ORIGINAL ARTICLE

Prevalence of ESBL and AmpC genes in *E. coli* isolates from urinary tract infections in the north of Iran

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Abstract

Beta-lactam resistance in Gram-negative bacteria, especially *Escherichia coli*, is a main clinical problem. It is often caused by the production of β-lactamases, particularly extended-spectrum β-lactamases (ESBLs) or AmpC enzymes. This study was undertaken to characterize ESBL and AmpC producers among *Escherichia coli* isolates from urine samples. During six months, 263 *E. coli* isolates were detected by standard biochemical tests. The isolates were screened for ESBL production by the double-disk synergy test using Ceftazidime (30 μg) and Cefotaxime (30 μg) disks and confirmed by combined disk diffusion test using Clavulanic acid. AmpC production was confirmed by an AmpC disk test based on filter paper disks impregnated with EDTA. The presence of genes encoding TEM, SHV, CTX-M, CIT, FOX, MOX, ACC, and EBC were detected by PCR. 263 *E. coli* isolates were selected for the combined disk (Ceftazidime, Cefotaxime, and Clavulanic acid) assay in the disk agar diffusion test. In the combined disk assay, among 263 isolates, 121 (46%) isolates were detected as ESBLs, and none of the isolates were AmpC producers. PCR performed on all ESBL producers and *blaSHV*, *blaTEM*, and *blaCTX-M* were detected in 42 (34.7%), 44 (36.4%), and 47 (38.8%) cases, respectively. Also, from 48 Isolates with zone diameters of less than or equal to 18 mm to Cefoxitin, 7 (14.6%), 4 (8.3%), and 9 (18.8%) cases contained MOX, EBC, and CIT genes, respectively. DHA, FOX, and ACC genes were not detected in any sample. Since pathogens evolve in the hospital setting, updating local data, such as this research, offers scientific evidence to improve the outcome of nosocomial infections.
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Introduction

Escherichia coli is one of the important causes of hospital-associated infections in humans, such as urinary tract infection (UTI), bloodstream infection (BSI), and gastrointestinal infection (GI) [1]. β-lactams have been used extensively to treat different types of human infections caused by *E. coli* [2]. However, the widespread use of antibiotics poses a selective pressure leading to the selection of resistant bacteria [3]. β-lactamase are the primary causes of resistance to β-lactam agents that hydrolyse the β-lactam ring [4]. Extended-spectrum β-lactamases (ESBLs) belonging to class A and AmpC β-lactamases belonging to class C Ambler classification are the two prevalent β-lactamases in Gram-negative bacteria, particularly in *Enterobacteriales* [5]. Both ESBLs and AmpC β-lactamases confer resistance to a broad spectrum of β-lactams includes Penicillins and Cephalosporins [6]. But, unlike ESBLs, plasmid-encoded AmpC β-lactamases are effectively active against cephamycins and are not inhibited by a β-lactamase inhibitor such as Clavulanic acid [7]. Harbouring these enzymes is usually associated with multiple antibiotic resistance (MDR) means that there are fewer antibiotic options available to treat [8]. Knowing the epidemiology of ESBLs and AmpC producing organisms is important to ensure effective therapy, as well as infection control measures [8]. Therefore,

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this study aimed to investigate the occurrence of ESBLs and AmpC producing *E. coli* isolates among hospitalized patients with UTI. Results of the present work can be used for the evidence-based improvement of available infection control policies and antimicrobial stewardship programs.

Materials and methods

Study design and bacterial isolation

From September 2018 to March 2019, 263 nonrepetitive *E. coli* strains (one per patient) were isolated from hospitalized patients with UTI in Razi hospital in the North of Iran. The study design was approved by the regional Ethics Committee of Guilan University of Medical Sciences (IR.GUMS.REC.1397.230) and was following the declaration of Helsinki. Briefly, each urine sample was streaked on the Blood agar and EMB agar (Merck, Germany) media, and plates were incubated aerobically at 37 °C for 24-48 h. After incubation, *E. coli* isolates were identified by routine microbiological tests and confirmed by API 20E strip (API-bioMérieux, France).

Antimicrobial susceptibility testing

An antimicrobial susceptibility test was performed on all isolates by disk-diffusion method on Mueller-Hinton agar medium (Merck, Germany) according to Clinical and Laboratory Standards Institute (CLSI) guidelines [9]. Antibiotic disks, including Ampicillin (10 μg), Co-amoxiclav (30 μg), Ceftazidime (30 μg), Ceftriaxone (30 μg), Cefepime (30 μg), Cefoxitin (30 μg), Aztreonam (30 μg), Gentamicin (10 μg), Amikacin (30 μg), Meropenem (10 μg), Imipenem (10 μg), Ciprofloxacin (5 μg), Levofloxacin (5 μg), Norfloxacin (10 μg), and Nalidixic acid (30 μg) (MAST, UK) were used. The plates were incubated aerobically at 37 °C for 16–18 h. *E. coli* ATCC 25922 was used for quality control purposes.

