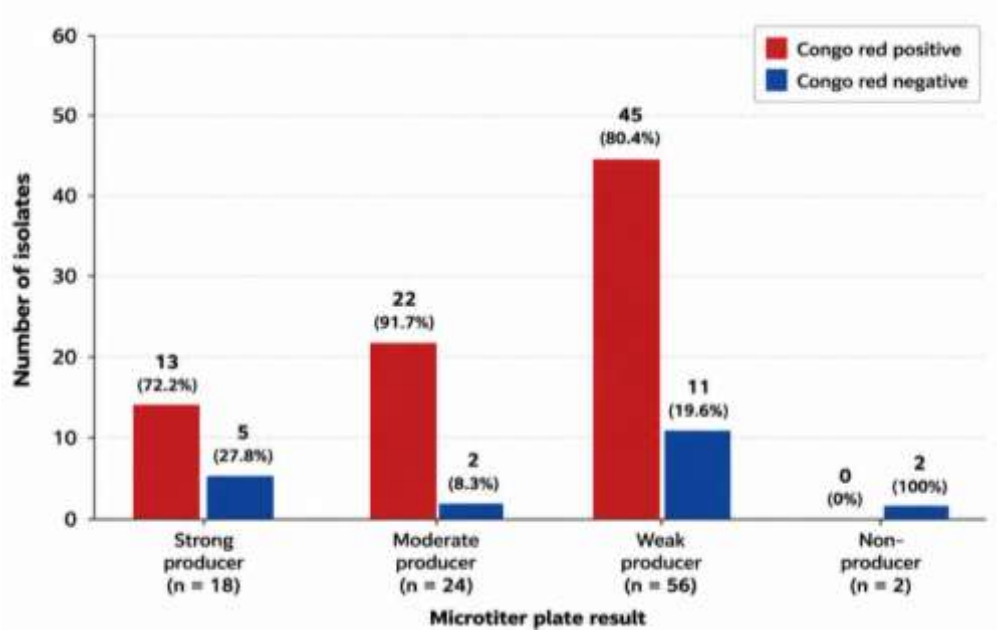

Figure 3. Comparison of Congo red agar detection among different biofilm production categories identified by the microtiter plate assay in *E. coli* isolates (n = 100).

Association between biofilm formation and antimicrobial resistance patterns

An analysis of the relationship between biofilm formation and antibiotic resistance patterns was done by applying Fisher's exact test. There was no statistically significant relationship between the biofilm formation status and resistance to any of the antimicrobial drugs used ($p > 0.05$ for all). In biofilm forming isolates, there were 94/98 (95.9%) isolates resistant to amoxicillin-clavulanate, in comparison with 1/2 (50.0%) non-biofilm-forming isolates ($p = 0.098$). Antibiotic resistance patterns of other antimicrobials depending on biofilm formation are presented in **Table 6**.

Table 6. Association between biofilm formation status and antimicrobial resistance patterns among *E. coli* isolates (n = 100)

Antibiotic	Antimicrobial class	Overall resistant n (%)	Biofilm producers resistant n (%) (n = 98)	Non-biofilm producers resistant n (%) (n = 2)	p-value
Chloramphenicol	Phenicols	18 (18.0)	17 (17.3)	1 (50.0)	0.329
Trimethoprim–sulfamethoxazole	Sulfonamides	71 (71.0)	69 (70.4)	2 (100.0)	1.000

Nitrofurantoin	Nitrofurans	8 (8.0)	8 (8.2)	0 (0.0)	1.000
Tetracycline	Tetracyclines	79 (79.0)	78 (79.6)	1 (50.0)	1.000
Ciprofloxacin	Fluoroquinolones	58 (58.0)	57 (58.2)	1 (50.0)	1.000
Nalidixic acid	Quinolones	66 (66.0)	65 (66.3)	1 (50.0)	1.000
Levofloxacin	Fluoroquinolones	54 (54.0)	53 (54.1)	1 (50.0)	1.000
Gentamicin	Aminoglycosides	40 (40.0)	39 (39.8)	1 (50.0)	1.000
Amikacin	Aminoglycosides	24 (24.0)	23 (23.5)	1 (50.0)	0.424
Imipenem	Carbapenems	39 (39.0)	38 (38.8)	1 (50.0)	1.000
Ceftriaxone	Third-generation cephalosporins	78 (78.0)	77 (78.6)	1 (50.0)	0.393
Cefixime	Third-generation cephalosporins	83 (83.0)	82 (83.7)	1 (50.0)	0.313
Ceftazidime	Third-generation cephalosporins	78 (78.0)	77 (78.6)	1 (50.0)	0.393
Cefoxitin	Cephamycins	32 (32.0)	32 (32.7)	0 (0.0)	1.000
Cefepime	Fourth-generation cephalosporins	77 (77.0)	76 (77.6)	1 (50.0)	0.409
Amoxicillin–clavulanate	Penicillins + β-lactamase inhibitor	95 (95.0)	94 (95.9)	1 (50.0)	0.098
Amoxicillin	Aminopenicillins	81 (81.0)	80 (81.6)	1 (50.0)	0.345

R: resistant isolates. Percentages for biofilm-producing and non-biofilm-producing groups were calculated based on the number of isolates within each group (n = 98 and n = 2, respectively). Statistical comparisons were performed using Fisher's exact test because of the limited number of non-biofilm-producing isolates.

Association between biofilm formation and multidrug resistance (MDR) phenotype among *E. coli* isolates

The relationship between biofilm production and the MDR phenotype was determined in the examined *E. coli* isolates. In total, MDR was found in 95 out of 100 (95%) isolates. MDR was observed in 94 out of 98 (95.9%) biofilm producing isolates, while the MDR phenotype was observed in 1 out of 2 (50.0%) non-biofilm-producing isolates. There was no statistically significant relationship between biofilm production and the MDR phenotype ($p = 0.098$).

On the basis of biofilm production intensity, MDR was observed in all strong biofilm producers (18/18, 100%) and in all moderate biofilm producers (24/24, 100%). MDR phenotype was observed in 52 out of 56 (92.9%) weak biofilm producers. Among the non-biofilm-producing isolates, MDR was observed in 1 out of 2 (50.0%) isolates. The distribution of the MDR phenotype according to biofilm production categories is presented in **Table 7** and **Figure 4**.

Table 7. Association between biofilm formation categories and MDR phenotype among *E. coli* isolates (n = 100)

Biofilm category	Total isolates (n)	MDR n (%)	Non-MDR n (%)
Strong producer	18	18 (100.0%)	0 (0.0%)
Moderate producer	24	24 (100.0%)	0 (0.0%)
Weak producer	56	52 (92.9%)	4 (7.1%)
Non-producer	2	1 (50.0%)	1 (50.0%)
Total	100	95 (95.0%)	5 (5.0%)

MDR was defined as resistance to at least one antimicrobial agent in three or more antimicrobial categories according to Magiorakos et al. Fisher's exact test was used because of the limited number of non-biofilm-producing isolates.

