CLINICAL SCIENCE

The prevalence of aminoglycoside-modifying enzyme genes *(aac (6′)-I, aac (6′)-II, ant (2″)-I, aph (3′)-VI)* in *Pseudomonas aeruginosa*

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INTRODUCTION: *Pseudomonas aeruginosa* (*P. aeruginosa*) is one of the primary opportunistic pathogens responsible for nosocomial infections. Aminoglycosides are an important component of antipseudomonal chemotherapy. The inactivation of drugs by modifying enzymes is the most common mechanism of aminoglycoside resistance.

OBJECTIVES: The inactivation of aminoglycosides by modifying enzymes is the primary resistance mechanism employed by *P. aeruginosa*. The aim of the present study was to investigate the occurrence of aminoglycoside resistance and the prevalence of four important modifying enzyme genes (*aac (6′)-I, aac (6′)-II, ant (2″)-I, aph (3′)-VI*) in *P. aeruginosa* in Iran.

METHODS: A total of 250 clinical isolates of *P. aeruginosa* were collected from several hospitals in seven cities in Iran. Antimicrobial susceptibility tests (using the disk diffusion method and E-tests) were performed for all 250 isolates. In addition, all isolates were screened for the presence of modifying enzyme genes by polymerase chain reaction.

RESULTS: The resistance rates, as determined by the disk diffusion method, were as follows: gentamicin 43%, tobramycin 38%, and amikacin 24%. Of the genes examined, *aac (6′)-II* (36%) was the most frequently identified gene in phenotypic resistant isolates, followed by *ant (2″)-I, aph (3′)-VI,* and *aac (6′)-I*.

CONCLUSIONS: Aminoglycoside resistance in *P. aeruginosa* remains a significant problem in Iran. Therefore, there is considerable local surveillance of aminoglycoside resistance.

KEYWORDS: *Pseudomonas aeruginosa*; Antibiotic; Resistance; *aac (6′)-II*; *ant (2″)-I.*

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INTRODUCTION

Pseudomonas aeruginosa (*P. aeruginosa*) is one of the primary opportunistic pathogens responsible for nosocomial infections. The most important problem in the eradication of *P. aeruginosa* is the frequently observed multi-drug resistance of the species.[1] In addition, *P. aeruginosa* can also acquire resistance to various antimicrobial agents, such as aminoglycosides, β-lactams[2] and fluoroquinolones.[3] Aminoglycosides are an important component of antipseudomonal chemotherapy,[4] and they exhibit synergy with β-lactams.[5] Resistance to aminoglycosides occurs via enzymatic modification, impermeability, the activity of efflux pumps (MexXY-OprM),[6] the PhoP-PhoQ system,[7] ndvB-dependent biofilm formation,[8] and the activity of 16s rRNA methylases.[9] Among these mechanisms, the inactivation of drugs by plasmid- or chromosome-encoded modifying enzymes is the most common. These modifying enzymes include aminoglycoside phosphoryl transferase (*aph*), aminoglycoside acetyltransferase (*aac*), and aminoglycoside nucleotidyl transferase (*ant*).[10-12] Four of these enzymes, encoded by *aac (6′)-I, aac (6′)-II, ant (2″)-I,* and *aph (3′)-VI*, are of particular significance because they are among the most common modifying enzymes present in *P. aeruginosa,* and their substrates are the most important antipseudomonal aminoglycosides. *aac* (6′)-I confers resistance to tobramycin and amikacin, *aac* (6′)-II and *ant* (2″)-I inactivate tobramycin and gentamicin, and amikacin is the substrate of *aph* (3′)-VI.[13,14]

The aim of the present nationwide study was to investigate the occurrence of aminoglycoside resistance and the prevalence of the resistance-modifying enzyme genes, *aac (6′)-I, aac (6′)-II, ant (2″)-I* and *aph (3′)-VI,* in *P. aeruginosa* isolated from several hospitals in seven Iranian cities.

