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Original Article

The Mechanism of Resistance in AmpC-Producing *Escherichia coli* Isolated from Urinary Tract Infections

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Abstract

Background and Aim: AmpC β-lactamases are capable of hydrolyzing all β-lactams except cefepime and carbapenems. The detection of AmpC-producing *Escherichia coli* has a high priority in infection management. This research is aimed to investigate the resistant AmpC- generating *E. coli* isolates and identify their genetic variety.

Methods: In this study, 230 *E. coli* isolates from patients having urinary tract infection symptoms were investigated in 2017-2018 to assess their susceptibility toward antimicrobial agents. AmpC genes were evaluated by PCR and molecular typing using the 10-loci MLVA method. MLVA images were examined by BioNumerics 6.6 software through the use of the UPGMA algorithms.

Results: The highest frequencies of susceptibility among *E. coli* isolates were to meropenem 96.08%, piperacillin-tazobactam 90.43%, followed by gentamicin 66.54%, ceftazidime 50%, ciprofloxacin 48.26%, ceftriaxone 41.74%. All *E. coli* isolates were resistant to amoxicillin-clavulanate. Thirty-eight AmpC-generating *E. coli* isolates were detected. The most abundant determinant was CIT and EBC, FOX, and DHA had the next ranks, respectively. Six major clusters and a singleton were identified by MLVA.

Conclusion: AmpC-generation ability is an effective feature in the resistance of *E. coli* isolates and its investigation is of crucial significance in infection management. The major mechanisms of AmpC beta-lactamase vary depending on time and geographical location.

Keywords: *Escherichia coli*; *E. coli*; Uropathogenic *Escherichia coli*; AmpC β-lactamases; Molecular Typing; Gene; Resistance; Urinary Tract Infections.

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Introduction

Escherichia coli is the first suspect in the development of urinary tract infection (UTI) (1) accounting for more than 85% of urinary tract infection cases (2). One hundred and fifty million cases of urinary tract infection are annually reported throughout the globe (3). It is 30 times more prevalent among females and almost 60% of women experience UTI at least once in their lives (3).

Antimicrobial agents can greatly contribute to the clinical control of urinary tract infections. Antimicrobial resistance, in particular, multidrug-resistant gram-negative bacilli, has posed a remarkable challenge for the hospital and the community settings (4). Diverse drug resistance mechanisms have been introduced in gram-negative bacteria among which, extended-spectrum β -lactamases (ESBL) production, AmpC β-lactamase generation, efflux, and porin deficiency can be mentioned. AmpC β-lactamases and ESBLs can be identified in the clinical laboratories.

AmpC β-lactamases are capable of causing resistance to both narrow- and broad-spectrum cephalosporins, β-lactam/β-lactamase inhibitor combinations, and aztreonam (5). They can be chromosomally modified or plasmid-mediated.

The plasmid-mediated AmpC β-lactamases can hydrolyze the entire β-lactam antibiotics, except cefepime and carbapenems. The plasmid-mediated AmpC genes are originated from inducible chromosomal genes mobilized in different organisms. ACC, FOX, MOX, DHA, CMY, CIT, and EBC genes are among the well-known gene determinants (6-8). AmpC detection plays a crucial role in the clinical management of infections as it can offer epidemiological data. Nonetheless, there is no clinical and experimental standard guideline for detecting AmpC resistance in gram-negative bacilli which can lead to misleading data, especially in the phenotypic tests (9). Considering the scarce epidemiological data, this study is aimed to assess the frequency of ACC, FOX, MOX, DHA, CIT, and EBC genes in the AmpC-generating *E. coli* strains collected from patients with UTI. The second goal is the determination of genetic diversity in the AmpC-generating strains.

Methods

In this study, 230 *E. coli* isolates were sampled in 2017-2018 from patients suffering UTI symptoms who were admitted at Milad Hospital.

The written informed consent was obtained from all participants prior to participating in this study.

The isolates were detected by standard biochemical assays (10). The isolates were kept at -80 C in tryptic soy broth containing 15% glycerol for more molecular investigations.

