Iraqi Journal for Applied Science (IJAS)
Vol. 3, No. 2, June, 2026, pp. 43-52
E-ISSN: 3006-5828 DOI: 10.69923/vz9kmf92

Research Article

Investigation of efflux pumps and biofilm formation in *Escherichia* coli isolated from various clinical sources in Baghdad, Iraq

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OPEN ACCESS

Article Information

Article history:

Received: 26, 08, 2025
Revised: 22, 12, 2025
Accepted: 01, 04, 2026
Published: 30, 06, 2026

Keywords:

Escherichia coli,
Efflux pump,
Biofilm,
Antibiotic resistance,
Phenotypic detection.

Abstract

The rapid emergence of antibiotic-resistant *Escherichia coli* poses a major global health challenge, particularly in clinical settings where infections are common and increasingly difficult to treat. Among the principal mechanisms contributing to antimicrobial resistance are efflux pumps, which reduce intracellular antibiotic concentrations, and biofilm formation, a structured survival strategy that promotes bacterial persistence and decreases susceptibility to antimicrobial agents. This study aimed to phenotypically detect efflux pump activity and biofilm formation in *E. coli* isolates obtained from various clinical sources in Baghdad, Iraq, and to evaluate their contribution to multidrug resistance and their association with antimicrobial susceptibility patterns. A total of 305 clinical specimens were collected from patients with urinary tract infections, diarrhea, bloodstream infections, wounds, burns, and respiratory tract infections. Fifty *E. coli* isolates were identified using conventional microbiological methods and the VITEK 2 automated system. Antimicrobial susceptibility testing was performed against 13 commonly used antibiotics using the disk diffusion method, and the minimum inhibitory concentration (MIC) of colistin was determined. Based on their resistance profiles, 24 multidrug-resistant isolates were selected for further phenotypic analysis. High resistance rates were observed for ampicillin (94%) and cefoxitin (84%), whereas fosfomycin demonstrated complete effectiveness against urinary isolates. Among the multidrug-resistant isolates, 79.1% exhibited biofilm-forming ability, classified as strong (20.8%), moderate (20.8%), and weak (37.5%). Furthermore, 95.8% of the isolates showed efflux pump activity. Both biofilm formation and efflux pump activity were strongly associated with resistance to β-lactams, tetracyclines, quinolones, and cephalosporins. These findings highlight the significant role of efflux pumps and biofilm formation in the development and persistence of multidrug resistance in *E. coli*. Routine detection of these resistance mechanisms may improve diagnostic accuracy, support appropriate antimicrobial therapy, and strengthen infection prevention and control strategies.

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1. INTRODUCTION

Antibiotic resistance has emerged as one of the most pressing global health threats in recent years, significantly compromising the effectiveness of available treatments and leading to increased morbidity, mortality, and healthcare costs [1]. E. coli is recognized as a key opportunistic Gram-negative bacterium and a part of the normal human intestinal flora [2]. However, it can act as a primary pathogen causing various infections, including urinary tract infections, sepsis, wound and burn infections, and respiratory tract infections. Certain E. coli strains have become significant contributors to the antimicrobial resistance crisis [3]. Over the past decades, the uncontrolled and inappropriate use of antibiotics has facilitated the emergence of multidrug-resistant strains, complicating treatment and increasing morbidity and mortality rates worldwide [4].Several mechanisms underlie antibiotic resistance in E. coli, among which efflux pumps and biofilm formation are particularly critical. Efflux pumps are specialized protein transporters embedded in the bacterial cell membrane that expel harmful substances, including antibiotics, from the cell. These transporters play a crucial role in resistance against β-lactams, macrolides, tetracyclines, chloramphenicol, and novobiocin, and also participate in physiological functions such as biofilm formation and quorum sensing [5,6]. Abiofilm is a common multicellular bacterial lifestyle characterized by cells adhering to solid surfaces and secreting extracellular polymeric substances (EPS), including polysaccharides, proteins, and extracellular DNA, which helps stabilize the biofilm structure [7]. Biofilms protect bacteria from antibiotics, host immune responses, and harsh environmental conditions while enhancing antibiotic resistance through quorum sensing and the activation of resistance-related genes [8]. This capability makes certain E. coli strains among the most pathogenic, particularly in gastrointestinal and urinary tract infections [9,10].

Despite extensive studies on E. coli resistance, there remains limited understanding of the combined contribution of efflux pump activity and biofilm formation in clinical isolates. This gap hinders the development of targeted diagnostic tools and effective therapeutic strategies against multidrug-resistant strains.

Therefore, the present study aims to phenotypically investigate the role of efflux pumps and biofilm formation in antibiotic resistance among clinical E. coli isolates, providing insights to inform the development of novel diagnostics and improved treatment approaches.

