

First Detection of 16S rRNA Methylase and blaCTX-M-15 Genes among *Klebsiella pneumoniae* Strains Isolated from Hospitalized Patients in Iran

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Abstract

Background: The increasing pattern of Multi-Drug Resistant (MDR) bacteria has limited number of therapeutic options especially for nosocomial isolates of *Klebsiella pneumoniae*. Therefore, in this study we aimed at molecular detecting of 16S rRNA methylase and bla_{CTX-M-15} among *K. pneumoniae* strains isolated from hospitalized patients in Mofid, Imam Hossein and Taleghani hospitals

Materials and Methods: This study was done on 110 *K.pneumoniae* isolates from hospitals in Tehran, Iran. Antibiotic susceptibility tests were carried out by Kirby-Bauer disc diffusion and broth microdilution methods according to CLSI guidelines. ESBL, AmpC and KPC enzymes were detected by Combined Disk Diffusion Test (CDDT) and Modified Hodge Test (MHT) methods. The *armA*, *rmtB*, *rmtC*, *rmtD* and bla_{CTX-M-15} genes were detected by PCR and sequencing techniques. Typing of antibiotic resistance isolates was carried out by PFGE technique.

Results: In this study, Fosfomycin, colistin and tigecycline were found to be more active than other antibiotics. Among the 110 *K. pneumoniae* strains, 60(54.5%), 33(30%) and 5(4.5%) were ESBL, Amp-C and KPC positive, respectively. The existence of bla_{CTX-M-15}, *armA* and *rmtC* was detected in 40(36.3%), 15 (13.6%) and 2 (1.8%), respectively. Of 15 representative armA-producing *K. pneumoniae* isolates analyzed by PFGE, 9 different pulsotypes (PF1–9) were identified by Dice coefficients of ≥90% similarity.

Conclusion: High-level aminoglycoside resistance in human pathogens result of due to 16S rRNA methylases is one of the serious concerns in Iran.

Keywords: *Klebsiella pneumoniae*; 16s rRNA methylase ; β-lactamases

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Introduction

Antibiotic resistance in bacterial pathogens is considered as a serious threat to hospitalized patients (1, 2). Therefore, the World Health Organization (WHO) called the year 2011 as the year of antibiotic resistance (3, 4). The increasing pattern of Multi-Drug Resistant (MDR) bacteria has created a therapeutic challenge especially for nosocomial isolates of *K. pneumoniae*. One of the resistance mechanisms in bacteria is beta-lactamase production. These enzymes are considered to act as the main defense mechanism of Gram-negative bacteria against antibiotics (5-7). According to Bush-Jacoby classification, β-lactamases are divided into four groups. Extended Spectrum β-

Lactamases (ESBLs) are one of the groups of enzymes produced by Gram-negative bacteria. These enzymes can hydrolyze aztreonam and cephalosporins but are inhibited by beta-lactamase inhibitors such as clavulanic acid. ESBL genes are often on various plasmids and many of them are conclusion of mutations in TEM (Temoneira) and SHV (Sulphydryl variable) genes specified by substitution of amino acid around the active site. Apart from SHV and TEM, *K. pneumoniae* isolates may additionally produce CTX-M (Cefotaximase-Munchen) enzyme (8, 9).