Antimicrobial susceptibility tests

Antimicrobial susceptibility tests were conducted on *E. coli* isolates according to the E-test method (Liofilchem® MIC Test Strips*)*. The MICs (minimum inhibitory concentration) of eight antibiotics (ceftazidime, ceftriaxone, gentamicin, meropenem, ciprofloxacin, piperacillin/tazobactam, and trimethoprim/sulfamethoxazole) were examined against the *E. coli* isolate. The results were recorded based on M100-CLSI 2019 guideline and categorized into three groups: resistant, intermediate, and susceptible.

Detection of AmpC-positive isolates

The screening tests were carried out on isolated *E. coli* strains to detect AmpC-positive isolates by the use of cefoxitin (30 µg) disk. Subsequently, the suspected strains were further verified by an AmpC detection set (MAST DISCS™ID, UK). AmpC detection set has been validated for the identification of AmpC β-lactamase. *E. coli* ATCC 25922 and *Enterobacter cloacae* ATCC 13047 served as negative and positive controls, respectively, for the production of AmpC β-lactamase. The findings of AST and AmpC detection tests were entered in WHONET software.

Detection of ESBL-positive isolates

The ESBL-resistance suspected *E. coli* isolates were confirmed using the combination disc based on CLSI guidelines (CLSI 2019). In a typical procedure, a ceftazidime disc (CAZ) (30 µg) alone and one in combination with clavulanic acid (CAC) (30/10 µg) were applied to confirm the isolates. Both discs were placed on a Mueller Hinton Agar (MHA) plate followed by overnight incubation at 37°C. The distance between the centers of the discs was set to 20 mm. ESBL-positive involved $A \geq 5$ mm increase in zone diameter of either antimicrobial agents combined with clavulanic acid as compared to their zone upon the use of antimicrobial agents alone. *K. pneumoniae* ATCC 700603 and *E. coli* ATCC 25922 were utilized as ESBL-positive and negative controls, respectively.

Molecular detection of AmpC-generating genes

Genomic DNA extraction was carried out by the High Pure PCR Template Preparation kit (Roche, Germany). Detection of AmpC- β-lactamase and ESBL genes were conducted by PCR employing the specific oligonucleotide primers as presented in Table 1. PCR amplification was achieved in a Peqlab PCR thermal cycler with the PCR Master Mix (Ampliqon Inc., Denmark) following the manufacturer's guidelines. The PCR reactions were carried out on 25 µL volume. The master mix included 12 µL Master Mix Red (Ampliqon Inc, Denmark) and 1 µL target DNA. Forward and reverse primers were added as mentioned in Table 1. The reaction volume was increased to 25 µL by adding sterile distilled water. The addition of forward and reverse primers and temperature profiles was performed by the Queenan's protocol (11).

Multi-locus variable number of tandem repeats analysis (MLVA)

The complete *E. coli* DNA was provided from overnight cultured samples utilizing a High Pure PCR Template Preparation Kit (Roche, Germany). *E. coli* MLVA was conducted by the seven tandem sequence repeats (CVN001, CVN002, CVN003, CVN004, CVN007, CVN014, CVN015) according to Lindstedt et al. (12). Moreover, CVN016, CVN017, and a regularly-interspersed short palindromic repeat (CCR001), which have been reported to enhance the discriminatory power of the MLVA, were included with 10-loci *E. coli* MLVA (13). The applied primers were shown in Table 2. Repeats were amplified by PCR and assessed on 3% agarose. Here, the size of PCR products was simply assayed on agarose gel with no complication using 100 bp and 20 bp size markers (Bio-Rad Laboratories Inc.).

The following formula was employed to determine the VNTR (Variable-Number Tandem Repeat) repeat numbers of each locus; ((NPS−OF)/RL, in which PS= product size, OF=offset region (region of repeat-free sequence) and RL=length of one repeat unit (14).

Table 1. Primers for AmpC genes

Target gene		Primer	Tm °C	Product size	Ref.
FOX	F	AAC ATG GGG TAT CAG GGA GAT G	56	190	[11]
	R	CAA AGC GCG TAA CCG GAT TGG	59.7		
MOX	F	GCT GCT CAA GGA GCA CAG GAT	59.6	520	
	R	CAC ATT GAC ATA GGT GTG GTG C	56.6		
EBC	F	TCG GTA AAG CCG ATG TTG CGG	60.5	302	
	R	CTT CCA CTG CGG CTG CCA GTT	62.7		
ACC	F	AAC AGC CTC AGC AGC CGG TTA	61.2	346	
	R	TTC GCC GCA ATC ATC CCT AGC	60.1		
DHA	F	AAC TTT CAC AGG TGT GCT GGG T	59.3	405	
	R	CCG TAC GCA TAC TGG CTT TGC	59.2		
CIT	F	TGG CCA GAA CTG ACA GGC AAA	59.3	462	
	R	TTT CTC CTG AAC GTG GCT GGC	60.1		

