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Prevalence of AmpC β-lactamase in Clinical Isolates of *Escherichia coli*, *Klebsiella* spp., and *Proteus mirabilis* in a Tertiary Hospital in Tehran, Iran

Hiva Saffar,[1,*] Neda Asgari Niaraki,[2] Arash Ghahroudi Tali,[1] Zohre Baseri,[1] Alireza Abdollahi,[1] and Rouzbeh Yalfani[2]

[1]Department of Pathology and Laboratory Medicine, Tehran University of Medical Sciences, Tehran, IR Iran
[2]Department of Microbiology, Islamic Azad University, Varamin Pishva, IR Iran

[*]*Corresponding author*: Hiva Saffar, Department of Pathology and Laboratory Medicine, Tehran University of Medical Sciences, Tehran, IR Iran. Tel: +98-2184902187, Fax: +98-2188633078, E-mail: hsaffar@sina.tums.ac.ir

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Abstract

Background: *AmpC* β-lactamase confers resistance to a variety of β-lactam agents, and all plasmid-mediated *AmpC* genes are considered clinically significant. The transfer of the *AmpC* gene to plasmid has resulted in dissemination among the *Enterobacteriaceae* family, including *Escherichia coli*, *Klebsiella* spp., and *Proteus mirabilis*.
Objectives: The prevalence of plasmid-mediated *AmpC* genes was determined in isolates of *E. coli*, *Klebsiella* spp., and *P. mirabilis* with reduced susceptibility to cefoxitin or extended-spectrum cephalosporins by the multiplex PCR method.
Methods: A total of 310 consecutive non-duplicate isolates of *E. coli*, *Klebsiella* spp., and *P. mirabilis* were obtained from various clinical specimens. Isolates with positive screening test results were subjected to further molecular evaluation.
Results: Fifty isolates were positive on the screening test. Among them, positive PCR reactions were identified in 35/221 and 12/77 isolates of *E. coli* and *Klebsiella* spp., respectively, including 16 (34.0%) for *CIT* only, 7 (14.8%) for *DHA* only, and 24 (51.0%) for both *DHA* and *CIT*. No isolate was positive for *FOX* or *MOX*. No *Proteus* organism was positive for *AmpC* genes.
Conclusions: Currently, phenotypic tests are unable to accurately and reliably recognize plasmid-mediated *AmpC* β-lactamase-producing organisms. Although not possible for routine testing, clinical laboratories, especially in referral centers, should employ molecular testing for surveillance studies.

Keywords: PCR, *AmpC* β-lactamase, *Escherichia coli*, *Proteus mirabilis*, *Klebsiella*

1. Background

The production of β-lactamases, including *AmpC* β-lactamase and extended-spectrum β-lactamase (ESBL), is an important mechanism of drug resistance in Gram-negative bacteria (1, 2). *AmpC* β-lactamase confers resistance to a variety of β-lactam agents, including oxyimino-cephalosporins, 7-α-methoxycephalosporins (cephamycin), monobactam, and the β-lactam/β-lactam inhibitor combination (3-6).

AmpC β-lactamase production can be chromosomal or plasmid-mediated (5, 6). Chromosomal *AmpC* genes are expressed constitutively at low-level mutations in the *AmpC* promotor/attenuator of *Escherichia coli* and can result in constitutive resistance. Certain other *Enterobacteriaceae*, such as *Citrobacter* spp., *Serattia* spp., and *Enterobacter* spp., carry inducible *AmpC* genes, which can be induced by cefoxitin and imipenem (6). However, the transfer of the *AmpC* gene to plasmid has resulted in dissemination among the *Enterobacteriaceae*, including *E. coli*, *Klebsiella* spp., *Proteus mirabilis* , and *Salmonella* spp. (7). All plasmid-mediated *AmpC* β-lactamase (PMABL) genes are considered clinically significant (6). Organisms such as *E. coli* or *Klebsiella* spp. that carry *AmpC* genes in plasmid are multidrug-resistant (5) and can cause problems for optimal clinical management and infection-control programs.

Although the exact prevalence of PMABL remains unknown in most countries, it is estimated to be lower than that of ESBL (4); although with increased frequency (8). This is due to limitations in laboratory methods for detecting PMABL. Molecular tests are required for accurate determination and confirmation (4); however, many laboratories have limited resources and prefer to achieve high pretest probability before performing polymerase chain reaction (PCR) analyses (9). Reduced susceptibility to cefoxitin has been an indicator for probable *AmpC* production (3, 4, 7, 9, 10) but it is non-specific and can be mediated by alterations in outer membrane permeability (7). Some authors also suggest that isolates with non-susceptibility

to ESBL cephalosporins should be suspected to carry *AmpC* genes (9).

