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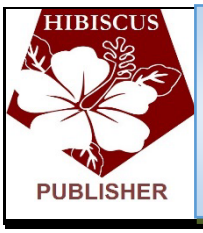

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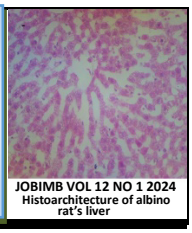

Molecular Studies on Quinolone Resistant MDR *E. coli* Detected in Urine Specimens

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ABSTRACT

Multidrug-resistant (MDR) *Escherichia coli* is a major cause of urinary tract infections (UTIs) and represents a growing public health concern due to its resistance to multiple antibiotics. This study aimed to investigate the prevalence of MDR *E. coli* in urine samples and to characterize the antibiotic resistance patterns and genetic mechanisms underlying resistance. A total of 40 urine samples were collected from patients at Mbarret El-Asafra Hospital in Egypt in 2021. Of these, 30 *E. coli* isolates were identified and tested for antibiotic susceptibility using the disk diffusion method across six antibiotics from four different classes. Molecular analysis was performed using polymerase chain reaction (PCR) to detect the presence of the *parC* gene, which is associated with quinolone resistance. Out of 30 *E. coli* isolates, 18 (60%) exhibited multidrug resistance. The majority of MDR isolates showed resistance to fluoroquinolones (levofloxacin and ciprofloxacin) and carbapenems (meropenem), with additional resistance observed against aminoglycosides and tetracycline. The *parC* gene was detected in 66.7% of MDR isolates, indicating a significant genetic basis for quinolone resistance. The resistance rates and presence of the *parC* gene were consistent with global trends, highlighting the widespread nature of antibiotic resistance among *E. coli* strains. The high prevalence of MDR *E. coli* in urine samples underscores the urgent need for enhanced antimicrobial stewardship and alternative treatment strategies. The detection of the *parC* gene in a substantial proportion of MDR isolates emphasizes the importance of molecular surveillance in understanding resistance mechanisms. These findings call for comprehensive approaches to mitigate the impact of MDR pathogens and ensure effective management of UTIs in clinical settings.

INTRODUCTION

The bacterium *Escherichia coli* falls within the Enterobacteriaceae family. It is a Gram-negative, rod-shaped microorganism that lacks spores, and its motility may be either absent or facilitated by peritrichous flagella. *E. coli* is characterized as chemoorganotrophic, demonstrating facultative anaerobic metabolism, capable of generating acid from glucose. It exhibits a positive catalase reaction, is negative for oxidase activity, and thrives in mesophilic conditions. Recognized as a prominent commensal bacterium, *E. coli* swiftly establishes itself in the gastrointestinal tract shortly after birth. Within the human gut, it competes effectively and stands as the most prevalent facultative anaerobe within the intestinal microbiota [1].

Over the last two decades, there has been a substantial rise in the prevalence and dissemination of antibiotic-resistant strains of *Escherichia coli*, exhibiting resistance to significant categories of antimicrobial agents [2]. Acquired resistance can arise from chromosomal point mutations or the acquisition of mobile resistance genes. This transformation typically occurs in bacterial populations that were once susceptible, often in response to exposure to antimicrobial substances [3].

Numerous factors contributing to resistance often coexist within a single R plasmid, which hosts multiple genes conferring resistance to various antibiotics, thus, the transfer of multiple resistances can occur through a single conjugation event among bacteria. Numerous R plasmids involved in this process carry resistance genes targeting primary classes of antibiotics,

including aminoglycosides, macrolides, phenicols, and tetracycline [4]. The current research aimed to identify and detect *E. coli* strains in clinical samples, focusing on those displaying multi-drug resistance (MDR), through biochemical reactions and antibiotic disc diffusion method. Moreover, the study focused on pinpointing the specific genes associated with multi-drug resistance using genotypic analysis via the PCR technique.

MATERIALS AND METHODS

Collection and preparation of urine samples

40 urine samples were promptly transported to the laboratory, adhering to strict guidelines: they should be stored for no longer than four hours at room temperature or up to 24 hours at four degrees Celsius. This protocol was implemented to prevent microbial proliferation and maintain the integrity of the samples, thus ensuring the accuracy of subsequent medical analyses [5]. The midstream or 'clean catch' specimen is widely regarded as the most commonly used non-invasive method for collecting urine samples [6].

Microbiological Examination

All clinical isolates underwent morphological examination to assess their colony characteristics on agar media.