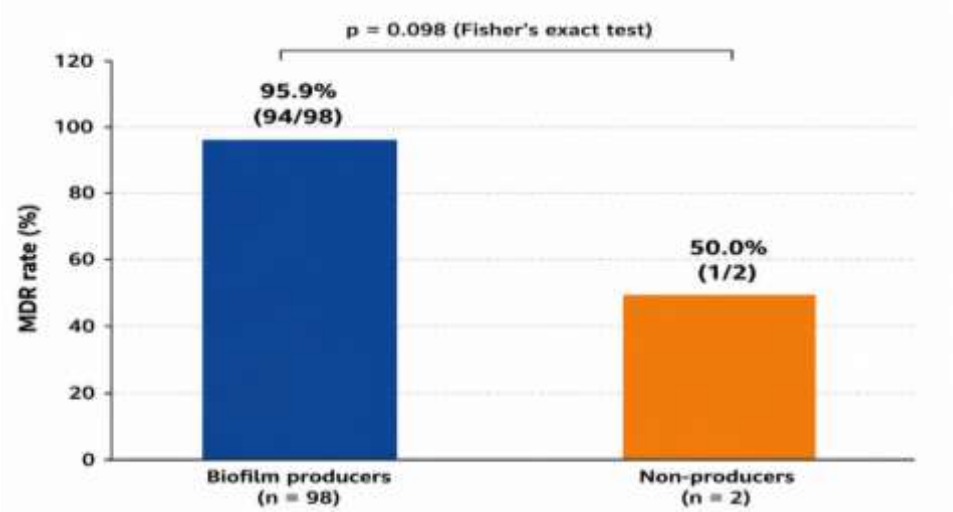

Figure 4. Distribution of MDR phenotype according to biofilm production status among *E. coli* isolates (n = 100).

Molecular detection of biofilm-associated and antimicrobial resistance genes among *E. coli* isolates

The molecular characterization study was conducted to assess the prevalence of biofilm-associated and antimicrobial resistance genes in the *E. coli* isolates.

The gene *fimH* was found in the highest number of *E. coli* isolates, which was 93 out of 100 (93%) isolates, followed by *csgA* gene in 77 out of 100 (77%) isolates, *luxS* gene in 72 out of 100 (72%) isolates, and *bcsA* gene in 58 out of 100 (58%) isolates.

In terms of antimicrobial resistance genes, the *acrB* gene was prevalent in 90 out of 100 (90%) isolates and the $bla_{CTX\text{-}M}$ gene in 69 out of 100 (69%) isolates. The prevalence of the investigated genes is illustrated in **Figure 5** and summarized in **Table 8**.

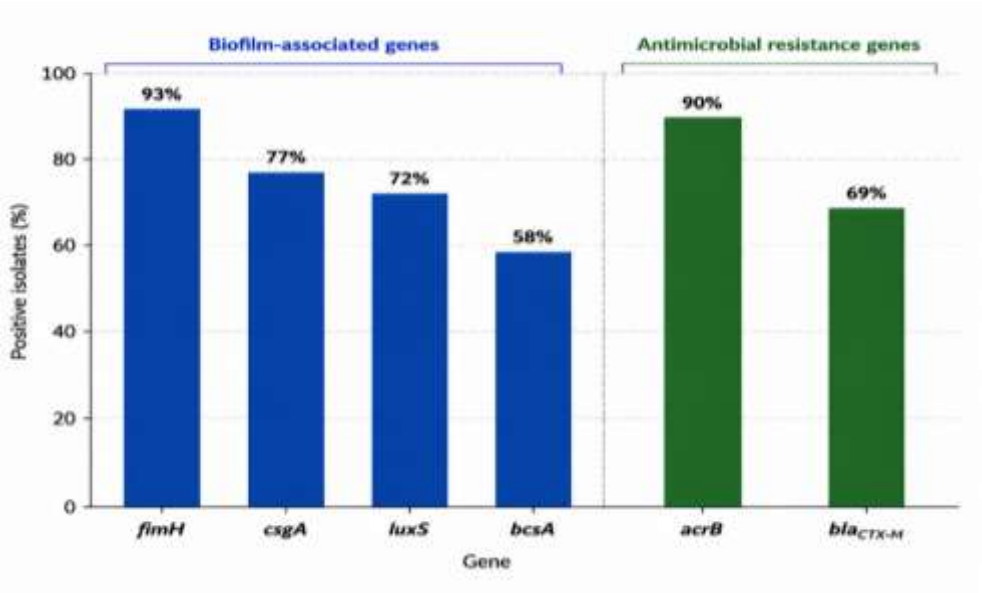

Figure 5. Prevalence of biofilm-associated genes (*fimH, csgA, luxS,* and *bcsA*) and antimicrobial resistance genes (*acrB* and $bla_{CTX\text{-}M}$ among *E. coli* isolates (n = 100).

Table 8. Distribution of biofilm-associated and antimicrobial resistance genes among *E. coli* isolates (n = 100)

Gene	Functional category	Positive isolates n (%)
fimH	Adhesion and type 1 fimbriae formation	93 (93%)
csgA	Curli fiber formation	77 (77%)
luxS	Quorum sensing / AI-2 signaling	72 (72%)
bcsA	Cellulose biosynthesis	58 (58%)
acrB	Multidrug efflux pump	90 (90%)
$bla_{CTX\text{-}M}$	ESBL-mediated β-lactam resistance	69 (69%)

Moreover, the frequency distribution of different types of combined biofilm-associated genes and antimicrobial resistant genes profile among the isolated isolates of *E. coli* was also studied. Seven different profiles were found. *FimH* + *csgA* + *luxS* + *bcsA* + *acrB* +$bla_{CTX\text{-}M}$ was found to be the most common profile present in 20/100 (20%) samples. Next in order came the *FimH* + *acrB* + *csgA* + *luxS* +$bla_{CTX\text{-}M}$ profile that was found in 16/100 (16%) samples, and the *fimH* + *acrB* +*csgA* + *luxS* + *bcsA* profile in 14/100 (14%) samples, as shown in **Table 9**.

Table 9. Distribution of common gene profiles among *E. coli* isolates (n = 100)

Detected gene profile	Isolates n (%)
fimH + *acrB*	6 (6%)
fimH + *acrB* + *csgA*	12 (12%)
fimH + *acrB* + *csgA* + *luxS*	18 (18%)
fimH + *acrB* + *csgA* + *luxS* + *bcsA*	14 (14%)
fimH + *acrB* + *csgA* + *luxS* + $bla_{CTX\text{-}M}$	16 (16%)
fimH + *acrB* + *csgA* + *luxS* + *bcsA* + $bla_{CTX\text{-}M}$	20 (20%)
Other combinations	14 (14%)
Total	**100 (100%)**

Gene co-occurrence analysis and hierarchical clustering of *E. coli* isolates

Co-occurrence analysis and hierarchical clustering were conducted depending on the presence and absence of the six tested genes in isolates. The result of co-occurrence showed that biofilm formation and resistance genes occurred simultaneously at a high frequency rate. The most common co-occurrence was between *fimH* and *acrB*, found in 88/100 (88%) isolates, *fimH* and *csgA* in 74/100 (74%), acrB and csgA in 70/100 (70%), *fimH* and *luxS* in 69/100 (69%), *luxS* and *acrB* in 68/100 (68%), and *acrB* and $bla_{CTX\text{-}M}$ in 65/100 (65%) isolates. The complete distribution of gene co-occurrence patterns is presented in **Table 10**.

Three hierarchical genetic groups were found for 100 studied isolates depending on their gene profile. Group 1 included 56 (56%) isolates which have five or six genes, namely *fimH, csgA, luxS, bcsA, acrB*, and, usually, $bla_{CTX\text{-}M}$. Group 2 had 31 (31%) isolates with intermediate gene profile having three or four investigated genes. Group 3 had 13 (13%) isolates with a minimal gene profile containing one or two genes. The results of hierarchical grouping of the isolates are summarized in **Table 11**.

