



# High Resistance to $\beta$ -Lactams but Sustained Susceptibility to Colistin and Carbapenems in *Escherichia coli* Isolated from Urinary Tract Infections

Lalan Rebaz Mohammed <sup>a, b, \*</sup> , Taib Ahmed Hama Soor <sup>a, c</sup>

<sup>a</sup>Department of Medical Laboratory, College of Health and Medical Technology, Sulaimani Polytechnic University, Sulaymaniyah, Iraq.

<sup>b</sup>Medical Laboratory Department, Kurdistan Technical Institute, Sulaymaniyah, Iraq.

<sup>c</sup>Department of Medical Laboratory Analysis, College of Health Sciences, Cihan University - Sulaimani, Sulaymaniyah, Iraq.

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\* Corresponding Author:

[lalan.moham-med.chmt@spu.edu.iq](mailto:lalan.moham-med.chmt@spu.edu.iq)

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**Abstract:** Multidrug-resistant bacterial strains represent a growing public health threat, particularly in clinical environments, as they significantly impair the success of treatment and control strategies for infectious diseases. This cross-sectional study investigated the antimicrobial resistance profiles of 100 *Escherichia coli* (*E. coli*) isolates derived from urine specimens of patients with symptomatic urinary tract infections (UTIs) in Sulaymaniyah City, Iraq. The samples were obtained from individuals visiting both public and private hospitals between November 2024 and February 2025. Bacterial identification and antimicrobial susceptibility testing were conducted using the VITEK 2 automated system at hospital laboratories, and molecular confirmation of the isolates was achieved through amplification of the *uidA* (glucuronidase) gene specific to *E. coli*. A total of ten antibiotics from various antimicrobial classes were tested. Colistin demonstrated complete effectiveness, with a 100% susceptibility rate, followed by doripenem (93%), imipenem (84%), tigecycline (71%), and amoxicillin-sulbactam (65%). In contrast, amoxicillin and amoxicillin-clavulanate showed high resistance rates of 83% and 82%, respectively. Resistance to cephalosporins was also considerable, with cefixime and ceftazidime exhibiting resistance rates of 70% and 60%. The findings highlight the continued effectiveness of colistin and carbapenems but draw attention to the concerning resistance to widely used  $\beta$ -lactams and cephalosporins. These results underscore the necessity of sustained antimicrobial resistance surveillance and improved antibiotic stewardship. The data generated in this study are critical for guiding empirical treatment decisions and enhancing the clinical management of *E. coli*-associated UTIs in the region.

## 1. Introduction

Urinary tract infections (UTIs) rank as the second most commonly occurring type of infection in humans, following respiratory tract infections, and represent a major concern across global healthcare systems [1]. Every year, they affect millions worldwide and burden healthcare infrastructures, making them a major worldwide health concern. A variety of bacterial pathogens can cause UTIs, with the most common culprits being *Escherichia coli* (*E. coli*), *Enterococcus faecalis* (*E. faecalis*), *Klebsiella pneumoniae* (*K. pneumoniae*), *Serratia marcescens* (*S. marcescens*), *Pseudomonas aeruginosa* (*P. aeruginosa*), *Staphylococcus saprophyticus* (*S. saprophyticus*), *Staphylococcus aureus* (*S. aureus*), and *Proteus mirabilis*. These microorganisms are frequently isolated from patients with UTIs and are considered the primary infectious agents responsible for the majority of UTIs diagnosed in both hospital and community settings [2]. Among these pathogens, *E. coli* is the most prevalent, responsible for approximately 50% of hospital-

acquired UTIs and 85% of community-acquired UTIs. Several factors influence the occurrence of UTIs, including age, gender (with females being more susceptible than males), immunosuppression, and the use of urological devices, all of which can increase the likelihood of infection. These factors contribute to the high incidence of UTIs in both healthcare and community settings, with *E. coli* being a significant causative agent in these cases [3]. UTIs can lead to severe health complications, including sepsis and kidney damage. These infections can vary from mild cystitis to more serious conditions like pyelonephritis [4]. A growing concern in UTI management is the growing incidence of antimicrobial resistance in uropathogens, which poses a significant challenge [5]. Resistance to common antibacterial agents has escalated to alarming level; this development is a major threat to public health worldwide [6]. Treatment, management, and control of infections are made more difficult by the rise of multidrug-resistant (MDR) types of pathogenic bacteria, which are especially problematic in healthcare settings [7].