Biochemical tests

Those exhibiting colonies were processed for biochemical testing. For the Catalase Test, a droplet of 3% hydrogen peroxide (H2O2) was applied to a clean slide, where bacterial growth from a loop was mixed with the H2O2 drops. A positive outcome was characterized by the rapid appearance of air bubbles.In the Oxidase Test, a few drops of a 1% solution of tetramethyl-p-phenylenediamine dihydrochloride reagent were placed on filter paper, and the colony of interest was touched with the reagent.

A positive reaction was indicated by the quick development of a deep blue color.Regarding the Urease Test, the tested organism was streaked onto the surface of a urea slant and then incubated at 37°C for 24 hours. A positive result was denoted by the emergence of a pink color.In the Indole Test, the organism under examination was introduced into peptone water and allowed to incubate at 37°C for 18-24 hours. Following this, Kovac's reagent was added to the inner wall of the tubes. A positive outcome was evident when a stable red color formed at the interface of the reagent and the peptone water within seconds [7]. For biochemical tests standard procedures were used [8].

Detection of multidrug resistant *E. coli* by Kirby- Bauer disc diffusion method

The antibiotic susceptibility of the isolated *E. coli* strains was assessed against six different antibiotics categorized into four distinct groups, carbapenems (meropenem 10µg), quinolones (levofloxacin 5µg and ciprofloxacin 5µg), aminoglycosides (gentamicin 10µg and tobramycin 10µg) and cyclines (tetracycline 30µg) (Oxoid, England).

An antibiotic resistance test of *E. coli* was done, the procedure involved placing discs containing known concentrations of antibiotics onto the surface of Mueller Hinton (MH) agar plates previously inoculated with standardized inoculums of the target organism. Using a separate sterile cotton-tipped applicator, the Mueller Hinton agar plates were streaked, ensuring complete coverage of the entire surface by rotating the plate between streaks and rimming it to guarantee uniform growth to the edges. Following a 2-3 minute interval, a mechanical dispenser was employed to apply the antibiotic discs onto the agar surface. The plates were subsequently incubated at 37°C for 24 hours. Antimicrobial activity was indicated by an inhibition zone. The diameter of the inhibition zones was measured in millimeter using a calibrated scale [9].

The size of the diameter of inhibition zone was interpreted based on criteria specified by CLSI (Table1). The results were categorized as susceptible (S) meaning highly sensitive, intermediate (I) meaning sensitive or (R) meaning resistant [10].

Table 1. Interpretative Zone Diameter of Antimicrobial Agents for Gram-Negative Bacteria According to CLSI Guidelines.

Antimicrobial Agent	Disc Cont./µg	Abbr.	Zone Diameters (mm)		
			Susceptible	Intermediate	Resistant
Meropenem	10	MEM	≥ 14	11 -13	≤ 10
Levofloxacin	5	LEV	≥ 16	13 – 15	≤ 12
Ciprofloxacin	5	CIP	≥ 21	16 – 20	≤ 15
Gentamicin	10	CN	≥ 15	13 – 14	≤ 12
Tobramycin	10	TOB	≥ 15	13 – 14	≤ 12
Tetracycline	30	TE	≥ 19	15 – 18	≤ 14

Molecular Examination

The study analyzed the makeup of genes to multiple drugs by using Polymerase Chain Reaction (PCR) after extracting DNA, from Escherichia coli strains that were found to be resistant to multiple drugs. The bacteria were grown in 5 mL of brain heart infusion broth at 37°C for a day. Following incubation, the cells were collected through centrifugation at 12,000 rpm for 2 minutes. The DNA extraction process involved employing the QIAamp DNA Mini Kit (QIAGEN, USA).

This process included spinning pellets at 7,500 rpm for 10 minutes re suspending the pellets in 180 µL Buffer ATL adding 20 µL of proteinase K and allowing them to incubate at 56°C for an hour for cell breakdown. Subsequently the samples were treated with 200 µL of Buffer AL heated at 70°C for ten minutes treated with ethanol and then subjected to a series of centrifugation steps to attach and cleanse the DNA on a spin column. The DNA was then withdrawn using 200 µL AE buffer. Kept at 20°C. The obtained DNA was used as a model in PCR to amplify genes linked with resistance, to drugs utilizing primer pairs under optimized conditions.

Polymerase Chain Reaction

Polymerase Chain Reaction (PCR) was utilized to identify the *parC* gene, which is associated with resistance to quinolone antibiotics in isolated samples. Specific primers for the *parC* gene were designed, with the forward primer sequence being GCCTTGCGCTACATGAATTT and the reverse primer sequence ACCATCAACCAGCGGATAAC, producing a 287 bp product size. These primers, prepared in nanoparticle form in Germany, were initially dissolved to a stock concentration of 100 pmol/µl and then diluted to a working concentration of 10 pmol/µl by mixing 10 µl of primer stock (forward and reverse) with 90 µl of RNase and DNase-free water.