While plasmid-mediated *AmpC* causes clinical concern for the improvement of the clinical management of infection and infection-control measures (7), the available phenotypic tests are not convenient and lack sufficient sensitivity and specificity (1). Presently, there is no standard phenotypic test to identify these organisms. The current Clinical and Laboratory Standard Institute (CLSI) guidelines do not describe any method for the detection of *AmpC* β-lactamase (8).

2. Objectives

The present study was designed to determine the prevalence of plasmid-mediated *AmpC*-producing isolates of *E. coli*, *Klebsiella* spp. and *P. mirabilis* with reduced susceptibility to cefoxitin or extended-spectrum cephalosporins, using the multiplex PCR method.

3. Methods

A total of 310 consecutive non-duplicate isolates of *E. coli*, *Klebsiella* spp. and *P. mirabilis* obtained from various clinical specimens, including blood, wounds (pus), urine, and respiratory tract excretions, were received in the microbiology laboratory of Shariati Hospital, affiliated with Tehran University of Medical Sciences, from January 1, 2015, to April 1, 2015. The isolates were identified by conventional microbiological procedures. Table 1 summarizes the number and percentage of isolates from the different specimen types. Antibiotic susceptibility was determined using the Kirby-Bauer disk diffusion method according to CLSI guidelines (11).

Screening for *AmpC* β-lactamase production was done by placing a cefoxitin disk (30 μg; Rosco, Denmark) on Mueller-Hinton agar (2, 3, 8, 12). Isolates showing an inhibition zone diameter of < 18 mm were considered positive on the screening test and were subjected to further molecular evaluation.

Screening and confirmation of ESBL production was also performed by the disk diffusion method as per the CLSI, using ceftazidime 30 μg, cefotaxime 30 μg, and ceftriaxone 30 μg disks for screening, followed by combined disks of ceftazidime (30 μg)/ceftazidime-clavulanate (30 μg/10 μg) and cefotaxime (30 μg)/cefotaxime-clavulanate (30 μg/20 μg) for confirmation (Rosco, Denmark) (11). Isolates with positive screen tests that failed to be confirmed as ESBL by the combined disk method were also considered for further molecular testing.

The genomic DNA of the isolates with positive screening tests for *AmpC* was extracted using extraction kits (Bioneer, Seoul, Korea) according to the manufacturer's instructions, followed by multiplex PCR assays to detect four family-specific *AmpC* genes carried on plasmid, including *FOX*, *MOX*, *DHA*, and *CIT*. PCR was carried out with final volume of 25 μL (Eppendorf thermocycler; Germany). The primers (13) used for PCR assay are listed in Table 2.

The cycling conditions were as follows: initial DNA release and denaturation at 94°C for 3 min followed by 35 cycles at 94°C for 1 minute, 53°C for 1 minute, and 72°C for one minute, with a final extraction at 72°C for 5 minutes. The amplicons were analyzed by gel electrophoresis (agarose gel), stained with ethidium bromide, and visualized by UV transillumination. Standard *E. coli* (ATCC25922) and *Klebsiella pneumoniae* (ATCC 700603), or previously known positive and negative isolates of *E. coli*, were included in each run.

4. Results

A total of 310 isolates were identified as *E. coli* (n = 221), *Klebsiella* spp., (n = 77), or *P. mirabilis* (n = 12). Among these, 50 isolates that showed inhibition zones of < 18 mm on the cefoxitin disk and/or had positive ESBL screening tests but failed to be confirmed were all subjected to PCR analysis. Of these 50 isolates, 47 showed a positive reaction (94%), including 16 (34.04%) for *CIT* only, 7 (14.89%) for *DHA* only, and 24 (51.06%) for both *DHA* and *CIT*. No isolate was positive for *FOX* or *MOX* (Figures 1 and 2)

The frequency distribution of plasmid-mediated *AmpC* and ESBL-producing isolates with regard to different medical wards and clinical specimens is summarized in Tables 3 and 4, respectively. Medical wards were categorized as emergency medicine (general, oncology, obstetrics), internal medicine (nephrology, respiratory tract, general, hematology/oncology, bone marrow transplant, renal transplant, endocrinology, gastrointestinal tract, heart and coronary care unit, surgical (general, orthopedics, urology, neurosurgery, gynecology, head and neck), and intensive care (general, neonatal, neurosurgical).