CTX-M β-lactamases are active against ceftriaxone and

and cefotaxime more than ceftazidime, although point mutations may enhance their activity against ceftazidime. Detection of ESBL is important in infection control processes and selecting appropriate antibacterial therapy (10). Carbapenem-hydrolyzing beta-lactamases are enzymes from Ambler classes A, B, and D that have been reported among *Enterobacteriaceae* globally. The prevalence of *K. pneumoniae* possessing the carbapenemase KPC is now a crucial problem in many medical centers in the North-Eastern USA and is being increasingly reported worldwide. The class C (Amp-C) beta-lactamases organize a group of enzymes widely distributed among *Enterobacteriaceae*. They preferentially deactivate narrow-spectrum cephalosporins (11). One of the important antibiotics that are still used widely for the treatment of serious bacterial infections are aminoglycosides. This agent often acts synergistic with beta-lactam antibiotics. These factors bind to A site in 30s ribosomal subunit of bacteria and interfere with protein synthesis (12). Most of aminoglycoside resistance mechanisms are due to enzyme inactivation by acetyltransferase enzyme and phosphodiesterase nucleotide transferase. Other known mechanisms of resistance to aminoglycosides, include defects in cell permeability, active transport and, rarely, nucleotide substitution in the target molecule (13). Since 2003, the aminoglycosides resistant strains produced by 16S rRNA Methylase plasmid genes have been reported (14). Up to now, types of 16s rRNA Methylase that have been identified are ArmA, RmtD, RmtB, RmtC, RmtD, RmtE, and NpmA. The armA is the most common among the *Enterobacteriaceae* family (15-17). NpmA is the only enzyme among them that causes the methylation of A1408, while others methylate the G1405 (18). Recently, the companionship of 16s rRNA Methylase such as rmtB and armA with KPC and ESBL producing pathogens have been reported that is very disturbing and their development restricts the treatment (12, 19). Further to the lack of sufficient information on the frequency and distribution pattern of these genes in Iran, additional studies can help physicians in prescribing more effective drugs and better treatment of infectious diseases caused by this group of bacteria. The aims of this study were detection of 16S rRNA methylase and *bla*_{CTX-M-15} genes among *K. pneumoniae* strains isolated from hospitalized patients in Mofid, Imam Hossein and Taleghani hospitals in Tehran, Iran.

Materials and Methods

From October 2011 to October 2013, 110 *K. pneumoniae* isolates were collected from hospitalized patients in Taleghani, Imam Hossein and Mofid Children Hospitals, Tehran, Iran. The isolates were

stored at -20 °C in trypticase soy broth medium containing 20% glycerol.

Antibiotic Susceptibility Testing

Antimicrobial susceptibility of *K. pneumoniae* isolates was performed by the Kirby-Bauer disk diffusion method (Mast Group, Merseyside, UK) on Mueller Hinton agar (Merck, Germany) and interpreted as suggested by Clinical and Laboratory Standards Institute (CLSI) or FDA breakpoints (Tigecycline) guidelines. Disks of Piperacillin (PIP, 100 µg), Piperacillin/Tazobactam (PTZ, 100/10 µg), Imipenem (IPM, 10 µg), Meropenem (MEM, 10 µg), Ertapenem (ETP, 10 µg), Doripenem (DOR, 10 µg), Ampicillin (AMP, 10 µg), Ceftazidime (CAZ, 30 µg), Cefotaxime (CTX, 30 µg), Cefepime (FEP, 30 µg), Ceftriaxone (CRO, 30 µg), Cefpodoxime (CPD, 30 µg), Aztreonam (ATM, 30 µg), Gentamicin (GEN, 10 µg), Amikacin (AK, 30 µg), Tetracycline (TE, 10 µg), Ciprofloxacin (CIP, 5 µg), Trimethoprim-sulfamethoxazole (TS, 2.5 µg), Fosfomycin/Trometamol (FOT, 200 µg) and Tigecycline (TGC, 15 µg) were used and *Escherichia coli* ATCC25922 was used as a control strain (20).

Minimum Inhibitory Concentration (MIC)

MIC was performed by broth microdilution method according to the guidelines of the CLSI. The imipenem, meropenem, Gentamicin, Amikacin, Ciprofloxacin, Cefepime, Ampicillin, Piperacillin/Tazobactam, cefotaxime, Ceftriaxone and ceftazidime (GLAXO England Co and Himedia) were used. *Escherichia coli* ATCC25922 was used as a control strain (20).

Phenotypic detection of ESBLs

ESBLs was tested for all isolates by Combination Disk Diffusion Test (CDDT) containing Cefotaxime (CTX) and Ceftazidime (CAZ) with Cefotaxime 30µg+CA 10µg and Ceftazidime 30µg+CA 10 µg per disc (Mast Group, Merseyside, UK) (21). The zone of inhibition was compared for the CTX, CAZ discs with that of the CAZ 30µg+ Clavulanic (CA) 10 µg and CTX 30µg+ CA 10 disc. The presence of ESBL in the test organism was confirmed with an increase in zone diameter of ≥5mm in the presence of clavulanic acid. *Escherichia coli* ATCC 25922 and *K. pneumoniae* ATCC700603 were used as negative and positive controls for ESBL production, respectively (15).

