Arch Clin Infect Dis. 2019 December; 14(6):e84372. doi: 10.5812/archcid.84372.

Published online 2020 January 4. **Research Article**

Assessment of 16srRNA Methylase Genes Among Non-ESBL and ESBL-Producing *Klebsiella pneumoniae* Isolates

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Received 2018 September 17; **Revised** 2019 December 15; **Accepted** 2019 December 17.

Abstract

The aim of our study was to investigate mechanisms of aminoglycoside resistance in extended-spectrum beta-lactamases (ESBL)-producing *Klebsiella pneumoniae* (*K. pneumoniae*) isolates from Iran. To this end, 154 clinical isolates of *K. pneumoniae* were collected from two hospitals in Ilam city, Iran. The Kirby-Bauer (agar diffusion) antibiotic testing method was used to determine the susceptibility pattern of the isolates against kanamycin, gentamicin, tobramycin, netilmicin and amikacin. Aminoglycoside acetyltransferases (*aac(3)-IIa*, aac(6')-Ib, and *aac(3)-Ia*), 16SrRNA methylase genes (*armA* and *rmtB*) and ESBL genes (bla_{TEM}, bla_{SHV}, and bla_{CTX-M}) were detected by PCR amplification. 59.1% (n = 91) of *K. pneumoniae* isolates were detected ESBL producers with the phenotypic test. Moreover, bla_{TEM}, bla_{SHV} and bla_{CTX-M} were detected in 83.5% (n=76), 52.7% (n=48) and 26.4% (n=24) of the ESBL-producing isolates, respectively. Among 52 resistant or intermediate isolates against aminoglycosides, the *aac(3)-IIa*, *aac(6')-Ib* and *rmtB* genes were detected in 55.8% (n = 29), 80.8% (n = 42) and 1.9% (n = 1) of the isolates, respectively; none of the isolates, however, had the *aac(3)-Ia* and *armA* genes. Therefore, the results showed the high prevalence of aminoglycosides resistance in the *K. pneumoniae* isolates. As observed, the acetyltransferase modifying enzymes (*aac* genes) played major roles in determining this resistance. However, the rate of 16srRNA methylase genes was extremely low in *K. pneumoniae*.

Keywords: *Klebsiella pneumonia*, Aminoglycosides, Antibiotic Susceptibility, Extended-Spectrum Beta-Lactamases, 16srRNA Methylase Genes

1. Background

The increase of antibiotic resistance in clinical pathogens is a global health-care problem. Description of antibiotic resistance determinants at genomic level serves an important role in understanding and controlling the spread of resistant pathogens. *Klebsiella pneumoniae* (*K. pneumoniae*) has become one of the most important bacteria, causing healthcare-associated infections. *K. pneumoniae* lives in natural environments and on mucosa of mammals, causing different opportunistic and hospital-acquired infections in humans. Extended-spectrum beta-lactamases (ESBLs) and other types of resistance are common in *K. pneumoniae* strains, as reported from different parts of Iran and Asian countries. The most dangerous consequence of the so-called drug-resistant strains is infections that mainly occur in debilitating patients, which cannot be treated with routine antibiotics (1).

Aminoglycosides are used for treatment of some serious infections of Gram-negative and Gram-positive bacteria, and are usually administered in combination with other antimicrobial agents. These antibiotics attach themselves to the 30S subunit of bacterial ribosomes and interfere in the synthesis of proteins. Mechanisms of resistance to aminoglycosides include production of modifying enzymes comprising aminoglycoside acetyltransferases (AACs), aminoglycoside nucleotidyltransferases (ANTs) and aminoglycoside phosphotransferases (APHs), modification of the bacterial ribosome, and the decrease

of drug accumulation by several mechanisms (2).

