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Prevalence of aac(3')-IIa and aac(6')-Ib Genes Incidence Involved in Aminoglycoside Resistance in *Klebsiella pneumoniae* Isolated from Clinical Samples in Urmia Hospitals, Iran

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ABSTRACT

Extensive use of antimicrobials such as aminoglycosides has been associated with increasing antimicrobial resistance. The aim of present study was to investigate prevalence of aminoglycoside resistance and frequency of resistance genes aac(3)-IIa and aac(6')-Ib involved in resistance of *Klebsiella pneumoniae* to aminoglycosides. 212 clinical strains of *K. pneumoniae* isolated from Urmia hospitals collected from May 2013 to January 2014 were assessed in the study. The Kirby-Bauer method was used for screening of resistant isolates to Gentamicin, Amikacin, Kanamycin, Tobramycin and Netilmicin. The aac(6')-Ib and aac(3)-IIa genes were amplified by PCR method. Among 212 *K. pneumoniae* isolates evaluated, the most resistance rate observed against Gentamicin (26.9%). In total, 85 (40.1%) were resistant at least for one of aminoglycosides used. Among bacteria with resistance or intermediate susceptibility to aminoglycoside, 62(72/9%) and 63(74/1%) of isolates had acc(3)-IIa and aac(6')-Ib genes respectively. Our study showed that aminoglycoside resistance rate in klebsiella isolated from hospitals in northwest of Iran (Urmia) is relatively high and acetyl transferase modifying enzymes have the major role in resistance.

Keywords: *Klebsiella pneumoniae*, aminoglycoside, resistance gene.

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INTRODUCTION

Antibiotic resistance and increasing rate of resistant pathogens in hospitals is a major concern throughout the world. Understanding of antibiotic resistance determinants at genomic level has a critical role in controlling the spread of resistant pathogens. *K. pneumoniae* has become one of the most important leading causes of health care-associated infections[1-3]. Because of overuse of antimicrobials such as aminoglycosides, resistance to this class of antibiotics become prevalent and has affect its therapeutic efficacy[4]. Aminoglycosides attach to 30S subunit of bacterial ribosome and interfere with protein synthesis[1-3]. The mechanisms of resistance to aminoglycosides include production of modifying enzymes comprising aminoglycoside phosphotransferases (APHs), acetyltransferases (AACs), and nucleotidyl transferases (ANTs), modification of the bacterial ribosome and decreasing drug accumulation by several mechanisms[5, 6]. Among the resistance mechanisms mentioned above, enzymatic modification is the most common mechanism of aminoglycoside resistance[4]. APHs and ANTs are the bisubstrate modifying enzymes and facilitate transfer of γ-phosphate and nucleotide monophosphate from a nucleotide substrate to hydroxyl groups of aminoglycosides respectively but AAC enzymes acetylate amino groups derived from acetyl coenzyme A (acetyl-CoA)[7]. Genes encoding the most general modifying enzymes in *K. pneumoniae* include *aac (6')-I, aac (6')-II, ant (2")-I*, and *aph (3')-VI*. Resistance to amikacin and tobramycin is induced by *aac* (6′)-I while gentamicin and tobramycin resistance is conferred by *aac* (6′)-II and *ant* (2″)-I genes. Beside that *aph* (3′)-VI gene inactivates amikacin[8]. Modification of 16S rRNA(posttranscriptional) leading to loss of affinity and gives high-level resistance arbekacin, amikacin, kanamycin, tobramycin and gentamicin[9]. Among 85 different aminoglycoside-modifying enzymes, only the 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 major leading cause of aminoglycoside resistance [10]. The aim of this study was to investigate occurrence of aminoglycoside resistance and prevalence of resistance-modifying enzyme encoding genes, aac(6')-Ib and acc(3)-IIa in *K. pneumoniae* isolated from Clinical samples in Urmia hospitals (Iran). This study is the first evaluation about aac(6')-Ib and aac(3)-IIa genes frequency in *K. pneumoniae* and aminoglycoside resistance in north west of Iran(Urmia).