Table 2. Primer sequences for MLVA technique

Target locus		Primer sequence	Tm °C	Ref.
CVN001	F	AACCGGCTGGGGCGAATCC	62.4	[12]
	R	GGCGGCGGTGTCAGCAAATC	62	
CVN002	F	AACCGTTATGAARGRAAGTCCT	53.8	
	R	TCGCCCAGTAAGTATGAAATC	52.4	
CVN003	F	AAAAATCCGGATGAGWTGGTC	54.9	
	R	TTGCGTTGTCAGTAATTTGTTCAG	55.3	
CVN004	F	MGCTGCGGCRCTGAAGAAGA	63.2	
	R	CCCGGCAGGCGAAGCATTGT	63.2	
CVN007	F	ACCGTGGCTCCAGYTGATTTC	57.8	
	R	ACCAGTGTTGCGCCCAGTGTC	61.1	
CVN014	F	TCCCCGCAATCAGCAAMACAAAGA	62.6	
	R	GCAGCRGGGACAACGGAAGC	63.8	
CVN015	F	TAGGCATAGCGCACAGACAGATAA	58.2	
	R	GTACCGCCGAACTTCAACACTC	58.6	
CVN016	F	GCTGCAGGAGAATGGGATGGTTTT	60.1	[13]
	R	GGTGAGGTGTCCGAGTGGCTGAAG	63.4	
CVN017	F	GCAATCACCGCCGCAATCTGTT	61.6	
	R	CGCCGCCGAAGCAAATCTC	59.8	
CCR001	F	CTCAGGGAAAAGGGAAGACACTAC	57	
	R	TTGCACTGAACACCGAATACG	56.2	

Results

Among 230 uropathogenic *E. coli* isolates, 87 samples were from men, while the remaining 142 were sampled from women.

The patients' age varied from one to 93 years (average: 58.81±25.07).

Antimicrobial susceptibility results

In this study, *E. coli* isolates revealed high susceptibility to meropenem 96.08% (221/230) and piperacillin-tazobactam 90.43% (208/230) followed by gentamicin 66.54% (153/230), ceftazidime 50% (115/230), ciprofloxacin 48.26% (111/230), ceftriaxone 41.74% (96/230), trimethoprim/sulfamethoxazole 39.56% (91/230). All *E. coli* isolates were resistant to amoxicillin-clavulanate.

Phenotypic detection of AmpC- and ESBL-producing isolates

Thirty-eight AmpC-positive E. coli isolates were determined by cefoxitin (30 µg) among which, 35 isolates were verified by the AmpC detecting test (MASTDISCS™ID, UK). Among these 35 AmpC-positive *E. coli* isolates, nineteen samples coproduced AmpC and ESBL; while sixteen samples only generated AmpC.

Also, 40% (14/35) of AmpC-producing isolates showed resistance toward gentamicin. 65.7% (23/35) and 74.2% (26/35) of the studied isolates were resistant against trimethoprim-sulfamethoxazole and ciprofloxacin, respectively.

Molecular analysis of AmpC genes

As suggested by Figure 1, no MOX and ECC genes were detected in uropathogenic *E. coli* isolates, while, 73.6% of the tested samples contained CIT. FOX (FOX-1 to FOX-5), DHA (DH7wwwqA-1 and DHA-2) and EBC were detected in 10.5 %, 10.5% and 15.8% of AmpC-producing *E. coli* isolates, respectively. Four isolates had both CIT and EBC. CIT and FOX were simultaneously present in four isolates. Two isolates also possessed both CIT and DHA.