Table 10. Gene co-occurrence analysis among *E. coli* isolates (n = 100)

Gene pair	Co-occurrence n (%)
fimH + *acrB*	88 (88%)
fimH + *csgA*	74 (74%)

acrB + bla_{CTX-M}	65 (65%)
fimH + *luxS*	69 (69%)
csgA + *luxS*	60 (60%)
acrB + *csgA*	70 (70%)
luxS + *acrB*	68 (68%)
fimH + *bcsA*	55 (55%)
csgA + bla_{CTX-M}	52 (52%)
luxS + bla_{CTX-M}	50 (50%)
bcsA + *acrB*	56 (56%)
bcsA + bla_{CTX-M}	40 (40%)

Table 11. Summary of cluster analysis based on gene profiles among *E. coli* isolates (n = 100)

Cluster	Number of isolates n (%)	Predominant gene profile	Genetic characteristics
Cluster 1	56 (56%)	*fimH* + *csgA* + *luxS* + *bcsA* + *acrB* ± bla_{CTX-M} (5–6 genes)	High biofilm–high resistance profile
Cluster 2	31 (31%)	*fimH* + *csgA* + *luxS* ± *bcsA* + *acrB* (3–4 genes)	Intermediate biofilm-associated genetic profile
Cluster 3	13 (13%)	*acrB* ± bla_{CTX-M} *with limited biofilm genes* (1–2 genes)	Low genetic burden / resistance-dominant profile
Total	100 (100%)	—	—

Association between biofilm-associated genes and biofilm production phenotype

Correlation of the biofilm-associated genes to categories of biofilm formation was assessed among the tested *E. coli* isolates. There is a statistically significant correlation between the categories of biofilm formation and the prevalence of all biofilm-associated genes studied ($p < 0.05$). The *fimH* gene was found in 18/18 (100%) of strong biofilm producers, 23/24 (95.8%) of moderate producers, 52/56 (92.9%) of weak producers, and not found in any non-biofilm producing isolates (0/2, 0%) ($p = 0.041$). The *csgA* gene was found in 17/18 (94.4%) strong producers, 21/24 (87.5%) moderate producers, 38/56 (67.9%) weak producers, and 1/2 (50.0%) non-producers ($p = 0.032$). In a similar manner, the *luxS* gene was found in 16 out of 18 (88.9%) strong producers, 19 out of 24 (79.2%) moderate producers, 36 out of 56 (64.3%) weak producers, and 1 out of 2 (50.0%) non-producers ($p = 0.048$). *bcsA* gene was found in 15 out of 18 (83.3%), 17 out of 24 (70.8%), 25 out of 56 (44.6%) and 1 out of 2 (50%) strong, moderate, weak producers and non-producers respectively ($p = 0.019$). Distribution of biofilm-associated genes based on biofilm producing categories is provided in **Table 12** and **Figure 6**.

Table 12. Association between biofilm-associated genes and biofilm production categories among *E. coli* isolates (n = 100)

Gene	Strong producer (n=18)	Moderate producer (n=24)	Weak producer (n=56)	Non-producer (n=2)	p-value
fimH	18 (100%)	23 (95.8%)	52 (92.9%)	0 (0%)	0.041
csgA	17 (94.4%)	21 (87.5%)	38 (67.9%)	1 (50%)	0.032
luxS	16 (88.9%)	19 (79.2%)	36 (64.3%)	1 (50%)	0.048
bcsA	15 (83.3%)	17 (70.8%)	25 (44.6%)	1 (50%)	0.019

Values represent the number and percentage of isolates positive for each gene within each biofilm category. Statistical significance was determined using Fisher's exact test.

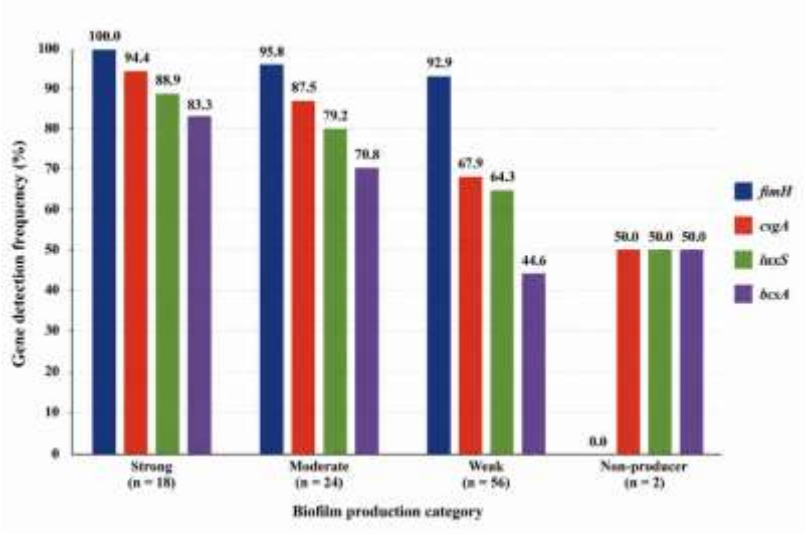

Figure 6. Distribution of biofilm-associated genes according to biofilm production categories among *E. coli* isolates (n = 100).

Association between antimicrobial resistance genes and MDR phenotype

Relationships between antimicrobial resistance genes and MDR phenotype were studied among the tested isolates of *E. coli*. The presence of the *acrB* gene was found in 88/95 (92.6%) of MDR isolates, while in only 2/5 (40.0%) of non-MDR isolates; this result was significant statistically (p = 0.012). The presence of the $bla_{CTX\text{-}M}$ gene was found in 69/95 (72.6%) of MDR isolates, while in none (0/5, 0.0%) of non-MDR isolates; this result was also significant statistically (p = 0.041).The distribution of antimicrobial resistance genes according to MDR phenotype is presented in **Table 13** and **Figure 7**.

Table 13. Association between antimicrobial resistance genes and MDR phenotype among *E. coli* isolates (n = 100)

Resistance gene	MDR isolates (n=95)	Non-MDR isolates (n=5)	*p*-value
acrB positive	88 (92.6%)	2 (40.0%)	0.012
$bla_{CTX\text{-}M}$ positive	69 (72.6%)	0 (0.0%)	0.041

MDR was defined according to international criteria. Fisher's exact test was used because of the limited number of non-MDR isolates.

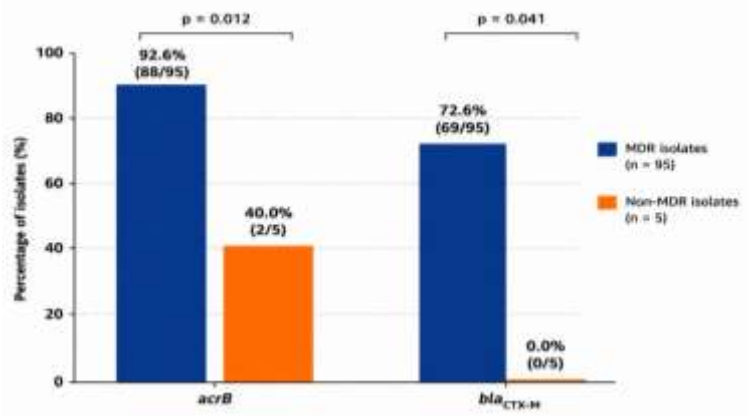

Figure 7. Association between antimicrobial resistance genes and MDR phenotype among *E. coli* isolates (n = 100).