MATERIALS AND METHOD

Bacterial isolates

From May 2013 to January 2014, 212 isolates of *K. pneumoniae* were collected from *Urmia* teaching hospitals. The isolates were obtained from different clinical specimens including urine,

trachea, wound, sputum, blood and various secretions. Isolates were obtained from patient in intensive care unit, internal, neurosurgery, infectious, neurology and general surgery wards. Bacterial identification was done by conventional microbiological tests.

Antimicrobial susceptibility testing

Antimicrobial susceptibility assay was done by disk diffusion method on Mueller-Hinton agar, according to the guidelines of the Clinical and Laboratory Standards Institute (CLSI)(11). The following antimicrobial agents were used: amikacin(30µg), gentamicin(10µg), kanamycin(30µg), netilmicin(30µg) and tobramycin(10µg) (Mast diagnostic, Bottle Merseyside, U.K.). 85 aminoglycoside resistant isolates were subjected to further investigations

Screening for aminoglycoside resistance encoding genes

Detection of aminoglycoside resistance encoding genes aac(6')-Ib and aac(3)-IIa was performed by PCR method using specific oligonucleotide primers (Table 1). Fresh bacterial colonies were suspended in 400 mL of sterile distilled water and boiled at 100^{0C} for 10 min, then stored in -20^{0C} for 15 min. After centrifugation in 10000rpm for 5 min, 2 µL of supernatant was used for PCR assays. Multiplex PCR steps were followed : Initial denaturation at 95^{o}C for 4 min followed by 35 cycles of denaturation at 94 ^{o}C for 45 sec, annealing at 55 ^{o}C for 35 sec, and extension at 72 ^{o}C for 60 sec with a final extension at 72 ^{o}C for 10 min. Amplification of DNA was performed in thermal cycler (Eppendrof, Germany). PCR elongation times and temperature conditions were described in Table 1. PCR products were visualized by electrophoresis in 1.5% agarose gel [12-15].

Table 1: Primers used for PCR detection of aac(6')-Ib and acc(3)-IIa genes

Gene	Primer	size	Annealing temperature	Ref:
Aac(6')-Ib	F: 5′-ATG ACT GAG CAT GAC CTT G-3′ R: 5′-AAG GGT TAG GCA ACA CTG-3′	524bp	53°C	This study
Acc(3)-IIa	F: 5- CGGAAGGCAATAACGGAG- 3 R: 5 -TCGAACAGGTAGCACTGAG -3	749 bp	53°C	This study

Abbreviations; aac(6')-Ib: The aminoglycoside 6'-N-acetyltransferase type Ib, aac(3)-IIa: The 3-N-aminoglycoside acetyl transferase type IIa.

RESULTS AND DISCUSSION

Bacterial isolates

A total of 212 clinical isolates were collected from patient admitted to different wards of Urmia teaching hospitals, Iran. The isolates were obtained from different clinical specimens including urine, trachea, wound, sputum, blood and various secretions at the rate of 180(84.9%), 3(1.4%), 2(0.9 %), 20(9.5%),6(2.8 %) and1(0.5 %) respectively. The isolates were obtained from patients

in intensive care unit, internal, neurosurgery, infectious, neurology and general surgery wards. 45.8% of patients were males and 54.2% were females. Eighty-five isolates were resistant to aminoglycosides including gentamicin 26.9%, tobramycin 21.2%, kanamycin 21.2%, netilmicin 4.7% and amikacin 6.1% (Figure 1). Only 5 isolates (2.36%) were resistant to all of aminoglycoside antibiotics. The results indicated that high rate of antibiotic resistance were seen in strains isolated from urine to Gentamicin (57 isolates), Kenamycin and tobramycin (45 isolates) (Table 2).

