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Analysis of β-lactams and Carbapenem Heteroresistance in Extra-intestinal Pathogenic *Escherichia coli* from Blood Samples in Iran

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ABSTRACT

Introduction: Heteroresistance is the phenomenon wherein subpopulations of microbes exhibit a range of resistance to an antibiotic and are prevalent in a broad range of microorganisms. Not much data on carbapenem and β-lactams heteroresistance among extra-intestinal pathogenic *Escherichia coli* (ExPEC) in blood infections is available. This study aimed to investigate the frequency of heteroresistance to β-lactams and carbapenems in ExPEC strains isolated from patients' blood in Tehran, Iran. **Methods**: Identification of *E. coli* isolates was performed using standard biochemical tests, and PFGE was employed to determine the *E. coli* isolates genotypes. Antibiotic resistance pattern to carbapenems (ertapenem, meropenem, and imipenem), cefotaxime, and cefepime was determined by measuring minimum inhibitory concentration (MIC). The results were used to assess β-lactam and carbapenem heteroresistance in *E. coli* isolates. **Results:** In this study, for the first time, heteroresistance to the antibiotics carbapenem (ertapenem, meropenem, and imipenem), cefotaxime, and cefepime was observed in *E. coli* isolates from blood samples. Sixteen samples had heteroresistance to antibiotics. Three and two isolates showed heteroresistance to imipenem and ertapenem, respectively. Simultaneous heteroresistance to two carbapenem family antibiotics in three isolates was detected. Also, three isolates showed heteroresistance to cefepime and five to cefotaxime. The imipenem-resistant isolates exhibited a higher level of heteroresistance than those resistant to other carbapenem antibiotics. **Conclusions:** The present study shows that heteroresistance is increasing in Iran. Furthermore, this study demonstrated increasing resistance to the carbapenem family.

INTRODUCTION

Heteroresistance is a phenomenon in which a preexisting subpopulation of resistant bacteria can rapidly replicate in the presence of a given antibiotic [1]. *Escherichia coli* is genetically a heteroresistance species and includes non-pathogenic and pathogenic strains. Non-pathogenic strains are part of the intestinal microbiota of humans and animals and serve the host benefits by contributing to producing vital elements such as vitamin K and B12. On the other hand, pathogenic strains are divided into intestinal and extra-intestinal pathogenic *E. coli* (ExPEC). ExPEC can cause various infections such as urinary tract infections, meningitis, and septicemia [2-4]. Increased resistance to antibacterial in ExPEC strains can complicate the treatment and increase the treatment cost [5].

The worldwide emergence of the multidrug-resistant (MDR) *E. coli* sequence type 131 (ST131) clone represents an excellent example of the importance of prevention and management of ExPEC infections [6]. This clonal group possesses numerous virulence factors, including *fimH*, *iutA*, *hly*, *cnf*, and *kpsMTII* genes [7, 8]. These genes are located either on chromosomes or mobile genetic elements, e.g., plasmids. This high level of genetic heterogeneity within the *E. coli* species, specifically the ST131 clone, reflects the genomic plasticity of this generally clonal group [9].

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Extended-spectrum ß-lactamases (ESBLs) are among the main factors that contribute to the resistance to the extended-spectrum third-generation cephalosporins (cefotaxime, ceftazidime), fourth-generation cephalosporins (cefepime), fifth-generation cephalosporins (ceftaroline), and carbapenems (imipenem and meropenem) commonly used for treating nosocomial infections [10, 11]. Since the late 1980s, the ESBL producing strains have become widespread in hospital settings resulting in nosocomial infections [12]. The genes encoding these ß-lactamases can be transferred to the other bacteria species by various mechanisms such as chromosomes, plasmids, and transposons [13]. Besides *E. coli*, ESBLS are produced by gram-negative bacteria such as *Klebsiella pneumoniae* [14], *Enterobacter* spp. [15], *Salmonella* spp. [16], *Pseudomonas aeruginosa* [17], *Acinetobacter baumannii* [18], *Citrobacter* spp. [18], and *Proteus* spp. [19]. The class A ESBLs include CTX-M-type enzymes, rapidly spreading among *E.coli* globally with more than 50 allotypes clustered into six sub-lineages[20].

This study was designed to analyze β-lactams and carbapenem heteroresistance in ExPEC isolates obtained from blood samples.