ESBL and AmpC screening tests

ESBLs production was investigated using the double-disk synergy test by Ceftazidime (30 μg) and Cefotaxime (30 μg) disks, and combination with Clavulanic acid (10 μg) disk-based on CLSI recommendation [9]. The presumptive AmpC β-lactamases producing isolates were screened by the standard disk diffusion test using 30 μg Cefoxitin disks. Cefoxitin non-susceptible isolates were selected for further investigation. The AmpC production was confirmed based on the method described by Black et al. based on the use of Tris-EDTA to permeabilize a bacterial cell and release β-lactamases into the external environment [6]. An AmpC positive clinical strain of *Pluralibacter gergoviae* was provided by a colleague (formerly *Enterobacter gergoviae*) for quality control purposes [10].

DNA extraction and polymerase chain reaction

The bacterial DNA was extracted from pure overnight cultures using the boiling method [11]. The ESBLs encoding genes (bla_{SHV}, bla_{TEM}, and bla_{CTX-M}) and AmpC genes (MOX, EBC, CIT, DHA, FOX, and ACC) were amplified individually on a SimpliAmp™ thermal cycler (Applied Biosystems, Foster City, CA) using the specific primers (Metabion Co, Germany). The list of primer sequences is displayed in Table 1. The amplification reaction was performed in a final volume of 20 μL containing Master Mix (Bioneer, South Korea), primers at concentrations of 10 pM, 50–100 ng of extracted DNA templates, and ddH_2O. The PCR conditions for the amplifications were as follows, 5 min at 95°C for the initial denaturation step; 30 cycles of 30 sec at 95°C for DNA denaturation, 30 sec for primer annealing). The temperature depended on the sequences of primers (primer extension at 72 °C for 1 min and a final extension of 5 min at 72°C. The PCR products were separated on 1.5% agarose gel prepared in 1X TBE (Tris/Boric/EDTA) buffer and visualized using ultraviolet light after staining with safe stain (CinaGen Co., Iran).

Statistical methods

Statistical analysis was performed using SPSS™ software, version 21.0 (IBM Corp., USA). The results were presented as descriptive statistics in terms of relative frequency. The Chi-square or Fisher's exact tests were used to analyse the data whenever appropriate. A p-value <0.05 was considered statistically significant.

Results

The antibiotic susceptibility results in Table 2 revealed that the majority of *E. coli* isolates were nonsusceptible to Ampicillin

TABLE 1. The primer sequences of the ESBL and AmpC genes amplified by PCR [36–38]

Primers		Sequences 5′→3′	Size (bp)	Reference
SHV	F	TCAGCGAAAAACACCTTG	471	[38]
	R	CCCGCAGATAAATCACCA		
TEM	F	GAGTATTCAACATTTCCGTGTC	861	[38]
	R	TAATCAGTGAGGCACCTATCTC		
CTX-M	F	TTTGCGATGTGCAGTACCAGTAA	544	[39]
	R	CGATATCGTTGGTGGTGCCATA		
DHA	F	AACTTTCACAGGTGTGCTGGGT	405	[40]
	R	CCGTACGCATACTGGCTTTGC		
FOX	F	AACATGGGGTATCAGGGAGATG	190	[40]
	R	CAAAGCGCGTAACCGGATTGG		
MOX	F	GCTGCTCAAGGAGCACAGGAT	520	[40]
	R	CACATTGACATAGGTGTGGTGTT		
ACC	F	AACAGCCTCAGCAGCCGGTTA	346	[40]
	R	TTCGCCGCAATCATCCCTAGC		
CIT	F	TGGCCAGAACTGACAGGCAAA	462	[40]
	R	TTTCTCCTGAACGTGGCTGGC		
EBC	F	TCGGTAAAGCCGATGTTGCGG	302	[40]
	R	CTTCCACTGCGGCTGCCAGTT		

F: Forward, R: Reverse.