Reports indicate that *E. coli*, a widely observed and leading uropathogenic bacterium, is increasingly developing resistance to antibiotics in both developed and developing countries, raising concerns about the effectiveness of standard treatment approaches [8]. This trend complicates infection treatment, as antibiotic resistance can lead to the failure of empirical therapies, which are commonly used in as many as 95% of severe UTI cases without prior bacteriological testing. There is considerable regional variation in both the rate and antibiotic sensitivity patterns of *E. coli*, influenced by differences across populations and environmental conditions [9]. Therefore, designing appropriate therapeutic approaches requires a detailed insight into the antibiotic resistance trends of uropathogenic organisms within a given region. To improve the effectiveness of empiric treatment protocols and curb the rise of drug-resistant bacterial strains, it is essential to precisely detect the causative agents of UTIs and evaluate their susceptibility to commonly administered antibiotics in healthcare settings [10].

Although multiple studies have reported on antimicrobial resistance (AMR) in *E. coli* from UTIs in different parts of Iraq, data specific to Sulaymaniyah remain limited. For example, a study conducted in Duhok province identified *E. coli* as the predominant uropathogen and highlighted significant resistance frequencies to commonly used antibiotics, including ampicillin and ceftriaxone [11]. Since antibiotic resistance patterns can vary significantly in different regions, localized data from Sulaymaniyah are needed to inform empirical treatment guidelines and support effective infection management.

The purpose of this research was to examine and identify the antibiotic sensitivity patterns of 100 *E. coli* isolates obtained from urine samples collected in Sulaymaniyah city, Iraq, using ten different antibiotics. Given the limited availability of updated regional data on AMR, particularly in Sulaymaniyah, this study seeks to investigate a significant local knowledge gap. The findings are expected to provide valuable insights into current resistance trends in the area, thereby enabling clinical practitioners to make informed and evidence-based decisions regarding antibiotic use for the effective treatment of UTIs.

## 2. Materials and Methods

### 2.1. Specimen Collection and Bacterial Recovery

Between November 2024 and February 2025, a total of 104 *E. coli* subcultures were obtained from urine samples collected from patients who presented with clinical symptoms suggestive of UTIs. The samples were previously confirmed as *E. coli* by the Vitek 2 automated system at the hospitals. Subcultures were then prepared for further analysis. All urine samples were collected using the midstream clean-catch method to reduce the risk of contamination. These samples were gathered from individuals attending both public and private hospitals throughout Sulaymaniyah city. Following collection, a single, pure bacterial swab from each sample was carefully streaked onto two different selective and differential culture media: Eosin-Methylene Blue (EMB) agar and MacConkey (MAC) agar. The EMB agar, manufactured by Liofilchem (Italy), and the MAC agar, produced by Accumedica LAB (UK), were used to facilitate the selective growth and preliminary identification of *E. coli*. The inoculated agar plates were then incubated at a constant temperature of 37°C for a period of 16 to 24 hours to allow for sufficient bacterial growth. After incubation, only well-isolated colonies showing morphological features consistent with *E. coli* were selected as a representative sample for further analysis. This colony was

subjected to molecular identification techniques to confirm and validate the presence of *E. coli* strains among the isolates. [12].

## **2.2. Ethical Statement**

This study was approved by the Ethics Committee of Sulaimani Polytechnic University. All procedures were conducted in accordance with institutional ethical standards and the principles of the Helsinki Declaration. Prior to collecting samples, consent was secured from all patients or their authorized representatives. participants confidentiality and anonymity were strictly maintained throughout the study.

## **2.3 Molecular Identification and Confirmation of *E. coli***

### **2.3.1. DNA Extractions**

To perform DNA extraction, a freshly grown bacterial colony was carefully selected and suspended in 100  $\mu$ L of sterile distilled water. The suspension was then subjected to a heat treatment process by incubating it at a temperature of 94°C for 15 minutes using a PCR ThermoCycler (Acculab, USA). This boiling step was used to lyse the bacterial cells and release their genomic DNA into the solution. After the heat treatment, the mixture was then centrifuged at a speed of 10,000 RPM to separate the cell debris from the soluble components. The resulting supernatant, which contained the crude DNA extract, was then collected. From this supernatant, 2 $\mu$ L was used as the DNA template in the subsequent molecular identification and characterization of the bacterial isolates [7, 13].

