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Aminoglycoside-modifying Enzymes in Carbapenem-resistant *Pseudomonas aeruginosa* Clinical Isolates

Karbapenem Dirençli *Pseudomonas aeruginosa* Klinik İzolatlarında Aminoglikozit-modifiye Edici Enzimlerin Araştırılması

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Abstract

Introduction: Aminoglycosides are the drug of choice for the treatment of *Pseudomonas aeruginosa* infections. Aminoglycoside resistance in *P. aeruginosa* often occurred via acquired aminoglycoside-modifying enzymes (AMEs). In this study, we aimed to investigate the presence of AME in *P. aeruginosa* in carbapenem-resistant and carbapenem-susceptible isolates.
Materials and Methods: A total of 98 isolates of *P. aeruginosa* from various clinical samples presenting resistance to amikacin and/or gentamicin were included in this study. Fifty-four were carbapenem-resistant isolates. Polymerase chain reaction amplification of six genes for AMEs (*aac(6')-Ib,aac(6')-IIa, aac(3')-IIa, aph(3')-Ia,aph(3')-VIa, ant(2'')-Ia*) was performed.
Results: The most frequent *AME* gene was *aac(6')-Ib* (n=13, 13.2%), followed by *ant(2'')-Ia* (n=7, 7.1%). *aac(6')-Ib* was the most common AME in carbapenem-resistant isolates (11/54, 20.3%); however *ant(2'')-Ia* was the most common AMEs in carbapenem-susceptible isolates (4/44, 9%). In 74 of the isolates, none of the AME genes was detected. *aac(6')-Ib* positivity in carbapenem-resistant isolates was significantly higher than that in carbapenem-susceptible isolates.
Conclusion: Aminoglycosides are one of the drug of choice in carbapenem-resistant *P. aeruginosa* isolates. However, given the transfer of multidrug resistance determinants, the presence of *AME* was significantly higher in carbapenem-resistant isolates, and monitoring resistance determinants among Gram-negative bacteria is crucial.
Keywords: Aminoglycoside-modifying enzyme, *P. aeruginosa*, carbapenem resistance

Öz

Giriş: Aminoglikozitler, *Pseudomonas aeruginosa* enfeksiyonlarının tedavisi için tercih edilen ilaçlardır. *P. aeruginosa*'daki aminoglikozit direnci, sıklıkla kazanılmış aminoglikozit modifiye edici enzimler (AME) yoluyla meydana gelmektedir. Bu çalışmada, karbapenem dirençli ve duyarlı izolatlarda *P. aeruginosa*'da AME varlığını araştırmayı amaçladık.
Yöntem: Amikasin ve/veya gentamisine direnç gösteren çeşitli klinik örneklerden alınan toplam 98 *P. aeruginosa* izolatı bu çalışmaya dahil edildi. İzolatların 54'ü karbapenem dirençliydi. PZR amplifikasyonu ile altı *AME* geninin (*aac(6')-Ib,aac(6')-IIa, aac(3')-IIa, aph(3')-Ia,aph(3')-VIa, ant(2'')-Ia)* varlığı araştırıldı.
Bulgular: En sık saptanan *AME* geni *aac(6')-Ib* (n=13, %13,2) olup, bunu *ant(2'')-Ia* (n=7, %7,1) izlemiştir. *Aac(6')-Ib*, karbapenem dirençli izolatlarda en yaygın AME (11/54, %20,3) iken, ancak karbapenem duyarlı izolatlarda *ant(2'')-Ia* en yaygın AME (4/44, %9) idi. İzolatların 74'ünde *AME* genlerinin hiçbiri saptanmadı. Karbapenem dirençli izolatlarda *aac(6')-Ib* pozitifliği, karbapenem duyarlı izolatlardan istatistiksel olarak daha yüksek bulundu.
Sonuç: Aminoglikozitler, karbapenem dirençli *P. aeruginosa* izolatlarında tercih edilen ilaçlardan biridir. Ancak, çoklu ilaç direnç belirleyicilerinin transferi nedeniyle, karbapenem dirençli izolatlarda AME varlığı olarak daha yüksektir ve Gram-negatif bakteriler arasında direnç belirleyicilerinin izlenmesi çok önemlidir.
Anahtar Kelimeler: Aminoglikozit modifiye edici enzimler, *P. aeruginosa*, karbapenem direnci

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Introduction

Pseudomonas aeruginosa is one of the nosocomial pathogens that cause infections with a high mortality and morbidity, especially in patients with immunocompromised status, burns and cystic fibrosis[1,2]. Aminoglycosides can be useful components of antipseudomonal chemotherapy, and resistance continues to be an issue[3].