2. METHOD

2.1. Specimens' collection:

A total of 305 clinical samples were collected from patients suffering from a variety of infectious conditions, including urinary tract infections (urine), gastrointestinal infections (diarrhea) in children under the age of three, wound and burn infections, bloodstream infections, and sputum. The samples were collected over the period from 1 September 2024 to 28 February 2025.They were obtained from various sources, including Baghdad Teaching Hospital, the Specialized Burns Hospital, Ghazi Al-Hariri Specialized Surgery Hospital, the National Center for Educational Laboratories, Al-Kindi General Hospital, Imam Ali Hospital, and Ibn Al-Baladi Hospital for Pediatrics and Gynecology. All samples were processed in accordance with standard microbiological procedures, and ethical approval was obtained from the Institutional Review Committee of the College of Education for Pure Sciences, University of Diyala.

2.2. Isolation of E. coli by traditional methods

In the laboratory and under aseptic condition, the collected samples were streaked directly on *MacConky agar*, *blood agar* and *Eosin methylene blue (EMB) agar* after preparation of this culture media according to guidelines of the manufacture and sterilization by autoclave. Samples were then incubated for 24h at 37°C to identify isolated colonies of *E. coli* based on the morphological characteristics, gram staining and some biochemical properties [11].

2.3. Identification of E. coli with VITEK- 2 system

To verify the diagnosis, the bacterial isolates were first inoculated and cultured, After incubation, the turbidity of the bacterial suspensions was adjusted using VITEK Densities (bioMérieux) to match the 0.5–0.6 McFarland standard. A single colony was then selected and suspended in the solution. The Gram-negative identification card was placed into the bacterial suspension, and the tubes were manually loaded into the VITEK-2 system. The remaining steps were performed using the software according to the manufacturer's instructions (bioMérieux) [12].

2.3. Antibiotic Susceptibility Testing

The antimicrobial susceptibility of the isolates was determined using the Kirby–Bauer disk diffusion method, following Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Standardized bacterial suspensions were inoculated on Mueller–Hinton agar, antibiotic disks were applied, and plates were incubated at 37°C for 24 hours. Inhibition zones were measured and interpreted according to CLSI breakpoints. [13].

The antibiotics used for testing are listed in table (1): Mueller-Hinton agar plates were prepared in advance and stored at room temperature until use. Bacterial colonies, grown for 24 hours on MacConkey agar plates, were taken and transferred to sterile tubes containing 2-3 mL of saline solution. The turbidity of each suspension was adjusted to match the 0.5 McFarland standard, which equals roughly 1.5×10^8 CFU/mL. Using sterile cotton swabs, the prepared inoculum was transferred to the surface of Mueller-Hinton agar and spread in three directions, rotating the plate each time to ensure even distribution. The plates were then left to dry at room temperature. To test antibiotic susceptibility 13 antibiotic discs were placed on agar plates using sterile forceps Among them, two antibiotics—fosfomycin and nitrofurantoin—were used exclusively for isolates from urinary tract infections (UTIs), whereas chloramphenicol was tested against all isolates except those from UTIs, five discs per plate. Each disc was placed approximately 15 mm from the edge to avoid overlapping areas and pressed lightly to hold it in place. The plates were incubated at 37°C for 24 hours. After incubation, the diameter of the inhibition zones around each disc was measured using a ruler and interpreted following the CLSI (2024) guidelines [13]. which determined whether the isolate was sensitive (S), resistant (R) or Intermediate (I).

Table (1): Antibiotic Disks and Inhibition Zones (CLSI, 2024)

Antibiotic	Symbol	Concentration (µg)	S (≥ mm)	I (mm)	R (≤ mm)	Country of Origin	Manufacturer
Amikacin	AK	30	≥20	17-19	≤12	Turkey	Bioanalyse
Ampicillin	AMP	10	≥17	14-16	≤13	India	Hi-Media
Piperacillin-tazobactam	TZP	100/10	≥25	21-24	≤20	Turkey	Bioanalyse
Cefepime	FEP	30	≥25	19-24	≤18	Turkey	Bioanalyse
Cefoxitin	CX	30	≥18	15-17	≤14	India	Hi-Media
Ofloxacin	OFX	5	≥16	13-15	≤12	Turkey	Bioanalyse
Doxycycline	DOX	10	≥14	11-13	≤10	India	Hi-Media
Trimethoprim-Sulfamethoxazole	SXT	25	≥16	11-15	≤10	Turkey	Bioanalyse
Meropenem	MEM	10	≥23	20-22	≤19	India	Hi-Media
Chloramphenicol	CHL	30	≥18	13-17	≤12	Turkey	Bioanalyse
Aztreonam	ATM	30	≥21	18-20	≤17	Turkey	Bioanalyse
Colistin	COL	-	-	-	-	India	MITS
Nitrofurantoin	NIT	300	≥17	13-16	≤12	India	Hi-Media
Fosfomycin	FOF	200	≥16	13-15	≤12	India	Hi-Media

- Determination of the Minimum Inhibitory Concentration (MIC) of Colistin Using the Microtiter Broth Dilution Method:

