RESEARCH ARTICLE

•Arch Hyg Sci 2019;8(4):253-258
•Journal Homepage: http://jhygiene.muq.ac.ir

# Frequency of *ant(2")-I* and *aac(6')-II* Genes in the Clinical Isolates of *Pseudomonas aeruginosa* from Shahid Sadoughi Hospital in Yazd, Iran

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**A-R-T-I-C-L-EI-N-F-O**

**Article Notes:**
Received: Nov 27, 2019
Received in revised form: Nov 27, 2019
Accepted: Nov 30, 2019
Available Online: Feb 01, 2020

**Keywords:**
Aminoglycoside
Antibiotic resistance
*Pseudomonas aeruginosa*
*aac(6')-II* and *ant (2")-I* genes

**A-B-S-T-R-A-C-T**

**Background & Aims of the Study:** *Pseudomonas aeruginosa* (*P. aeruginosa*) is the common cause of nosocomial infections, especially among patients. The aim of the present study was to investigate the occurrence of aminoglycoside resistance and prevalence of the resistance-modifying enzyme genes *aac(6')-II* and *ant(2")-I* in *P. aeruginosa* isolates from the clinical samples of hospitalized patients in Yazd, Iran.
**Materials and Methods:** This cross-sectional study was carried out on *P. aeruginosa* isolates during March 2016 to March 2017. All clinical samples were initially identified by the standard biochemical method, and their aminoglycoside resistance was studied using the disc diffusion method according to Clinical and Laboratory Standards Institute recommendations. Polymerase chain reaction was conducted for the detection of aminoglycoside resistance using the specific primers of *aac(6')-II* and *ant(2")-I* genes.
**Results:** A total of 144 isolates were evaluated for antibiotic susceptibility testing. The resistance of *P. aeruginosa* isolates to the tested antibiotics was reported as 118 (81.9%) isolates to kanamycin, 91 (63.2%) isolates to gentamicin, 80 (55.6%) isolates to tobramycin, and 84 (58.3%) isolates to amikacin. The *aac(6')-II* and *ant(2")-I* genes were detected in 93 (64.6%) and 114 (79.2%) *P. aeruginosa* isolates, respectively.
**Conclusion:** Aminoglycoside resistance in *P. aeruginosa* remains a significant problem. Therefore, there will be considerable local surveillance of aminoglycoside resistance profile.

**Please cite this article as:** Zarei Yazdeli M, Alipanah H, Ciamak G. Frequency of *ant(2")-I* and *aac(6')-II* Genes in the Clinical Isolates of *Pseudomonas aeruginosa* from Shahid Sadoughi Hospital in Yazd, Iran. Arch Hyg Sci 2019;8(4):253-258

## Background

*Pseudomonas aeruginosa* (*P. aeruginosa*) is a common gram-negative pathogenic bacteria linked to nosocomial infections responsible for 10-15% of nosocomial infections all over the world (1-2). Antibiotic resistance is a worldwide emerging issue, and among *P. aeruginosa* strains, the extensive use of antibiotics may be the main cause of the increase in multidrug resistance. Aminoglycosides are among the very effective series of antibiotics against a wide range of gram-negative and gram-positive aerobic bacteria.

In spite of the popular use and clinical prosperity of aminoglycosides, the adminis-tration of these antibiotics to control bacterial


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infections has been challenging due to the emergence of resistant strains (3-4). The enzymatic change, efflux pumps activity (MexXY-OprM), impermeability, ndvB dependent biofilm formation, PhoP-PhoQ system, as well as the activity of 16S rRNA methylases, make resistance to aminoglycosides (5-6). Among these, the inactivation of medications through plasmid or modifying enzymes encoded by chromosomes is the most common mechanism.

Aminoglycosides are inactivated by aminoglycoside-modifying enzymes through a number of molecular mechanisms, such as acetylation, phosphorylation, and adenylation, catalyzed by actyl-CoA dependent N-acetyltransferase, ATP-dependent O-phosphotransferase, and ATP-dependent O-nucleotidyltransferase, respectively (7). The *ant(2'')-I* and *aac(6')-II* are two important enzymes modifying aminoglycosides in *P. aeruginosa*. The *aac(6')-II* of the enzymes provides resistance to tobramycin and gentamicin, and *ant(2'')-I* provides resistance to kanamycin, tobramycin, and gentamicin.