Aminoglycosides are one of the older groups of antibiotics with broad-spectrum activity against many Gram-negative and Gram-positive bacteria. However, the emergence of resistance has limited their use in recent years.

Resistance to aminoglycosides may be due to (i) chemical modification by aminoglycoside-modifying enzymes (AMEs), (ii) efflux, (iii) reduced permeability, and (iv) alteration of the target by 16S rRNA methyltransferases (16S RMTases). Among these, the presence of AMEs is the most common mechanism of resistance to aminoglycosides. These enzymes modify the aminoglycoside molecule by acetylation [aminoglycoside acetyltransferase (AAC)], phosphorylation [aminoglycoside phosphoryltransferases (APH)], or adenylation [aminoglycoside nucleotidyltransferases (ANT)], and their occurrence and frequency vary by geographical region and hospital depending on the selective pressure exerted by the use of specific aminoglycoside(s)[4-7].

Aminoglycoside resistance in *P. aeruginosa* has often arisen via acquired AMEs and 16S rRNA methylases that confer high level of resistance, and the MexXY-OprM efflux pump generally contributes to the low-moderate level of antimicrobial resistance[1,3,6-8]. Of these mechanisms, the enzymatic modification of aminoglycosides by plasmid or chromosome-encoded genes is a more prevalent mechanism found in *P. aeruginosa*[9-12].

Aminoglycoside inactivation in resistant strains involves their modification by enzymes that phosphorylate (APH), acetylate (AAC), or adenylates (ANT) these compounds. These enzymes commonly cause aminoglycoside resistance in *P. aeruginosa*[3].

A growing concern is the emergence and spread of multidrug-resistant *P. aeruginosa*. Such resistance is due partly to the dissemination of carbapenemases in this species. Although still rare, colistin resistance is of particular concern among patients with burns and cystic fibrosis[2]. While acquisition of resistance genes (e.g., those encoding *β-lactamases* and *AMEs*) via horizontal gene transfer can and drive antimicrobial/multidrug resistance development in *P. aeruginosa*, more common mutations of chromosomal genes (target site and efflux mutations) explain resistance in this organism[1].

The aim of this study was to investigate the prevalence of AMEs in carbapenem-susceptible and resistant isolates.

Materials and Methods

Bacterial Isolates

P. aeruginosa clinical isolates (n=98) resistant to amikacin and/or gentamicin were enrolled in the study. Of the 98 isolates, 54 (55.1%) were resistant to carbapenem.

Antimicrobial Susceptibility Testing

The identification of the isolates was performed using Vitek MS (BioMérieux, France) automated system, and analysis of the antimicrobial susceptibility of the isolates was performed in Vitek2 Compact system (BioMérieux, France). The disc diffusion method was also performed for amikacin, gentamicin, netilmicin, and tobramycin. Antimicrobial susceptibility results of the isolates were interpreted according to the EUCAST criteria[13]. *E. coli* ATCC 25922 and *P. aeruginosa* ATCC 27853 were used as quality control strains. The gradient diffusion method was used for carbapenem-resistant isolates that were determined to be resistant using the Vitek2 Compact system.

Molecular Characterization of Aminoglycoside Resistance Determinants

Isolates that were resistant to amikacin or gentamicin were tested by polymerase chain reaction (PCR) for six *AME* genes. The specific primers for the following genes were included in the PCR assay: *aac(3')-IIa*, *aac(6')-Ib*, *ant(2'')-Ia*, *aph(3')-VIa*, *aac(6')-IIa*, and *aac(3')-Ia* (Table 1).

DNA preparation was performed by the boiling method. Then, 2 µL of DNA was added to a reaction mixture containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM deoxynucleoside triphosphate, 0.5 µM of each primer, and 1 U of Taq DNA polymerase. The amplification conditions were as follows: 94 °C for 5 min, followed by 30 cycles at 94 °C for 30 s, 55 °C for 30 s and 72 °C for 1 min, and 10 min at 72 °C for the final extension[14]. PCR products were analyzed on 1.5% (w/v) agarose gels stained with ethidium bromide.

Statistical Analysis

The chi-square test was used to examine the association of aminoglycoside resistance with genes encoding AMEs. A p-value of <0.05 was considered significant.