Phenotypic detection of KPC

KPC production was tested for all isolates by Modified Hodge Test. Briefly a 0.5 McFarland dilution of the *E. coli* ATCC 25922 in 5 ml of saline was prepared and diluted 1:10 by adding 0.5 ml of the 0.5 McFarland to 4.5 ml saline. A lawn of the 1:10 dilution of *E. coli* ATCC 25922 was streaked on

Mueller Hinton agar plate and was allowed to dry for 3–5 minutes. A 10 µg ertapenem disk was laid on the center of the test area. Test germs were streaked from the edge of the disk to the edge of the plate in a straight line. The plates were incubated at 37 °C

overnight. After incubation, the plate was examined for a clover leaf-type indentation at the intersection of the test organism and the *E. coli* ATCC25922, within the zone of inhibition of the carbapenem susceptibility disk (15).

Table 1. Primer sequences for CTX-M-15 and 16S rRNA methylase genes.

Primers name		primer sequences	Product size (bp)	Primer annealing	Reference
CTX-M-15	F	5'-CGTCGGTGACGATTTTAGCC-3'	823	59	This study
	R	5'-ACCGTCACGCTGTTGTTAGG-3'			
armA	F	5'-ATTCTGCCTATCCTAATTGG-3'	846	55	Yan 2004(23)
	R	5'-ACCTATACTTTATCGTCGTC-3'			
rmtB	F	5'-GCTTCTGCGGGCGATGTAA-3'	769	55	Doi 2007(13)
	R	5'-ATGCAATGCCGCTCGTAT-3'			
rmtC	F	5'-CGAAGAAGTAACAGCCAAAG-3'	711	57.5	Doi 2007(13)
	R	5'-ATCCCAACATCTCTCCACT-3'			
rmtD	F	5'-CGGCACGCGATTGGGAAGC-3'	401	56	
	R	5'-CGGAAACGATGCGACGAT-3'			

Phenotypic detection of Amp-C

The Amp-C detection was carried out using a kit developed by Mast Group Ltd that comprises three discs-labeled A, B and C. Disc A contained cefpodoxime and an Amp-C inducer, disc B contained cefpodoxime, an Amp-C inducer and an ESBL inhibitor and disc C contains cefpodoxime, an Amp-C inducer, an ESBL inhibitor, and an AmpC inhibitor. The presence of an ESBL inhibitor into the disc set (discs B and C) is included to detect Amp-C Production when the isolate is also co-producing ESBL. All isolates were tested employing Mueller Hinton agar (Merck, Germany). The discs were located on the inoculated agar surface and incubated overnight at 37 °C ± 1 °C for 18-24 hours. Following incubation, the inhibition zones were measured and zones with a ≥5mm increase in diameter around disc C compared with both discs A and B were considered positive for Amp-C production (22).

Detection of 16S rRNA methylase genes by PCR and sequencing methods

Total DNAs of the different bacterial isolates were extracted by the DNA extraction kit (Bioneer Company, Korea, Cat. number K-3032-2). The *armA*, *rmtB*, *rmtC*, *rmtD* and *bla*_{CTX-M-15} genes were identified by PCR using specific primers listed in Table1. PCR was carried out using the AccuPower® HotStart PCR PreMix (Bionner, Korea). Amplification was carried out under following thermal cycling condition: 5 minute

at 94 °C and 36 cycles of amplification consisting of 1 minute at 94 °C, 1 minute at 55 °C-59 °C, and 1 minute at 72 °C, with 5 minutes at 72 °C for the final extension. Next step was electrophoreses of PCR products on 1% agarose gels. The PCR purification kit (Bioneer Co., Korea) was used to purify PCR products and sequencing was performed by the Bioneer Company (Korea). The nucleotide sequences were analyzed by FinchTV software and compared with sequences in GenBank using the NCBI basic local alignment search tool (www.ncbi.nlm.nih.gov/BLAST).

Epidemiological typing

ArmA-positive *K. pneumoniae* isolates were subsequently typed by PFGE of XbaI-digested total DNA as described for *E. coli*. XbaI-macrorestriction patterns were interpreted according to the recommendations of Tenover et al. The GelCompare II software, (version 5.1; Applied Maths) was used to calculate the Dice similarity coefficients and generate dendrograms by cluster analysis with the unweighted-pair group method using average linkages. Pulsotype designations were assigned at the ≥90% profile similarity level.

Statistical methods

The Minitab Software (Student's t-test for paired samples) was used for data analysis. A *P* value of 0.05 was considered statistically significant.

Ethics Statement

All patients were accepted to participate in this study and it was approved by the Shahid Beheshti University of Medical Sciences Ethics Committee.