Logistic regression analysis of predictors associated with MDR phenotype

Binary logistic regression analysis was done to determine the predictors that were associated with MDR phenotypes in the tested isolates of *E. coli*. Production of biofilms was significantly associated with MDR (OR = 23.5; 95% CI: 1.23-447.59, p = 0.036). Also, strong biofilm phenotype was significantly associated with MDR (OR = 15.2; 95% CI: 1.42-162.4, p = 0.024). *acrB* was significantly associated with MDR (OR = 18.3; 95% CI: 2.07-161.3, p = 0.009) while positivity of bla_{CTX-M} was significantly associated with MDR (OR = 5.1, 95% CI: 1.09–23.5, p = 0.038), as presented in **Figure 8** and **Table 14**.

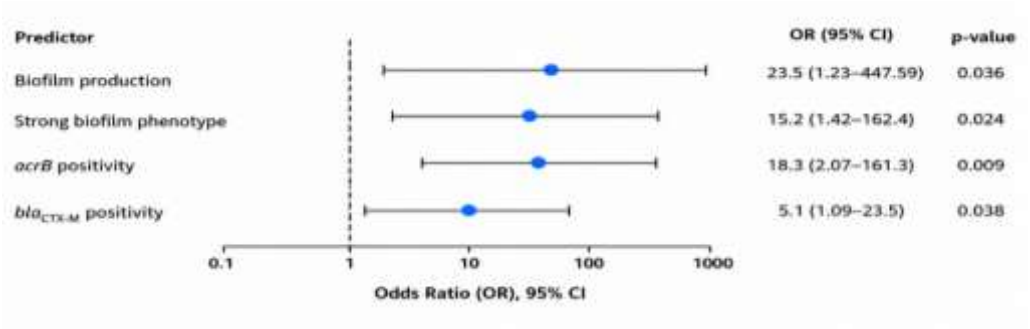

Figure 8. Forest plot of binary logistic regression analysis for predictors associated with MDR among *E. coli* isolates (n = 100).

Table 14. Regression coefficients and statistical parameters for MDR predictors

Predictor	β coefficient	Standard error (SE)	OR	95% CI	p-value
Biofilm production	3.157	1.504	23.5	1.23–447.59	0.036
Strong biofilm phenotype	2.721	1.201	15.2	1.42–162.4	0.024
acrB positivity	2.906	1.112	18.3	2.07–161.3	0.009
bla_{CTX-M} positivity	1.624	0.781	5.1	1.09–23.5	0.038

β: regression coefficient; SE: standard error; OR: odds ratio; CI: confidence interval. Logistic regression was performed using MDR status as the dependent variable.

DISCUSSION

The present study demonstrated a substantial burden of antimicrobial resistance among *E. coli* isolates, characterized by high resistance to multiple commonly prescribed antimicrobial agents and an exceptionally high prevalence of MDR. These findings underscore the increasing challenges associated with empirical management of UTIs and highlight the consequences of inappropriate antimicrobial use and inadequate antimicrobial stewardship.

From the tested antibiotics, nitrofurantoin showed the greatest in vitro efficacy, making it the most effective antimicrobial agent against the investigated UPEC isolates despite the widespread resistance observed to several other antibiotic classes. T *E. coli* his result is in agreement with the findings of Abo-alella *et al.* (2024) [29]. The sustained effectiveness is due to the limited use in non-complex cases of lower UTIs, the unique mechanism of action by targeting different bacteria, and the number of chromosomal mutations required before resistance becomes significant. On the other hand, the high level of resistance to β-lactams, tetracycline, and trimethoprim-sulfamethoxazole is consistent with the results obtained by Hadi *et al.* (2023) [30]. The particularly high resistance observed against third- and fourth-generation cephalosporins may reflect the extensive dissemination of CTX-M-type extended-spectrum β-lactamases (ESBLs), which are commonly carried on transferable plasmids and facilitate the rapid spread of resistance among Enterobacterales. The resistance to fluoroquinolones was high, which further limits their suitability for empirical treatment in the study setting. This resistance is likely multifactorial and has been attributed to chromosomal mutations in the *gyrA* and *parC* genes, plasmid-mediated quinolone resistance determinants, and overexpression of multidrug efflux pumps, as reported by Choudhury *et al.* (2024) [31]. Another significant result that should be highlighted is the intermediate susceptibility to imipenem, which is significantly less compared to the data reported in the majority of earlier

studies. Despite the relevance of carbapenems as drugs used in the treatment of complicated cases of MDR infections, this finding might suggest the development of resistance mechanisms toward carbapenems, such as the presence of carbapenemases, outer membrane protein reduction, or active multidrug efflux systems.

The extremely high rate of occurrence of MDR isolates in the current study suggests that the prevalence of *E. coli* multidrug-resistance has increased tremendously in the population studied, thus creating a major problem for the empirical treatment of UTIs. This result is similar to findings made in several recent studies carried out in areas where there is rampant use of antimicrobials with increasing selection pressures due to improper antibiotic use. The increased MDR occurrence may be linked to the propagation of mobile genetic elements such as plasmids encoding multiple antimicrobial resistance genes that can be shared by HGT as indicated by Nasrollahian *et al.* (2024) [32]. Clinically, a high level of MDR imposes serious restrictions on therapeutic opportunities and leads to treatment failures, chronicity of infection, recurrent UTI, and increased cost of health care. In case of recurrent or complicated infection, empiric antimicrobial therapy should be based on the updated information on local resistance patterns. The ability to form biofilm is among the key virulence factors of *E. coli*, which allows bacteria to persist in the urinary tract and cause chronic and recurrent UTIs. By means of forming biofilms, bacteria increase their resistance to host immune response and antimicrobial agents.

In this study, 98% of the *E. coli* isolates have been found to be biofilm producers, where the weak biofilm producers (56%) constitute the majority of the phenotypes. These results are generally similar to the observations made by Saman *et al.* (2026) [33]. The extremely high prevalence rate of biofilm formation in this study could be attributed to the predominance of highly virulent clinical isolates, variations in the population of patients studied, local epidemiology, or increased sensitivity of the microtiter plate assay for quantification of the biofilm. However, dominance of isolates that produce poor biofilms implies that while the majority of isolates had capability of forming biofilms, few isolates had formed mature biofilms with significant amounts of extracellular matrix formation. This could be attributed to different regulation of genes involved in biofilm formation, environmental conditions of in vitro culture, and expression of quorum-sensing system.

Antimicrobial resistance in biofilms is a well-coordinated biological phenomenon consisting of multiple defense systems that work together. As pointed out by Nasrollahian *et al.* (2024), biofilm formation decreases the effectiveness of antibiotics due to multiple factors such as limited penetration of antimicrobials into the biofilm due to the extracellular polymer matrix, decreased metabolic rate of bacteria, persister cell formation, and increased horizontal gene transfer in the biofilm population [32]. Indeed, in comparison to the CRA method, the MTP assay showed significantly better performance for identifying biofilm formation as the phenotypic method of choice for the evaluation of biofilms. Despite the fact that the percentage agreement between the two assays was relatively good (82%), the value of Cohen's kappa coefficient was quite low (0.15). It is known that sometimes there is a paradox when the kappa coefficient turns out to be very low despite the high agreement rate due to an extreme imbalance in the number of positive/negative cases, called "kappa paradox". Thus, the extremely high proportion of biofilm-forming isolates (98%) in the current study may explain why the value of kappa turned out to be rather low. Moreover, discordant isolates were categorized as biofilm producers using the MTP test but as non-producers using the CRA test, implying that the CRA technique was mainly unable to detect isolates with relatively poor capacity to produce biofilms rather than producing false positive results. This inference is also supported by the fact that CRA has relatively lower analytical sensitivity compared to weak biofilm producers. These results validate earlier studies that consider the microtiter plate assay as the phenotypic gold standard for biofilm detection while CRA should only be used as a cheap and rapid screening test as described by Mahale *et al.* (2025) [34]. In view of this practical laboratory setting, CRA technique can still be useful in terms of its simplicity and low cost although quantitative assays like the MTP test are preferred if accurate biofilm determination is needed.