The study was approved by Clinical Ethics Committee of Ondokuz Mayıs University Medical Faculty (B.30.2.ODM.0.20.08/121). The study followed the Declaration of Helsinki principles. Informed consent was not obtained because study-only isolates were tested, and patients' electronic data were used without ID information.

Results

In this study, 98 *P. aeruginosa* isolates were tested, and 54 of them were resistant to carbapenem. The respiratory tract specimens (42.8%) were the most common specimen with *P. aeruginosa* isolates (Table 2).

The lowest resistance rate was detected for amikacin (10.2%). The highest resistance rates were observed for gentamicin (44.9%). Netilmicin and tobramycin resistance rates were 27.5% and 37.7%, respectively. However, resistance rates to all tested antimicrobials were higher in carbapenem-resistant isolates. Resistance rates are shown in Table 3. Regarding the statistical analyses, the resistance rates to gentamicin, tobramycin, and netilmicin were significantly higher in carbapenem-resistant isolates than in carbapenem-susceptible isolates ($p<0.05$).

PCR screening for *AME* genes showed that *aac(6')-Ib* (n=13, 13.2%) was the most prevalent *AME* gene, followed by *ant(2'')-Ia* (n=7, 7.1%). The combination of *aac(6')-Ib* + *aac(3')-IIa* and *ant(2'')-Ia* + *aph(3')-VIa* was detected in one isolate.

The *aac(6')-Ib* was the most common AME in carbapenem-resistant isolates (11/54, 20.3%); however, *ant(2'')-Ia* was the most common AME in carbapenem-susceptible isolates (4/44, 9%). The distribution of AME is given in Table 4. In 74 of the isolates, no *AME* genes were detected. Seven of the isolates were resistant to all of the tested aminoglycosides. *aac(6')-Ib* positivity in carbapenem-resistant isolates was statistically higher than that in carbapenem-susceptible isolates.

The resistance phenotype of the isolates is given in Table 4; 11/12 of the *aac(6')-Ib*-positive isolates had resistance against gentamicin, which is an unexpected resistance. One of the

Table 1. Distribution of clinical specimens

Specimen	Carbapenem-susceptible (n=44)	Carbapenem-resistant (n=54)	Total (n=98)
Respiratory tract specimen	14 (31.8%)	28 (51.8%)	42 (42.8%)
Wound	12 (27.2%)	12 (22.2%)	24 (24.5%)
Urine	12 (27.2%)	7 (12.9%)	19 (19.4%)
Sterile body fluid	4 (9%)	2 (3.7%)	6 (6.1%)
Blood	2 (4.5%)	3 (5.5%)	5 (5.1%)
CSF	-	2 (3.7%)	2 (2%)

CSF: Cerebrospinal fluid

Table 2. Resistance rates of aminoglycoside in carbapenem-resistant and carbapenem-susceptible isolates

	Carbapenem-susceptible (n=44)	Carbapenem-resistant (n=54)	Total (n=98)
Amikacin	3 (6.8%)	7 (13%)	10 (10.2%)
Gentamicin	11 (25%)	33 (61.1%)	44 (44.9%)
Netilmicin	7 (15.9%)	20 (37%)	27 (27.5%)
Tobramycin	11 (25%)	26 (48.1%)	37 (37.7%)

Table 3. *AME* genes in carbapenem-resistant and carbapenem-susceptible isolates

AME	Carbapenem-susceptible (n=44)	Carbapenem-resistant (n=54)	Total (n=98)
aac(6')-Ib	1 (2.3%)	11 (20.3%)	12 (12.2%)
ant(2'')-Ia	4 (9%)	2 (3.7%)	6 (6.1%)
aac(3')-IIa	-	-	-
aph(3)-VIa	-	-	-
aac(3')-IIa + *aac(6')-Ib*	1 (2.3%)	-	1 (1%)
ant(2'')-Ia + *aph(3')-VIa*	-	1 (1.8%)	1 (1%)

Table 4. Resistance phenotypes of isolates that *AME* was detected

	No. of isolates (%)	Expected resistance phenotype	Observed resistance phenotypes (no. of isolates)
aac(6')-Ib	12 (13.2%)	A, T, N	Unexpected resistance to G (11)
ant(2'')-Ia	6 (7.1%)	G, T	Unexpected resistance to N (1)
aac(3')-IIa + *aac(6')-Ib*	1 (1%)	A, G, N, T	As expected
ant(2'')-Ia + *aph(3')-VIa*	1 (1%)	G, A, N,T	As expected

ant(2'')-Ia-positive isolate was resistant to netilmicin, which is an unexpected resistance[10].