MATERIALS AND METHODS

Sample collection. In this study, from Sep. 2018 to Oct. 2019, we collected 188 *E. coli* isolates from blood specimens of patients hospitalized in Shariati and Imam Khomeini hospitals in Tehran, Iran

Isolation and identification of *E. coli*. Gram-negative bacteria were identified from other non-lactose fermenting coliforms by lactose fermentation on MacConkey agar. Standard biochemical tests including catalase, oxidase, lysine decarboxylase, indole, motility, Voges-Proskauer (VP), Simmons' citrate agar, and hydrogen sulfide (TSI) were performed to confirm the identity of the isolates [21]. The *E. coli* isolates were inoculated into the Tryptic soy broth (TSB) (Merck, Germany) containing glycerol and stored at -70° C for further investigations.

Antibiotic susceptibility testing. Antibacterial susceptibility was performed by the Kirby-Bauer disk diffusion assay according to the clinical and laboratory standards institute (CLSI, 2019) guidelines on Mueller-Hinton agar plates, using a panel of 13 antibiotic disks including ciprofloxacin 10 mg (CIP), gentamicin 10 mg (GM), amikacin 30 mg (AK), aztreonam 10 mg (ATM), piperacillin-tazobactam 100/10 mg (PTZ), ampicillin-sulbactam 20/10 mg (SAM), ceftriaxone 30 mg (CRO), cefoxitin 30 mg (FOX), nalidixic acid 30 mg (NAL), trimethoprim-sulfamethoxazole 20 mg (SXT), tetracycline 30 mg (TET), ertapenem 10 mg (ETP), and cefepime 30 mg (CPM). (Mast Group Ltd., Merseyside, United Kingdom).

Population Analysis Profiling (PAP). Broth microdilution (BMD) and agar dilution (AD) methods were deployed for PAP. The broth microdilution with two-fold concentrations of the antibiotics, including carbapenem (ertapenem, meropenem, and imipenem), cefotaxime, and cefepime ranging from 0.25 to 512 µg/ml was used to determine minimum inhibitory concentrations (MICs) in isolates. The broth microdilution method was performed in 96-well plates (SPL Life Sciences Co) according to the CLSI 2019 guidelines. Bacterial suspensions without antibiotics were included as positive controls and medium cultures without bacteria as negative controls. The results were measured at 620 nm using an ELISA reader (iMark™ Microplate Absorbance Reader, Bio-Rad). Non-inhibitory concentration (NIC) was determined as the highest concentration of the antibiotic with no inhibitory effect.

In the agar dilution method, two-fold dilutions of the antibiotics were prepared as 20 µl of 0.5 McFarland standard suspension and inoculated into Mueller-Hinton agar followed by incubation at 37 °C for 48 h. The colony-forming unit (CFU) defined by the number of colonies on each plate was used for drawing the graph. Usually, CFU/ml $\leq 10^5$ shows the susceptibility of *E. coli* isolates to the antibiotic. In both methods, the bacterial isolates were considered heteroresistance if the ratio of MIC/NIC was ≥8 [22, 23].

Detection and characterization of ESBL resistance genes. The genomic DNA from *E. coli* isolates was extracted using a high pure PCR template purification kit (Roche Applied Science) followed by multiplex PCR using the primers that amplify the bla_{TEM}, bla_{SHV}, and $bla_{CTX\text{-}M}$ genes (Table 1). The amplification program included an initial denaturation for 5 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 51°C, and 40 s at 72°C, with a final extension for 3 min at 72°C.

Table 1. The primers used for amplification of *blaCTX-M*, *blaTEM*, and *blaSHV* genes.

Target gene	Primers sequence (5' to 3')	size (bp)	annealing temperature	Reference
$bla_{\text{CTX-M}}$	F: AAGCACGTCAATGGGACGAT R: CCTTAGGTTGAGGCTGGGTG	478	55	[36]
bla_{TEM}	F: CCGCATACACTATTCTCAGAATG R: CTCACCGGCTCCAGATTTATC	440	56	[37]
bla_{SHV}	F: TGTATTATCTCCCTGTTAGCCACC R: GTATCCCGCAGATAAATCACCA	767	56	[37]