The tested strains were inoculated into Mueller-Hinton broth and incubated for 24 hours; the stock solution of the selected antibiotic was prepared using the following equation:

$$C1 \times V1 = C2 \times V2$$

- C1 = 0.33mg Concentration of the antibiotic as provided in the vial
- V1 = Initial volume in which the concentration is dissolved
- C2 = Desired concentration for the experiment (2048 µg/mL)
- V2 = Final volume required to dilute the initial concentration

Sterile Mueller-Hinton broth was prepared, and 100 µL was added to all wells of the microtiter plate. Notably, the first well already contains 100 µL of broth; therefore, when the target concentration C_2 is added, it is effectively halved to 1024 µg/mL in the well. A serial two-fold dilution of colistin was performed, starting from 1024 µg/mL to 2 µg/mL, by adding 100 µL of the stock antibiotic solution to the wells in the first column (leftmost of the plate). The antibiotic was thoroughly mixed in the wells by aspirating and dispensing the solution 5–8 times. Subsequently, 100 µL was transferred from the first column to the second column to achieve a two-fold dilution. This procedure was repeated for columns 1 to 10, while column 12 was used as a negative control, 100 µL from column 10 was discarded and not used. Then, 50 µL of bacterial suspension standardized to 0.5 McFarland (approximately 1×10^8 cells/mL) was distributed into wells in columns 1–10 (column 12 was used as a negative control containing only the isolates and Mueller-Hinton broth). The plate was incubated at 37°C for 18–24 hours. The absorbance of the plate was measured using an ELISA reader at a wavelength of 630 nm for all wells.

The Minimum Inhibitory Concentration (MIC) was defined as the according to Hancock [14].

2.4. Phenotypic Detection of Biofilm Formation

E. coli isolates were cultured in Brain Heart Infusion (BHI) broth and incubated at 37°C for 24 hours. Following incubation, bacterial suspensions were inoculated into microtiter plate wells, BHI broth without bacterial inoculation was used as the negative control to establish the baseline optical density (OD) for comparison. After incubation, wells were washed three times with sterile distilled water to remove non-adherent cells. Sterile distilled water was used instead of PBS because the biofilms had already been fixed with methanol, rendering cell viability irrelevant; the primary purpose of washing was to remove non-adherent cells without affecting the fixed biofilm structure.

Biofilms were subsequently fixed with 99 % methanol for 15 minutes and left to air-dry. Wells were then stained with 1% crystal violet for 45 minutes, rinsed, and allowed to dry at room temperature. The bound dye was solubilized using 95% ethanol, and biofilm formation was quantified by measuring the optical density (OD) at 630 nm using an ELISA reader [15].

Interpretation	**Absorption value**
Non – adherent	$OD_C \geq OD$
Weakly adherent	$OD_C < OD \leq 2_X OD$
Moderately adherent	$2_X OD_C < OD \leq 4_X OD_C$
Strong adherent	$4_X OD_C < OD$

OD = Optical density of tsted isolate (OD_I)
OD_C = Optical density of control (OD_C)

2.5. Phenotypic Detection of Efflux pumps

Decimal dilutions of all bacterial isolates were prepared using sterile physiological saline, and their turbidity was compared using the standard turbidity apparatus (McFarland standard). This test was conducted on bacterial isolates that exhibited antibiotic resistance, with a minimum resistance to at least five antibiotics, selected based on the antibiotic susceptibility test, using a modified version of the EtBr–Agar Cartwheel Method., selected based on the antibiotic susceptibility test, using a modified version of the EtBr–Agar Cartwheel Method. This method employed Tryptone Soy Agar medium and ethidium bromide dye at varying concentrations as described in [16], as follows: Different concentrations of ethidium bromide dye (5, 10, 15, 20, 25 µg/mL) were prepared by adding various amounts of the dye to the Tryptone Soy Agar medium after sterilization and slight cooling. The media were mixed well and poured into sterile Petri dishes that had been previously divided radially, and stored in the refrigerator until use. Sterile cotton swabs were immersed in the diluted bacterial suspension, then rotated and pressed against ، the inner wall of the tube to remove excess inoculum. The swabs were then streaked radially from the edge, of the plate toward the center for each isolate and each concentration. The plates were then incubated at 37°C for 18–24 hours. The bacterial isolates were examined using an ultraviolet light source to quantitatively observe the intensity of fluorescence. This was achieved by selecting isolate No. 2 from the UTI isolates as a positive control, since it exhibited the highest fluorescence among the tested isolates, while ***E. coli*** ATCC 25922 was used as the negative control.

