Frequency of Aminoglycoside Resistance Genes in Clinical Isolates of *Pseudomonas aeruginosa* from Sistan, Iran

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**Abstract**

**Introduction:** *Pseudomonas aeruginosa* is a prevalent healthcare-acquired infection. It is a global concern due to its potential for multidrug resistance and for posing significant challenges to healthcare providers. Two critical processes that lead to aminoglycoside resistance are the methylation of 16S ribosomal RNA (16S rRNA) and aminoglycoside-modifying enzymes (AMEs). Aminoglycosides are potent antimicrobial medicines often used to eliminate pseudomonas infections efficiently. This research aimed to assess the frequency of the occurrence of 16S rRNA methylation and AME genes in clinical isolates of *P. aeruginosa* in Sistan, Iran.

**Methods:** A total of 60 clinical isolates of *P. aeruginosa* were collected. The isolates were identified from July 1, 2020, to May 28, 2022. A multiplex polymerase chain reaction (PCR) was carried out to amplify the 16S rRNA methylase genes (armA and rmtA) and AME genes ([Aph(3′)-Ib, Aph(6′)-VI, aadA1, aadB, and aac]) in clinical isolates of *P. aeruginosa* in Sistan, Iran.

**Results:** The most prevalent AME gene was aadB, which was present in 48.33% of the isolates (29/60). The least prevalent genes were aac, aadA1, and Aph(3′-Ib) with frequencies of 6 (10%), 6 (10%), and 7 (11.66%), respectively. It was found that 85% (51/60) of the *P. aeruginosa* isolated were resistant to gentamicin, 65% (39/60) were resistant to amikacin, and 23.33% (14/60) were resistant to tobramycin.

**Conclusion:** Overall, this study determined that aadB was the most prevalent AME gene. In contrast, the genes aac, aadA1, and Aph(3′)-Ib had the lowest occurrence in clinical isolates of *P. aeruginosa* from southeastern Iran.

**Keywords:** *Pseudomonas aeruginosa*, AME genes, Aph (3′)-Ib, Aph (6′)-VI, aadA1, aadB, aac

**Introduction**

*Pseudomonas aeruginosa* is a rod-shaped aerobic gram-negative bacterium. It has a larger genome (5.5-7Mb) compared to other bacteria and a remarkable genetic capacity, enabling it to thrive in many habitats and withstand a broad spectrum of physical circumstances to generate a range of harmful characteristics and exhibit resistance to the majority of presently accessible antibiotics.

The biological adaptability enables *P. aeruginosa* to cause various infections in people with severe underlying medical problems, significantly contributing to healthcare-associated illnesses globally. *P. aeruginosa* is capable of causing a range of illnesses in individuals with both regular and weakened immune systems. The tendency of the organism to cause infections in individuals with weakened immune systems, its ability to adapt to different conditions, its resistance to antibiotics, and its diverse range of defensive mechanisms make it a complicated organism to treat in contemporary medicine.

Aminoglycoside antibiotics are often used in medical settings, especially to treat severe infections caused by *P. aeruginosa*. The A-site of the bacterial 30S ribosomal subunit is strongly attracted to these antibiotics. This site checks to see if codon-anticodon interactions are correct. Additionally, these antibiotics disrupt the activity of the 16S rRNA ribosomal subunit, hinder protein synthesis, and ultimately cause the death of bacteria. Regrettably, improper and excessive use of these medicines has reduced their effectiveness.
Due to the increasing versatility of Gram-negative bacteria like P. aeruginosa, researchers should focus more on understanding aminoglycoside resistance. The increasing frequency of P. aeruginosa strains resistant to antibiotics is a cause for concern because these bacteria cause various hospital-acquired infections (HAIs). The use of antibiotics during the COVID-19 pandemic likely contributed to the rapid rise in resistance rates. One major reason why P. aeruginosa is resistant to aminoglycosides is that medicines are not working because of aminoglycoside-modifying enzymes (AMEs), which can be found on plasmids or chromosomes.