### **2.3.2. Polymerase Chain Reaction for *E. coli* Detection**

To confirm the presence of *E. coli* in the collected bacterial isolates, a Polymerase Chain Reaction (PCR) assay was performed targeting the  $\beta$ -glucuronidase (*uidA*) gene, a commonly used housekeeping gene specific to *E. coli*. The amplification of this gene was achieved using a set of gene-specific primers: the forward primer (F) with the sequence 5'-TGGTAATTACCGACGAAAACGGC-3' and the reverse primer (R) with the sequence 5'-ACGCGTGGTTACAGTCTTGCG-3' as previously described by Molina *et al.* [14]. The PCR conditions were adapted from the methods previously established by Alaa *et al.* [15] and Chen and Griffiths [16]. The PCR reaction mixture had a final volume of 20 $\mu$ L, which included 10  $\mu$ L of 2X HotStart Taq PCR Master Mix (SBS Genetech, Beijing, China), 1  $\mu$ L of primers (0.5  $\mu$ L each of forward and reverse primers at a final concentration of 0.25  $\mu$ M), 7  $\mu$ L of sterilized distilled water, and 2  $\mu$ L of the extracted DNA template. The thermal cycling settings began with an initial denaturation carried out at 94°C for 5 minutes. This was followed by 35 amplification cycles, each consisting of denaturation at 94°C for 30 seconds, primer annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. The cycling ended with a final extension phase at 72°C for 7 minutes to ensure complete amplification.

A 2% agarose gel was used to separate the PCR products by electrophoresis, which was run at 100V for 75 minutes. After electrophoresis, Visualization of DNA bands was performed under UV light via a Gel Documentation System (Model: CSL-MDOCUV3121D, Cleaver Scientific, UK).

## **2.4. Antimicrobial Susceptibility Assessment**

The Kirby-Bauer disk diffusion method was used to evaluate the antimicrobial susceptibility of *E. coli* isolates. This method was conducted according to the standardized procedures and interpretative criteria recommended by the Clinical and Laboratory Standards Institute (CLSI, 2020). The assay was carried out to evaluate the response of the bacterial isolates to a total of ten antibiotics. The antibiotics tested in this study included: Colistin (10  $\mu$ g), Amoxicillin/Sulbactam (30/15  $\mu$ g), Doripenem (10  $\mu$ g), Cefixime (5  $\mu$ g), Ceftazidime (30  $\mu$ g), Cefixime/Clavulanic acid (5/10  $\mu$ g), Amoxicillin/Clavulanate (20/10  $\mu$ g), Amoxicillin (25  $\mu$ g), Imipenem (10  $\mu$ g), and Tigecycline (15  $\mu$ g). All antibiotic disks used in the testing were supplied by Bioanalyse (Ankara, Turkey). During the procedure, a swab of 500  $\mu$ L bacterial suspension was uniformly swabbed onto the surface of Mueller-Hinton agar plates. Antibiotic-impregnated disks were then carefully placed on the inoculated agar surfaces, and the plates were

incubated at 37°C for 18–24 hours. Following incubation, the diameters of the zones of inhibition around each disk were measured in millimeters and interpreted according to CLSI guidelines to classify the isolates as susceptible, intermediate, or resistant. This testing provided critical insights into the antibiotic resistance patterns of *E. coli* strains isolated in the region [17].

### 2.5. Statical Analysis

Data analysis was performed using SPSS version (25) (IBM Corp., Armonk, NY, USA). Descriptive statistics, including mean inhibition zone diameters, standard deviations, and the proportions of isolates classified as resistant, intermediate, or susceptible, were calculated to summarize the antimicrobial susceptibility patterns.