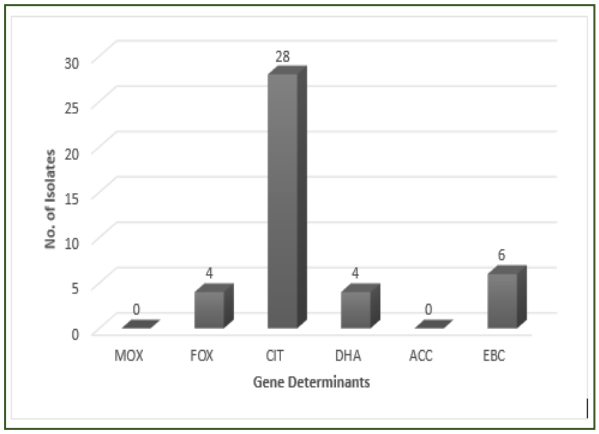

Figure 1. The frequency of AmpC genes in *Escherichia coli* isolates

Typing of AmpC-producing *E. coli* isolates by MLVA

A dendrogram was formed based on 10-loci using BioNumerics software ver.6.6 and the 31 MLVA types created six major clusters (labeled A to F) by 10-loci based according to on their genetic similarity (cutoff of 80%). Moreover, a singleton was detected in AmpC-producing *E. coli* isolate sampled from patients with UTI. In the present research, 30 distinct genotypes were categorized by 10- loci MLVA typing of 35 AmpC-producing *E. coli* isolates (Figure 2).

As mentioned in Table 3, the resistance profile exhibited no significant relationship with MLVA pattern and the AmpC or ESBL producing *E. coli*.

Table 3. Characterization of AmpC Positive *E. coli* Isolates

Isolate Code	MLVAProfile	Gene profile	ESBL	CRO	CIP	SXT	TZP	MEM	AMC	GEN	CAZ
452	A	CIT	+	R	S	R	R	S	R	R	R
85	A	CIT, FOX	+	R	R	R	R	S	R	S	R
89	A	CIT, EBC	+	R	R	R	R	S	R	S	R
267	A	CIT, EBC	+	R	R	R	R	S	R	R	R
478	A	EBC	+	R	R	R	I	S	R	R	R
408	B	CIT, FOX	+	R	R	R	R	S	R	S	R
460	B	CIT	+	R	R	S	I	R	R	R	R
367	B	CIT, EBC	+	R	R	R	I	I	R	R	R
409	B	CIT, EBC	+	R	R	R	R	S	R	R	R
419	B	CIT, FOX	+	R	R	R	R	S	R	R	R
421	B	CIT	+	S	S	S	S	S	R	S	S
463	B	CIT, FOX	+	R	S	R	S	S	R	R	R
405	B	CIT	+	R	R	S	S	S	R	R	R
47	B	CIT	+	R	R	R	S	S	R	R	R
498	B	DHA	+	R	R	S	R	R	R	R	R
474	C	EBC	-	R	R	S	S	S	R	S	S
74	C	CIT	-	S	S	S	S	S	R	S	S
485	C	CIT	+	R	R	R	R	S	R	R	R
434	C	CIT	-	R	R	S	S	S	R	S	R
427	C	CIT	+	R	R	R	S	S	R	S	R
396	D	DHA	-	R	R	S	R	S	R	S	S
59	D	CIT	-	S	R	R	S	S	R	S	S
448	D	CIT	-	S	R	S	S	S	R	R	R
410	D	CIT	-	R	R	R	S	S	R	S	R
206	D	DHA	+	R	R	R	R	S	R	S	R
27	D	CIT	-	S	R	R	S	S	R	S	I
263	D	DHA	+	R	S	R	S	S	R	S	R
40	D	CIT	-	S	S	S	S	S	R	S	S
179	E	CIT	-	S	S	S	S	S	R	S	S
18	E	CIT	-	S	S	R	S	S	R	S	S
46	F	CIT	+	R	R	R	R	S	R	S	R
22	F	CIT	-	R	S	S	I	S	R	S	R
64	F	CIT	+	R	R	R	S	S	R	R	R
174	F	CIT	+	R	R	R	S	S	R	S	R
180	Singleton	CIT	+	S	R	R	S	S	R	R	R

MLVA: Multi Locus VNTR Analysis, ESBL: Extended Spectrum Beta-Lactamase, CRO: Ceftriaxone, CIP: Ciprofloxacin, SXT: Trimethoprim/Sulfamethoxazole, TZP: Piperacillin-Tazobactam, MEM: Meropenem, AMC: *Amoxicillin*/clavulanic acid, GEN: Gentamicin, CAZ: Ceftazidime

