

Original Article

Prevalence of Genes Encoding Aminoglycoside Modifying Enzymes in Clinical Isolates of *Klebsiella Pneumoniae* in the Hospitals of Borujerd

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Background and Aims: Given the importance of aminoglycoside resistance in nosocomial and community infections caused by bacterial pathogens such as *Klebsiella pneumoniae* (*K. pneumoniae*), the aim of this study was to determine the frequency of *aac (6')- Ib* and *aac (3)- IIa*, the genes encoding aminoglycoside modifying enzymes involved in aminoglycoside resistance.

Material and Methods: A total of 100 *K. pneumonia* isolates were collected from hospitalized patients from April to September 2015 in Borujerd hospitals. Conventional microbiological tests were carried out to detect and confirm *K. pneumonia* isolates. Antibiotic susceptibility of isolates was detected by disk diffusion methods. The presence of the *aac(6')-Ib* and *aac(3)-IIa* genes which encode aminoglycoside modifying enzymes was determined by polymerase chain reaction.

Results: Among 100 *K. pneumonia* isolates, 34% showed resistance to gentamicin and 21% to amikacin. Resistance to both gentamicin and amikacin was detected in 18% of the isolates. Multi-resistance phenotypes were detected in 71% of the isolates. The *aac (3)-IIa* and *aac(6')-Ib* genes were found in 71% (n=24) and 5.8% (n=2) of aminoglycoside resistant isolates, respectively. Simultaneous carriage of *aac (3)-IIa* and *aac(6')-Ib* was detected in 64% (n=22) of the aminoglycoside resistant isolates.

Conclusions: The results of this study showed the presence of *aac (3)-IIa* genes in more than 70% of the aminoglycosides resistant *K. pneumoniae* strains; this may be due to the transmission of this gene through mobile genetic elements that create a high risk of rapid spread of these genes in hospitals.

Introduction

Nowadays, the opportunistic pathogen, *Klebsiella pneumoniae* (*K. pneumoniae*) is considered as the main bacteria involved in nosocomial infections [1]. Infections caused by *K. pneumoniae* including urinary tract infections, septicemia, pneumonia and intra-abdominal infections in hospitalized patients are responsible for a high rate of mortality [2]. Antibiotic resistance has always been regarded as a serious problem for human health by affecting patients in hospitals around the world. [3]. Considering that *K. pneumoniae* causes disease in patients with a weakened immune system thereby increasing the rate of resistance to antibiotics in bacteria, it can be a serious threat for health care settings [2, 3]. Therefore, the determination of antibiotic resistance patterns in common pathogenic bacteria aimed at conducting empirical and specific therapy against a particular pathogen is important [4]. One of the most common antibiotic resistances in gram negative bacteria such as *K. pneumoniae* is aminoglycoside resistance [5]. Antibiotics such as streptomycin, gentamicin, tobramycin, amikacin, and kanamycin are known as the family of aminoglycoside antibiotics. All aminoglycosides inhibit protein synthesis in bacteria by binding to the 30S subunit of 16S rRNA and thus show bactericidal effect [4, 5]. Production of aminoglycoside modifying enzymes (AMEs) is the most common type of resistance to aminoglycosides which results in a high level of bacterial resistance [6, 7]. The common encoding genes for aminoglycoside modifying

enzymes in the Enterobacteriaceae family are *aac (3)- II* and *aac (6')- Ib* [8]. N-acetyltransferases *aac (6)* and *aac (3)* are most frequently found in clinical isolates in Iran and certain other countries [3,8-10]. Aminoglycoside 6'-N-acetyltransferases of type Ib [*aac(6')-Ib*] are widespread among members of the Enterobacteriaceae family including *K. pneumoniae* [11, 12]. There are limited studies on the AMEs in *K. pneumoniae* in Iran, Considering the role of *K. pneumoniae* in nosocomial infections as well as the importance of identifying resistance to aminoglycosides particularly resistances caused by mobile genetic elements, the aim of this study was to determine the two genes *acc (6')- Ib* and *acc(3)- II* in clinical isolates of *K. pneumoniae* from patients admitted to hospitals in the city of Borujerd in the west of Iran.

Materials and Methods

K. pneumoniae isolates

In a cross-sectional study, a total of 100 *K. pneumoniae* strains were isolated from clinical samples (blood, wound, urine and trachea) of patients in the hospitals of Borujerd from April to September 2015. *K. pneumoniae* isolates were identified and confirmed by conventional microbiological tests: Gram staining and standard biochemical tests such as lactose fermentation, indole test, motility, citrate and urease test, lysine decarboxylase and Methyl Red Voges Proskauer (MR-VP).

Antimicrobial susceptibility testing

The antimicrobial susceptibility of *K. pneumoniae*

isolates to gentamicin (10 µg), amikacin (30 µg), ampicillin (10 µg), cephalothin (30 µg) aztreonam (30 µg) ceftriaxone (30 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), imipenem (10 µg), and nalidixic acid (30 µg) disks (Rosco company, Denmark) was determined by disk diffusion method, according to clinical and laboratory standards institute guidelines (9). *K. pneumoniae* ATCC 13883 was used as the control strain for disk susceptibility testing.