3. RESULTS AND DISCUSSION

3.1. assessment of cultural characteristics:

On MacConkey agar, the colonies appeared lactose-fermenting with distinct pink coloration and a dry morphology. colonies grown on blood agar showed exhibiting β- hemolysis patterns around the colonies, Colonies appeared circular, convex, and smooth in morphology. Eosin Methylene Blue (EMB) agar displaying characteristic green metallic sheen colonies typical of strong lactose fermenters but mentioning the surrounding dark centers would provide a more complete description, when the isolates were cultured in a semi-solid medium using the stabbing technique, diffuse zones appeared around the inoculation site. Under a light microscope the bacterial cells appeared rod-shaped cells exhibiting motility by the polar flagella, Upon Gram staining and examination under a light microscope, the bacterial isolates appeared pink to red with safranin indicating that they are Gram-negative, [11]. Diagnosis of fifty *E. coli* isolates from different clinical sources was conducted through microscopic examination, cultural characteristics, biochemical testing and VITEK- 2 system, Table (2).

Table (2): Distribution of *E. coli* isolates according to their source: number of isolates and corresponding percentages

No	Sample Source	Samples	*E. coli* Isolates	Proportion (%)
1	Urine	40	25	62.5%
2	Burns and Wounds	75	7	9.3%
3	Diarrhea	40	9	22.5%
4	Sputum	50	3	6.0%
5	Blood	100	6	6.0%
	Total	305	50	16.39%

Note: Samples: refers to the number of specimens collected from each source.

The samples were collected randomly from the patients.The analysis confirmed that urinary tract infections (UTIs) represented the highest infection rate caused by *E. coli* 62.5% ، followed by gastrointestinal infections (diarrhea) at 22.5%, then burn and wound infections at 9.3%, while the lowest rates were recorded in respiratory tract infections (sputum) and bloodstream infections, each at 6.0%. The increase in UTIs may be attributed to environmental factors, weak immune systems, and the short urethral tract, especially in females [17]. These findings are consistent with a previous study conducted in Ethiopia by Yenehun Worku et al. [18], where the infection rate was 63.6%. The variation in infection rates may be due to differences in sample size, sample characteristics, and patients' health conditions, especially in those with chronic diseases such as diabetes. In such cases, the increased infections are not primarily due to bacterial virulence but to changes in the epithelial cells of the urinary tract and misuse of antibiotics [19]. The current study indicated that gastrointestinal infections (diarrhea) caused by *E. coli* reached 22.5%, which aligns with a study conducted in Tikrit by Alrifai, *et al* [20]. where the infection rate was 25.9%. These infections frequently occur in children under five years of age, with

ETEC (Enterotoxigenic *E. coli*), EPEC **(**Enteropathogenic *E. coli***)**, strains being the most common causative agents in developing countries. Infection rates in Iraq fall within the global range. The differences in these rates are likely related to water quality, consumption of contaminated food or unpasteurized milk, and the use of antibiotics (which may alter infection patterns). Children are particularly vulnerable to such infections due to their weaker immune systems and the occasional absence of breast feeding.Regarding burn and wound infections, the current study found a rate of 9.3%, consistent with a study conducted in Maysan province by Hateet, [21]. which reported a rate of 7.6%? These variations may be due to differences in regional healthcare systems, contamination at wound treatment centers, use of non-sterile tools, or direct contact with wounds. Additionally, the depth and extent of burns or wounds may increase the likelihood of *E. coli* contamination.As for bloodstream infections, the rate was 6.0%, which disagrees with a study conducted in Baghdad by Hassan, *et al* [22].

Which reported a higher rate of 18%, This discrepancy may be due to differences in isolate numbers and population characteristics. The lower infection rate may also reflect the focus in other studies on high-risk patients in intensive care units or those with chronic conditions like diabetes or long-term intravenous catheters, which increase the risk of bloodstream infections. *E. coli* bacteremia usually results from bacterial translocation due to chronic urinary or gastrointestinal infections (diarrhea) or from surgical procedures. From an epidemiological perspective, the 6.0% rate indicates a relatively low risk of. *E. coli* bacteremia in Baghdad and suggests that other Gram-negative and Gram-positive organisms may be more dominant. The results of the current study also showed that *E. coli* infections in the respiratory tract (sputum) accounted for 6.0%, which is lower than the rate reported in a study from Nepal by Raghubanshi and Karki, [23].

Which was 15.5%, This may be due to geographic factors and the presence of specific mutated strains in those regions. Respiratory tract infections caused by *E. coli* are primarily associated with artificial respiration or the use of respiratory catheters.

3.2. The antimicrobial susceptibility testing:

Antibiotic susceptibility testing has conducted on 50 clinical isolates of *E. coli* obtained from several sources, using the disc diffusion method (Bauer-Kirby) on Mueller-Hinton agar. The results had interpreted by measuring the diameters of the inhibition zones in consistent with the Clinical and Laboratory Standards Institute guidelines [13]. The isolates showed varying levels of resistance to the tested antibiotics, Figure 1**.**