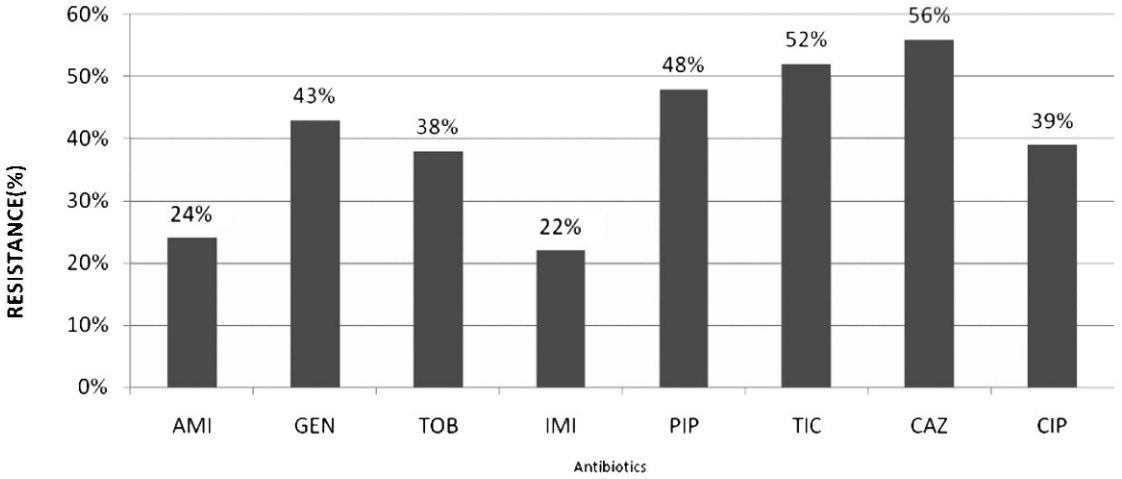

Figure 1 - The antimicrobial resistance rate of 250 *P. aeruginosa* isolates as determined by the disk diffusion method AMI: amikacin, GEN: gentamicin, TOB: tobramycin, IMI: imipenem, PIP: piperacillin, TIC: ticarcillin, CAZ: ceftazidime, CIP: ciprofloxacin.

METHODS AND MATERIALS

Collection of bacterial isolates

A total of 250 non-duplicate, clinical isolates of *P. aeruginosa* were collected from a nationwide distribution of several hospitals in seven cities in Iran (Tehran, Shiraz, Zahedan, Tabriz, Sannandaj, Sari, and Ahvaz) between May 2007 and January 2008. Strain data and the demographic and clinical data for each patient were regularly forwarded to our laboratory. The study population was 62% male and 38% female. The specimens were isolated from urine (38%), wounds (18%), the trachea (18%), blood (10%), sputum (9%), and other sources (7%). Isolate confirmations were conducted using conventional biochemical tests, and then the isolates were stored at –76℃ in glycerol skim milk broth.

Antimicrobial susceptibility testing

Antimicrobial susceptibility tests were performed using the disk diffusion method according to Clinical and Laboratory Standards Institute (CLSI) guidelines[15] for three aminoglycosides [gentamicin (10 μg), amikacin (30 μg), and tobramycin (10 μg)] and for five other antibiotics [imipenem (10 μg), piperacillin (100 μg), ticarcillin (75 μg), ceftazidime (30 μg), and ciprofloxacin (5 μg)]. All drugs were obtained from Mast laboratories (Merseyside, United Kingdom). For all 250 isolates, the minimum inhibitory concentrations (MIC) of amikacin, gentamicin, and tobramycin were determined using the E-test (Biodisk, Dalvagen, Sweden) according to CLSI guidelines. *P. aeruginosa* (ATCC 27853) served as a control for the disk diffusion and E-tests.

Polymerase chain reaction amplification

Polymerase chain reaction (PCR) was used to screen all 250 isolates for the presence of the modifying enzyme genes, *aac (6′)-I, aac (6′)-II, ant (2″)-I* and *aph (3′)-VI*. The total template DNA for the PCR amplification was extracted from the supernatant of a mixture of *P. aeruginosa* cells produced by the boiling method. PCR amplification was performed using 2.5 μL of the template DNA, 1 μL of each primer,[16] 19.5 μL master mix, and 1 μL of Taq DNA polymerase (CinnaGen) in a total volume of 25 μL. A thermocycler (Mastercycler gradient; Eppendorf, Hamburg, Germany) was programmed

Table 1 - Aminoglycoside susceptibility profiles according to the disk diffusion method and MIC results (E-test) for 250 *P. aeruginosa* isolates (according to CLSI guidelines).[15]

	Disk Diffusion[1]			E-test[2]						MIC 50% (ug/mL)	MIC 90% (ug/mL).
				Resistant			Intermediate	Susceptible			
	No. (%) of isolates			MIC(μg/ml)			MIC(μg/ml)	MIC(μg/ml)			
	Resistant	Intermediate	Susceptible	–1024 128	64–128	16–64	8	2–4	<2		
Gentamicin	108(43.2)	8(3.2)	134(53.6)	10	41	49	8	98	44	4	64
Tobramycin	95(38)	7(2.8)	148(59.2)	8	45	37	5	103	52	4	64
				MIC(μg/ml)			MIC(μg/ml)	MIC(μg/ml)		MIC 50% (ug/mL)	MIC 90% (ug/mL).
				>256	–256 128	64–128	32	4–16	<4		
Amikacin	59(23.6)	7(2.8)	184(73.6)	4	17	32	6	134	57	8	128

1. Zone diameter (mm) for gentamicin and tobramycin, R:<12, I:13-14, S:>15; for amikacin, R:<14, I:15-16, S:>17.
2. MIC breakpoint(μg/ml) for gentamicin and tobramycin, R:>16, I:8; S:<4; for amikacin, R:>64, I:32, S:<16.