Detection of *aac(6')-Ib*, *aac(3)-II* genes

Genomic DNA was extracted from all aminoglycoside resistance *K. pneumoniae* isolates using a DNA extraction kit (Cinapure DNA, CinaClon, Iran) according to the manufacturer's instructions. Amplification of the genes encoding aminoglycoside modifying enzymes, *aac(6')-Ib*, and *aac(3)-II*, was performed using specific primers (Table 1) by polymerase chain reaction (PCR). The PCR mixture was prepared in a final volume of 25 µl consisting of template DNA (1 µl), 0.25 µM of the respective primers [13], 2.5 µl PCR buffer, 0.5 µM deoxynucleotide triphosphates, 0.75 µM of MgCl₂, 0.25 U Taq DNA polymerase (Cinna Gene, Tehran, Iran), and 19.5 dd H₂O. A thermocycler (PEQ STAR, Germany) was programmed with the following parameters: after an initial denaturation for 5 min. at 94°C, 30 cycles of amplification were performed with denaturation at 94°C for 30 sec, annealing at 55°C for 30 sec, and DNA extension at 72°C for 30 sec, followed by a final extension at 72°C for 5 min. Then, PCR products were visualized by electrophoresis on 1% agarose gel. This study was approved

by Ethics Committee of Hamadan university of medical sciences, Hamadan, Iran.

Results

The results of this study showed a significant difference between the types of the clinical samples (urine) as regards resistant to aminoglycosides. A total of 79% (27 isolates) of aminoglycosides resistant isolates were isolated from urine and other clinical samples including trachea 11.7% (4 isolates), wounds 5.8% (2 isolates) and blood 2.9% (1 isolate).

Based on the results of antibacterial susceptibility testing, out of 100 samples of *K. pneumoniae*, 34% of the isolates were resistant to gentamicin and 21% to amikacin. Moreover, resistance to both gentamicin and amikacin was detected in 18% of the isolates.

According to the results of the susceptibility tests, of the 34 gentamicin resistant isolates, 94% (32 isolates) were resistant to ampicillin and 51% to amikacin and ceftriaxone. The results of the antibiogram for the 34 gentamicin resisting isolates are shown in Figure 1.

The amplification of aminoglycoside resistant genes by PCR showed that 71% (n=24) and 5.8% (n=2) of the aminoglycoside resistant strains harbored the *aac(3)-IIa* and *aac(6')-Ib* genes; simultaneous harboring of the *aac(3)-IIa* and *aac(6')-Ib* genes was found in 64% (n=22) of the aminoglycoside resistant isolates (Fig. 2). In this study we could also detect the simultaneous presence of the *aac(6')-Ib* and *aac(3)-IIa* genes in one PCR reaction (duplex PCR).

Table 1. Primer sequences of *aac (6')-Ib* and *aac (3)-II*

Gene	Primers Sequences: (5-'3')	Fragment
<i>aac (6')-Ib</i>	F: CCGACACTTGCTGACGTACAG	61 bp
<i>aac (6')-Ib</i>	R: TGACGGACTCTTGCGCTAAA	
<i>aac (3)-II</i>	F: TGAAACGCTGACGGAGCCT	370 bp
<i>aac (3)-II</i>	R: GTCGAACAGGTAGCACTGAG	

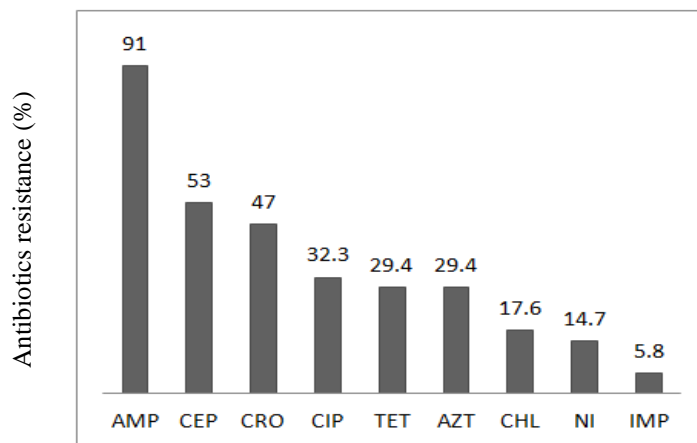


Fig. 1. frequency (%) resistance to antibiotics among gentamicin resistant *K. pneumoniae* strains. AMP= ampicillin; CEP= cephalothin; CRO= ceftriaxone; CIP= ciprofloxacin; TET= tetracycline; AZT= aztronam; CHL= chloramphenicol; NI= nitrofurantoin; IMP= imipenem