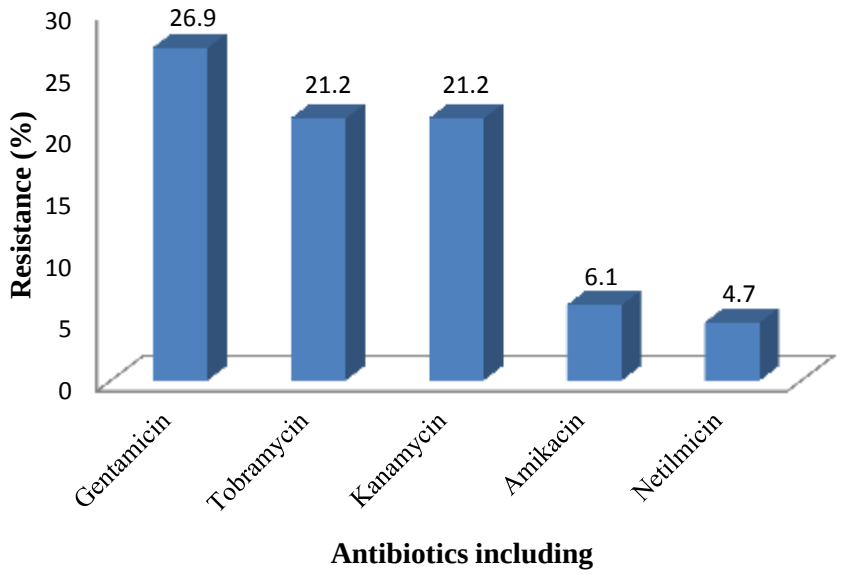

Figure 1: The antimicrobial resistance rate of 212 *K. pneumoniae* isolates to antibiotics: amikacin, gentamicin, tobramycin, netilmicin and kanamycin determined by the disk diffusion method

Table 2: Resistance to single and multiple antibiotics and resistance genes

Antibiotic	Percent of resistance*	Percent of Genes in resistant strain**		
		IIa	Ib	IIa & Ib
GM	26.9	80.7	72.2	71.93
K	21.2	62.2	68.9	48.89
AK	6.1	42.2	30.8	23.1
TOB	21.2	80	88.9	77.77
NET	4.7	60	60	50
GM&K	14.15	70	66.7	56.67
GM&AK	6.13	46.2	30.8	23.08
GM&TOB	17.45	86.5	89.2	86.5
GM&NET	4.72	60	60	50
GM&K&AK	5.66	41.7	25	16.67
GM&K&AK&TOB	2.83	33.3	50	33.3

GM&K&AK&TOB&NET	2.35	40	60	40
K&AK&TOB&NET	2.35	40	60	40
K & AK & TOB	2.83	33.3	50	33.3
K&TOB&NET	3.3	57.1	71.4	57.1
K&AK	5.66	41.7	25	16.67

* Percent of resistance to antibiotics is calculated from all samples (212 sample). ** Percent of Genes is calculated in resistant strain for mentioned antibiotic.(resistant samples isolates with screening method for antibiotics).

PCR for detection of aminoglycoside resistance genes

All of Eighty five resistant isolates to aminoglycosides were tested for detection of aac(6')-Ib and aac(3)-IIa in multiplex procedure. aac(6')-Ib was amplified by PCR with primers F: 5′-ATGACTGAGCATGAC CTT G-3′ and R: 5′-AAGGGTTAGGCAACACTG-3′ to produce a 524 bp product. aac(3')-IIa was amplified by PCR with primers F: 5′-CGGAAGGCAATAACGGAG-3′ and R: 5′- TCGAACAGGTAGCACTGAG -3′ to produce a 749 bp product. Our results indicated that Gentamicin resistant isolates had high frequency of resistance genes aac(6')-Ib (n=44,72.2%), aac(3)-IIa(n=46,80.7%). 80 %(n=36) and 88.9 % (n=40) of resistant isolates to Tobramycin had aac (3)-IIa and aac(6')-Ib genes respectively. In total 72.9 % (n=62) and 74.1 % (n=63) of resistance strains had aac (3)-IIa and aac(6')-Ib genes respectively and 58.8 % (n=50) of strains had both aac (3)-IIa and aac(6')-Ib genes.