The greatest number of *AmpC*-producing organisms were recovered from emergency departments (21/47); however, considering the number of submitted specimens, the intensive care units showed the highest prevalence of *AmpC*-producing organisms (19.23%), while surgical wards revealed the lowest frequency (11.11%). The specimen type most often positive for *AmpC*-producing organisms was urine (29/47). However, again considering the number of samples, the highest percentage came from wound cultures (8/38, 21.05%).

Positive PCR reactions were identified in 35/221 (15.83%) and 12/77 (15.58%) of the *E. coli* and *Klebsiella* spp. isolates, respectively. None of the *Proteus* organisms were positive

Table 1. Frequency of Isolates with Regard to Specimen Type

Isolate	Specimen Type						
	Urine	Blood	Body Fluid	Respiratory Excretion	Wound and Pus	Catheter	Total
E. coli	163/206 (79.13%)	8/14 (57.14%)	18/19 (94.74%)	11/21 (52.38%)	14/38 (36.84%)	7/12 (58.33%)	221/310 (71.29%)
Klebsiella spp.	33/206 (16.02%)	4/14 (28.57%)	1/19 (5.26%)	10/21 (47.62%)	24/38 (63.16%)	5/12 (41.67%)	77/310 (24.84%)
P. vulgaris	10/206 (4.85%)	2/4 (14.29%)	0/19 (0.00%)	0/21 (0.00%)	0/38 (0.00%)	0/12 (0.00%)	12/310 (3.87%)

Table 2. Primers Used for Characterization of *AmpC* β-lactamase

Target Gene	Primer	Sequence (5' to 3', as Synthesized)	Amplicon Size (bp)
MOX-1, MOX-2, CMY-1, CMY-8* to *CMY-11	MOXMF	GCT GCT CAA GGA GCA CAG GAT	520
	MOXMR	CAC ATT GAC ATA GGT GTG GTG C	
LAT-1* to *LAT-4, CMY-2* to *CMY-7, BIL-1	CITMF	TGG CCA GAA CTG ACA GGC AAA	462
	CITMR	TTT CTC CTG AAC GTG GCT GGC	
DHA-1, DHA-2	DHAMF	AAC TTT CAC AGG TGT GCT GGG T	405
	DHAMR	CCG TAC GCA TAC TGG CTT TGC	
FOX-1* to *FOX-5b	FOXMF	AAC ATG GGG TAT CAG GGA GAT G	190
	FOXMR	CAA AGC GCG TAA CCG GAT TGG	

Table 3. Frequency of *AmpC*- and ESBL-producing isolates with regard to different wards

Isolate	Hospital Ward				
	Emergency Department	Internal Medicine Wards	Surgery Wards	Intensive Care Units	Total
Plasmid-mediated *AmpC* producer	21/143 (14.68%)	12/79 (15.18%)	4/36 (11.11%)	10/52 (19.23%)	47/310
ESBL-producing organisms	68/143 (47.55%)	44/79 (55.69%)	16/36 (44.44%)	24/52 (46.15%)	152/310

Table 4. Frequency of *AmpC*- and ESBL-producing isolates with regard to different sample types

Isolate	Specimen Type						
	Urine	Blood	Body Fluid	Respiratory Excretion	Wound and Pus	Catheter	Total
Plasmid-mediated AmpC producer	29/206 (1.15%)	2/14 (14.28%)	3/19 (15.78)	4/21 (19.04%)	8/38 (21.05%)	1/12 (8.33%)	47/310
ESBL-producing organisms	116/206 (56.31%)	8/14 (57.14%)	12/19 (63.15%)	6/21 (28.57%)	10/38 (26.31%)	0/12 (0.00%)	152/310

for the *AmpC* gene. The frequencies of different genes are summarized in Table 5. Of the total of 310 organisms, 86/221, 64/77, and 2/12 isolates of *E. coli*, *Klebsiella* spp., and *P. vulgaris*, respectively, were positive for ESBL production. Twenty-nine and 7 isolates of *E. coli* and *Klebsiella* spp., respectively, were positive for both ESBL and *AmpC* production. All of the isolates were susceptible to imipenem.