F: Forward; R: Reverse

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Pulsed-Field Gel Electrophoresis (PFGE). PFGE was performed to evaluate the correlation of genotypes with heteroresistance [24]. The isolates were cultured on the Mueller Hinton agar plates followed by incubation at 37°C for 18 h. For macrorestriction digestion, the *Xba*I enzyme (50 units/sample) was added to the extracted genomic DNA in microtubes with the method described previously and kept at 37 ºC, 24h [25]. The digestions were resolved in a 1% SeaKem® Gold agarose gel (Lonza Group, Rockland, ME, USA) in a 0.5 X TBE buffer at 6 V/cm on a CHEF DR III SYSTEM (Bio-Rad, USA). A linear ramping factor with pulse times ranging from 5 to 35 s at 14°C, and an inclined angle of 120° was applied for 20 h. The gels were stained with ethidium bromide (30 µg/ml) and then destained in distilled water for 45 min and visualized under UV light in a Gel Documentation system (Bio-Doc). PFGE images were digitally captured, and the banding patterns were analyzed with Pyelph software [26]. Also, clustering was performed by the unweighted pair group average method (UPGMA) using the Dice coefficient.

Statistical analysis. The data analysis (antimicrobial resistance patterns, virulence traits, and PFGE profiles) was compared in different groups using chi-square (χ^2) or Fisher's exact test using SPSS software.

RESULTS

Antibiotic susceptibility testing. According to antibiotic susceptibility testing out of 16 *E. coli* isolates, the resistance against to CPM, TET, CRO, SXT, NAL, GM, CIP, ATM, SAM, FOX, PTZ, ETP and AK was observed in 68.7% (11/16), 62.5% (10/16), 62.5% (10/16), 62.5% (10/16), 62.5% (10/16), 50% (8/16), 43.7% (7/16), 43.7% (7/16), 37.5% (6/16), 18.7% (3/16), 12.5% (2/16), 6.2% (1/16), and 6.2% (1/16) of isolates, respectively (Table 2). The results of this study showed the multidrug-resistant (MDR) profile in 14 out of 16 isolates (87%).

Population Analysis Profiling (PAP). Antibiotic resistance analysis revealed β-lactam and carbapenem heteroresistance in 16 pathogenic ExPEC isolates from blood samples (Fig. 1). The bacterial count for each clone was performed, and the PAP pattern was defined (Table 2).

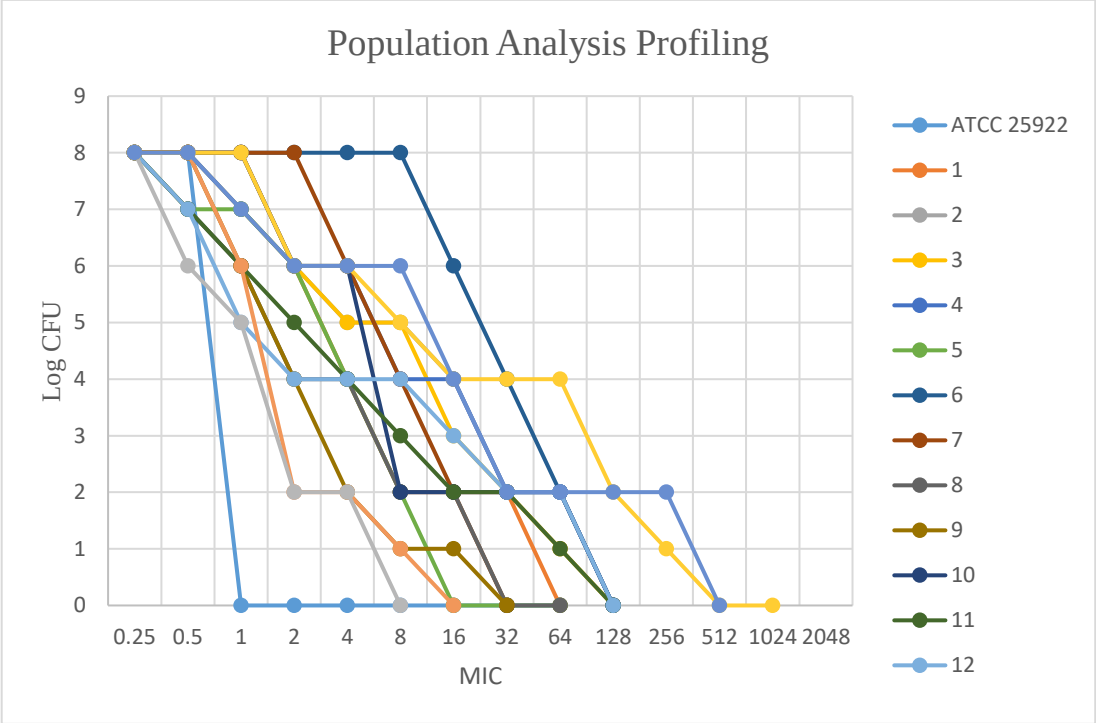

Fig. 1. The PAP results for the 16 heteroresistant isolates. Each color represents an isolate, and the two ends of each line show the spectrum of the bacterial growth in various concentrations of antibiotics, showing the bacterial heteroresistance spectrum. 1-16, the antibiotics carbapenem (ertapenem, meropenem, and imipenem), cefotaxime, and cefepime heteroresistant isolates; ATCC 25922, non-heteroresistant isolate.