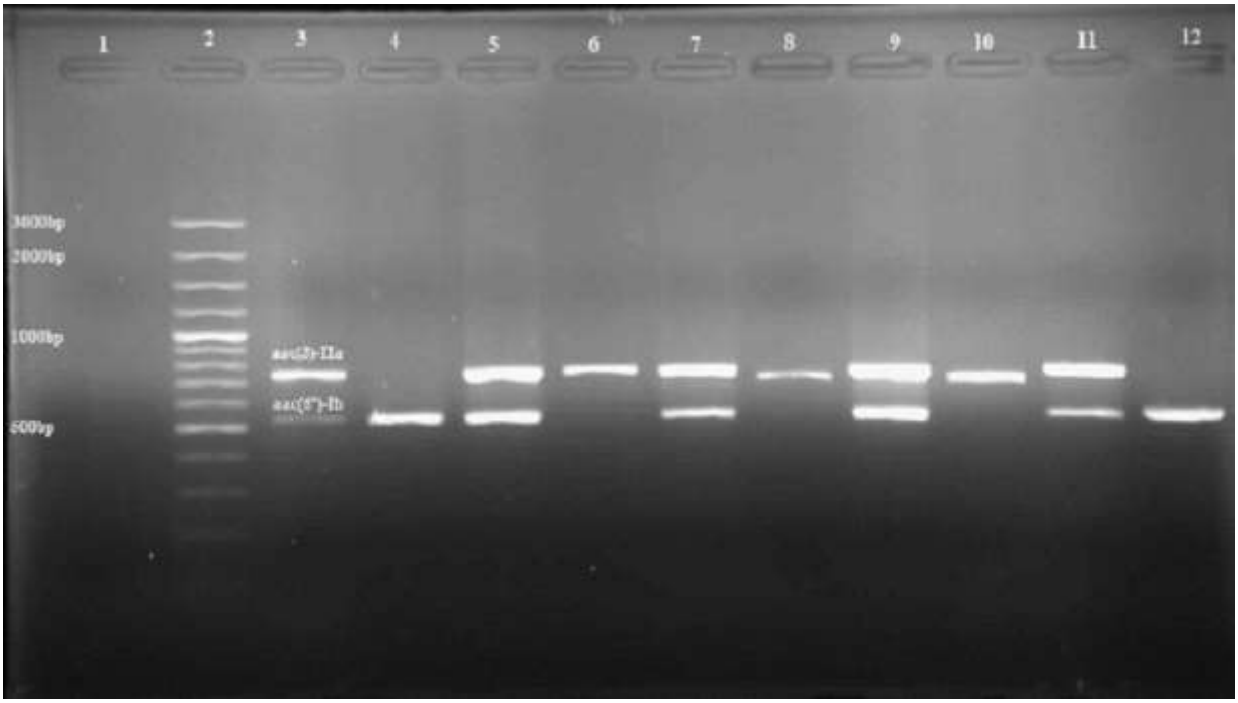

Figure 2: Electrophoresis of PCR product of resistant genes from *k. pneumoniae* resistant isolates on 1% agarose gel, L (Ladder 100 bp), column 4 of *aac(6')-Ib* gene(524bp), column 6

***aac(3')-IIa gene* (749bp). Column 3 is positive control and column 1 is negative control for two mentioned genes.**

The increasing prevalence of multidrug-resistant Gram-negative nosocomial bacteria has created severe therapeutic challenges. Resistance to aminoglycosides is also common, particularly among *K. pneumoniae*, due to the acquisition of several aminoglycoside-modifying enzymes. In current study, the highest antibiotic resistance occurred to Gentamicin (n=57, 26.9%) and the lowest was to Netilmicin (n=10, 4.7%). Previous studies about mechanisms of aminoglycoside resistance have indicated that aminoglycoside-modifying enzymes, including AAC, APH and ANT are among the primary mechanism of resistance[16]. It should be noted that, because of narrow efficiency against substrate and low specifity, these enzymes alone cannot induce resistance to all aminoglycosides. Because enzymes that modify gentamicin have weak activity against amikacin and because amikacin was developed from kanamycin for prevention the access of various kanamycin-modifying enzymes to their target points, amikacin resistance is observed at low prevalence among members of *Enterobacteriaceae*. In study conducted by Shah cheraghi et al., resistance to Gentamicin and Amikacin was 11% and 14% respectively[17] and in Willikie et al research, the resistance rate to Gentamicin, Amikacin, Tubramycin and Netilmicin was 48%, 0.8%, 39.8% and 13% respectively that are in agreement with the results of present study[18].