The PCR amplification mixture included 2 µl of each primer (10 pmol/µl), 25 µl of Master Mix (2X concentration), 4 µl of DNA crude extract, and sterile H2O DEPC treated to bring the total volume to 50 µl. The amplification process was performed using an Applied Biosystems Veriti 96-well Thermal Cycler. The program consisted of an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds, and a final extension at 72°C for 10 minutes. After PCR amplification, the products were analyzed on a 1.5% agarose gel to confirm the presence of the *parC* gene.

Gel electrophoresis

Gel electrophoresis and visualization of DNA bands were performed using several prepared stock solutions and reagents. The TBE buffer (10X) was prepared by dissolving 108 g of Tris base, 55 g of boric acid, and 40 mL of 0.5 M EDTA (pH 8) in deionized distilled water to a final volume of 1 liter. A 1X working solution was made by diluting the 10X stock buffer with DNase-free deionized water in a 1:9 ratio. Ethidium bromide, used as a DNA stain, was prepared at a concentration of 10 mg/mL by dissolving 1 gram of ethidium bromide in 100 mL of double-distilled water. The agarose gel loading buffer consisted of 0.25% (W/V) bromophenol blue, 0.25% (W/V) xylene cyanol FF, and 30% (W/V) glycerol in water. The agarose gel itself was prepared at a concentration of 1.5% by mixing 1.5 g of agarose (Vivantis, USA) with 100 mL of 1X TBE buffer. This gel was used for the electrophoretic separation of DNA, with the DNA bands visualized under UV light following staining with ethidium bromide.

RESULTS

The study began with the collection of 40 urine specimens from Mbarret El-Asafra Hospital in Egypt in 2021. From these specimens, 30 isolates were confirmed as *Escherichia coli*, of which 18 exhibited multidrug resistance (MDR). The demographic details of the patients, including gender and age, are summarized in **Table 2**. The isolates showed a varied distribution across different age groups and genders, with a slight predominance in females.

Table 2. Types of *E. coli* collected categorized in correlation with patient gender and age.

Isolate Code	Sex	Age (Years)
EC-1	Male	39
EC-2	Female	40
EC-3	Male	42
EC-4	Female	20
EC-5	Female	42
EC-6	Female	41
EC-7	Male	37
EC-8	Female	29
EC-9	Female	40
EC-10	Female	33
EC-11	Female	41
EC-12	Male	39
EC-13	Female	30
EC-14	Male	52
EC-15	Female	35
EC-16	Female	44
EC-17	Male	23
EC-18	Male	38
EC-19	Female	30
EC-20	Female	39
EC-21	Female	22
EC-22	Male	23
EC-23	Female	44
EC-24	Male	57
EC-25	Female	39
EC-26	Female	40
EC-27	Female	42
EC-28	Female	32
EC-29	Female	28
EC-30	Female	44

The phenotypic characterization of the 30 *E. coli* isolates was performed using standard microbiological techniques. Morphologically, the isolates displayed typical characteristics of *E. coli* on MacConkey agar, presenting as pink to dark pink colonies with a dry, donut-shaped appearance after 24 hours of incubation at 37°C (**Fig. 1**). Gram staining confirmed the isolates as Gram-negative short bacilli, characteristic of *E. coli*. Biochemical tests further validated the identity of these isolates, with results shown in **Table 3**, including positive reactions for indole and methyl red tests and negative reactions for Voges-Proskauer, H2S production, citrate utilization, oxidase, and urease tests.

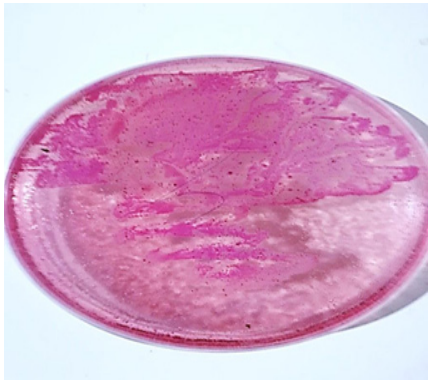

Fig. 1. *E. coli* on MacConkey agar medium.

Gram staining was performed for the morphological identification of *E. coli*, revealing Gram-negative short bacilli. Further characterization of these isolates was conducted through the study of their biochemical characteristics using biochemical test reactions, as detailed in (**Table 3**).

Table 3. Biochemical reactions of *E. coli* isolates.