Detection and characterization of the ESBL genes. Out of 188 isolates, 16 showed heteroresistance; nine isolates were positive for ESBL, ten for CTX-M, 2 for SHV, and 1 for TEM with PCR and CLSI phylogenic tests (Table 2).

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Pulsed-Field Gel Electrophoresis (PFGE). By using XbaI, 16 singletons were detected according to the PFGE typing method among the 188 isolates. The study revealed five distinct restriction profiles, considering a significant difference in a single band (Fig. 2). The dendrogram for the 16 *E. coli* isolates classification was done using the pyElph software method (Fig. 3 and Table 1).

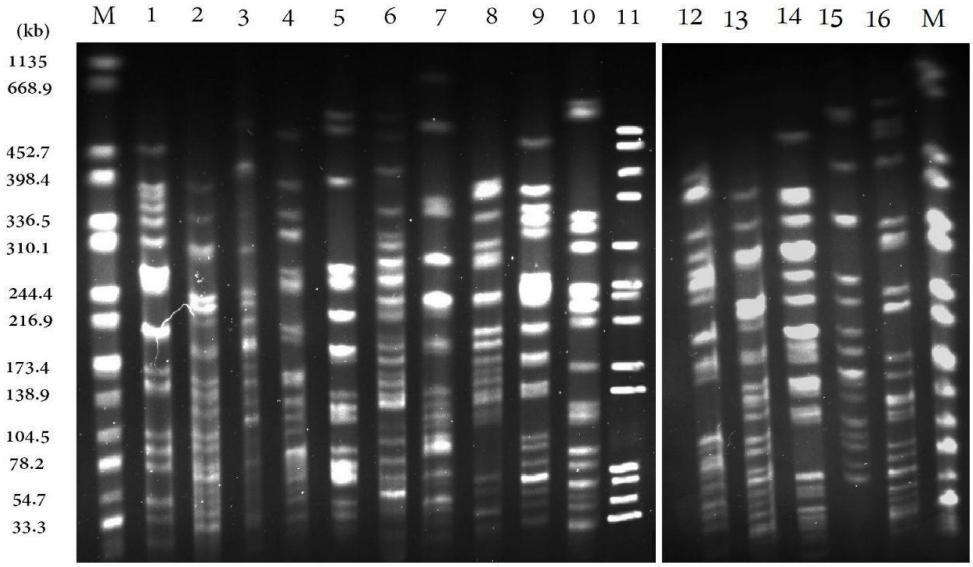

Fig. 2. The PFGE patterns of the 16 heteroresistant *E.coli* isolates. Lane M shows *Salmonella* serotype Braenderup H9812 and lanes 1-16 represent the isolates with different genotypes.