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TABLE 2. Results of antibiotic resistance pattern of *E. coli* isolates by ESBL

		Total *E. coli* N = 263		Non-ESBL-producing *E. coli* N = 142		ESBL-producing *E. coli* N = 121		
Class	Antibiotics	No.	%	No.	%	No.	%	P value
β-lactam/β-lactamase inhibitor	Amoxicillin/clavulanat	154	58.6	58	40.8	96	79.3	<0.001
β-lactam	Ampicilin	220	83.7	99	69.7	121	100	<0.001
Monobactams	Aztreonam	137	52.1	19	13.4	118	97.5	<0.001
Cephalosporins II	Cefoxitin	36	13.7	12	8.5	24	19.8	0.006
Cephalosporins III	Ceftazidime	145	55.1	30	21.1	115	95	<0.001
Cephalosporins III	Ceftriaxone	148	56.3	28	19.7	120	99.2	<0.001
Cephalosporins IV	Cefepime	104	39.5	12	8.5	92	76	<0.001
Carbapenem	Meropenem	2	0.8	1	0.7	1	0.8	0.709
Carbapenem	Imipenem	11	4.2	4	2.8	7	5.8	0.187
Aminoglycosides	Amikacin	37	14.1	20	14.1	17	14	0.569
Aminoglycosides	Gentamicin	54	20.5	11	7.7	43	35.5	<0.001
Quinolones	Norofloxacin	135	51.3	42	29.6	93	76.9	<0.001
Quinolones	Levofloxacin	134	51	41	28.9	93	76.9	<0.001
Quinolones	Ciprofloxacin	148	56.3	50	35.2	98	81	<0.001
Quinolones	Nalidixic acid	203	77.2	90	63.4	113	93.4	<0.001

(83.7%), Nalidixic acid (77.2%), and Co-amoxiclav (58.6%). In comparison, most of the isolates were susceptible to carbapenems, including Meropenem (99.2%) and Imipenem (95.8%). Based on phenotypic results, of 263 *E. coli* isolates, 121 (46%) isolates were ESBLs producers. Moreover, none of the isolates showed AmpC production. According to the results of Table 2, among 121 ESBL-producing *E. coli*, the highest resistance was observed to Ceftriaxone (99.2%) and the lowest resistance to Meropenem (0.8%). Also, antibiotic resistance was significantly higher among ESBL-producing isolates compared to non-ESBLs ($P < 0.05$), except for Amikacin, Imipenem and Meropenem. PCR for detection of ESBL encoding genes was performed on all of the 121 ESBL-isolates, of which the prevalence of SHV, TEM, and CTX-M were 42 (34.7%), 44 (36.4%), and 47 (38.8%) isolates, respectively. AmpC genes, including MOX, EBC, and CIT among 48 cefoxitin-resistant isolates were 7 (14.6%), 4 (8.3%), and 9 (18.8%), respectively. Meanwhile, DHA, FOX, and ACC genes were not found in any isolates. The occurrence patterns of ESBL and AmpC genes in studied isolates are shown in Tables 3 and 4, respectively. As shown, seven different patterns were identified for each of them. The most prevalent pattern for ESBL genes were bla_{TEM} (14%), and bla_{SHV} + bla_{TEM} + $bla_{CTX\text{-}M}$ (14%). While for AmpC genes, bla_{CIT} (10.4%) was the predominant pattern. Also, 48 and 33 of *E. coli* isolates had no genes for ESBLs and AmpC, respectively.

Discussion

Trends of resistance to β-lactam antibiotics in Gram-negative bacteria isolated from clinical samples have been increased over recent years [12]. The main mechanism of β-lactam resistance in *Enterobacteriales*, particularly *E. coli*, is often due to the production of ESBL or AmpC enzymes [13]. In this study, the phenotypic data indicated that in the north of Iran, a large number of *E. coli* isolates were ESBLs producers (46%). Despite the heterogeneity in reported rates, our results are consistent with the average reported in these studies.

The prevalence reported in our study (46%) is higher than those reported, Semnan (Central, 26.6%) [14], Shiraz (South, 34.6%) [15], Kermanshah (West, 24.5%) [16], and Kerman (South, 41%) [17]. In contrast, it is lower than those reported

TABLE 3. The occurrence patterns of genes encoding ESBL-betalactamase

	ESBL-P	
Pattern	No	%
bla_{SHV}	4	3.3
bla_{TEM}	17	14
$bla_{CTX\text{-}M}$	9	7.4
bla_{SHV} + bla_{TEM}	5	4.1
bla_{SHV} + $bla_{CTX\text{-}M}$	16	13.2
bla_{TEM} + $bla_{CTX\text{-}M}$	5	4.1
bla_{SHV} + bla_{TEM} + $bla_{CTX\text{-}M}$	17	14
No gene	48	40
Total	121	100

TABLE 4. The occurrence patterns of genes encoding AmpC-betalactamase

	Cefoxitin-R	
Pattern	No	%
bla_{MOX}	4	8.3
bla_{EBC}	2	4.2
bla_{CIT}	5	10.4
bla_{MOX} + bla_{EBC}	0	0
bla_{MOX} + bla_{CIT}	2	4.2
bla_{EBC} + bla_{CIT}	1	2.1
bla_{MOX} + bla_{EBC} + bla_{CIT}	1	2.1
No gene	33	69
Total	48	100