The results of Diaze PQ et al showed that the resistance rate to Gentamicin and Amikacin was 65% and 47% respectively[19] and Rastegar lari et al reported that 72%, 71% and 86% of *Klebsiella* isolates was resistant to Gentamicin, Amikacin and Tobramycin respectively[20]. In comparison of mentioned authors works about aminoglycoside resistance with the results of current study, we can concerned about differences in geographical positions, diagnostic methods, type of isolates and pattern of prescription of Antibiotics specially Aminoglycosides by physicians. In our study 13(6.1%) isolates were amikacin resistant that 6 (42.2%) isolates contained the *aac(3')-IIa* and 4 (30.8%) isolates contained *aac(6')-Ib* Genes. The gene *aac(3)-IIa* presents the AAC(3)-II aminoglycoside resistance pattern phenotype, The gene *aac(3)-IIa*, originally identified in R plasmids[21], accounts for 85% of the AAC(3)-II phenotype, according to reports[10, 22]. The AAC (3)-VI resistance pattern includes resistance to Gentamicin[5,23]. *aac(3)-VI* gene that occurs rarely among clinical isolates was initially cloned from a conjugative plasmid of *E. cloacae*[23]. The amino acid sequence that raised from this gene, shares 50% amino acid similarity with the *aac(3)-IIa* gene[24]. In Diaz et al study, the prevalence of aac(3)-IIa and aac(6')-Ib genes has been reported 36% and 69% respectively[(19)]. In Liang et al study, the positive rates of AEMs genes, such as *aac(3)-IIa* and *aac(6')-Ib* were 30.2% and 19.8% respectively (4). Among various studies, there

were increases and decreases between these genes prevalence, maybe because of difference in strains and resources those strains evaluated from them.

CONCLUSION

The present study revealed a high prevalence of aac (3)-IIa and aac(6')-Ib genes among *K. pneumoniae* isolates in Urmia. Resistance to aminoglycosides is affected by many factors, including amount of enzyme that modify the drug, penetration rate of antibiotic into bacterial site and efflux pumps. Our results showed the high prevalence of aminoglycoside resistance in *K. pneumoniae* isolates. The acetyl transferase modifying enzymes have the major role in resistance and there is a notable relationship between incidence of aminoglycoside resistance and presence of aminoglycoside resistance genes.

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REFERENCES

1. Mingeot-Leclercq MP, Glupczynski Y, T.PM. Aminoglycosides: activity and resistance. Antimicrob Agents Chemother. 1999;43(4):727-37.
2. Kotra LP, Haddad.J, Mobashery S. Aminoglycosides: perspectives on mechanisms of action and resistance and strategies to counter resistance. Antimicrob Agents Chemother. 2000;44(12):3249-56.
3. Fluit AC, Visser MR, Schmitz FJ. Molecular detection of antimicrobial resistance. Clin Microbiol Rev. 2001;14(4):836-71, table of contents.
4. Liang C, Xing B, Yang X, Fu Y, Feng Y, Zhang Y. Molecular epidemiology of aminoglycosides resistance on Klebsiella pneumonia in a hospital in China. International journal of clinical and experimental medicine. 2015;8(1):1381-5.
5. Shaw KJ, Rather PN, Hare RS, Miller GH. Molecular genetics of aminoglycoside resistance genes and familial relationships of the aminoglycoside-modifying enzymes. Microbiol Rev. 1993;57(1):138-63.
6. Yamane K, Wachino J, Doi Y, Kurokawa H, Arakawa Y. Global spread of multiple aminoglycoside resistance genes. Emerg Infect Dis. 2005;11(6):951-3.
7. Toth M, Frase H, Chow JW, Smith C, Vakulenko SB. Mutant APH(2")-IIa enzymes with increased activity against amikacin and isepamicin. Antimicrob Agents Chemother. 2010;54(4):1590-5.