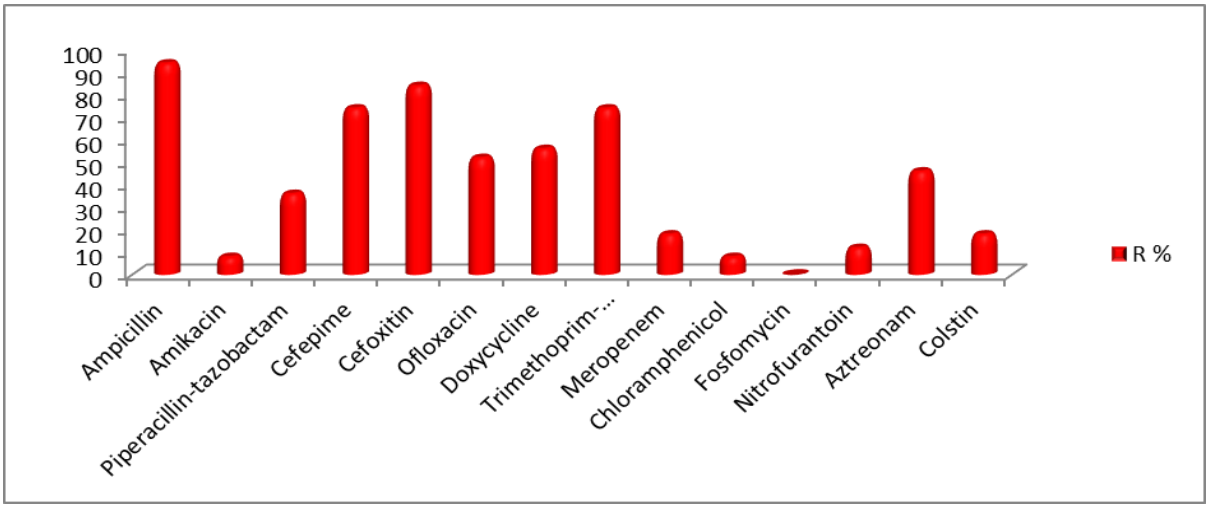

Figure (1). represents the results of the antibiotic susceptibility test for. *E coli* isolates.

The results of antibiotic susceptibility testing, conducted using the Kirby-Bauer disk diffusion method, revealed that the highest resistance rate was observed against Ampicillin (94%). This finding aligns with a study conducted in Iraq by Halah and AL-Hasani, [24]. ◌hich reported a resistance rate of 95%. The resistance of E. coli to Ampicillin is associated with the widespread, unnecessary use of this antibiotic and its extensive use within the community without medical consultation. Meanwhile, the resistance rate to Piperacillin-tazobactam was 32%, which is consistent with a study conducted in India by Das, *et al*[25] here it was reported at 38%. However, it was lower than the resistance rate recorded in a study conducted in Zakho by Polse, *et al* [25]. Which was 41%. Regarding Cefepime, the resistance rate observed in the current study was 67%, which did not agree with the findings of [24]in Baghdad, where the rate was 84%, but it was higher than that reported by Polse, *et al* [26]which was 52%.As for Aztreonam, the resistance rate in this study was 52%, which was lower than the rate reported by Halah and AL-Hasani, [27] in Baghdad (67%), but it agreed with the findings of a study conducted in India by Govindaswamy, *et al*[27]which reported a rate of 56.3%.The current study recorded an 18% resistance rate to Meropenem, which is in agreement with a study conducted by Al-Haddad, [28]where the resistance rate to Imipenem was 19.5%.

Regarding Cefoxitin, the resistance rate was 84%, which did not agree with the study conducted in Zakho by [26]where it was 52%, .The resistance of *E. coli* to these groups of β-lactam antibiotics is attributed to the production of β-lactamase enzymes, which deactivate these antibiotics before they reach the enzymes responsible for constructing the bacterial cell wall [29]. The resistance rate to amikacin was 8%, consistent with a study by a group of researchers in Iran and Azerbaijan [30], which also reported 8% resistance among *E. coli* isolates, indicating the effectiveness of this aminoglycoside antibiotic, possibly due to its limited usage, For the lip polypeptide group, colistin showed a resistance rate of 18%, comparable to a study conducted in Duhok [31], which found a 16% resistance rate.

This resistance is attributed to enzymes encoded by mcrgenes. The resistance rate to Trimethoprim-sulfamethoxazole was 74%, close to that reported in a study from Jordan [32], which was 63%, Ofloxacin, a member of the fluoroquinolones group, showed a resistance rate of 52%. This result aligns with a study in Baghdad [33], where levofloxacin resistance was 73%, %. This variation may be due to the widespread use of fluoroquinolones, especially in urinary tract infections. All isolates showed 100% sensitivity to fosfomycin, in agreement with [32], which reported a 95% sensitivity rate. This may be due to its infrequent use. Nitrofurantoin resistance was 12%, which aligns with a study conducted in Vietnam [34], where resistance was 18%, Doxycycline resistance was 56%, lower than that reported by [35] at 100%**.** This variation may be attributed to the diversity of sample sources. . While the resistance to Chloramphenicol antibiotic (8%) was consistent with a study conducted by [36] where the resistance rate was 7.8% for *E. coli* isolates obtained from various sources excluding UTI isolates.