Biochemical test	Result
Indole	+
Catalase	+
Voges–Proskaure	-
Production of H2S	-
Methyl Red	+
citrate utilization	-
oxidase, and urease	-

(+) positive reaction, (-) negative reaction.

Susceptibility testing of *E. coli* to the different antibiotics and detection of MDR isolates

Antibiotic susceptibility testing was conducted on all 30 *E. coli* isolates using the disk diffusion method across six different antibiotics. The results, detailed in **Table 4**, demonstrated varied resistance profiles. Notably, a significant proportion of isolates exhibited resistance to carbapenems and quinolones, two classes of antibiotics critical for treating *E. coli* infections. For example, isolates EC-1 and EC-2 showed complete resistance (indicated as 'R') to carbapenems (MEM) and quinolones (LEV, CIP), while others, such as EC-3 and EC-4, displayed intermediate susceptibility ('I') to certain antibiotics like quinolones (LEV, CIP) and aminoglycosides (CN, TOB).

Table 4. Susceptibility of *E. coli* to the different antibiotics.

Sample No.	Antibiotic (mm)					
	Carbapenem	Quinolone		Aminoglycosides		Cycline
	MEM	LEV	CIP	CN	TOB	TE
EC-1	9	0	8	25	16	14
	R	R	R	S	S	R
EC-2	0	0	7	29	18	10
	R	R	R	S	S	R
EC-3	0	12	16	12	5	15
	R	R	I	R	R	I
EC-4	5	15	0	0	13	15
	R	I	R	R	I	I
EC-5	7	13	0	16	20	10
	R	I	R	S	S	R
EC-6	0	0	16	0	8	7
	R	R	I	R	R	R
EC-7	10	15	12	22	13	8
	R	I	R	S	I	R
EC-8	0	0	10	20	14	7
	R	R	R	S	I	R
EC-9	6	14	0	13	25	7
	R	I	R	I	S	R
EC-10	9	15	9	19	14	10
	R	I	R	S	I	R
EC-11	0	10	0	8	0	17
	R	R	R	R	R	I
EC-12	0	0	12	13	13	14
	R	R	R	I	I	R
EC-13	5	15	0	8	9	8
	R	I	R	R	R	R
EC-14	8	0	5	12	0	17
	R	R	R	R	R	I
EC-15	10	0	0	0	0	7
	R	R	R	R	R	R
EC-16	3	0	8	9	7	15
	R	R	R	R	R	I
EC-17	0	13	0	0	0	17
	R	I	R	R	R	I
EC-18	0	12	9	14	22	5
	R	R	R	I	S	R

Genotypic analysis for the *parC* gene was conducted using Polymerase Chain Reaction (PCR)

Genotypic analysis was conducted to detect the presence of the *parC* gene, known for its association with quinolone resistance among MDR *E. coli* strains. PCR assays identified the *parC* gene in 12 out of the 18 MDR *E. coli* isolates (**Table 5**), indicating a high prevalence of this resistance mechanism. The PCR amplification results revealed a 287 base pair DNA fragment corresponding to the *parC* gene (**Fig. 2**), confirming the presence of this resistance gene in the tested isolates. The detection of the *parC* gene highlights its potential role in the observed multidrug resistance patterns and underscores the need for ongoing surveillance and targeted antibiotic therapy to manage MDR *E. coli* infections effectively.

Table 5. The frequency of MDR gene in the selected MDR *E. coli*.

E. coli No.	parC Gene
EC-1	+ve
EC-2	+ve
EC-3	-ve
EC-4	+ve
EC-5	+ve
EC-6	-ve
EC-7	+ve
EC-8	-ve
EC-9	+ve
EC-10	-ve
EC-11	+ve
EC-12	+ve
EC-13	+ve
EC-14	+ve
EC-15	+ve
EC-16	-ve
EC-17	+ve
EC-18	-ve

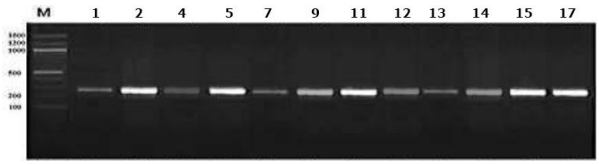

Fig. 2. An amplified 287 base pair DNA fragment of the parC gene of multi-drug-resistant E. coli ;M as marker & 1,2,4,5,7,9,11,12,13,14,15, 17 as no. of sample.