## 3. Results

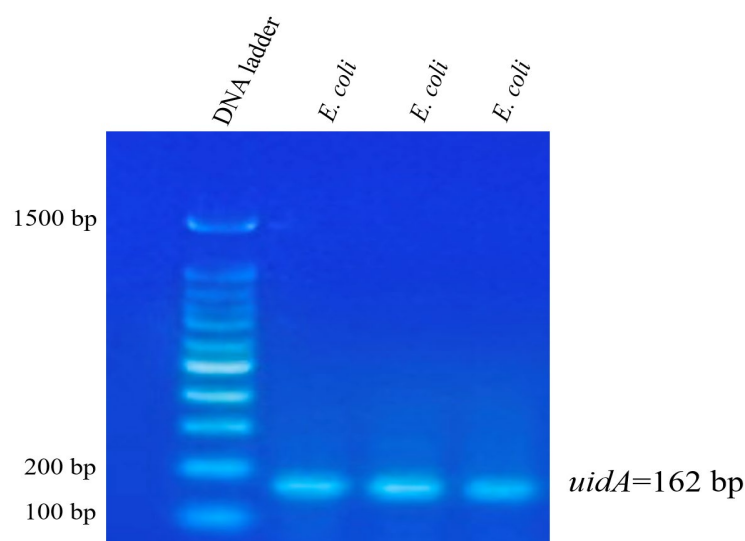
### 3.1. Isolation and Preliminary Identification

Over a three-month period spanning from November 2024 to February 2025, a substantial number of urine samples were received at the laboratory for microbiological culture and analysis. These samples were obtained from patients exhibiting clinical signs and symptoms suggestive of UTIs. Out of the total submissions, 104 isolates of *E. coli* were successfully subcultured and selected for further investigation. The identification of these *E. coli* isolates was performed using the VITEK 2 automated system.

In addition to *E. coli*, several other bacterial pathogens were isolated from the urine cultures. These included *S. aureus*, species of *Proteus*, and *Pseudomonas*. Some urine specimens, however, did not exhibit any visible bacterial growth following incubation and were categorized as culture-negative. Despite the presence of multiple bacterial types, the current study focused exclusively on the *E. coli* isolates due to the organism's well-established association with UTIs and its clinical significance in the region. Consequently, all non-*E. coli* bacterial isolates were excluded from subsequent analysis and not considered for molecular or antibiotic susceptibility testing.

### 3.2. Molecular Detection and Characterization of *E. coli* Bacterial Isolates

From the total of 104 *E. coli* isolates obtained from various hospital sources, molecular testing was carried out to confirm their identity by targeting the *uidA* gene, a widely recognized genetic marker specific to *E. coli*. This confirmatory step was essential to ensure the accuracy of bacterial identification beyond conventional culture-based methods. The existence of the *uidA* marker gene was verified through PCR, figure 1 shows an illustration of the outcomes of this molecular analysis.



**Figure 1:** PCR Amplification of the *uidA* (162 bp) Gene for *E. coli* Confirmation.

Following the PCR amplification, it was observed that 100 among the 104 *E. coli* isolates collected produced a positive result for the *uidA* marker gene, thereby confirming them as true *E. coli* strains. The

remaining four samples were excluded from further analysis due to their negative or ambiguous molecular results. As a result, the study proceeded with a focused investigation on the 100 genetically confirmed *E. coli* isolates. These confirmed samples were then subjected to antibiotic susceptibility testing to assess their resistance patterns against a selected panel of antibiotics. This step was crucial in determining the effectiveness of commonly used antimicrobial agents, contributing to a better understanding of local resistance trends in the Sulaimaniyah region.