Among the resistance mechanisms mentioned above, enzymatic modification is the most common one for aminoglycoside resistance (3). ANTs and APHs are modifying enzymes affecting bisubstrate and facilitating transfer of γ-phosphate and nucleotide monophosphate from a nucleotide substrate to hydroxyl groups of aminoglycosides, respectively. However, Acetylated amino groups of AAC enzyme are derived from acetyl coenzyme A (acetyl-CoA) (4).

The most general modifying enzymes in *K. pneumoniae* are *aac(6')-I*, *aac(6')-II*, *ant(2")-I* and *aph(3')-VI* (5). Resistance to amikacin and tobramycin is induced by *aac(6')-I*, while gentamicin and tobramycin resistance is conferred by the *aac(6')-II* and *ant(2")-I* genes. In addition, the *aph (3')-VI* gene inactivates amikacin (6).

Modification of 16S rRNA by post-transcriptional methylation leads to the loss of affinity and high-level resistance to arbekacin, amikacin, kanamycin, tobramycin and gentamicin (7). Among more than 85 different aminoglycoside-modifying enzymes, only some enzymes including *ant(2")-I*, *aac(6')-I*, *aac(3)-I*, *aac(3)-II*, *aac(3)-III*, *aac(3)-IV* and *aac(3)-VI* appear to be the major cause of aminoglycoside resistance (8).

2. Objectives

The present present study aims to investigate aminoglycosides resistance patterns and presence of aminoglycoside modifying enzyme genes of clinical ESBL-producing *K. pneumoniae* isolates in Iran.

3. Methods

3.1. Bacterial Isolates

In this cross-sectional study, 154 clinical *K. pneumoniae* isolates were collected from patients admitted to various wards of two hospitals (Emam Khomeini and Shahid Mostafa, as two teaching and treatment hospitals in Ilam) from April to September, 2014. The isolates were stored in a trypticase soy broth containing 15% glycerol at -20°C for further experiments. The code of ethics was not mandatory at the time of the study. The whole research work was carried out on the bacteria according to standard methods.

3.2. Antibiotic Susceptibility Assay and Verification of ESBL-Production

Antibiotic susceptibility assay was carried out by the disk diffusion method on the Mueller-Hinton agar (Difco, Germany), based on the guidelines of the Clinical and Laboratory Standards Institute (CLSI) (9). Aminoglycoside antibiotics including amikacin, gentamicin, tobramycin, netilmicin and kanamycin were used for phenotypic screening of aminoglycoside resistant isolates. The ESBL-producing isolates were verified by the double disc diffusion method. Phenotypic screening of the ESBL-producing isolates was performed with cefotaxime-clavulanic acid (30/10 μg), cefotaxime (30 μg), ceftazidime-clavulanic acid (30/10 μg), and ceftazidime (30 μg) discs (Mast, England). An increase of $\geq$ 5 mm in the inhibition diameter zone of clavulanic acid-supplemented discs, as compared with the inhibition diameter zone of plain discs was consider as the ESBL-producer. *K. pneumoniae* ATCC 700603 was used for the ESBL positive control.

3.3. Detection of Aminoglycoside Resistance and ESBL Genes

Aminoglycoside resistant genes (*aac(3)-IIa, aac(6')-Ib, and aac(3)-Ia*) and 16S rRNA methylase (*armA* and *rmtB*), as well as ESBL-producing genes (bla_{TEM} (beta-lactamase temoneira), bla_{SHV} (beta-lactamase sulfhydryl variable) and $bla_{CTX\text{-}M}$ (beta-lactamase cefotaxime)) were detected by the polymerase chain reaction (PCR) method. To extract DNA, fresh bacterial colonies were suspended in 100 mL of sterile distilled water and boiled at 100°C for 10 min; then, they were stored at -20°C for 15 min. After centrifugation in 1000 rpm for 5 min, 3 mL of the supernatant was used for PCR assays with the primers shown in Table 1. Amplification of DNA was performed in a thermal cycler (Eppendorf, Germany). PCR processes and conditions are explained in Table 2.