5. Discussion

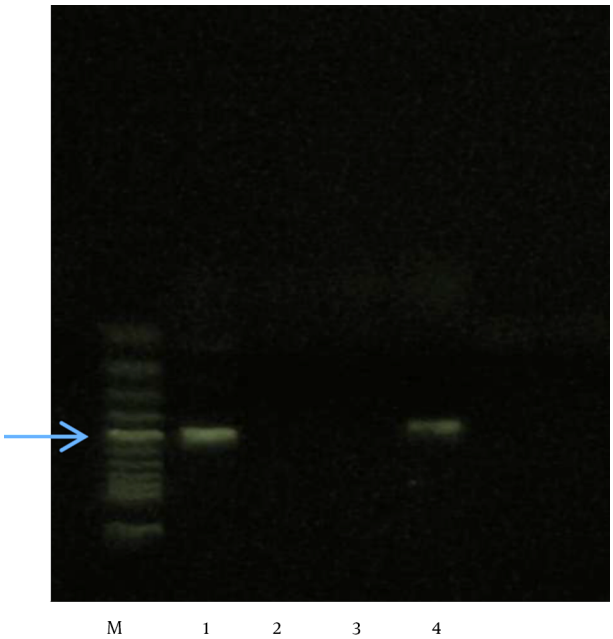
Plasmid-mediated *AmpC* β-lactamases are becoming more important clinically (9), and their recognition will be beneficial for both surveillance and for epidemiological measures and infection control (2), in order to avoid nosocomial outbreaks and treatment failures (6). Enzyme-extraction methods have been introduced for the phenotypic detection of *AmpC* activity (7); however, these are not suitable for routine clinical use (7). Various inhibitory-based methods, such as boronic acid compounds, cloxacillin, M3D (5, 7, 8), a modified double-disk test (5, 8, 14), and an *AmpC* disk test (5), have also been employed. However, as mentioned above, these methods have limited sensitivity and specificity (1). Moreover, they are unable to differentiate between chromosomal and plasmid-mediated *AmpC* producers (7). Therefore, molecular tests are still required for reliable identification of organisms carrying *AmpC* genes on plasmid (4).

Using multiplex PCR as a confirmatory test in the present study, the prevalence of plasmid-mediated *AmpC* genes in isolates of *E. coli*, *Klebsiella* spp., and *P. mirabilis* was 15.16%. A 2004 report from the United States documented that 7% - 8.5% of *Klebsiella* spp. and 4% of *E. coli* isolates contained plasmid-mediated *AmpC* genes (4, 15). In Asia, using different screening and confirmatory methods, the prevalence of plasmid-mediated *AmpC* is variable. A study

Table 5. Frequency of *AmpC* genotypes in clinical isolates

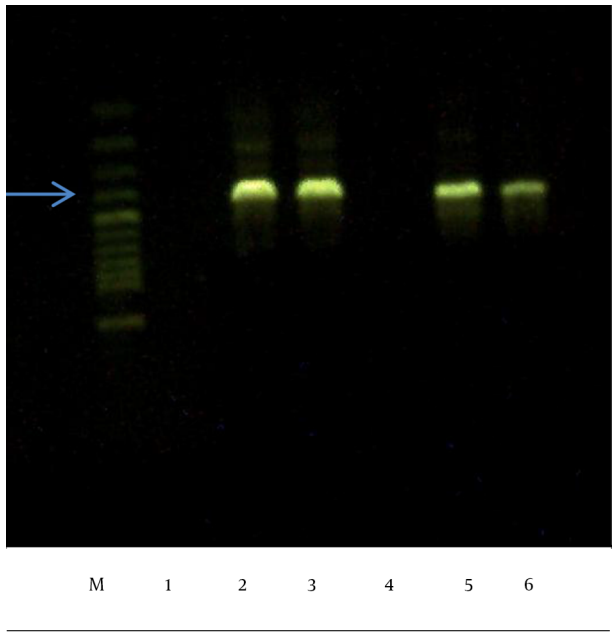
Isolate	Gene					
	CIT	DHA	*CIT-DHA*	*FOX*	*MOX*	Total
***Klebsiella* spp.**	3	4	5	0	0	12
E. coli	13	3	19	0	0	35

Figure 1. PCR amplification of *CIT* (462 bp)



Lane M: ladder; Lane 1: positive control; Lane 2: negative control; Lane 3: negative test isolate; Lane 4: positive test isolate.