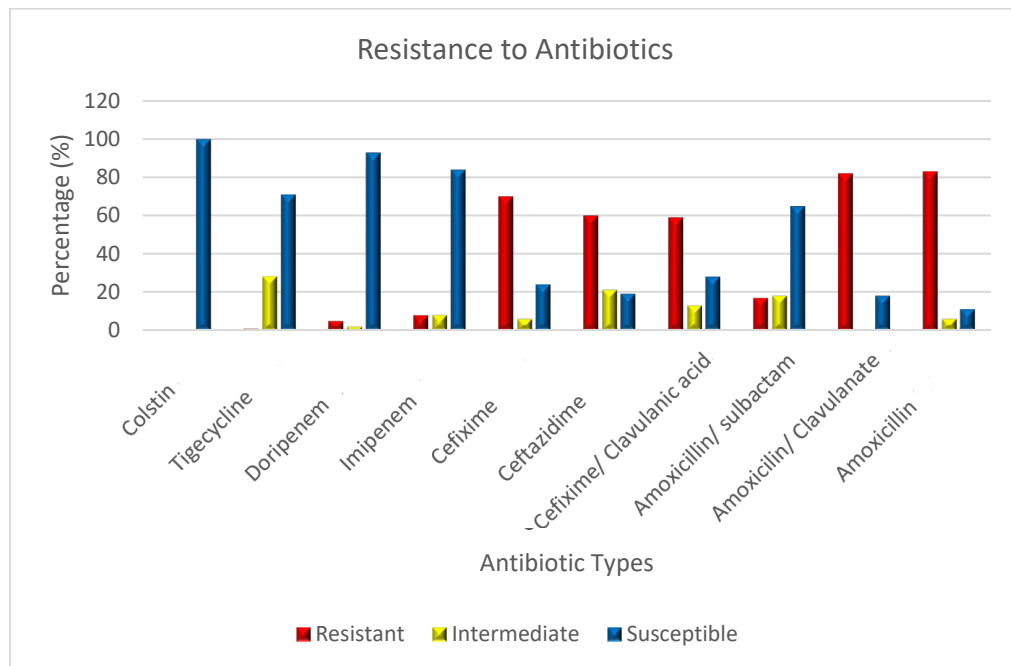
### 3.3. Antimicrobial Susceptibility Test

The evaluation of antibiotic sensitivity reveals significant variability in susceptibility patterns across the tested antibiotics, highlighting both highly effective and resistant profiles. The findings of this study are presented in table 1.

**Table 1:** Antimicrobial resistance profile of *E. coli* Isolates, including mean zone diameters, ranges, and susceptibility rates (resistant, intermediate, and susceptible).

	Antibiotics	Mean Zone ± Standard Deviation (mm)	Range (mm)	Resistant (n, %)	Intermediate (n, %)	Susceptible (n, %)
<b>Polymyxins</b>	Colistin (10 µg)	14.42 ± 0.87	14-19	0 (0%)	0 (0%)	100 (100%)
<b>Glycylcyclines</b>	Tigecycline (15 µg)	19.11±1.89	9-24	1 (1%)	28 (28%)	71 (71%)
<b>Carbapenems</b>	Doripenem (10 µg)	26.48±3.63	8-31	5 (5%)	2 (2%)	93 (93%)
	Imipenem (10 µg)	25.16±3.23	9-21	8 (8%)	8 (8%)	84 (84%)
<b>Cephalosporins</b>	Cefixime (5 µg)	11.39±7.12	0-27	70 (70%)	6 (6%)	24 (24%)
	Ceftazidime (30 µg)	14.96±5.84	0-25	60 (60%)	21 (21%)	19 (19%)
	Cefixime/ Clavulanic acid (5/10 µg)	13.83±7.48	0-28	59 (59%)	13 (13%)	28 (28%)
<b>Beta-lactams</b>	Amoxicillin/ sulbactam (30/15 µg)	16.26 ± 5.55	0-27	17 (17%)	18 (18%)	65 (65%)
	Amoxicilin/ Clavulanate (20/10 µg)	8.89±5.09	0-24	82 (82%)	0 (0%)	18 (18%)
	Amoxicillin (25 µg)	8.94±4.89	0-23	83 (83%)	6 (6%)	11 (11%)

Colistin demonstrated complete efficacy, with all isolates (100%) being susceptible. The mean inhibition zone diameter and standard deviation were 14.42 ± 0.87 mm, ranging from 12 to 19 mm, indicating that colistin remains a highly effective treatment option against *E. coli* in this study. Carbapenems, including doripenem and imipenem, also exhibited strong activity, with susceptibility rates of 93% and 84%, respectively. The mean zone diameters for doripenem and imipenem were 26.48 mm and 25.16 mm, these findings affirm the sustained efficacy of certain antibiotics as dependable therapeutic agents against *E. coli* infections. Conversely, a notably high level of resistance was detected for both amoxicillin and amoxicillin/clavulanate, with resistance rates reaching 83% and 82%, respectively, among the tested isolates. The mean inhibition zones were 8.94 mm for amoxicillin and 8.89 mm for amoxicillin/clavulanate, highlighting the limited efficacy of these antibiotics against the tested isolates. Cephalosporins also exhibited notable resistance trends. Cefixime and ceftazidime resistance rates were 70% and 60%, respectively. The mean zone diameters for these antibiotics were 11.39 mm and 14.96 mm, suggesting that *E. coli* in this study demonstrates significant resistance to these β-lactam antibiotics. Tigecycline exhibited moderate activity, with 71% susceptibility. However, 28% of isolates showed intermediate susceptibility, indicating potential variability in bacterial response to this antibiotic. Similarly, amoxicillin/sulbactam demonstrated a 65% susceptibility rate, with Resistance and intermediate rates recorded at 17% and 18%, respectively. The resistance rate for all antibiotics used in this study is summarized in figure 2.