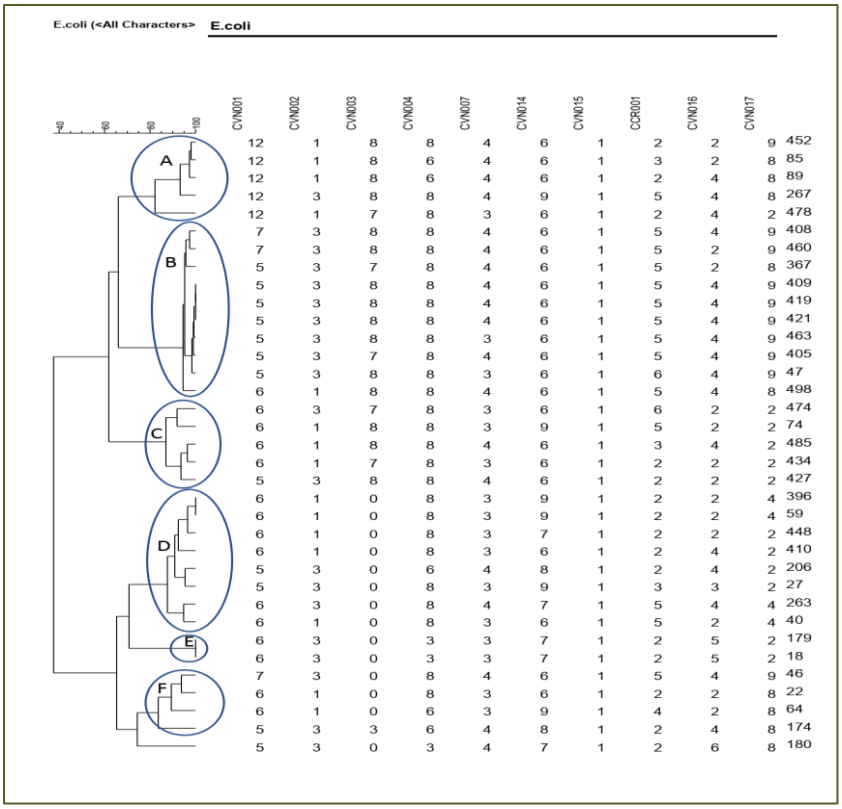

Figure 2. Clustering results of β -lactamase -producing *Escherichia coli* using 10-loci MLVA

Discussion

Plasmid-mediated AmpCs have clinical significance due to causing serious challenges in the treatment procedure, drug resistance surveillance, epidemiological process, and infection management programs. In this context, the determination of the AmpC-producing isolates can greatly contribute to both surveillance and infection control for preventing nosocomial outbreaks and treatment failure (15). β-lactamase resistance is more prevalent in *Enterobacteriaceae* compared to other gram-negative bacilli (16). Some factors can explain this condition: the extensive use of β-lactamase antibiotics, in particular, broad-spectrum cephalosporins (17). No strategy is currently available to detect and confirm AmpC β-lactamases in clinical microbiology labs (18). Nonetheless, the molecular evaluation of these isolates highlights its clinical detection. Thus, the molecular study of β-lactamases, in particular, AmpC type, is of crucial significance in terms of gene determinants and molecular epidemiology. Here, more than 54% (19/35) of *E. coli* isolates coproduced AmpC and ESBL and the remaining 46% of isolated *E. coli* showed only AmpC-producing features. It was previously reported that AmpC β-lactamase is capable of hiding the second resistance phenotypes like ESBL (19). As a result, the production of plasmid-mediated AmpC in an isolate may lead to false-negative results concerning ESBL. Based on the latest version of the CLSI guideline (M100-29), despite the unnecessity of the AmpC and ESBL tests, their results can be helpful in epidemiological studies or infection management. ESBL-suspected isolates, which have negative confirmatory results, should be tested for AmpC β-lactamases.

In this research, screening tests on 16 *E. coli* isolates showed positive AmpC β-lactamase findings but negative-ESBL results based on confirmation test (CAZ-CLA versus CAZ). 40% (14/35) of AmpC-producing isolates exhibited resistance upon exposure to gentamicin. Moreover, 65.7% (23/35) and 74.2% (26/35) isolates were resistant against trimethoprim-sulfamethoxazole and ciprofloxacin, respectively. While 51.74% of AmpC-negative isolates were resistant to ciprofloxacin.