The number of *P. aeruginosa* strains resistant to aminoglycosides is increasing (8). The aim of the current widespread study was to examine the incidence of aminoglycoside resistance, as well as the prevalence of the resistance-modifying enzyme genes *aac(6')-II* and *ant (2'')-I*, in *P. aeruginosa* isolates from the clinical samples of hospitalized patients in Yazd, Iran.

## Materials & Methods

### *Sample collection and isolation*

This descriptive study was carried out on 144 clinical isolates of *P. aeruginosa* collected from Shahid Sadoughi Hospital in Yazd during March 2016 to March 2017. A questionnaire was used for recording patients' demographic data, such as name, age, gender, type of sample, and ward. The bacterial strains were isolated from different clinical specimens, such as blood, bronchial fluid, urine, cerebrospinal fluid, catheter, pleural fluid, ear swap, sputum, and wound, consequently inoculated on sheep blood agar (Merck, Germany) and EMB agar media (Merck, Germany) incubated at 37°C for 24 h. All *P. aeruginosa* isolates were identified using standard biochemical tests, such as growth on Cetrimide agar (Liofilchem, Italy), growth at 42°C, oxidase test, gram staining, pigment production, and Oxidative/fermentation test.

### *Antibiotic susceptibility testing*

Antibiotic susceptibility tests were performed using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines for four aminoglycosides (i.e., gentamicin [10 µg], amikacin [30 µg], kanamycin [5 µg], and tobramycin [10 µg]). All antibiotic disks were purchased from Mast Company (UK). E-test method (Liofilchem, Italy) was used to determine the minimum inhibitory concentration (MIC) of gentamicin. Based on CLSI guideline, MIC > 16 was considered resistant isolates to gentamicin (9). *P. aeruginosa* ATCC 27853 was used as a control in this study.

### *Genomic DNA extraction*

In this study, genomic DNA was extracted by the salting-out method (10). The quality and quantity of obtained DNA were measured by 0.7% agarose gel electrophoresis and nanodrop, respectively. The DNA samples were stored at -20°C until the examination day.

### *Polymerase Chain Reaction*

Polymerase chain reaction (PCR) was carried out for the detection of *ant(2'')-I* and *aac(6')-II* genes on a thermal cycler (Eppendorf, Germany). Primers were developed for each gene using Primer3 (version 0.4.0). The primer pair sequences used in this study and PCR conditions are detailed in Table 1 (11). The


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**Table 1) Sequences of used primers for detection of aminoglycosides genes**

| Primer name | Sequence | Polymerase chain reaction condition | | | | |
|---|---|---|---|---|---|---|
| | | Denaturing | Anneal | Extension | Cycles | Size (bp) |
| ***ant(2'')-I*** | 5'-CACAACGCAGGTCATT-3'<br>5'-CGCTAAGAATCCATAGTCCAA-3' | 94 | 52 | 72 | 30 | 220 bp |
| ***aac(6')-II*** | 5'-GCCGATGCTCCATGAT TG-3'<br>5'-TCGAAGGCTTGTCGTGTT-3' | 94 | 52 | 72 | 30 | 480 bp |

final 20 µl volume for PCR reaction consisted of water 5 µl, PCR Master Mix (Ampliqon, Denmark; 10 µl), 2 µl of the primers, and a volume of 3 µl of the DNA template. Moreover, 1% agarose gel electrophoresis was performed on PCR products and sequenced for further confirmation (11).

### *Statistical analysis*

The correlation between the genes and resistance to aminoglycosides were analyzed using the Chi-square test by SPSS software (version 16.0).

## Results

Totally, 144 strains of *P. aeruginosa* were isolated from the patients within the age range of 7 months to 80 years who were referred to Shahid Sadoughi Hospital of Yazd. The mean age of the subjects was 22.7±34.9 years. The majority of the isolates were collected from the patients hospitalized in burn ward (n=68; 47.2%) and intensive care unit (ICU) (n=27; 18.8%) followed by surgery (n=24; 16.7%), infectious disease (n=18; 12.5%), and neurology (n=7; 4.9%) wards. Most of the strains were isolated from the male subjects with 34 years of age.

Among all 144 *P. aeruginosa* isolates, aminoglycosides resistance rates were 81.9%, 63.2%, 55.6%, and 58.3% for kanamycin, gentamicin, tobramycin, and amikacin, respectively. Generally, 74.6% of the isolates were resistant at least to one aminoglycoside. Phenotypic results showed the highest simultaneous resistance of 69.44% to gentamicin-tobramycin, followed by 63.2% to gentamicin-kanamycin, 52.8% to kanamycin-tobramycin, and 47.2% to gentamicin-tobramycin-kanamycin (Table 2). The MICs of 91 isolates resistant to gentamicin identified by the disc diffusion method were 80 resistant isolates (87.91%) and 11 sensitive isolates (12.07%) (Table 3).