DISCUSSION

The research examined 40 urine samples, of which 30 were identified as Escherichia coli (*E. coli*). Among these isolates 18 (60%) showed resistance to drugs (MDR) indicating a presence of MDR strains, in the sample group. A demographic analysis revealed that 70% of the isolates were from participants aligning with studies like the one by Pallett et al. which also found a higher incidence of urinary tract infections (UTIs) in females compared to males constituting about 85.7% of UTI cases in their research [13]. This gender difference is likely influenced by factors, such as the urethra in females that makes it easier for bacteria to reach the bladder. These results underscore the nature of UTI prevalence influenced by variables like age, gender and the presence of devices consistent with earlier studies conducted by Iqbal et al. [14].

Testing *E. coli* isolates for susceptibility against six antibiotics, from classes—Carbapenems (Meropenem) Quinolones (Levofloxacin, Ciprofloxacin) Aminoglycosides (Gentamicin, Tobramycin) and Cyclines (Tetracycline)—revealed concerning resistance patterns. All 18 isolates of MDR exhibited resistance to antibiotics showing resistance, to Quinolones (Levofloxacin and Ciprofloxacin) and Carbapenems (Meropenem). Additionally, a notable percentage of the isolates displayed resistance to Aminoglycosides (50% to Gentamicin and 44.4% to Tobramycin). Tetracycline (66.7%).

The resistance patterns observed align with trends reported in the decade consistent with studies conducted in Jordan and other regions that have noted a rise in resistance among *E. coli* causing UTIs [15 17]. The high rates of resistance to ciprofloxacin found in this study support research findings [18]. In Karachi, Pakistan, Saeed et al. Reported that 92% of Gram isolates including various *E. coli* strains showed resistance to one or more antibiotics highlighting the widespread issue of antibiotic resistance in clinical environments [19]. Likewise Al Mardeni et al. in Jordan discovered that 59.9% of *E. coli* isolates were resistant to three or more antimicrobials emphasizing the level of multidrug resistance [20]. Mathai et al. Also noted rates of resistance among commensal *E. coli* isolates in Tamil Nadu, India with 42% displaying resistance, to one or more drugs—an indication that antibiotic resistance extends beyond strains [21].

In Saudi Arabia research conducted by Al Tawfiq uncovered varying levels of resistance, among *E. coli* strains showing resistance rates to antibiotics ranging from 2% to 29% indicating regional resistance patterns. The rapid spread of resistance in *E. coli* presents significant challenges in clinical treatment. These bacteria are a cause of UTIs worldwide. Show high resistance to commonly used antibiotics such as amoxicillin, tetracycline and trimethoprim/sulfamethoxazole. Although fluoroquinolones like ciprofloxacin and ofloxacin were initially effective in treating UTIs studies have reported an increasing resistance to these drugs, which's consistent with the results of this study.

Through analysis using polymerase chain reaction (PCR) it was found that the parC gene associated with resistance was present in 66.7% of the multidrug resistant isolates. This discovery aligns with research identifying mutations in the parC gene linked to quinolone resistance highlighting the importance of targeted strategies to address these resistant mechanisms. Dehbanipour et al.s study also revealed that all multidrug resistant isolates showed resistance to quinolones (Levofloxacin and Ciprofloxacin) and many displayed resistance to antibiotics such, as meropenem, aminoglycosides and tetracycline.

The significant occurrence of the parC gene, in isolates in this research (12 out of 18 isolates) emphasizes its role in quinolone resistance. This aligns with Dehbanipour et al.s discoveries, where the parC gene was widespread in 61 out of 135 isolates. Additionally, Oloruntoba et al. Observed that all five *E. coli* isolates in their study carried parC genes during analysis emphasizing the genetic foundation of antibiotic resistance in *E. coli* populations.

The high prevalence of multi drug MDR) *E. coli* strains in urine samples (60% of *E. coli*) highlighted in this study emphasizes the immediate requirement for effective antimicrobial stewardship and infection control measures. Given the rates of resistance, against fluoroquinolones it is essential to implement alternative treatment strategies and strengthen surveillance efforts to combat the increasing menace of antibiotic resistance.

CONCLUSION

Based on the results of this study it's clear that dealing with multidrug MDR) Escherichia coli presents a challenge, to public health especially when it comes to urinary tract infections (UTIs). With 60% of E. Samples showing resistance to antibiotics including important types like carbapenems and fluoroquinolones it's crucial to have strong antimicrobial stewardship programs in place. The significant presence of the parC gene in MDR samples further emphasizes the factors behind resistance highlighting the importance of monitoring and specific interventions. These discoveries emphasize the pressing need for treatment approaches and enhanced infection control methods to address the growing problem of resistance ensuring effective UTI management and reducing the impact of MDR bacteria in healthcare settings. Ongoing surveillance and research are vital, for adapting to changing resistance trends and developing strategies for treatment and prevention.

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