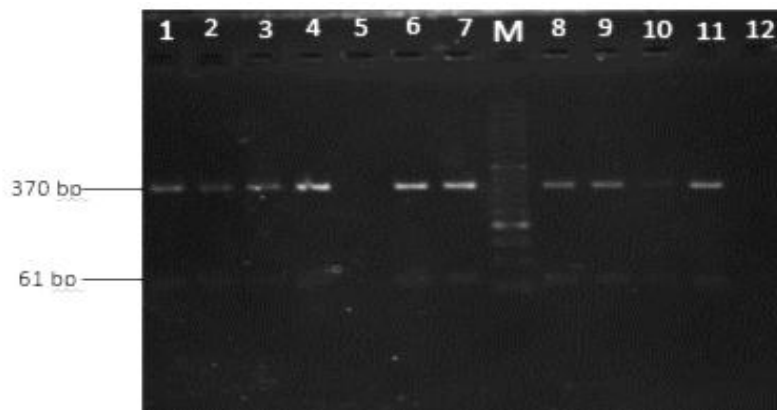


Fig. 2. Agarose gel electrophoresis of amplified DNA fragments of *aac (6')-Ib* and *aac (3)-II* by duplex PCR from reference strains and clinical isolates of *K. pneumoniae*. Wells: M, 50 bp Plus DNA ladder; 5 and 12, as negative control; 1, as positive control; 4, 6, 7, 8, 9, 10, 11, clinical isolates of *K. pneumoniae*

Discussion

Emergence of resistant strains to aminoglycosides can be a serious threat against public health in hospitals and society, causing difficulties for medical treatment and imposing additional

costs on the healthcare system. In this study, antibiotic resistance patterns and the occurrence frequency of the aminoglycoside resistance genes *aac (6')-Ib* and *aac (3)-IIa*

in *K. pneumoniae* strains isolated from hospitalized patients in Borujerd, in the west of Iran, was investigated by PCR. These genes encode aminoglycoside modifying enzymes for gentamicin and amikacin. In fact, acetyltransferase enzymes of gentamicin and amikacin are encoded by these genes [10]. Different results concerning resistance to aminoglycosides have been published in Iran; however, few studies have been carried out on the prevalence of aminoglycoside resistant genes in *K. pneumoniae* [11, 12, 14, 15]. Linderman et al. from Norway reported that 90% of *K. pneumoniae* strains are resistant to aminoglycosides [16]. The results of our research indicated a relatively high resistance (34%) to gentamicin. Resistance to both gentamicin and amikacin was observed in 18% of the cases. Antibiotics that inhibit cell wall synthesis increase the transfer of aminoglycosides into bacteria cells, hence the combination of cell wall synthesis-inhibitor antibiotics such as beta-lactams and aminoglycosides can be used to treat infections caused by *K. pneumoniae* [7, 12, 14]. In recent years, strains of bacteria resistant to beta-lactams and aminoglycoside antibiotics, especially in hospitals, have increased [17]. In this study, over 90% of the isolates resistant to gentamicin were also resistant to ampicillin. These results suggest that combining these two classes of antibiotics is not effective in treating resistant strains of *K. pneumoniae* while the imipenem showed the highest activity against the most resistant strains to aminoglycosides. In contrast to our results, Peerayeh, et al. reported a higher prevalence (42.5%) of *aac*

(6')- *Ib* genes among *K. pneumoniae* isolates in Tehran's hospitals. They also detected the *aac* (3)- *II* gene in 35.1% of these isolates [18]. In addition, Lindermann reported a higher frequency for the *aac* (3)-*II* gene found in 79.3% of *K. pneumoniae* isolates in comparison with the *aac* (6')- *Ib* gene (found in 37.9% of the isolates) [16]. In 2015, Liang et al. studied *aac* (6')- *Ib* in *K. pneumoniae* isolates and found 19% of the isolates containing this gene [19]. In a study, Almaghrabi et al., reported resistance to gentamicin and amikacin in 40%, and 16% of *K. pneumoniae* isolates, respectively. In the United States' hospitals, 98% of aminoglycoside resistant strains possessed AMEs, including *aac* (6')-*Ib*. These results have shown that the location of sampling may affect the distribution of aminoglycoside genes among *K. pneumoniae* isolates [20]. Other isolates with negative PCR results for AME genes have probably become resistant to aminoglycosides through other resistance mechanisms such as the reduction of uptake or change in the ribosome binding site [18, 21].

Conclusions

The results of this study indicate moderate resistance to aminoglycosides in comparison with the results achieved by other researchers. Furthermore, the presence of the *aac* (6')- *Ib* and *aac* (3)-*IIa* genes was observed in more than 50% of nosocomial *K. pneumoniae* strains resistant to aminoglycosides. This may be due to the transmission of this gene through mobile genetic elements such as plasmids and transposons that create a high risk of rapid

spread of these genes among *K. pneumoniae* isolates in hospitals.

Conflict of Interest

The authors declare no conflict of interest.

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