3.3 Multidrug Resistance in *Escherichia coli*:

The results of the present study showed that, out of fifty (50) clinical isolates, forty-six (46) isolates (92%) of *E. coli* exhibited multidrug and extensive drug resistance (MDR and XDR). The findings revealed that four (4) isolates were classified as extensively drug-resistant (XDR), with one isolate showing resistance to eleven (11) antibiotics out of thirteen (13) tested antibiotics for urinary tract infection (UTI) isolates. The remaining isolates in this group consisted of twenty-one (21) MDR isolates, including two isolates resistant to ten (10) antibiotics, one isolate resistant to nine (9) antibiotics, three isolates resistant to seven (7) antibiotics, four isolates resistant to six (6) antibiotics, four isolates resistant to five (5) antibiotics, four isolates resistant to four (4) antibiotics, and three isolates resistant to three (3) antibiotics, with resistance to at least one antibiotic from each class. The remaining isolates, representing 12% of the total, were susceptible to most antibiotics, Additionally, three (3) isolates from various sources were identified as XDR, showing resistance to ten (10) antibiotics out of twelve (12) tested. The remaining isolates were classified as MDR, with two isolates resistant to nine (9) antibiotics, four

isolates resistant to seven (7) antibiotics, five isolates resistant to six (6) antibiotics, four isolates resistant to five (5) antibiotics, five isolates resistant to four (4) antibiotics, and one isolate resistant to three (3) antibiotics, with resistance to at least one antibiotic from each class. Only one isolate was susceptible to most antibiotics, as shown in Table (4). The current study demonstrated that 92% of the isolates were multidrug-resistant (MDR), of which 8.6% were extensively drug-resistant (XDR). These findings are consistent with a study conducted in Baghdad by Halah and AL-Hasani, [24]. where MDR prevalence was 100% and XDR prevalence was 24%. The emergence of *E. coli* strains exhibiting multidrug resistance represents a growing global concern, particularly in regions with weak healthcare systems, where clinical isolates show substantial variability in resistance and susceptibility patterns depending on geographical location, as well as significant differences across various populations and clinical or environmental samples [37]. Depending on Antibiotic Susceptibility Test results, 24 isolates showing the highest resistance to antibiotics had selected for phenotype detection of biofilm and efflux pumps from different sources (Table-3).

Table (3): Antibiotic resistance level of 24 selected *E. coli* isolates

N	source	Amp	CFE	CX	PTZ	ATM	MR	AK	OFX	DOX	SXT	CHL	FOF	NIT	COL	Number of antibiotics
1	urine 1	R	I	R	R	R	S	S	R	R	S	-	S	I	S	7
2	urine 2	R	R	R	R	I	R	R	R	R	R	-	S	R	R	11
3	urine 5	R	R	R	R	S	R	I	R	R	R	-	S	R	R	10
4	urine 6	R	R	R	R	R	R	S	R	R	R	-	S	I	R	10
5	urine 10	R	R	R	R	R	R	S	S	R	R	-	S	S	R	9
6	urine 12	R	R	R	S	R	R	I	R	S	R	-	S	S	S	8
7	urine 18	R	R	R	I	I	S	I	R	R	R	-	S	S	S	7
8	urine 20	R	R	R	R	I	I	R	R	I	R	-	S	S	S	8
9	urine 24	R	R	S	I	R	S	S	S	R	R	-	S	R	S	7
10	urine 25	R	I	R	R	R	S	S	R	R	R	-	S	S	S	8
11	Stool29	R	R	R	S	S	S	S	S	I	R	S	-	-	S	5
12	Stool32	R	R	R	R	R	S	S	R	S	R	S	-	-	S	8
13	Stool33	R	R	R	R	R	S	I	S	R	R	I	-	-	S	8
14	Stool34	R	R	R	R	R	S	I	R	R	R	I	-	-	R	9
15	Blood36	R	R	R	S	R	S	S	S	R	R	S	-	-	S	7
16	Blood37	R	R	R	S	R	S	I	R	I	R	S	-	-	S	7
17	Blood40	R	R	R	R	R	R	S	R	R	R	S	-	-	R	10
18	Burn42	R	R	R	I	R	S	I	R	S	R	S	-	-	S	7
19	Burn43	R	R	R	R	S	R	I	R	R	R	I	-	-	R	9
20	Sputum45	R	R	R	I	R	S	R	R	I	R	S	-	-	S	8
21	Sputum46	R	R	R	S	I	R	I	R	R	R	S	-	-	S	8
22	Sputum47	R	R	R	R	R	S	R	R	R	R	S	-	-	R	10
23	Wound49	R	I	R	I	R	S	S	R	R	S	S	-	-	S	6
24	Wound50	I	I	S	I	R	S	S	R	I	R	R	-	-	S	5
	S	0	0	2	5	3	15	11	5	3	2	10	10	5	16	
	R	23	20	22	13	17	8	4	19	16	22	1	0	3	8	
	I	1	4	0	6	4	1	9	0	5	0	3	0	2	0	