Electrophoresis was performed in 1.5% agarose gel, which was stained with safe-stain and visualized in a gel document system. The QIA quick PCR purification kit (QIAGEN, Inc., Chatsworth, CA, USA) was used for purification of the PCR product of the *rmtB* gene. Moreover, the ABI PRISM 3100 Genetic analyzer (Applied Biosystems) was employed for sequencing the both strands. Then, the sequences were compared with the nucleotide database in GenBank at NCBI (www.ncbi.nlm.nih.gov/blast/).

4. Results

The *K. pneumoniae* isolates were collected from patients admitted to the intensive care unit, as well as to internal, neurosurgery, infectious diseases, neurology and general surgery wards. The isolates were obtained from different clinical specimens including urine (88.3%), trachea (4.5%), ulcer (6.5%), and sputum (0.6%), belonging to male (30.5%) and female (69.5%) patients. The susceptibility pattern showed that of the 154 isolates, 34%were resistant to aminoglycosides, 14.9% were gentamicin resistant,

Table 1. Primers Used for the PCR Detection of *aac(3)-IIa*, *aac(6')-Ib*, and *aac(3)-Ia*; 16S rRNA Methylases (*armA* and *rmtB*); and *blaTEM*, *blaSHV*, and *blaCTX-M* Genes

Gene	Primer	Amplified Size, bp	Annealing Temperature, °C	References
aac(3)-Ia	F: 5'-ATGGGCATCATTCGCACA-3'	484	55	(10)
	R: 5'-TCTCGGCTTGAACGAATTGT-3'			
aac(6')-Ib	F: 5'-ATGACTGAGCATGACCTTG-3'	524	53	(10)
	R: 5'-AAGGGTTAGGCAACACTG-3'			
aac(3)-IIa	F: 5'- CGGAAGGCAATAACGGAG-3'	749	55	(11)
	R: 5' -TCGAACAGGTAGCACTGAG-3'			
armA	F: 5'-AGGTTGTTTCCATTTCTGAG-3'	591	53	(12)
	R: 5'- TCTCTTCCATTCCCTTCTCC-3'			
rmtB	F: 5'-CCCAAACAGACCGTAGAGGC-3'	585	56	(12)
	R: 5'-CTCAAACTCGGCGGGCAAGC-3'			
blaTEM	F: 5'- ATGAGTATTCAACATTTCCGT-3'	861	53	In this study
	R: 5'- TTACCAATGCTTAATCAGTGA-3'			
blaSHV	F: 5'-GGGTTATTCTTATTTGTCGC-3'	927	53	In this study
	R: 5'- TTAGCGTTGCCAGTGCTC-3'			
blaCTX-M	F: 5'-ACGCTGTTGTTAGGAAGTG-3'in	759	55	In this study
	R: 5'-TTGAGGCTGGGTGAAGT-3'			

Table 2. PCR Conditions for the Detection of Aminoglycoside Genes

Number of Cycles	Cycle Name	Temperature and Time	
1	Primary denaturation	94°C for 5 minutes	
35	Denaturation	94°C for 45 seconds	
	Annealing	*aac(3)-Ia*	55°C for 30 seconds
		aac(6')-Ib	53°C for 30 seconds
		aac(3)-IIa	55°C for 30 seconds
		armA	53°C for 30 seconds
		rmtB	56°C for 30 seconds
	Primary extension	72°C for 1 minutes	
1	Final extension	72°C for 10 minutes	

and 8.4%, 20.1%, 7.8%, and 9.1% were resistant to tobramycin, kanamycin, netilmicin, and amikacin, respectively; only three isolates (1.9%) were resistant to all the aminoglycoside antibiotics. Resistance to single and multiple aminoglycosides and the rate of resistance genes in phenotypically resistant strains are described in Table 3. The results indicated a high rate of kanamycin (25, 16.23%) and amikacin (13, 8.44%) resistance in the urine isolates.