Plasmids possessing AmpC-encoding genes and resistance genes against other antibiotics could warn the outbreak of nosocomial resistance in a treatment setting (20). Based on antimicrobial susceptibility assays, AmpC-positive isolates exhibited a great resistance to cephalosporins when compared with their AmpC-negative counterparts, some of them showed resistance to aminoglycosides and quinolones (Table 3). This highlights the significance of detecting AmpC-producing isolates. This important issue should be considered by clinicians when using cephalosporins. The detection of the AmpC-producing isolates could influence antimicrobial therapy.

The AmpC-encoding genes such as FOX, CIT, and EBC have been found in previous works (21, 22), while ACC class was not reported in any of them (21, 22). FOX family showed the highest prevalence in these works. While MOX class was not detected in Mansouri's research, Hoseini reported the presence of FOX and CIT. Other studies in different countries (e.g. Egypt) indicated 57.7% gram-negative AmpC-producing bacilli among which, 22 isolates carried MOX, FOX, and CIT families (21-23). In Egypt, Helmy (2014) reported the CMY homologs as the most dominant gene (86.9%); DHA (21.7%), FOX (17.3%), EBC (13%), and MOX (13%) had the next ranks (23). Helmy did not detect any members of the ACC class. In this research, however, CIT had the highest frequency (73.7%) among the AmpC-encoding families; EBC (15.8%), FOX (10.5%), and DHA (10.5%) allocated the subsequent ranks, respectively. No members of the ACC class were found. This enzyme could not be fully inhibited by cefoxitin (23, 24). Then et al. (25) found that screening to find cefoxitin resistance has a lower sensitivity toward the *ACC* family. Therefore, cefoxitin resistance could be exploited as a screening approach for the differentiation of ACC enzymes.

The geographical region and the duration of the research also can affect the prevalence and type of plasmid-mediated AmpCs (26, 27). Numerous clinical microbiological Laboratories fail in diagnosing the resistance mechanisms (28). In the cases of simultaneous various antibiotics-resistance mechanisms, the phenotypic detection of AmpC enzymes could be very difficult; thus, molecular methods are recommended (29). AmpC-producing isolates should be detected by laboratories as AmpC-β-lactamases have shown some associations with false cephalosporin susceptibility; therefore, the isolates with the potential to be falsely reported as ESBL-negative will be recognized (29, 30).

The MLVA method was also employed to determine the genotypes of AmpC-producing *E. coli*. Three isolates were lost, hence, the remaining thirty-five AmpC- producing *E. coli* isolates were evaluated to indicate their clonal association by MLVA approach. MLVA showed thirty-one distinct patterns of AmpC-producing *E. coli* isolates which can be classified into six clusters and one singleton (Cutoff 80%). The singleton and MLVA type E isolates may be transmitted to the hospital by the patients, visitors, or medical staff as they had not been established in the hospital. MLVA type B and MLVA type D showed the highest prevalence and MLVA type A, MLVA type C, and MLVA type F had the subsequent ranks, respectively. The mentioned MLVA types could be nosocomial infections; highlighting the necessity of evaluating MLVA types in the strains isolated from the patients and hospital staff. The two most prevalent MLVA types (Type A and B) exhibited two determinant genes of AmpC enzymes (CIT-EBC and CIT-FOX), whereas the other MLVA types only held one AmpC-encoding gene (Table 1). These results suggest the existence of the plasmids containing two genes in MLVA type A and B which can be transmitted by conjugating with other isolates. Thus, it is essential to identify these isolates and determine their transmission routes to establish a proper infection control strategy to prevent the release of resistance genes.

Conclusion

The determination of the frequency of AmpC-producing urinary *E. coli* isolates plays an important role in the antibiotic resistance and infection management programs in hospitals. The appearance of plasmid-mediated AmpC-producing *E. coli* can result in the spread of antibiotics resistance in clinical settings. This dissemination

can occur because of plasmid-mediated genes that may serve as the reservoir for antibiotic resistance. Therefore, the study of common gene determinants of AmpC resistance and its distribution in the bacterial species is of urgent necessity.

Conflict of Interest

The authors declared that they have no conflict of interest.

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Ethics

All procedures performed in this study was approved by the Ethics Committee of Islamic Azad University, Iran (Registration No.: IR.IAU.PS.REC.1397.306).

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