3.4 Phenotypic Detection of Biofilm Formation:

The study revealed that 19 out of 24 clinical *E. coli* isolates (79.1%) were biofilm-forming. Among these, 5 isolates (26.3%) produced strong biofilms, 5 isolates (26.3%) produced moderate biofilms, and 9 isolates (47.3%) produced weak biofilms, as illustrated in Figure 2. These findings are consistent with a study conducted in Baghdad by Kadhim Mohammed [38], which reported a biofilm production rate of 82%, and with a study in Isfahan, Iran, by Poursina et al. [39], which reported a rate of 80%. However, the present results did not align with a study in Baghdad Governorate by Hamid and Khoshabeh [40] regarding strong biofilm production, reported at 38%, though moderate and weak biofilm-producing isolates were comparable (22% and 32%, respectively). Similarly, the study by Abo-Alella et al. [41] in Egypt reported moderate biofilm production at 22.4%, while strong biofilm production was lower (10.5%).

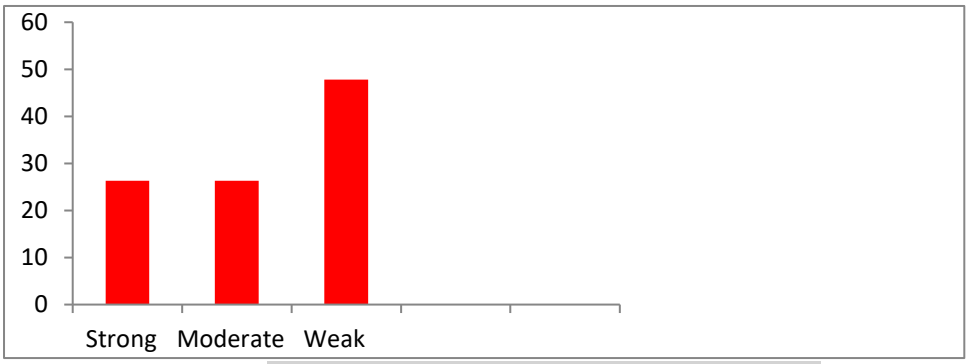

Figure 2 illustrates the percentage of biofilm production in *E. coli* isolates

Biofilms play a significant role in bacterial resistance to a wide range of antibiotics and disinfectants and are implicated in up to 60% of human infections, which are often difficult to eradicate with conventional therapy [42]. Numerous studies have shown that biofilm-producing *E. coli* isolates exhibit higher resistance rates compared to non-biofilm producers. This resistance is primarily attributed to the extracellular polymeric substances (EPS), which act as a protective barrier against antibiotics, reduced metabolic activity of cells within the biofilm, and horizontal gene transfer of resistance genes among bacterial populations. Biofilms contribute to increased bacterial resistance to antibiotics and disinfectants, leading to treatment failure and delayed patient recovery, facilitating chronic and recurrent infections, and increasing the spread of healthcare-associated infections. They also impact the selection of the most appropriate treatment to manage infections effectively.

The formation of biofilms is influenced by several environmental and physiological factors, including optimal temperature (37°C), composition and concentration of growth medium, pH, oxygen availability, and the source of the clinical isolate. Detection and characterization of biofilms can be performed using various methods: the tube method for observing biofilm deposition, Congo red agar for identifying biofilm-producing colonies, and the microtiter plate assay, which is considered the most accurate for quantifying biofilm intensity. Additionally, molecular detection (PCR) can identify biofilm-associated genes such as *fimH*, while advanced techniques like electron microscopy allow direct visualization of the three-dimensional biofilm structure. In summary, these findings highlight the clinical relevance of biofilm formation in *E. coli* infections, emphasizing its role in antibiotic resistance, treatment failure, and infection persistence. The results underscore the need for the development of targeted therapeutic strategies and novel anti-biofilm agents, as well as the importance of future research to better understand environmental and genetic factors influencing biofilm formation and to guide effective clinical management (Figure 3).

Figure 3: Biofilm production of *E. coli* isolates.

3.5 Phenotypic Detection of Efflux pumps:

Phenotypic detection of efflux pumps and biofilm production was performed in 24 multidrug-resistant (MDR) *E. coli* isolates using the EtBr-Ew method, which relies on ethidium bromide staining as an indicator. The results revealed that 95.8% of the isolates were positive for efflux pumps, determined by the lowest concentration at which no fluorescence was observed under UV light. These findings indicate that efflux pumps play a major role in antibiotic resistance among the studied isolates, consistent with a study conducted in Diyala by Ibrahimi and Al-Zubaid [43], which reported a similar rate of 95%. Broad-spectrum efflux pumps can expel a variety of antimicrobial agents, including β-lactams, tetracycline, and quinolones, thereby reducing intracellular concentrations below the effective threshold [44]. In addition to efflux activity, biofilm formation further enhances resistance. Biofilms act as physical barriers that limit antibiotic penetration and reduce bacterial growth rates, providing a protective environment where efflux pumps and other resistance mechanisms can operate more effectively. The combined action of efflux pumps and biofilm formation creates a synergistic effect, leading to multidrug resistance and making infections harder to treat. These adaptations often arise under selective pressure from repeated use of antibiotics and disinfectants and may involve regulatory mutations, plasmid-encoded efflux genes, or reduced cell permeability. In light of these findings, it is recommended to employ PCR techniques for genomic confirmation of efflux pump genes and associated biofilm-related factors. This approach improves diagnostic accuracy and provides a deeper understanding of the interplay between efflux mechanisms and biofilm formation in promoting antibiotic resistance, Figure 4.