The results of the present study showed that frequencies of *aac(6')-II* and *ant(2'')-I* genes in the studied isolates were 93 (64.6%) and 114 (79.2%), respectively (Figure 1). A significant correlation was observed between the presence of *aac(6')-II* gene and resistance against

**Table 2) Results of antibiotic resistance and studied genes in 144 *P. aeruginosa* isolates**

| Antibiotic | Resistant n (%) | *aac(6')-II* gene n (%) | *ant(2'')-I* gene n (%) |
|---|---|---|---|
| **GM-TOB-K- AK** | 59 (41.0) | 44 (74.6) | 53 (89.8) |
| **GM-TOB-K** | 68 (47.2) | 53 (77.9) | 62 (91.2) |
| **GM-AK-K** | 59 (41.0) | 44 (74.6) | 53 (89.8) |
| **GM-K** | 91 (63.2) | 66 (72.5) | 77 (84.6) |
| **K-TOB** | 76 (52.8) | 61 (80.3) | 70 (92.1) |
| **GM-TOB** | 100 (69.44) | 55 (38.19) | 63 (43.75) |
| **K** | 118 (81.9) | 85 (72.0) | 99 (83.9) |
| **GM** | 91 (63.2) | 66 (72.5) | 77 (84.6) |
| **TOB** | 80 (55.6) | 64 (80.0) | 73 (91.2) |
| **AK** | 84 (58.3) | 53 (63.1) | 71 (84.5) |

K: Kanamycin; TOB: Tobramycin; GM: Gentamicin; AK: Amikacin.


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**Table 3) Results of minimum inhibitory concentration of gentamicin by E-test method**

| Minimum inhibitory concentration range (µg/ml) | 2> | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 |
|---|---|---|---|---|---|---|---|---|---|---|---|
| Number | 2 | 3 | 6 | 0 | 11 | 23 | 6 | 7 | 3 | 5 | 25 |
| Percentage | 1.2 | 3.2 | 6.4 | 0 | 12.1 | 25.2 | 6.4 | 7.6 | 3.2 | 5.4 | 27.4 |

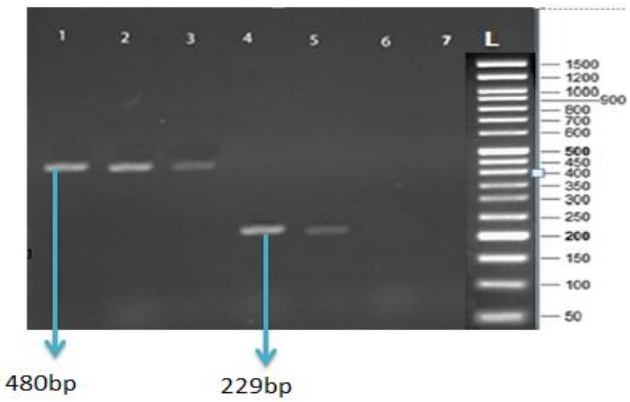


**Figure 1) Polymerase chain reaction amplification of *aac(6)-II* and *ant(2")-I* genes in clinical *P. aeruginosa* isolates; lane 1: positive controls for *aac(6')-II* (480 bp); lane 2-3: isolates positive for *aac(6')-II* (480 bp); lane 4: positive controls for *ant(2")-I* (229 bp); lane 5: isolates positive for *ant(2")-I* (229 bp); lane 6: negative control for *ant(2")-I*; lane 7: negative control for *aac(6)-II*; lane L: 50 bp DNA ladder**

kanamycin and tobramycin, and *ant(2'')-I* gene and resistance against gentamicin, amikacin, kanamycin, and tobramycin. Moreover, there was a significant correlation between burn ward and presence of *aac(6')-II* and *ant(2'')-I* genes.

## Discussion

Antibiotic therapies are extensively used for the treatment of infectious diseases (10). According to the literature, it has been revealed that *P. aeruginosa* is the most common bacterial isolate in burn wards in Tehran, Iran (11-12). Results of multiple studies have shown that *P. aeruginosa* is highly prevalent in Iranian hospitals. The findings of this study revealed that most of the *P. aeruginosa* isolates were reported in the burn ward (n=68; 47.2%). The obtained results of the present study are in line with those of other studies (13-12).