Among the isolates, 36.4% and 31.8% were resistant to ceftazidime and cefotaxime, respectively. Moreover, 59.1% (n = 91) of the isolates were identified as ESBL-producing *K. pneumoniae*. All the ESBL-producing *K. pneumoniae* isolates were checked for the ESBL genes, and 83.5% (n = 76), 52.7% (n = 48) and 26.4% (n = 24) were harbored as bla_{TEM}, bla_{SHV} and bla_{CTX-M}, respectively. Co-existence of bla_{TEM}-bla_{SHV}, bla_{TEM}-bla_{CTX-M} and bla_{SHV}-bla_{CTX-M} was detected in 20.9% (n = 19), 15.4% (n = 14) and 8.8% (n = 8) of the isolates, respectively, and 6.6% (n = 6) of the isolates had all the three genes.

The results showed that the *aac(3)-Ia* and *armA* genes were absent in all the resistant isolates. Moreover, the kanamycin resistant isolates (n = 31) showed the highest prevalence of *aac(6')-Ib* (n=23, 74.2%) and *aac(3)-IIa* (n = 18, 58.1%). Overall, 11.7% (n = 18) isolates showed resistance to the both kanamycin and gentamicin and among them, 61.1% (n = 11) had both *aac(6')-Ib* and *aac(3)-IIa*. All the aminoglycoside resistant isolates were collected from urine, which had both the *aac(6')-Ib* and *aac(3)-IIa* resistance genes; only one isolate with gentamicin resistance had *rmtB*.

5. Discussion

The outbreak of nosocomial multidrug-resistant Gram-negative bacteria has caused severe therapeutic problems. Various drug resistance genes such as aminoglycoside, sulfonamide, beta-lactamase and carbapene-

Table 3. Phenotypic and Genotypic Patterns of *K. pneumoniae* Against Aminoglycoside Antibiotics[a]

Antibiotics	Resistance Number, %	Presence of Genes					
		acc(3)-Ia	*acc(3)-IIa*	*aac(6')-Ib*	*IIa and Ib*	*armA*	*rmtB*
GM	23 (14.9)	0	16 (10.4)	19 (12.3)	14 (9.1)	0	1 (0.6)
K	31 (20.1)	0	18 (11.7)	23 (14.9)	14 (9.1)	0	0
AK	14 (9.1)	0	9 (5.8)	10 (6.5)	8 (5.2)	0	0
TOB	13 (8.4)	0	10 (6.5)	10 (6.5)	8 (5.2)	0	0
NET	12 (7.8)	0	9 (5.8)	10 (6.5)	8 (5.2)	0	0
GM and K	19 (12.3)	0	11 (7.1)	14 (9.1)	9 (5.8)	0	0
GM and AK	6 (3.9)	0	5 (3.2)	5 (3.2)	4 (2.6)	0	0
GM and TOB	12 (7.8)	0	8 (5.2)	9 (5.8)	7 (4.5)	0	0
GM and NET	11 (7.1)	0	8 (5.2)	9 (5.8)	7 (4.5)	0	0
GM, K, and AK	6 (3.9)	0	8 (5.2)	9 (5.8)	7 (4.5)	0	0
GM, K, AK, and TOB	4 (2.6)	0	3 (1.9)	3 (1.9)	2 (1.3)	0	0
GM, K, AK, TOB, and NET	3 (1.9)	0	2 (1.3)	2 (1.3)	1 (0.6)	0	0
K, AK, TOB, and NET	3 (1.9)	0	2 (1.3)	2 (1.3)	1 (0.6)	0	0
K, AK, and TOB	5 (3.2)	0	4 (2.6)	3 (1.9)	2 (1.3)	0	0
K, TOB, and NET	10 (6.5)	0	8 (5.2)	8 (5.2)	7 (4.5)	0	0
K and AK	13 (8.4)	0	9 (5.8)	8 (5.2)	7 (4.5)	0	0