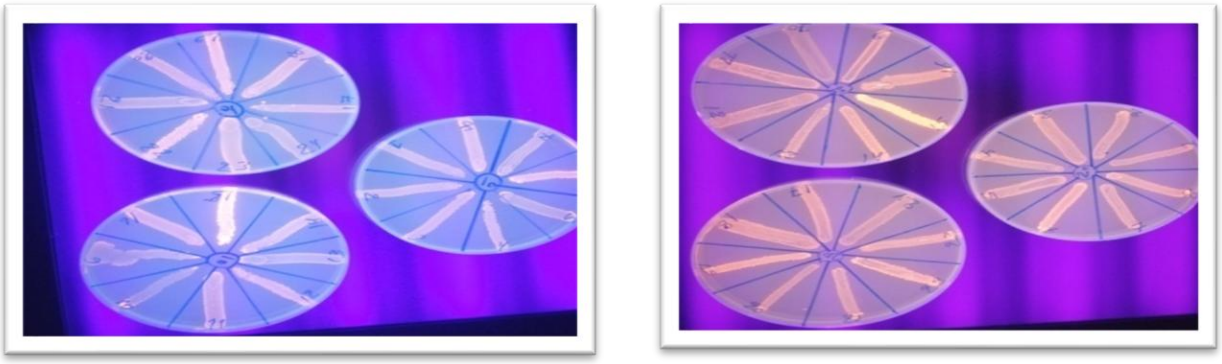

Figure (4): *E. coli* isolates were cultured on Tryptic Soy Agar (TSA) medium supplemented with different concentrations of ethidium bromide (5, 10, 15, 20, and 25 µL/mL)to detect efflux pumps.

Table (4): The results detection biofilm and efflux pumps of 24 selected *E. coli* isolates

Efflux pump					Biofilm	Resistance type	N
25	20	15	10	5			
+	+	-	+	+	+ (M)	XDR	E1
+	+	+	+	+	+ (W)	MDR	E2
+	-	+	+	+	-	MDR	E3
+	+	-	+	+	+ (S)	XDR	E4
+	-	+	+	+	+ (S)	MDR	E5
-	+	+	+	+	+ (W)	XDR	E6
-	+	-	+	+	+ (W)	XDR	E7
+	-	-	-	+	+ (M)	MDR	E8
+	+	-	+	+	-	MDR	E9
+	+	+	-	+	+ (W)	MDR	E10
+	+	+	+	+	+ (W)	MDR	E11
+	+	-	-	-	+ (M)	XDR	E12
+	+	+	+	+	-	XDR	E13
+	-	+	-	-	+ (S)	XDR	E14
-	-	-	-	-	+ (S)	MDR	E15
+	+	+	+	+	+ (M)	MDR	E16
+	-	-	+	-	-	MDR	E17
+	+	-	-	-	+ (W)	MDR	E18
-	-	-	+	-	+ (W)	XDR	E19
+	+	+	+	+	+ (S)	XDR	E20
-	+	+	+	+	-	XDR	E21
+	+	-	+	+	+ (W)	MDR	E22
+	-	+	+	-	+ (M)	MDR	E23
+	+	+	+	+	+ (W)	MDR	E24

4. CONCLUSION

The current investigation established that all E. coli isolates from diverse clinical sources displayed multidrug resistance, with 92% exhibiting MDR or XDR phenotypes. Ampicillin resistance was highest (94%), while fosfomycin retained full efficacy (0% resistance among UTI isolates). Furthermore, 79.1% of isolates were biofilm producers and 95.8% exhibited efflux pump activity, underscoring the multifactorial basis of antibiotic resistance. Collectively, these findings highlight the urgent need for targeted diagnostic strategies such as molecular confirmation (PCR), alongside stewardship programs to regulate antibiotic use. Continuous surveillance of resistance mechanisms is essential to guide therapeutic decisions and to mitigate the spread of MDR and XDR strains.

ACKNOWLEDGEMENTS

The authors appreciate the College of Education for Pure Sciences - University of Diyala - for using laboratories with the necessary equipment to carry out the experiment.

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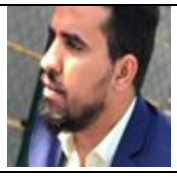

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