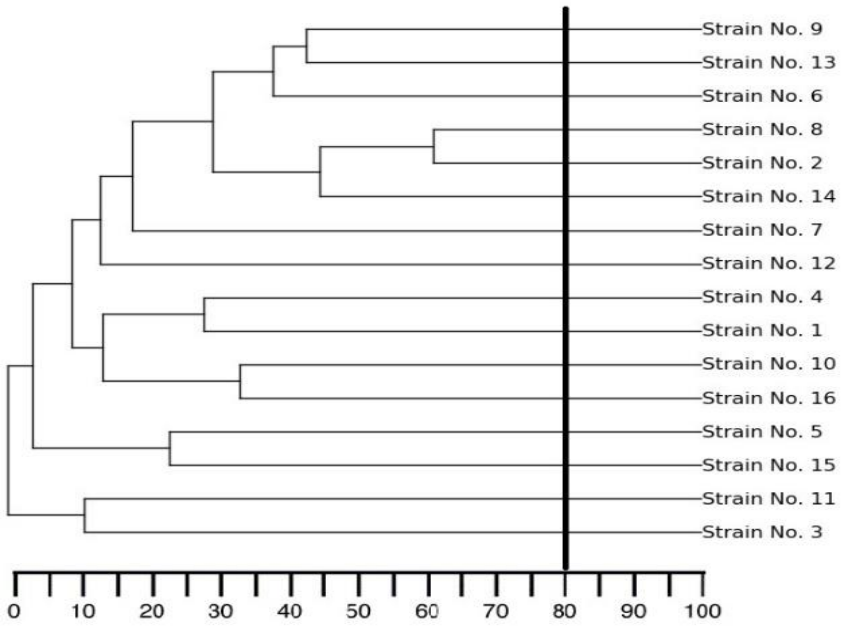

Fig. 3. The classification of the *E. coli* isolates with the pyElph software method, The UPGMA tree (Unweighted Pair Group Method with Arithmetic Mean) shows the results of PFGE and the similarity of the bands.

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DISCUSSION

Research in the heteroresistance field has become an essential and attractive topic for researchers due to the increased antibiotic resistance of bacteria and the associated mortality rate [27].

The overuse of antibiotics for treating bacterial infections can lead to therapeutic failure and extended hospitalization, finally resulting in the emergence of isolates resistant to a wide range of antibiotics [28, 29].

To the best of our knowledge, this present study is the only research on analyzing heteroresistance in *E. coli* isolates that originated from blood samples in Iran. Thus, our findings can significantly enhance the accuracy of the heteroresistant isolates diagnosis. In this study, we detected 16 heteroresistance *E. coli* isolates in blood samples. The PFGE patterns varied among the isolates, indicating different genotypes and origins (Fig. 2).

Table 2. Detection of the ESBL, the type of ESBL, PFGE analysis profile, MDR, and MIC (AD, BMD)

Strain no.	ESBL test	Carbapenem associated resistance genes	Composite BMD (g/mg)	Composite AD (g/mg)	PAP	Resistance to other antimicrobials*	PFGE Type	†MIC after 1-week passage	Frequency of subclones
1	+	CTX-M	64	16	Heteroresistance carbapenemase imipenem	TET, CRO, CPM, SXT	VIII	64	3.1×10^{-7}
2	-	-	64	16	Heteroresistance carbapenemase imipenem	SAM, GM, TET, CRO, ATM, CPM, NAL	X	128	5×10^{-7}
3	+	CTX-M	128	8	Heteroresistance carbapenemase imipenem	SAM, CIP, TET, SXT	VII	128	2.3×10^{-8}
4	+	CTX-M	128	16	Heteroresistance carbapenemase ertapenem	GM, TET, CRO, ATM, CPM, NAL, SXT	VII	128	5×10^{-5}
5	+	CTX-M	128	16	Heteroresistance carbapenemase ertapenem	CPM	VII	256	3×10^{-6}
6	-	-	12	4	Heteroresistance carbapenemase meropenem	TET	X	32	1×10^{-4}
7	+	CTX-M	128	8	Heteroresistance carbapenemase Co-resistance	SAM, CIP, GM, TET, FOX, CRO, ATM, CPM, NAL, SXT	VII	128	3×10^{-7}
8	-	-	32	8	Heteroresistance carbapenemase Co-resistance	NAL, SXT	VII	64	7×10^{-6}
9	-	SHV	32	4	Heteroresistance carbapenemase Co-resistance	CIP, GM, AK, TET, FOX, CRO, PTZ, ATM, CPM, NAL, ETP	VIII	64	7×10^{-5}
10	-	SHV	32	16	Heteroresistance cefepime	NAL, SXT	VII	128	2×10^{-6}
11	-	TEM	128	32	Heteroresistance cefepime	CIP, GM, CRO, PTZ, CPM, NAL, SXT	X	128	3×10^{-8}
12	+	CTX-M	128	8	Heteroresistance cefotaxime	CIP, GM, TET, CRO, ATM, CPM, NAL, SXT	VII	128	5×10^{-4}
13	+	CTX-M	128	16	Heteroresistance cefotaxime	TET, CRO, CPM, SXT	VII	128	3×10^{-4}
14	-	CTX-M	16	4	Heteroresistance cefotaxime	SAM, GM, TET, CRO, ATM, CPM, NAL	VIII	256	4×10^{-6}
15	+	CTX-M	1024	1024	Heteroresistance cefotaxime	SAM, CIP, TET, SXT	X	64	5×10^{-8}
16	+	CTX-M	512	512	Heteroresistance cefotaxime	SAM ,CIP, GM, FOX, CRO, ATM, CPM, NAL	VII	64	8×10^{-7}

*CIP, ciprofloxacin; GM, gentamicin; AK, amikacin; ATM, aztreonam; PTZ, piperacillin-tazobactam; SAM, ampicillin-sulbactam; CRO, ceftriaxone; FOX, cefoxitin; NAL, nalidixic acid; SXT, Trimethoprim-Sulfamethoxazole; TET, tetracycline; ETP, ertapenem; CPM, chlorpheniramine;†, the drug-free culture.