8. Poole K. Aminoglycoside resistance in Pseudomonas aeruginosa. Antimicrob Agents Chemother. 2005;49(2):479-88.
9. Yu FY, Yao D, Pan JY, Chen C, Qin ZQ, Parsons C, et al. High prevalence of plasmid-mediated 16S rRNA methylase gene rmtB among Escherichia coli clinical isolates from a Chinese teaching hospital. BMC Infect Dis. 2010;10:184.
10. Vakulenko SB, Mobashery S. Versatility of aminoglycosides and prospects for their future. Clin Microbiol Rev. 2003;16(3):430-50.
11. Wayne PA. Performance standards for antimicrobial susceptibility testing. Twenty-third informational supplement. M100-S23. Clinical and Laboratory Standards Institute. January 2013;33(1).
12. Doi Y, Wachino J, Yamane K, Shibata N, Yagi T, Shibayama K, et al. Spread of novel aminoglycoside resistance gene aac(6')-Iad among Acinetobacter clinical isolates in Japan. Antimicrob Agents Chemother. 2004;48(6):2075-80.
13. Ma L, Lin CJ, Chen JH, Fung CP, Chang FY, Lai YK, et al. Widespread dissemination of aminoglycoside resistance genes armA and rmtB in Klebsiella pneumoniae isolates in Taiwan producing CTX-M-type extended-spectrum beta-lactamases. Antimicrob Agents Chemother. 2009;53(1):104-11.
14. Bogaerts P, Galimand M, Bauraing C, Deplano A, Vanhoof R, De Mendonca R, et al. Emergence of ArmA and RmtB aminoglycoside resistance 16S rRNA methylases in Belgium. Journal of antimicrobial chemotherapy. 2007;59(3):459-64.
15. Yu F, Wang L, Pan J, Yao D, Chen C, Zhu T, et al. Prevalence of 16S rRNA methylase genes in Klebsiella pneumoniae isolates from a Chinese teaching hospital: coexistence of rmtB and armA genes in the same isolate. Diagn Microbiol Infect Dis. 2009;64(1):57-63.
16. Samadi N, Pakzad I, Monadi Sefidan A, Hosainzadegan H, Tanomand A. Study of aminoglycoside resistance genes in enter oC oCcus and salmonella strains isolated from ilam and milad hospitals, iran. Jundishapur journal of microbiology. 2015;8(4):e18102.
17. Ramirez MS, Tolmasky ME. Aminoglycoside modifying enzymes. Drug resistance updates : reviews and commentaries in antimicrobial and anticancer chemotherapy. 2010;13(6):151-71.
18. Shah cheraghi F, Moazzi H. Extended-spectrum β-lactamase (ESBL) in klebsiella pneumoniae isolated from clinical samples of Tehran hospitales. Journal of Infectious Diseases Ass oCiation Specialists of Infectious Disease 2008;39:57-60.
19. Willke A, Tural D. Susceptibility of gram-negative bacilli to aminoglycosides. Mikrobiyol Bul. 1987;21(2):98-102.

20. Díaz PQ, Bello HT, Domínguez MY, Trabal NF, Mella SM, Zemelman RZ. Resistance to gentamicin, amikacin and ciprofloxacin among nos oComial isolates of klebsiella pneumoniae subspecie pneumoniae producing extended spectrum beta-lactamases. Rev Med Chil. 2004;132(10):1173-8.
21. Rastegar Lari A, Azimi L, Rahbar M, Fallah F, Alaghehbandan R. Phenotypic detection of Klebsiella pneumoniae carbapenemase among burns patients: First report from Iran. Burns. 2013;39(1):174-6.
22. Allmansberger Rudolf, Bräu Barbara, Piepersberg Wolfgang. Genes for gentamicin-(3)-N-acetyl-transferases III and IV. Molecular and General Genetics MGG. 1985;198(3):514-20.
23. Miller GH, Sabatelli FJ, Hare RS, Glupczynski Y, Mackey P, Shlaes D, et al. The most frequent aminoglycoside resistance mechanisms--changes with time and geographic area: a reflection of aminoglycoside usage patterns? Aminoglycoside Resistance Study Groups. Clin Infect Dis. 1997;24 46-62.
24. Rather PN, Mann PA, Mierzwa R, Hare RS, Miller GH, Shaw KJ. Analysis of the aac(3)-VIa gene encoding a novel 3-N-acetyltransferase. Antimicrob Agents Chemother. 1993;37(10):2074-9.
25. Wright GD, Berghuis AM, Mobashery S. Aminoglycoside antibiotics. Structures, functions, and resistance. Adv Exp Med Biol. 1998;456:27-69.