Figure 2. PCR amplification of *DHA* (405 bp)



Lane M: ladder; Lane 1: negative control; Lane 2: positive control; Lane 3: positive test isolate; Lane 4: negative test isolate; Lanes 5 and 6: positive test isolates.

conducted in northern Iran reported a rate of 7.7% based on phenotypic methods (16). In another study in Tehran, 10.2% of *E. coli* isolates were *AmpC*-positive according to the PCR method (17). The lower prevalence compared to our results could be due to differences in PCR assay design, as the abovementioned studies did not apply primers for detecting *DHA* and *MOX* genes in the assay protocol (17). Peter-Getzlaff et al. (6) reported that 21/51 (41%) of *E. coli* isolates in their study were *AmpC* producers, among which 9 (19.6%) carried plasmid-mediated genes (6). In another study from India, 312/909 isolates were positive on the cefoxitin screening test, of which 114 (36.5%) were confirmed on PCR (4); however, the prevalence of plasmid-mediated *AmpC* was approximately 12.5%, which is rather similar to our findings.

In the present study, the prevalence rates of PMABL in *E. coli* and *Klebsiella* spp. isolates were 15.83% and 15.58% respectively, which are quite similar. In a study by Shafiq et al. (2) in Pakistan, of 55/103 positive-screen *AmpC*-producing isolates, 7.9% of *E. coli* and 12.37% of *Klebsiella* isolates carried *AmpC* genes. In another study in Pakistan (2, 18), the rates were 18% and 14% in isolates of *E. coli* and *Klebsiella*, respectively, which were closer to our findings. However, Fam et al. (10) reported that the prevalence of plasmid-mediated *AmpC* genes were significantly higher in *Klebsiella* isolates compared to *E. coli* (43.5% versus 17.7%) (10). It has been suggested that some PMABLs may be clinically more important (1). In a study by Black et al. (1), the inducible DHA-1 enzyme and the constitutively produced XMY-1-like enzymes were associated with 46% versus 14.3% mortality rates, respectively. However, the authors mentioned that due to the small size of the specimens, the significance of their findings should be further investigated (1).

In our study, genes belonging to the *CIT* family were more common in *E. coli* (n = 13) than in *Klebsiella spp.* (n = 3), while for *DHA*, the number was rather equal (n = 3

and n = 4, respectively). However, the most prevalent genotype in both types of isolates was *CIT-DHA*. In a study by Mohamudha et al. (5), *DHA* was more common in both *Klebsiella* spp. and *E. coli* isolates (46.7% and 38%, respectively). In another report, all *E. coli* isolates were positive for the *CIT* family (17) and no *FOX* was detected. Fam et al. (10) and Manoharan et al. (4) found that the *CIT* and CIT-*FOX* genes were more frequent. In other studies as well, no *AmpC* producers carried *FOX* or *MOX* (7, 10). The *ACC* gene also appears to be uncommon according to different studies (4, 5, 7, 10). The latter is important because using cefoxitin disks as a screening method is unable to detect isolates producing plasmid-encoded *AmpC* β-lactamase of the *ACC* family (6). Our study was limited due to a lack of primers able to amplify genes belonging to the *ACC* family, preventing us from evaluating the *ACC* genotype status in our isolates.

Ten out of 52 (19.23%) of the isolates from ICUs carried plasmid-mediated *AmpC* genes. This is unsurprising because patients can be compromised or exposed to previous cephalosporin therapy. However, the most specimens were isolated from the emergency departments (21/47, 44.6%), mainly due to referral cases admitted in our hospital. This needs special attention because it may indicate the spread of PMABL-producing strains in the community, as some other studies have suggested (2, 8). The prevalence of ESBL-producing isolates was 49% in our study, which is a little lower than the rates of 57% and 52% previously reported in Iran (16, 17).

Plasmid-mediated *AmpC* genes have the capacity to transfer and spread to other organisms within hospital settings, leading to nosocomial infections and treatment failures. To date, phenotypical tests are not able to accurately and reliably recognize PMABL organisms. Although not feasible for routine testing, clinical laboratories, especially in referral centers, should employ molecular testing for surveillance studies.

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Footnote

Authors' Contribution: Study concept and design: Hiva Saffar and Rouzbeh Yalfani; data analysis and interpretation: Neda Asgari Niaraki and Zohreh Baseri; statistical analysis: Neda Asgari Niaraki; drafting of the manuscript: Arash Ghahroudi Tali; revision of the manuscript for important intellectual content: Hiva Saffar, Alireza Abdollahi

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