**Figure 2:** Graphical representation of resistance rates for each antibiotic.

#### 4. Discussion

Pathogenic microorganisms are the primary contributors to UTIs, with bacteria being the most prevalent and responsible agents behind these infections [18]. Usually, the urethra allows these bacteria to enter the urinary tract. They then travel to the bladder and kidneys, where they multiply and cause illness [19]. This study's results corroborate those of previous research, reinforcing *E. coli* as a predominant infectious agent in clinical settings. The identification of other bacterial species further highlights the polymicrobial nature of infections and emphasizes the necessity for comprehensive microbiological evaluation. These observations concur with the findings of Abejew *et al.* [20], which documented comparable trends in the frequency of *E. coli* and other bacterial species in urine samples.

Among the 104 samples collected from hospitals for testing in this study, 100 yielded a positive result for the *uidA* gene, confirming their identity as *E. coli*. A notable finding was the discrepancy between the VITEK 2 automated system and molecular confirmation methods, as the VITEK 2 system failed to correctly identify four samples. This inconsistency underscores the limitations of automated biochemical identification systems, which, despite their efficiency and widespread use in clinical laboratories, may occasionally produce misidentifications, as reported in studies conducted in the India [21]. In contrast, molecular methods, such as *uidA* gene detection, offer a more reliable approach by targeting specific genetic markers, ensuring greater accuracy in bacterial identification, as demonstrated in studies from Iraq [22].

In this research, the rate of resistance to colistin was 0% for all isolates, indicating complete susceptibility. This finding contrasts sharply with a study conducted in Egypt involving 150 samples, where 78% of isolates demonstrated resistance to colistin, with no isolates showing susceptibility [23]. This discrepancy may be attributed to differences in regional antibiotic usage patterns, infection control practices, or the prevalence of colistin-resistant genes such as *mcr-1* and *mcr-2*. Furthermore, carbapenems, including doripenem and imipenem, exhibited strong activity in this study, with susceptibility rates of 93% and 84%, respectively. These outcomes agree with those of a recent study carried out in China [24], which also reported high sensitivity rates of *E. coli* to carbapenems. The 84% susceptibility rate to imipenem observed in our study further supports its clinical effectiveness.

Notably, this result aligns closely with data from the Duhok city in Kurdistan Region of Iraq, where a 96.4% susceptibility rate to imipenem among *E. coli* isolates was reported [25]. This agreement between our findings and both international and regional studies reinforce the continued reliability of carbapenems as an effective therapeutic option for *E. coli* infections, particularly in light of rising resistance to other antibiotic classes. Similarly, another study from Duhok, which focused exclusively on

female patients, reported elevated *E. coli* sensitivity to carbapenems [11]. These results support the effectiveness of carbapenems in treating UTIs in this population. Additionally, a three-year study conducted in Zakho also demonstrated high levels of sensitivity of *E. coli* to imipenem and meropenem, further confirming the potent activity of carbapenems against uropathogenic strains in the region [26].

Despite the consistently high sensitivity to carbapenems across various studies, emerging resistance trends should not be overlooked. Notably, neighboring countries such as Iran and Turkey have reported increasing prevalence of carbapenem-resistant *E. coli* strains, often associated with *bla*NDM and *bla*OXA-48 like carbapenemases. This underscores the importance of ongoing surveillance to detect resistance early and prevent widespread dissemination [27- 28].