Discussion

Regarding aminoglycosides (amikacin, gentamicin, and tobramycin) used in clinical practice, the CAESAR annual report 2016 showed that the rates of aminoglycoside (gentamicin, tobramycin) resistance were 18% and 17% for *P. aeruginosa* among blood and cerebrospinal fluid isolates in Turkey in 2014 and 2015, respectively[2].

Overall, *aac(6')-Ib* was the most prevalent *AME* gene in our study in both carbapenem-susceptible and carbapenem-resistant isolates and was detected in a total of 12 (12.2%) isolates. In a recent study, as part of the SENTRY Antimicrobial Surveillance Program, a prevalence of 46.2% for *aac(6')-Ib* was found among *P. aeruginosa* isolates[15]. Similarly, in another study from France, Dubois et al.[9] showed that the *aac(6')-Ib* gene was the most frequent (36.5% of the 52 resistant strains).

In a study from Iran, the prevalence of aminoglycoside resistance genes in 135 resistant isolates 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* in 7% of the resistant isolates[10]. According to Kashfi et al.[16], the prevalence values of *Aph (3')-Ib*, *Aph (6')-VI*, *rmtA*, *aac (6')-IIa*, *aadA*, *aadB*, and *armA* were 60%, 85%, 45%, 10%, 87.5%, and 55%, respectively, according to the PCR method.

The *aac(6')* family, of which two major subfamilies have been described in *P. aeruginosa*, is the major aac family contributing to aminoglycoside resistance in *P. aeruginosa*. AAC(6') enzymes are major determinants of resistance to tobramycin and amikacin (subfamily I) and tobramycin and gentamicin (subfamily II), although some subfamily I variants lack activity against amikacin[1]. In the present study, *aac(6')-Ib* resistance was higher in carbapenem-resistant isolates. This finding supports the fact that genes for AMEs are typically found on integrons with other resistance genes; thus, AMEs harboring isolates are often multidrug resistant[1]. Among the isolates of *P. aeruginosa* (n=150), *ant(2'')-I* (40%) was the most common AME, followed by *aac(6')-II* (29.3%) in Turkey[17]. In another study in Turkey (n=300), *aac(6')-Ib* was detected in six of the isolates, but *aac(6')-Ib-cr* was not detected in any isolates[18]. The *cr* variant of *aac(6')-Ib* is known to confer reduced susceptibility to ciprofloxacin[19].

In the present study, the second most common *AME* gene was *ant(2'')-Ia*, which is widely distributed as a gene cassette in integrons and causes resistance to gentamicin, tobramycin, kanamycin, and dibekacin[6]. All isolates containing this gene were both resistant to gentamicin and tobramycin, which is in agreement with the expected phenotype; however, one isolate was resistant to netilmicin.

In this study, *ant(2'')-Ia* + *aph(3')-VIa* was found in only one isolate. This finding is in contrast to study conducted in Korea, where the *aph(3')-VI* gene was the most frequently found gene (37 isolates)[20]. The *aph(3')-VI* subclass shows a resistance profile including amikacin and isepamicin[3].

In 74 isolates (75.5%), none of the investigated *AME* genes were present. This might be due to the action of other resistance mechanisms, such as 16s rRNA methylases, efflux, and impermeability.

Study Limitations

The study is limited by the use of isolates from a single center and testing only *AME* genes that are common in *P. aeruginosa* isolates. Moreover, aminoglycoside-susceptible isolates were not tested in the study.

Conclusion

In conclusion, in both carbapenem-resistant and carbapenem-susceptible isolates, *aac(6')-Ib* was the most common gene among the isolates tested in the study. To our knowledge, this is the first study that investigate AMEs in our region. Further studies are needed to monitor aminoglycoside resistance in the Mediterranean region and determine the mechanisms of AMEs.

Ethics

Ethics Committee Approval: The study was approved by Clinical Ethics Committee of Ondokuz Mayıs University Medical Faculty (B.30.2.ODM.0.20.08/121).

Informed Consent: Informed consent was not obtained because study-only isolates were tested, and patients' electronic data were used without ID information.

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Authorship Contributions

Concept: Y.T.Ç., O.S.C., Design: Y.T.Ç., O.S.C., Data Collection or Processing: İ.B., C.Ç., Analysis or Interpretation: Y.T.Ç., O.S.C., İ.B., C.Ç., D.G.V., K.B., A.B., Literature Search: Y.T.Ç., İ.B., C.Ç., D.G.V., K.B., Writing: Y.T.Ç., O.S.C.

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