Abbreviations: AK, amikacin; GM, gentamicin; K, kanamycin; NET, netilmicin; TOB, tobramycin.
[a]Values are expressed as No. (%).

mase genes have been studied in Gram-negative bacteria such as *K. pneumoniae* in Iran and other countries of the world (13-17). The highest antibiotic resistance rate in the present study belonged to ceftazidime whereas the lowest rate was observed for tobramycin. Phenotypically, 59.1% (n = 91) of the *K. pneumoniae* isolates were ESBL-producers. The prevalence of bla_{TEM} was higher than that of bla_{SHV} and bla_{CTX-M}. Resistance to aminoglycosides has been attributed to the acquisition of several aminoglycoside-modifying enzymes (18). Previous studies on mechanisms of aminoglycoside resistance have also indicated that aminoglycoside-modifying enzymes, including *aac*, *aph*, and *ant* are the primary mechanisms of resistance to these antibiotics (19). In this study, the highest aminoglycosides resistance occurred in kanamycin (n = 31, 20.1%) whereas the lowest one was found in netilmicin (n = 12, 7.8%). It should be noted that because of their narrow efficiency against the substrate and low specificity, these enzymes alone cannot induce resistance to all aminoglycosides. Since some enzymes modifying gentamicin had a weak activity against amikacin (19) and amikacin was developed from kanamycin, the access of various kanamycin modifying enzymes to their target was observed at a low prevalence among the members of Enterobacteriaceae (20).

PCR analysis disclosed that susceptible isolates had no resistance genes. In our study, 9.1% (n = 14) of the isolates were amikacin resistant. Among the isolates, nine contained the *aac(3)-IIa* gene, 10 contained the *aac(6')-Ib* gene, and eight had the both genes (Table 3). However, four amikacin resistant isolates had no aminoglycoside-modifying enzymes, as observed in the study. Therefore, it is needed to evaluate other aminoglycoside-modifying enzymes in future. The prevalence rate of amikacin resistance within ESBL-producing *K. pneumoniae* isolates from the United States, Latin America, Europe, the Western Pacific region and Canada was found to be 11.1%, 66.1%, 54.2%, 37.7% and 5.6%, respectively (21).

The *aac(3)-IIa* gene was primarily detected in R plasmids, delegating the *aac(3)-II* pattern phenotype (22). Reportedly, the rate of this gene is 85% in the *aac(3)-II* pattern phenotype (8, 23). The *aac(3)-VI* resistance pattern can cause resistance to gentamicin (3, 22). The *aac(3)-VI* gene is primarily detected from a conjugative plasmid in Enterobacter cloacae. This gene is rarely observed in clinical isolates (3). It can be deduced that there is 50% similarity between the amino acid sequences of the *aac(3)-VI* and *aac(3)-IIa* genes (24). According to Chinese reports, in pediatric

patients with clinical isolates, including *qnr* and *aac(6')-Ib-cr*, and ESBL-encoding genes were transferred together in ESBL- or AmpC-producing *Escherichia coli*. Since the identification of the *armA* gene in *K. pneumoniae* BM4536 strains from France in 2000 and the primary identification of the *rmtB* gene in *S. marcescens* S-95 strains from Japan in 2002, the two mentioned genes have been detected in *P. aeruginosa*, *A. baumannii* and Enterobacteriaceae in many areas (25-30).

In our study, the overall rate of 16S rRNA methylase genes (*armA* and *rmtB*) in the clinical *K. pneumoniae* isolates was 0% and 0.6%, respectively, which was lower than the previously declared rates in a Taiwanese research (0.9% and 0.3%) and a research conducted in Shanghai, China (3% and 1%) (29, 31). Our data, in agreement with other studies reporting on Enterobacteriaceae, showed that *rmtB* could be more prevalent than *armA*. Indeed, the *rmtB* gene is the most prevalent 16S rRNA methylase gene among Enterobacteriaceae isolates. In the present study, *armA* was not detected, which is compatible with other studies (29, 32). This report further highlights the low dissemination of 16S rRNA methylase genes among *K. pneumoniae*.