While biofilm-producing isolates were more resistant to most of the studied antimicrobial drugs, there were no statistically significant links found between biofilm formation and resistance to particular antibiotics ($p > 0.05$). The results are not reliable due to the small number of non-biofilm producing isolates (only two isolates), which greatly decreases the power of comparison and makes a Type II error more probable.

Despite the lack of statistical significance, the consistent higher frequencies of resistance among biofilm-forming isolates can be considered biologically meaningful. For instance, 95.9% of biofilm producing isolates were resistant to amoxicillin-clavulanate as opposed to 50.0% of non-biofilm producers. Such patterns were observed in most cases for other antimicrobial drugs though without statistical significance. The described biofilm-induced resistance mechanisms can clarify the consistent higher frequencies of resistance among biofilm-producing isolates despite the absence of statistically significant correlations for

separate antimicrobial drugs. The same results have been observed in the latest systematic review and meta-analysis conducted by Garousi *et al.* (2023), in which it was revealed that *E. coli* isolates forming biofilms usually possess higher resistance to antibiotics compared to isolates not forming biofilms; however, the strength of this correlation depends on the way of biofilm determination, the geographical location, and other epidemiological factors [35]. Hence, it should not be considered as an evidence of the absence of any biological association between these two variables; instead, it may indicate the very small number of isolates without the ability to form biofilms.

Though no statistically significant relationship between biofilm formation and the MDR phenotype was found using Fisher's exact test (p=0.098), there was a noticeable biological tendency. The MDR phenotype was found in 94 out of 98 (95.9%) isolates producing biofilm compared to just one out of two (50.0%) non-biofilm forming isolates. Most probably, the lack of statistical significance is explained by the very small number of non-biofilm forming isolates that decreased the statistical power of the analysis. Thus, the obtained p-value has to be considered carefully since it can be treated as an indirect evidence for the absence of the potential relationship. It should be pointed out that all the strong (18/18, 100%) and moderate (24/24, 100%) biofilm producers possessed the MDR phenotype while somewhat smaller percentage of weak biofilm producers (52/56, 92.9%) had this phenotype as well. This trend appears biologically plausible and can be justified through the use of biofilm-mediated tolerance mechanisms mentioned earlier that contribute together towards increased survival of bacteria and formation of multidrug-resistant colonies.

It should be noted that the subsequent binary logistic regression analysis revealed that biofilm formation was an independent predictor of MDR regardless of other predictors (OR = 23.5, p = 0.036). The seeming discrepancy with the results of the univariate Fisher's exact analysis can be attributed to differences in the statistics employed rather than to any contradiction. Unlike Fisher's exact test which examines the relationship between the two variables, logistic regression determines the independent effect of each predictor regardless of other factors included in the model. Similar findings were found by Liu *et al.* (2024), who pointed out that the increased biofilm formation contributes to bacterial persistence and promotes the occurrence of antimicrobial resistance determinants [36]. In terms of clinical implications, the emergence of MDR biofilm-forming *E. coli* isolates represents particular concern since it increases the chances of persistent bacteriuria and recurrent UTIs.

The molecular characterization revealed a high prevalence of biofilm-associated and antimicrobial resistance genes in the examined *E. coli* isolates, underscoring the complex genetic background behind their pathogenesis and survival strategies. The prevalence of *fimH* (93%) followed by *csgA* (77%), *luxS* (72%), and *bcsA* (58%) suggests that there are several different mechanisms associated with bacterial adhesion, biofilm development, quorum sensing, and exopolysaccharide production that are present simultaneously in the circulation of *E. coli* isolates.

From all the studied virulence factors, *fimH* was the most prevalent gene, which highlights its importance in the early stages of *E. coli* pathogenesis. The *fimH* adhesin helps bacteria adhere to mannosylated receptors of urothelium, colonize the urinary tract, and act as a crucial prerequisite for further biofilm formation. Thus, the high prevalence found in the current study is justified since the importance of *fimH* as one of the highly conserved virulence factors of *E. coli* was proven earlier by Ballesteros-Monrreal *et al.* (2023) [37]. In this regard, the relatively high prevalence rates of *csgA, luxS,* and *bcsA* clearly indicate that the development of biofilms is not a simple process controlled by a single gene but a well-regulated network comprising numerous virulence factors. *csgA* encodes curli fibers responsible for bacterial adherence and cell aggregation; *luxS* regulates quorum sensing via the AI-2 signaling pathway, while *bcsA* participates in cellulose synthesis. This suggests that the co-presence of these genes significantly increases the likelihood of forming a biofilm among *E. coli* isolates under investigation.

As far as resistance determinants are concerned, the most common resistance gene was *acrB* (90%), followed by $bla_{CTX\text{-}M}$ (69%). The high rate of *acrB* is indicative of the great contribution made by the AcrAB-TolC multidrug efflux pump to the intrinsic and acquired antimicrobial resistance of *E. coli* isolates, while the high rate of $bla_{CTX\text{-}M}$ is an explanation of the broad-spectrum resistance to third-generation cephalosporins. Our findings are in agreement with the available data on the widespread distribution of ESBL-producing *E. coli* lineages, as stated by Nasrollahian *et al.* (2024) and Ballesteros-Monrreal *et al.* **(**2023) [32, 37]. From a general perspective, it is evident that the analyzed *E. coli* isolates have been found to harbor a number of virulence and antimicrobial resistance factors that contribute to the increased colonization and survival of bacteria under antimicrobial pressure, leading to more recurrent and harder-to-treat UTIs.

Besides the independent frequency of occurrence of biofilm-associated and antimicrobial resistance factors, this study has revealed that those two types of genetic elements often coexist in the same *E. coli* isolates, reflecting the development of complementary virulence and antimicrobial resistance mechanisms rather than random presence of them. The most common genotype was represented by *fimH, csgA, luxS, bcsA, acrB,* and $bla_{CTX\text{-}M}$, meaning that successful *E. coli* isolates acquire both adhesin-related, biofilm-maturation-related, multidrug-efflux, and β-lactam resistance determinants. The concurrent presence of these genes can confer a significant adaptive benefit during UTI because the former promote bacterial attachment, persistence, and protection from immune system of the host while the latter increase survival under antimicrobial therapy.

The co-occurrence of genes also confirmed the hypothesis. A high incidence of *fimH-acrB*, *fimH-csgA*, *acrB-csgA*, and *acrB-* $bla_{CTX\text{-}M}$ indicates that the virulence and resistance factors are maintained frequently in the same bacterial isolates. Despite the fact that it was not in the scope of this study to identify the localization of those determinants, it is possible that this co-localization reflects their dissemination via evolutionary successful isolates or mobile elements, or selection for antibiotic resistance. The hierarchical clustering analysis also gave an interesting insight into the genetic structure of the studied bacteria. Almost half of all analyzed isolates (56%) belong to Cluster 1, which included bacteria with five or six studied genes. It implies that evolutionarily successful *E. coli* isolates in the study population have developed multiple virulence and resistance factors.