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TABLE 5. **The co-occurrence patterns of genes encoding ESBL and AmpC-betalactamase**

Pattern	Total		Cefoxitin-R		ESBL-P	
	No	%	No	%	No	%
bla_{MOX} + bla_{CIT} + bla_{TEM}	2	0.76	2	4.16	2	1.65
bla_{MOX} + bla_{EBC} + bla_{CIT} + bla_{SHV} + bla_{CTX-M}	1	0.38	1	2.08	1	0.82
bla_{MOX} + bla_{TEM}	2	0.76	2	4.16	2	1.65
bla_{MOX} + bla_{SHV} + bla_{CTX-M}	1	0.38	1	2.08	1	0.82
bla_{MOX} + bla_{SHV} + bla_{TEM} + bla_{CTX-M}	3	1.14	3	6.25	3	2.47
bla_{EBC} + bla_{SHV} + bla_{TEM}	1	0.38	1	2.08	1	0.82
bla_{EBC} + bla_{SHV} + bla_{CTX-M}	1	0.38	1	2.08	1	0.82
bla_{CIT} + bla_{TEM}	2	0.76	2	4.16	2	1.65
bla_{CIT} + bla_{SHV} + bla_{CTX-M}	2	0.76	2	4.16	2	1.65
bla_{CIT} + bla_{SHV} + bla_{TEM}	1	0.38	1	2.08	1	0.82
Total	263	100	48	100	121	100

from Ahvaz (South, 46.1%) [18], Shiraz (South, 69.2%) [19], and Mashhad (East, 72.9%) [20]. Same heterogeneity was observed from other countries include Brazil (7.1%) [21], India (41.6%) [22], Pakistan (40%) [23], France (69.4%) [24], and Nepal (91.7%) [25]. The difference observed in the prevalence of ESBL in UroPathogenic *Escherichia coli* (UPEC) isolates in Iran and other parts of the world is probably due to differences in geographical distribution, infection control policies, source and size of the sample [26]. The lack of a standard phenotypic method for detecting AmpC producing bacteria is the biggest obstacle to comparing the results of this enzyme. In this study, we identified 48 presumptive AmpC producing isolates, While none of them was an AmpC producer with a double disk test. Previously, the prevalence of AmpC beta-lactamase in *E. coli* isolates was noted from Zahedan (East of Iran, 2013) (5%) [12], Tehran (Capital city of Iran, 2015) (25%) [27], Brazil in 2016 (1.8%) [28], India (14.6%) [29], and Uganda (22.9%) [30].

In our study, the investigated ESBL genes were detected with almost a significant equal frequency. Previously, similar to our results, Naziri et al. Shiraz (South, 2020) reported the high prevalence of SHV (47.4%) and then CTX-M (37.2%) and TEM (15.4%) among UPEC isolates [15]. In another study in Kermanshah (West, 2013), the frequency of the CTX-M (93.3%) gene was reported more than the other ESBL genes, TEM (68.2%) and SHV (43.2%) among UPEC isolates [31]. Also, several studies in other parts of the world, such as Saudi Arabia, Vietnam, China, and Mexico, introduced the CTX-M, SHV, and TEM genes as the most important mechanisms of ESBL production in *E. coli* strains [32–35]. Based on molecular analysis, we found three types of AmpC genes in 31.2% isolates of cefoxitin-resistant strains, indicating the low prevalence of AmpC β-lactamases in the north of Iran. Also, the CIT gene was the most prevalent plasmid-mediated AmpC enzyme in our region, followed by MOX and EBC. Maleki et al. in Ilam (West of Iran) introduced CIT and DHA as the most frequent AmpC genes in *E. coli* isolates [36]. Ghanavati et al. in Tehran (North of Iran) also reported the clusters of CITM, EBCM, and DHAM genes as the most abundant genes in *Klebsiella* isolates, respectively [37].

According to the results, it seems that the CITM gene is the most important factor in the plasmid dissemination of AmpC-producing isolates in Iran. The significant rate of ESBL-producing UPEC isolates indicates the need for infection control policies to prevent the further spread of resistant strains. Due to meagre resistance to carbapenems and aminoglycosides, these antibiotics can be the choice for complicated UTIs. Identifying resistant strains and updating bacterial susceptibility pattern information will prevent the over-administration of antibiotics and the development of new resistant strains. Furthermore, to avoid treatment failure and infection control, ESBLs and AmpC production monitoring is recommended. Updating of local data, such as this study, provides empirical evidence to improve the outcome of nosocomial infections because the evolution of pathogens continues in the hospital environment.

Author contributions

All authors contributed to data analysis, drafting or revising the article, finalizing the version to be published, and agreed to be accountable for all aspects of the work.

All raw data are available by corresponding author on reasonable request.

Transparency declaration

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