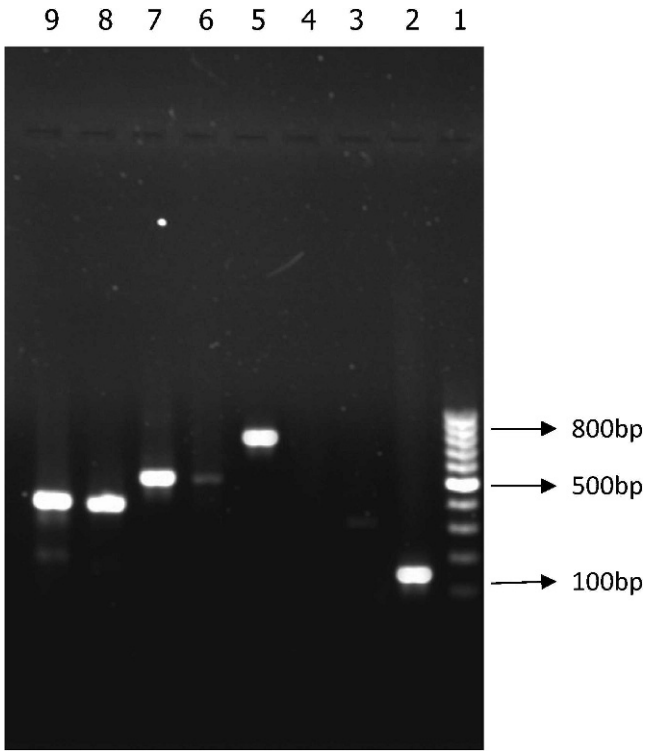

Figure 2 - Lane 1, 1000 bp DNA size marker; Lane 2, *aac*(6′)-II (125 bp); Lane 5, *aph*(3′)-VI (800 bp); Lane 7, *ant*(2″)-I (524 bp); Lanes 8 and 9, *aac*(6′)-I (392 bp).

with the appropriate conditions. Then, 5 µl of each PCR product was analyzed by electrophoresis on a 1% (w/v) TAE agarose gel (Fermentas UAB, Vilnius, Lithuania) containing 0.1 µl/ml ethidium bromide. The amplicons were then visualized on a UV transilluminator and photographed (BioDoc-Analyse; Biometra, Goettingen, Germany).

Statistical analysis

All statistical analyses were performed using Statistical Package for Social Sciences (SPSS) software (version 11.5) for Windows (χ^2-test and Fisher's exact test). *P*-values of <0.05 were considered significant.

RESULTS AND DISCUSSION

The resistance rates as determined using the disk diffusion method are presented in Figure 1. According to the disk diffusion method, 135 isolates were resistant to aminoglycosides. The resistance rates according to the E-test (base on MICs) were as follows: gentamicin 40%, tobramycin 36%, and amikacin 21%. The aminoglycoside susceptibility profiles according to the disk diffusion and E-test results for the 250 isolates are listed in Table 1.

PCR analysis revealed the absence of resistance genes in susceptible isolates. The prevalence of aminoglycoside resistance genes in the 135 resistant isolates (as determined by the disk diffusion method) was as follows: *aac (6′)-II* was detected in 36% of the resistant isolates, *ant (2″)-I* was detected in 28%, *aph (3′)-VI* in 11%, and *aac (6′)-I* was found in 7% of the resistant isolates (Figure 2). Interestingly, individual aminoglycoside-resistant isolates carried multiple (two to four) modifying enzyme genes. Six isolates harbored *ant(2″)-I* and *aac*(6′)-II; three harbored *aph(3′)-VI* and *ant(2″)-I*; two harbored *ant(2″)-I* and *aac(6′)-I;* and three isolates harbored *ant(2″)-I*, *aac(6′)-II* and *aph(3′)-VI.* Only one isolate harbored all four genes.