On the contrary, Clusters 2 and 3 harbored isolates containing fewer numbers of the investigated genes, showing high genetic diversity among the *E. coli* isolates circulating within the community. Such heterogeneity shows the dynamic evolutionary processes within *E. coli* strain population and implies that different genetic lineages have adopted different survival strategies within the UTI environment. On the whole, the analysis of the genetic profile, the co-occurrence patterns, and the clustering suggest that the pathogenic success of *E. coli* cannot be attributed to one virulence or resistance factor but is likely to be due to the collective activity of several complementary genetic mechanisms.

A significant correlation was found between the investigated biofilm-related genes and the biofilm formation intensity phenotype, suggesting a molecular link between the increased ability of *E. coli* isolates to form biofilm and their acquisition of particular virulence factors.

Among the examined genes, *fimH* exhibited the highest and most consistent presence in the set of biofilm-forming isolates, with the presence in all strong biofilm formers (100%), 95.8% in moderate formers and 92.9% of weak biofilm formers, and no presence in non-biofilm forming isolates. It highlights once again the primary role of *fimH* adhesion as the first step of the biofilm formation process and confirms its importance as a key factor in *E. coli* pathogenicity, which was confirmed by Ballesteros-Monrreal et al. (2023) [37]. Likewise, the gradual increase of *csgA, luxS,* and *bcsA* presence in weak to strong biofilm formers indicates that biofilm maturation is controlled by several genetic routes rather than a single virulence factor. The curli fibers synthesis (*csgA*), quorum sensing regulation (*luxS*), and cellulose biosynthesis (*bcsA*) ensure biofilm structure, strength, and coordination between the cells of *E. coli*. Similarly, the presence of a gene-dosage type of relationship, which indicates an increased prevalence of the genes with increasing intensity of biofilms, means that the more the number of biofilm-associated determinants in the isolates, the greater their capability to form biofilm. While no gene expression studies were done in the present study, the strong phenotypic and genotypic correlations make it evident that the above-mentioned virulence determinants play a biological role in biofilm development by *E. coli*. Such findings are compatible with the current knowledge that biofilm formation is a complex and multistep process, consisting of such phases as bacterial adherence, microcolony formation, quorum sensing, extracellular matrix development, and biofilm maturation. In the present study, the significant associations have been found, providing molecular basis for the extremely high prevalence of biofilm-producing isolates.

In the present study, the significant associations have been revealed between the investigated genes and the MDR phenotype, thus proving that particular resistance genes play a crucial role in the extensive antimicrobial resistance of the *E. coli* isolates. Detection of *acrB* gene was higher in 92.6% of MDR isolates as opposed to only 40% of non-MDR isolates, implying that AcrAB-TolC efflux pump is strongly related to MDR. This is biologically possible because the AcrAB-TolC efflux pump is capable of expelling different types of antimicrobials from bacteria cells leading to reduced intracellular concentration and therefore improving the bacterial survival rate. Furthermore, the overexpression of AcrAB-TolC results in enhanced biofilm tolerance and development of other resistance mechanisms, thus, contributing to MDR. Like the acrB gene, $bla_{CTX\text{-}M}$ was detected only in MDR isolates (72.6%) but not in non-MDR isolates. In addition, the blaCTX-M had a statistical relationship with MDR. Detection of the $bla_{CTX\text{-}M}$ in such a high frequency explains the presence of resistance in third generation cephalosporins. This is consistent with previous studies done by Nasrollahian *et al.* (2024), who described the vital role of the

multidrug efflux systems and ESBL-plasmid in the occurrence of MDR *E. coli* [32]. This is because the resistance factors can be found in transferable plasmids along with other resistance genes such that acquiring bla_{CTX-M} may lead to resistance to several antimicrobial classes and not only to β-lactams. All these data suggest that the MDR phenotype observed in the current study is due to a number of mechanisms and not just one. The combination of multidrug efflux systems, ESBL-producing, and the presence of several resistance genes in the same *E. coli* isolates causes MDR phenotype.

Binary logistic regression analysis was used in order to find out which parameters independently predict the MDR phenotype in the studied *E. coli* isolates. In contrast to univariate analysis, binary logistic regression allows for evaluating the impact of each variable independently, taking into account the effect of other variables included in the model.

It was found that biofilm production was independently associated with MDR (OR = 23.5, 95% CI: 1.23–447.59, $p = 0.036$). The obtained result proves that biofilm-positive isolates have about 23 times higher odds to be multidrug-resistant isolates compared to the isolates lacking biofilm-forming ability after controlling for other factors. The confidence interval was quite large probably due to the low number of non-biofilm producers; however, the result was statistically significant, which speaks in favor of the biological relevance of the phenomenon under study. High biofilm production was independently associated with MDR (OR = 15.2, $p = 0.024$). It means that high level of biofilm production increases the likelihood of MDR development biologically logically since mature biofilms can protect bacteria from antimicrobials by increasing production of extracellular matrix, reduction in metabolic activity and persistence of dormant bacteria. Among the studied molecular markers, *acrB* was found to be independently associated with MDR (OR = 18.3, 95% CI: 2.07–161.3, $p = 0.009$). The found result proves the crucial role of AcrAB-TolC multidrug efflux pump in the emergence of multidrug-resistance. The high odds ratio observed for *acrB* further supports its potential value as a molecular marker for predicting multidrug-resistant *E. coli* isolates. In addition, bla_{CTX-M} was still a significant predictor of MDR (OR = 5.1, 95% CI: 1.09-23.5, $p = 0.038$), further underscoring the role of ESBLs and their mechanisms of resistance to the development of MDR. In summary, the current results suggest that the development and maintenance of MDR among *E. coli* involves not only the presence of molecular markers of resistance but also phenotypic characteristics of resistance.

Generally, according to the regression model presented above, the MDR phenotype in *E. coli* is achieved via several complementing mechanisms of adaptation which emerge as a result of the interplay of virulence factors and antibiotic resistance mechanisms [38]. Similar adaptive responses have been reported in Gram-negative pathogens, where antibiotic exposure can modulate the expression of biofilm-associated determinants, suggesting that antimicrobial stress may contribute to bacterial persistence through alterations in biofilm-related pathways [39].

The current results will be useful for future studies aiming at high-risk *E. coli* identification as well as in terms of using molecular markers for antimicrobial resistance surveillance. The results obtained in the current study have important implications from the perspective of empirical antimicrobial therapy for UTIs caused by *E. coli*. Namely, the combination of widespread antimicrobial resistance, biofilm formation capacity and presence of virulence-and resistance-related genes suggests that empirical treatment is unlikely to be the best choice in the area under consideration, especially for recurrent or complicated UTIs. Antimicrobial therapy should, thus, always be based on urine cultures and antimicrobial susceptibility testing whenever possible. This will result in better therapeutic results without the unnecessary exposure to broad-spectrum antibiotics. The preserved in vitro activity of nitrofurantoin makes the drug a good choice as the preferred first-line treatment for uncomplicated UTIs. Nevertheless, this is only the case when the resistance situation remains unchanged through regular monitoring of the local resistance patterns. On the other hand, the high level of resistance against widely used β-lactams, third- and fourth-generation cephalosporins, trimethoprim–sulfamethoxazole, and fluoroquinolones is alarming and suggests that there might be a problem with the empirical use of these drugs without susceptibility testing. This underscores the need for regular updating of antimicrobial treatment protocols locally.