In Argentina, the 16S rRNA methyltransferase gene *rmtD2* was observed in 0.7% of Enterobacteriaceae. The incidence rate of the *rmtD2* gene was 13.3% in *Citrobacter* spp. and 9.3% in *Enterobacter* spp. Moreover, a correlation was reported between the presence of *rmtD2* and resistance to both amikacin and gentamicin (33).

In the study of Miro et al., among 330 various Enterobacteriaceae, 26.3%, 18%, 16.9%, 3.6% and 1.5% were resistant to kanamycin, gentamicin, tobramycin, netilmicin and amikacin, respectively, and 12.4% and 4.2% had *aac(3)-IIa* and *aac(6')-Ib* genes, respectively. Their results are consistent with our findings, showing the highest resistance to kanamycin (10). Furthermore, the study of carbapenemase and ESBL-producing Enterobacteriaceae (51 *E. coli* and 36 *K. pneumoniae*) isolates in Tunisian and Libyan hospitals showed that ESBL-producers and aminoglycoside resistance were 66.6% and > 60%, respectively, which are higher than those in our study (11). In Malaysia, 93 multidrug-resistant *K. pneumoniae* isolates had 91.3% and 67.7% of $bla_{CTX-M15}$ and *aacC2* genes, respectively (12).

In China, from 162 aminoglycoside resistant isolates of *K. pneumoniae*, 47.5% (n = 77) were ESBL-positive, and 30.2% (n = 49), 19.7% (n = 32), 11.1% (n = 18), and 6.2% (n = 10) had *aac(3)-IIa*, *aac(6)-Ib*, *armA* and *rmtB* genes, respectively. However, in the present study, the rate of 16S rRNA methylase genes (*armA* and *rmtB*) were higher (34). In another study, 17%, 37%, 68%, 53%, and 42% of *K. pneumoniae* isolates were resistant to ceftriaxone, ceftazidime, kanamycin, gentamicin and tobramycin, respectively. In addition, 66%, 11% and 13% of the isolates had *blaSHV*, *rmtB*, and *rmtC*, respectively. This is consistent with our results, showing the highest resistance to kanamycin; however, the range of bla_{SHV} and *rmtB* was reported to be higher than that in our study (35).

The present study revealed the high prevalence of *aac(3)-IIa* and *aac(6')-Ib* genes and the low prevalence of *aac(3)-Ia* and 16S rRNA methylase genes (*armA* and *rmtB*) among ESBL-producing *K. pneumoniae* isolates in Iran. Moreover, the rate of 16srRNA methylase genes was low in *K. pneumoniae*.

Acknowledgments

The authors would like to thank the Deputy of Research and Technology of Ilam University of Medical Sciences, Iran, for the funding and equipment provided to the study.

Footnotes

Authors' Contribution: Study concept and design: Naser Samadi, Iraj Pakzad, and Morovvat Taherikalani. Analysis and interpretation of data: Naser Samadi, Iraj Pakzad, and Morovvat Taherikalani. Drafting of manuscript: Iraj Pakzad, Hasan Hosainzadegan, and Naser Samadi. Critical revision of manuscript for important intellectual content: Mohammad Imanieini, Mohammad Rahbar, Karimolah Qasemigermi, and Reza Heidari. Statistical analysis: Iraj Pakzad and Naser Samadi.

Conflict of Interests: There are no conflicts of interest between authors and materials or hospitals used in the manuscript.

Ethical Approval: The project was approved by the Deputy of Research and Technology of Ilam University of Medical Sciences.

Funding/Support: The project was financially supported by the Research Deputy of Ilam University of Medical Sciences.

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