Results

Sixty-one strains were collected from Taleghani (55.45%), 41 from Mofid (37.27%) and eight (7.27%)

from Imam Hossein hospitals. Fosfomycin, colistin and tigecycline were more active than other antibiotics. Other results of antibiotic susceptibility of isolates are shown in Table 2 and MIC of 110 *K. pneumoniae* isolates is sorted in Table 3.

Table 3. Minimum Inhibitory Concentration of different antimicrobial agents against 110 *K. pneumoniae* isolates.

Antibiotic	Resistant No (%)	Intermediate No (%)	Sensitive No (%)
Gentamicin (10 µg)	46 (41.9%)	4 (3.6%)	60 (54.5%)
Amikacin (30 µg)	35 (31.9%)	4 (3.6%)	71 (64.5%)
Imipenem (10 µg)	25 (22.7%)	12 (10.9%)	73 (66.4%)
Meropenem (10 µg)	30 (27.3%)	10 (9%)	70 (63.7%)
Ertapenem (10 µg)	30 (27.3%)	10(9%)	70 (63.7%)
Doripenem (10 µg)	30 (27.3%)	10 (9%)	70 (63.7%)
Cefotaxime (30 µg)	67 (61%)	3 (2.7%)	40 (36.3%)
Ciprofloxacin(30 µg)	63 (57.4%)	7 (6.3%)	40 (36.3%)
Cefepime (30 µg)	47 (42.8%)	10 (9%)	53 (48.2%)
Ceftazidime (30 µg)	64 (58.2%)	6 (5.4%)	40 (36.4%)
Ceftriaxone (30 µg)	66 (60%)	4 (3.6%)	40 (36.4%)
Cefpodoxime (30µg)	74 (67.3%)	6 (5.4%)	30 (27.3%)
Ampicillin (10 µg)	83 (75.5%)	7 (6.3%)	20 (18.2%)
Tetracycline (10 µg)	60 (54.5%)	7 (6.3%)	43 (39.2%)
Fosfomycin/Trometamol (200 µg)	13 (11.8%)	12 (10.9%)	85 (77.3%)
Piperacillin/Tazobactam (100/10µg)	31 (28.2%)	11 (10.8%)	68 (61.9%)
Piperacillin (100 µg)	67 (60.9%)	3 (2.7%)	40 (36.4%)
Aztreonam (10 µg)	66 (60%)	8 (7.2%)	36 (32.8%)
Tigecycline (15µg)	7 (6.4%)	32 (29.1%)	71(64.5%)
Colistin (10µg)	0 (0%)	0 (0%)	110 (100%)

Among the 110 *K. pneumoniae* strains, 60(54.5%), 33(30%) and 5(4.5%) were ESBL, Amp-C and KPC positive, respectively. *bla*_{CTX-M-15}, *armA* and *rmtC* genes were detected in 40(36.3%), 15 (13.6%) and 2 (1.8%), respectively whereas none of them were positive for *rmtB* and *rmtD* genes (Figure 1).

Fifteen isolates were positive for *armA* and two were positive for *rmtC*. Coexistence of *rmtC* and *armA* genes was observed in one of the isolates. Only one of the 16S rRNA methylase genes (*armA* gene) was identified in the isolates susceptible to amikacin and

gentamicin. Fourteen isolates of *armA*-positive *K. pneumoniae* showed high-level resistance to amikacin and gentamicin ($p < 0.05$). Of 15 representative *armA*-producing *K. pneumoniae* isolates that were analyzed by PFGE, 9 different pulsotypes (PF1–9) were identified with Dice coefficients of $\geq 90\%$ similarity.

Nucleotide sequence accession numbers

The data of nucleotide sequence reported in this paper have been submitted to the GenBank sequence database and assigned accession numbers KF513160

for bla_{CTX-M-15}, KJ670494 for rmtC and KJ670493 for armA.

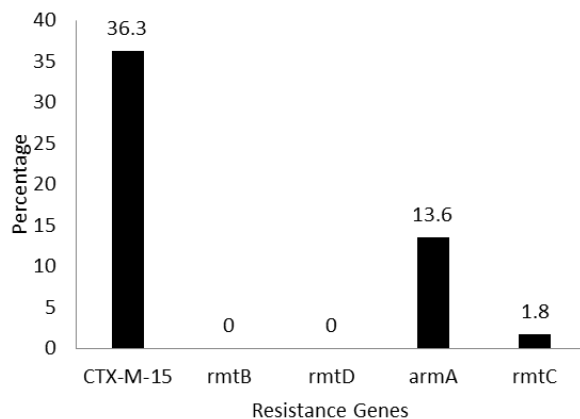


Figure 1. The prevalence of resistance genes in *K. pneumoniae* isolated from patients.