In addition to the choice of appropriate antimicrobials, the extremely high prevalence of biofilm-producing isolates implies that treatment of chronic UTI will pose even more challenges using the traditional approach of antimicrobial therapy alone. In view of this, in the future, treatment approaches should consider employing drugs that interfere with biofilm formation, quorum sensing mechanisms, or MDR efflux pumps in combination with conventional antimicrobials. All these results confirm the need for immediate actions to improve antimicrobial stewardship initiatives, increase antimicrobial resistance surveillance, and conduct molecular epidemiology investigations in order to reduce further spread of MDR *E. coli*.

One of the key advantages of this study lies in the integrated approach to phenotypic, genotypic and statistical analysis using one sample of clinical *E. coli* isolates. Through concurrent testing of antimicrobial resistance, biofilm formation, virulence factors and resistance genes, the paper offers more detailed understanding of the complicated interconnection between the pathogenicity and MDR mechanisms. Multiple phenotypic approaches used in biofilm detection, together with molecular characterization of the genes responsible for the biofilm formation and resistance mechanisms, increased the reliability and biological validity of the results. In addition, advanced statistical analysis of co-occurrence, clustering and multivariate regression made it possible to detect the genetic patterns associated with MDR.

CONCLUSION

This study proves that *E. coli* isolates are characterized by several virulence factors, the ability to form biofilms, and antimicrobial resistance mechanisms that promote persistence of the bacteria and decrease the efficacy of treatment. The close connection between the genes associated with biofilm formation, biofilm formation itself, and MDR confirms the dependence between the virulence and antimicrobial resistance. These results reveal the significance of using the approach based on the culture therapy, monitoring the resistance to antimicrobials, and implementing the programs of antimicrobial stewardship. In general, this research offers important information about the dependence of biofilm formation and resistance.

Study Limitations and Future Perspectives

The current study offers several important conclusions concerning the connection between biofilm production, virulence traits, and antibiotic resistance in *E. coli* isolates. Despite the fact that the samples were collected from the same geographical area and the number of comparative groups was small, the results of the research have high importance and may serve as the foundation for future research. It is recommended that future research involves large multicentric collections of samples and molecular methods such as whole genome sequencing, transcriptome analysis, and plasmid profiling. Also, research on the anti-biofilm strategies, regulation of resistance, and surveillance should be conducted.

Acknowledgments

The authors express their gratitude to Mustansiriyah University, Baghdad, Iraq for providing the support and help necessary to complete this work.

Data Availability

Available from corresponding author upon request.

Conflict Of Interest

None declared.

Funding

No external funding.

REFERENCES

[1] Zeadan AM, Saleem AJ, Abbas SM. Detection and analysis of resistance genes in *Escherichia coli* bacteria isolated from children in Baghdad. *Advanced Gut & Microbiome Research*. 2022;2022:1450019. doi:10.1155/2022/1450019.

[2] Sun D, Lv D, Mi Z, Hu L, Huang Y, Gao X, et al. Investigation of antibiotic resistance determinants and virulence factors of uropathogenic *Escherichia coli*. *Journal of Antibiotics (Tokyo)*. 2020;73:314-319. doi:10.1038/s41429-020-0284-7.

[3] Abbas SM, Dhahi MAR. Evaluation of some virulence factors and drug resistance of bacteria isolated from the urine of patients with TCC-bladder cancer. *Baghdad Science Journal*. 2022;19(3):0469. doi:10.21123/bsj.2022.19.3.0469.

[4] Mohsin AS, Alsakini AH, Ali MR. Outbreak of drug-resistant *Escherichia coli* phylogenetic group F in UTIs. *Iranian Journal of Microbiology*. 2022;14(3):341-350. doi:10.18502/ijm.v14i3.9771.

[5] Behzadi P, Urbán E, Gajdács M. Association between biofilm-production and antibiotic resistance in uropathogenic *Escherichia coli* (UPEC): an in vitro study. *Diseases*. 2020;8(2):17. doi:10.3390/diseases8020017.

[6] Dadi BR, Abebe T, Zhang L, Mihret A, Abebe W, Amogne W. Distribution of virulence genes and phylogenetics of uropathogenic *Escherichia coli* among urinary tract infection patients in Addis Ababa, Ethiopia. *BMC Infectious Diseases.* 2020;20(1):108. doi:10.1186/s12879-020-4844-z.

[7] Pantel A, Dunyach-Remy C, Ngba Essebe C, Mesureur J, Sotto A, Pagès JM, et al. Modulation of membrane influx and efflux in *Escherichia coli* sequence type 131 has an impact on bacterial motility, biofilm formation, and virulence in a *Caenorhabditis elegans* model. *Antimicrobial Agents and Chemotherapy.* 2016;60(5):2901-2911. doi:10.1128/AAC.02872-15.

[8] Bazgosha M, Saberi GA, Hormozi M, Shirangi K, Shaghaghi Bahri F, Zahmatkesh H, et al. Augmentation of antibacterial activity of ciprofloxacin by bisabololoxide A encapsulated in polymeric nanoparticles against uropathogenic *Escherichia coli*. *BMC Biotechnology.* 2025;25(1):140. doi:10.1186/s12896-025-01070-5.

[9] Dessartine MM, Kosta A, Doan T, Cascales É, Côté JP. Type 1 fimbriae-mediated collective protection against type 6 secretion system attacks. *mBio.* 2024;15(4):e0255323. doi:10.1128/mbio.02553-23.

[10] Wang Y, Zhang R, Wang P, Zhang W, Li Z, Pang X, et al. Biofilm-mediated resistance to berberine in *Escherichia coli*. *Frontiers in Cellular and Infection Microbiology.* 2025;15:1565714. doi:10.3389/fcimb.2025.1565714.

[11] Bunduki GK, Heinz E, Phiri VS, Noah P, Feasey N, Musaya J. Virulence factors and antimicrobial resistance of uropathogenic Escherichia coli (UPEC) isolated from urinary tract infections: a systematic review and meta-analysis. BMC Infectious Diseases. 2021;21(1):753. doi:10.1186/s12879-021-06435-7.

[12] Mohsin A, Alsakini A, Ali M, Samir A. Molecular characterization of Dr/Afa genes in multidrug-resistant Escherichia coli from urinary tract infections. Biomedicine. 2022;42:523-529. doi:10.51248/.v42i3.1632.

[13] Mirzahosseini HK, Najmeddin F, Najafi A, Ahmadi A, Sharifnia H, Khaledi A, et al. Correlation of biofilm formation, virulence factors, and phylogenetic groups among Escherichia coli strains causing urinary tract infection: a global systematic review and meta-analysis. Journal of Research in Medical Sciences. 2023;28:66. doi:10.4103/jrms.jrms_637_22.

[14] Mohsin AS, Ali MR, Alsakini AH. Biofilm-associated genes and their role in antibiotic resistance among clinical Staphylococcus aureus isolates. Journal of Infection in Developing Countries. 2026;20:416-424. doi:10.3855/jidc.22026.

[15] Alshaikh SA, El-Banna T, Sonbol F, Farghali MH. Correlation between antimicrobial resistance, biofilm formation, and virulence determinants in uropathogenic Escherichia coli from Egyptian hospital. Annals of Clinical Microbiology and Antimicrobials. 2024;23(1):20. doi:10.1186/s12941-024-00679-2.