In the present study, the highest frequency of *P. aeruginosa* isolation after the burn ward was related to ICU (n=27; 18.8%). The results of a study by Shakib et al. in 2014 carried out in the ICU of Besat Hospital in Sanandaj, Iran, confirmed the results of the present study (14). *P. aeruginosa* is resistant to a spectrum of antibiotics, such as macrolides, beta-lactams, and aminoglycosides (13). Aminoglycoside resistance in *P. aeruginosa* is often associated with the development of different enzymes (8). This resistance has become a worldwide issue (14), particularly in Asia (15).

Based on the disk diffusion findings, 81.9% and 55.6% of the isolates were resistant to kanamycin and tobramycin, respectively. This shows that the rate of resistance has increased, compared to those in other studies (11-15). Constraints in phenotypic methods made researchers prove their phenotypic results by means of molecular methods. It was shown in PCR results that 114 (79.2%) and 93 (64.6%) isolates harbored *ant(2")-I* and *aac(6')-II* genes, respectively. The prevalence of genes in the present study suggested an increasing trend of resistance to antibiotics in comparison to that of the previous years.

In a study performed in Iran (Tehran), the prevalence rates of *ant(2")-I* and *aac(6')-II* genes were 10% and 45%, respectively (16). Furthermore, in another study carried out in Iran (Tehran), the prevalence rates of *ant(2")-I* and *aac(6')-II* genes were 28% and 36% (17) respectively. However, in other countries, the prevalence of the genes was different (18). In a study performed in France in 2008, 1.9% of isolates were positive for *aac(6')-II* gene (19). In another study in Mecca, Saudi Arabia, among 65 isolates, *aac(6')-II* gene was observed in four of them (6.1%) (20).


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In a study conducted in South Korea, the prevalence rate of *ant(2")-I* gene was 43.6% (21). Therefore, controlling and treating the infections caused by *P. aeruginosa* is complex. It is essential to revise the treatment protocols in order to prevent from transferring resistant genes to the clinical isolates. In this study, the expression of *aac(6')-II* and *ant(2")-I genes* has a very high prevalence in comparison to that of other studies.

Considering the fact that most of the samples were collected from the burn ward, the probability of harboring *aac(6')-II and ant (2")-I genes* is also higher due to the high aminoglycoside resistance of these strains. Of course, it should be noted that the prevalence of these enzymes is geographically diverse, leading to distinct prevalence among countries. Most of the isolates with phenotypic resistance to aminoglycosides (80%) have *ant(2")-I* gene, and the presence of this enzyme is one of the most common mechanisms of resistance to aminoglycosides.

It is worth noting that since there was no gene concerning these enzymes in the phenotypic sensitive isolates, the significance of these genes in inducing the resistance of *P. aeruginosa* can be noted against aminoglycosides. In this study, there was a significant relationship between antibiotic resistance and *ant(2")-I* gene. Due to the high prevalence of *ant(2")-I* gene observed in the experiments of this study and its effect on increasing the resistance to aminoglycosides, this correlation seems argumentative.

Another important point to be noted is that, in addition to the mechanism of aminoglycoside-modifying enzymes, which is the main subject of the present study, other mechanisms, such as impenetrable and efflux pump, in *P. aeruginosa* are prevalent. In fact, if the isolates without the gene modifying enzymes are phenotypically resistant, they are probably resistant to aminoglycosides through other mechanisms (8).

## Conclusion

Resistance to aminoglycosides continues to be a major concern in Iran. Since these aminoglycoside resistance genes are transmissible by genetic elements, they can be spread and disseminated among other bacteria. Aminoglycoside resistance among clinical isolates of *Pseudomonas aeruginosa* is indicated to be a major significance in the future; therefore, the surveillance of aminoglycoside resistance genes and use of aminoglycosides with stronger affinity to their targets and resistance to these modifying enzymes are crucial.

## Footnotes

**Acknowledgements**

The authors would like to thank all those who participated in this study and the laboratory of the Department of Microbiology in the Faculty of Medicine in Shahid Sadoughi University of Medical Sciences of Yazd.

**Funding**

This project was financially supported by Shahid Sadoughi University of Medical Sciences of Yazd.

**Conflict of Interest**

The authors declare that there is no conflict of interest.

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