Discussion

Nosocomial infections caused by MDR *K. pneumoniae* isolates have become a serious problem in patients (24). In recent years, *K. pneumoniae* isolates have been developing resistance against fluoroquinolones, beta-lactams and aminoglycosides (10). In this study, the minimum rates of resistance in isolates were identified for imipenem, meropenem, ertapenem and Doripenem (27.3%), fosfomycin 13(11.8%), tigecycline 7 (6.4%), and Colistin 0 (0%). Nobari et al conducted a study in which 42 isolates (23.3%) were found resistant to meropenem, 29 isolates (16.1%) resistant to ertapenem, and 14 isolates (7.7%) resistant to imipenem(25).Also, Bina et al. reported the highest and lowest resistance against piperacillin (60.6%) and imipenem (13.9%), respectively(26). 16S rRNA methylases can consult high-level resistance to aminoglycosides (27). Among the 110 *K. pneumoniae* isolates, the prevalence of *armA* and *rmtC* were 13.6% and 1.8%, respectively, and *rmtB* and *rmtD* genes were not identified in the isolates. Fifteen isolates were positive for *armA* and two were positive for *rmtC*. Coexistence of *rmtC* and *armA* genes was observed in one of the isolates. Only one of the 16S rRNAmethylase genes (*armA* gene) was identified in the isolates susceptible to amikacin and gentamicin. Fourteen isolates of *armA*-positive *K. pneumoniae* showed high-level resistance to amikacin and gentamicin (MIC₅₀>64 µg/ml; MIC₉₀>128 µg/ml) and (MIC₅₀=128 µg/ml; MIC₉₀>256 µg/ml), respectively. Several *armA* or *rmtB*-positive strains have also been isolated in Asian and European countries, thus, there is a possibility of further dissemination (24). The *armA* gene was found to be more prevalent than *rmtB* gene in selected hospitals, which is contrary to a

report from China (12). ESBL and *Amp-C* enzymes were detected in fourteen and seven of *armA*-positive *K. pneumoniae* isolates, respectively. In our study, KPC enzymes were not identified in *armA*-positive *K. pneumoniae* isolates by Modified Hodge Test. In this study, 40 isolates of *K. pneumoniae* were positive for the bla_{CTX-M-15} gene. CTX-M-15 enzyme, which belongs to the CTX-M-1 group, is the most prevalent CTX-M allele with a worldwide distribution (28). In our study, 14 (2.7%) and 2 (1.8%) of *armA*- and *rmtC*-positive *K. pneumoniae* isolates harboured bla_{CTX-M-15} gene, respectively. However, in a study from Korea, only one 16S rRNA methylase-positive *K. pneumoniae* isolate harbored the bla_{CTX-M} allele (29). Bueno et al reported that three *K. pneumoniae* clinical strains with high-level resistance to aminoglycoside produced an *rmtD* group 16S rRNAmethyl transferase and five strains produced an *rmtG* gene (30).Yan et al reported that the *rmtB* and *armA* genes were detected in 58.2% and 40.0% of MDR *K. pneumoniae* isolates, respectively (31). Ma et al reported that among 235 isolates of *K. pneumoniae* which produce Extended-Spectrum β-Lactamase (ESBL), 102 (43.4%) were resistant to amikacin. The *rmtB* and *armA* alleles were individually detected in 37 and 44 of these 92 isolates, respectively. One isolate contained both *rmtB* and *armA* genes, also CTX-M-type β-Lactamase genes were found in all *rmtB*- or *armA*-bearing ESBL-producing *K. pneumoniae* isolates (32). High-level aminoglycoside resistance is of serious concern in Iran. If β-lactamases and 16S rRNA methylase-positive bacterial isolates disseminate extensively, the high level of pan-aminoglycoside and cephalosporins resistance will undoubtedly have an adverse effect on illness, costs of care and deaths of patients.

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Authors' contributions

AF, HA, ES and TS: analysis, interpretation of data and wrote the manuscript, AF and AS performed the microbiological and molecular studies. FF and HA advised the search. All authors approved the final manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest in this article.

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