[16] Kobayashi T, Ikeda M, Okada Y, Higurashi Y, Okugawa S, Moriya K. Clinical and microbiological characteristics of recurrent Escherichia coli bacteremia. Microbiology Spectrum. 2021;9(3):e0139921. doi:10.1128/Spectrum.01399-21.

[17] Noie Oskouie A, Hasani A, Ahangarzadeh Rezaee M, Soroush Bar Haghi MH, Hasani A, Soltani E. A relationship between O-serotype, antibiotic susceptibility and biofilm formation in uropathogenic Escherichia coli. Microbial Drug Resistance. 2019;25(6):951-958. doi:10.1089/mdr.2018.0330.

[18] Naziri Z, Kilegolan JA, Moezzi MS, Derakhshandeh A. Biofilm formation by uropathogenic Escherichia coli: a complicating factor for treatment and recurrence of urinary tract infections. Journal of Hospital Infection. 2021;117:9-16. doi:10.1016/j.jhin.2021.08.017.

[19] Zhao F, Yang H, Bi D, Khaledi A, Qiao M. A systematic review and meta-analysis of antibiotic resistance patterns, and the correlation between biofilm formation with virulence factors in uropathogenic *Escherichia coli* isolated from urinary tract infections. *Microbial Pathogenesis.* 2020;144:104196. doi:10.1016/j.micpath.2020.104196.

[20] Sheikhi R, Amini ME, Alidoust L, Atrkar Roushan Z, Nikokar I. Evaluation of adhesin genes and risk factors associated with urinary tract infections by drug-resistant uropathogenic Escherichia coli in North of Iran. Journal of Infection in Developing Countries. 2024;18(5):761-769. doi:10.3855/jidc.18566.

[21] Merradi M, Chebaani FZ, Heleili N, Alvanou MV, Giantsis IA. Plasmid-mediated colistin resistance in ESBL-producing Enterobacterales based on both phenotypic and molecular analysis in companion and farm animals from Algeria. Comparative Immunology, Microbiology and Infectious Diseases. 2025;120:102342. doi:10.1016/j.cimid.2025.102342.

[22] Begum S, Prabhu V, Mohanty V, Upadhyaya KK, Abdulla R. Unveiling the arcanum of formalin-fixed paraffin-embedded archival tissue blocks: a valuable resource for genomic DNA extraction. Journal of Oral and Maxillofacial Pathology. 2022;26(2):289. doi:10.4103/jomfp.jomfp_424_20.

[23] Sabat G, Rose P, Hickey WJ, et al. Selective and sensitive method for PCR amplification of Escherichia coli 16S rRNA genes in soil. Applied and Environmental Microbiology. 2000;66:844-849.

[24] Johnson JR, Stell AL. Extended virulence genotypes of Escherichia coli strains from patients with urosepsis in relation to phylogeny and host compromise. Journal of Infectious Diseases. 2000;181(1):261-272.
[25] Gualdi L, Tagliabue L, Bertagnoli S, Ierano T, De Castro C, Landini P. Cellulose modulates biofilm formation by Escherichia coli through activation of the cellulose synthase bcs genes. Environmental Microbiology. 2008;10(10):2665-2678.
[26] Wang Y, Wang X, Wang Y, et al. Biofilm formation plays a role in the formation of multidrug-resistant Escherichia coli toward nutrients in microcosm experiments. Frontiers in Microbiology. 2018;9:1840.
[27] Swick MC, Morgan-Linnell SK, Carlson KM, Zechiedrich L. Expression of multidrug efflux pump genes acrAB-tolC, mdfA, and norE in Escherichia coli clinical isolates as a function of fluoroquinolone and multidrug resistance. Antimicrobial Agents and Chemotherapy. 2011;55(2):921-924. doi:10.1128/AAC.00996-10.
[28] Moubareck C, Daoud Z, Hakimé NI, et al. Countrywide spread of community- and hospital-acquired extended-spectrum β-lactamase (CTX-M-15)-producing Enterobacteriaceae in Lebanon. Journal of Clinical Microbiology. 2005;43(7):3309-3313.
[29] Abo-alella D, Abdelmoniem W, Tantawy E, et al. Biofilm-producing and carbapenem-resistant Escherichia coli nosocomial uropathogens: a cross-sectional study. International Microbiology. 2024;27:1633-1640. doi:10.1007/s10123-024-00495-w.
[30] Hadi HA, Al-Hail H, Aboidris LE, Al-Orphaly M, Ahmed MAS, Samuel BG, et al. Prevalence and genetic characterization of clinically relevant extended-spectrum β-lactamase-producing Enterobacterales in the Gulf Cooperation Council countries. Frontiers in Antibiotics. 2023;2:1177954. doi:10.3389/frabi.2023.1177954.
[31] Choudhury D, Alanbari R, Saveliev P, Sokurenko E, Fuzi M, Tchesnokova V. Clonal and resistance profiles of fluoroquinolone-resistant uropathogenic Escherichia coli in countries with different practices of antibiotic prescription. Frontiers in Microbiology. 2024;15:1446818. doi:10.3389/fmicb.2024.1446818.
[32] Nasrollahian S, Graham JP, Halaji M. A review of the mechanisms that confer antibiotic resistance in pathotypes of Escherichia coli. Frontiers in Cellular and Infection Microbiology. 2024;14:1387497. doi:10.3389/fcimb.2024.1387497.
[33] Saman AB, Siddique AB, Aslam B, Nawaz Z. Biofilm formation and antibiotic resistance in uropathogenic Escherichia coli: a molecular characterization and antibiogram study. Microbial Drug Resistance. 2026;32(1):24-30. doi:10.1177/10766294251389587.
[34] Mahale RP, KA, Princy A, Maheshwarappa YD, Sumana MN. Comparative evaluation of biofilm-forming capacity in uropathogenic and commensal Escherichia coli. Frontiers in Cellular and Infection Microbiology. 2025;15:1570422. doi:10.3389/fcimb.2025.1570422.
[35] Garousi M, Monazami Tabar S, Mirazi H, Asgari P, Sabeghi P, Salehi A, et al. A global systematic review and meta-analysis on correlation between biofilm producers and non-biofilm producers with antibiotic resistance in uropathogenic Escherichia coli. Microbial Pathogenesis. 2022;164:105412. doi:10.1016/j.micpath.2022.105412.
[36] Liu HY, Prentice EL, Webber MA. Mechanisms of antimicrobial resistance in biofilms. npj Antimicrobials and Resistance. 2024;2:27. doi:10.1038/s44259-024-00046-3.
[37] Ballesteros-Monrreal MG, Mendez-Pfeiffer P, Barrios-Villa E, Arenas-Hernández MMP, Enciso-Martínez Y, Sepúlveda-Moreno CO, et al. Uropathogenic Escherichia coli in Mexico, an overview of virulence and resistance determinants: systematic review and meta-analysis. Archives of Medical Research. 2023;54(3):247-260. doi:10.1016/j.arcmed.2023.01.001.
[38] Firoozeh F, Zibaei M, Badmasti F, Khaledi A. Virulence factors, antimicrobial resistance and the relationship between these characteristics in uropathogenic Escherichia coli. Gene Reports. 2022;27:101622. doi:10.1016/j.genrep.2022.101622.
[39] Khalaf, M. J., Ibrahim, H. A., Abd, H. A., Mohammed, M. Q., & Mohsin, A. S. (2025). Gentamicin modulates *pgaA* and *pgaB* expression in *Klebsiella pneumoniae*: Insights into biofilm-associated adaptation. *Microbes and Infectious Diseases*. https://doi.org/10.21608/mid.2025.432058.3325