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Various mutations in efflux pumps resulting in overexpression may cause heteroresistance in different bacteria. e.g., AcrAB–TolC pump overexpression causes *Salmonella typhimurium* heteroresistant tigecycline. In the presence of mecillinam (a β-lactam antibiotic) due to several mutations in *E.coli*, heteroresistance was observed to this antibiotic (Fig. 1) [30].

The imipenem-resistant isolates showed a higher level of heteroresistance than other carbapenems (e.g., ertapenem and meropenem) resistant isolates. The reason for this observation could be due to more strong selection pressure on imipenem than ertapenem and meropenem. In China, out of 319 *E. coli* isolates from different specimens, e.g., blood, bile, and drainage, 70 (29%) showed heteroresistance to cefepime [14]. In another study in this country, 25%, 17.2%, and 3.9% of *E. coli* isolates showed heteroresistance to imipenem, ertapenem, and meropenem, respectively [15].

In our study, concurrent resistance to two carbapenem family antibiotics, a co-heteroresistance phenomenon, was also observed. The overuse of ertapenem has created a great deal of concern in clinics due to the side effects such as the potential to cause hypertension and changes in the bacteria's sensitivity pattern to antibiotics. The latest research has outlined that a better, more accurate introduction of ertapenem could cause a reduction in the use of other antibiotics of the carbapenem family, resulting in less resistance to these antibiotics [15]. Due to the lack of adequate data and research on heteroresistance to carbapenem antibiotics, more studies are required to clarify the precise mechanisms causing heteroresistance. The main challenge in clinical laboratories is the lack of accurate diagnosis methods for detecting heteroresistance and misdiagnosis of heteroresistant strains as sensitive strains. As a result, further analysis is required to come up with appropriate treatment protocols for these strains.

The presence of ESBL genes is an independent factor that contributes to heteroresistance in *Enterobacteriaceae*. Previous works have also shown the role of ESBL genes, such as the CTX-M-14 gene, in expanding heteroresistance in *E. coli*. Concerning other *Enterobacteriaceae* family members, e. g., *Klebsiella* spp., TEM and SHV genes were identified in 49% (64/130) isolates of this bacteria, which showed high resistance in antimicrobial susceptibility testing [31, 32]

In this study, for the first time, we investigated the heteroresistance phenomenon in *E. coli* isolates from blood samples in Tehran, Iran. Moreover, the present study showed a significant relation between MDR and heteroresistance, proving that MDR could contribute to the occurrence of heteroresistance. Further work is required to analyze the enzymes involved in heteroresistance and the difference between the heteroresistance strains and the non-heteroresistant.

In a study, among 93 *E. coli* isolates from female patients with *E. coli* urinary tract infection, no significant differences were observed in the distribution of antibiotic resistance among *E. coli* isolates, and the isolates were more sensitive to nitrofurantoin (1%) and imipenem (2%) [33]. In another study, one of the highest resistance rates belonged to cefotaxime (32%), and the most prevalent among ESBL genes was $bla_{\text{CTX-M15}}$, with an 87% rate [34]. Among the 63% detected ESBL-positive ciprofloxacin-resistant isolates in the Czech Republic, 84% contained the CTX-M1 enzyme [35]. Overall, our results agree with the above studies; however, further studies are needed to clarify the association between the enzymes and antibiotic resistance.

The present study showed that heteroresistance is increasing in Iran. Our study also revealed increased resistance to the carbapenem family. Consequently, imipenem is one of the most suitable antibiotics based on our results. Above all, more attention needs to be paid to the prescription and use of these antibiotics since inappropriate prescriptions can lead to more resistance, making it a substantial issue for healthcare professionals, clinical laboratories, and clinics.

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CONFLICT OF INTERESTS

The authors declare that there are no conflicts of interest associated with this manuscript.

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