The observed high resistance rates to amoxicillin and amoxicillin/ clavulanate in this study, with 83% and 82% of *E. coli* isolates classified as resistant respectively, are consistent with global trends, several studies have documented increasing resistance of *E. coli* to amoxicillin and amoxicillin/ clavulanate. For instance, studies analyzing *E. coli* isolates in western Algeria and Spain [29- 30]. Similarly, a study from Iraq also documented high resistance levels [31], further highlighting the widespread nature of this issue. The primary mechanism of resistance is likely due to the production of extended-spectrum  $\beta$ -lactamases, particularly *bla*TEM, *bla*SHV, and *bla*CTX-M genes, as described in studies from Nepal [32] and Guatemala [33]. These  $\beta$ -lactamases break down the  $\beta$ -lactam ring of these antibiotics and make them ineffective. The effectiveness of amoxicillin/ clavulanate combinations is compromised by the development of inhibitor-resistant  $\beta$ -lactamases by some *E. coli* bacteria, even though clavulanic acid acts as an inhibitor of  $\beta$ -lactamases. Furthermore, the uncontrolled and improper application of these antibiotics in both agriculture and healthcare has accelerated the emergence of resistant strains [34].

Cephalosporins showed significant resistance patterns in this study, with cefixime and ceftazidime exhibiting resistance rates of 70% and 60%, respectively. Our findings align with studies from other Iraqi cities, including Basra [35] and Baghdad [36], suggesting a widespread prevalence of extended-spectrum  $\beta$ -lactamases throughout Iraq. Moreover, these results also align with the findings of Seo *et al.* [37] and Afsharikhah *et al.* [38], who also reported high resistance rates to beta-lactam antibiotics.

Tigecycline demonstrated moderate activity in this study, with 71% susceptibility, 1% resistance, and 28% intermediate susceptibility. The susceptibility rate here is lower compared to studies in Morocco and China, where rates were 76% and 94.9%, respectively [39, 40]. This variation may be due to different prescribing habits or the presence of efflux pump overexpression and ribosomal protection mechanisms, now recognized as major drivers of tigecycline resistance in *Enterobacterales* [41].

From a clinical standpoint, these findings emphasize the need to revise empirical treatment protocols for UTIs in the region. Given the high resistance to amoxicillin and cephalosporins, these agents may no longer be suitable for first-line treatment. Instead, nitrofurantoin or fosfomycin, and carbapenems in severe or MDR cases, should be prioritized. Colistin should be reserved strictly for last-resort use. Implementation of local antibiotic stewardship programs and updated clinical guidelines is essential.

This study offers significant insights into the antimicrobial resistance profile of *E. coli* recovered from UTIs in Sulaymaniyah, Iraq, addressing a critical public health concern in a region where such data remain limited. The use of 100 molecularly confirmed *E. coli* isolates provides a reliable representation of the local bacterial population, and the combination of automated (VITEK 2) and molecular (*uidA* gene) identification enhances the accuracy of bacterial classification. The study also applied a comprehensive panel of ten antibiotics, including WHO-priority agents such as carbapenems and colistin, enabling a well-rounded assessment of resistance trends relevant to clinical practice. However, certain limitations must be acknowledged. Commonly used antibiotics such as fluoroquinolones and nitrofurantoin were not included, as this study is part of an ongoing Master's thesis project. The antibiotic panel was intentionally selected to complement the molecular component of the thesis, which focuses on detecting the *bla*CTX-M, *bla*TEM, and *mcr* resistance genes. Data related to these genes were excluded from this manuscript due to insufficient results available at the time of the manuscript prep-



aration. Despite these constraints, the findings remain robust and clinically relevant, providing an evidence base for guiding local empiric therapy and future regional surveillance studies. Further research will expand the antibiotic spectrum and integrate additional molecular and clinical variables to build on these initial findings.

## 5. Conclusions

The present study assessed antimicrobial resistance in 100 *E. coli* isolates collected from UTIs in Sulaymaniyah city, Iraq. Using the VITEK2 system and molecular confirmation via the *uidA* gene, high resistance was observed to amoxicillin, amoxicillin/ clavulanate, and several cephalosporins, while colistin and carbapenems remained effective. These findings highlight the importance of ongoing antimicrobial susceptibility testing, stewardship efforts, and molecular tools for accurate diagnosis. The results contribute valuable local data to guide UTI treatment, and future work should focus on identifying specific resistance mechanisms.

**Author contribution:** Lalan Rebaz Mohammed: Conceptualization, Investigation, Writing – original draft. Taib Ahmed Hama Soor: Methodology, Project administration, Writing – review & editing.

**Data availability:** Data will be available upon reasonable request.

**Conflicts of interest:** The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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