Several previous studies have examined the occurrence of aminoglycoside resistance mechanisms in *P. aeruginosa* isolated from different countries. The overall incidence of aminoglycoside resistance found in our study (according to the disk diffusion test and the E-test) was much higher than the incidence that has been reported previously in different countries worldwide.[14,16,17] However, Estahbanati and coworkers reported that 53.3% of clinical isolates from Iranian burn patients were resistant to amikacin, and 90.7% were resistant to gentamicin, a result that reveals a high level of aminoglycoside resistance in their study.[18]

The aminoglycoside resistance rate was almost as high in our isolates, and most of the resistant isolates harbored modifying enzyme genes. In addition, none of the susceptible isolates harbored these resistance genes. These results highlight the importance of aminoglycoside-modification-related mechanisms in aminoglycoside resistance in *P. aeruginosa*. Two genes, *aac (6′)-II* and *ant (2″)-I,* were the most frequent resistance genes observed in these isolates. These results are similar to what has been observed in different studies in other countries.[19-21] All isolates harboring the *aac (6′)-II* gene were resistant to gentamicin and tobramycin (100% concordance), which indicates that *aac* (6′)-II is a significant determinant of gentamicin and tobramycin resistance in *P. aeruginosa*. It is important to mention that we encountered an unexpected phenotype in some isolates (Table 2). For example, when an isolate harbored only the *aph (3′)-VI* gene, which has amikacin as a substrate, resistance to gentamicin, tobramycin, and amikacin was observed. We presume that the reason for

Table 2 - Prevalence of aminoglycoside modifying enzymes genes and the correlation between these genes and phenotypic patterns in aminoglycoside resistant *P. aeruginosa* isolates.

Gene	no. of isolates (%)[1]	Expected resistance[2]	Observed resistance phenotypes (no. of isolates)[3]
aac(6′)-I	9(7%)	TOB,AMI	Unexpected resistance to GEN(2)
aac(6′)-II	49(36%)	GEN,TOB	Unexpected resistance to AMI(16)
ant(2″)-I	38(28%)	GEN,TOB	As expected(38)
aph(3′)-VI	15(11%)	AMI	Unexpected resistance to GEN and TOB(2)
aac(6′)-II+ ant(2″)-I	6(4%)	GEN,TOB	As expected(6)
ant(2″)-I+ aph(3′)-VI	3(2%)	AMI,GEN,TOB	As expected(3)
aac(6′)-I+ ant(2″)-I	2(1%)	AMI,GEN,TOB	As expected(2)
ant(2″)-I + aac(6′)-II +aph(3′)-VI	3(2%)	AMI,GEN,TOB	As expected(3)
aac(6′)-II *ant*(2″)-I+ + *aph*(3′)-VI + *aac*(6′)-I	1(~1%)	AMI,GEN,TOB	As expected(1)

1. According to the disk diffusion method, a total of 135 resistant isolates were identified.
2. AMI: amikacin, GEN: gentamicin, TOB: tobramycin.
3. Described in greater detail in the text.

this phenomenon might be the action of other resistance mechanisms, such as impermeability, efflux pumps, or other types of modifying enzymes. On the other hand, we detected 15 isolates which co-harbored two, three, or four aminoglycoside-modifying enzyme genes simultaneously, which is in contrast to several studies conducted in the USA and Europe, which reported that the majority of isolates exhibit only a single aminoglycoside modifying gene.[22]

Miller et al. brought together the results of several studies of aminoglycoside resistance in *P. aeruginosa* carried out worldwide.[22] Their results indicated that in Europe, *aac(6′)-II* was the most prevalent resistance gene (32.5%), followed by *ant(2″)-I* (16.9%). These results are in concordance with our study. In contrast to our results, a Korean nationwide study of 250 isolates of *P. aeruginosa* reported that *aph(3′)-VI*, *ant(2″)-I*, and *aac(6′)-I* were all prevalent, but none harbored *aac(6′)-II*.[16] The difference in the distribution of modifying enzymes may derive from differences in aminoglycoside prescription patterns, the selection of bacterial population or geographical differences in the occurrence of aminoglycoside resistance genes.

CONCLUSIONS

In conclusion, although aminoglycosides remain useful antipseudomonal agents, resistance to these drugs continues to be a major issue, especially in Iran. Because these aminoglycoside resistance genes are usually located on mobile genetic elements (i.e., plasmid, transposon, or integrons),[23,24] there is a growing concern that they could easily spread and be disseminated among other bacteria. Integrons that carry gene cassettes encoding both *aac*s and carbapenemases will only exacerbate this problem.[25] The design of novel aminoglycosides with stronger affinity for their targets and resistance to these modifying enzymes is inevitable,[26] and the new generation of anti-*Pseudomonas* therapy is forthcoming.[27] Aminoglycoside resistance among clinical isolates of *P. aeruginosa* promises to become a major clinical concern in the future, and continuous local surveillance of aminoglycoside resistance is crucial.

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