There are proteins in plasmids that are not affected by aminoglycosides, which include acetyltransferases (aac), phosphotransferases (aph), and nucleotidyl transferases (ant). These genes are called antibiotic resistance genes. The production of 16S rRNA methylases (RMTs) by clinical isolates of P. aeruginosa is another plasmid-mediated mechanism of aminoglycoside resistance. The presence of 16S rRNA methylation enzymes was previously believed to be limited to environmental bacteria. However, the discovery of the armA gene-mediated aminoglycoside resistance in Klebsiella pneumoniae in 2003 refuted this idea. Ten different 16S rRNA methylase genes have been found so far in different types of Enterobacteriaceae, which include armA, rmtA, rmtB, rmtC, rmtD, rmtE, rmtF, rmtG, rmtH, and rnpmA. These genes produce 16S rRNA methylases that reduce the efficacy of several aminoglycosides. Since they are located on plasmids, 16S rRNA methylase genes may be readily transmitted to other bacteria. Preventative screening of these genes is advised for public health.

There is a lack of data on the prevalence of AME, armA, and rtmA genes in P. aeruginosa isolates during the COVID-19 pandemic in Sistan, southeastern Iran. This study aimed to identify the prevalence of 16S rRNA methylase genes and AME genes in clinical isolates of Pseudomonas aeruginosa in Sistan, southeastern Iran.

Materials and Methods

Bacterial Isolation

This cross-sectional study was undertaken on diverse clinical samples from July 1, 2020, to May 28, 2021, at the teaching hospitals affiliated with Zabol University of Medical Sciences in Sistan, southeastern Iran. A total of 274 clinical samples (including urine, pus or wound swabs, blood, vaginal swabs, sputum, and endotracheal specimens) were collected from patients. Of 274 samples, 183 (66.78%) belonged to males and 91 (32.21%) belonged to females, attending Amir-al-Mommenin hospital, Zabol University of Medical Sciences, Zabol, Iran. All clinical isolates of P. aeruginosa were identified in patients who were suspected of having infection by conventional biochemical tests. Only one isolate of P. aeruginosa per patient was collected, to avoid duplicates.

Isolation of Bacterial DNA Using the Boiling Method

The extraction of total DNA from P. aeruginosa isolates was performed using the boiling process, as previously reported. Briefly, all P. aeruginosa isolates were grown overnight (18-24 hours) in 5 mL of nutrient broth in a shaking incubator (200 rpm) at 37 °C. Then, 2 mL of bacterial culture broth was pelleted, resuspended in 200 µL of sterile double-distilled water, and boiled at 95 °C for 10 minutes. The suspension was then chilled on ice for 5 minutes, and the supernatant was collected after centrifugation at 13,000 rpm for 5 minutes. Finally, the supernatants were kept at a temperature of -20 °C until use for polymerase chain reaction (PCR).

Antimicrobial Susceptibility Testing

The antimicrobial susceptibility of all clinical isolates was examined using the disc diffusion method with three aminoglycoside antibiotics, including gentamycin, amikacin, and tobramycin. Antibiotic discs were placed on the inoculated Mueller-Hinton agar plates. Plates were incubated for 18–24 hours at 37 °C. The zones of growth inhibition around each of the antibiotic discs were measured to the nearest millimeter.

Designing Primers

The genes that encode AMEs and 16S rRNA methylases were identified using primers previously published (Table 1).

Multiplex PCR

The frequency of methylase genes (armA and rmtA) and AME genes [Aph(3′)-Ib, Aph(6′)-VI, aadA1, aadB, and aac] in clinical isolates of P. aeruginosa were determined in two separate Multiplex PCR assays and one single PCR assay (Table 1). In this study, the Multiplex TEMPase 2x Master Mix (Ampliqon, Denmark, Cat. No. A260301) was used to perform Multiplex PCR. The mixture included 25 µL of 2 X Master Mix, 25 mM of MgCl₂, 10 µM of each forward and reverse primer in nuclelease-free water (Ampliqon, Denmark, Cat. No. A340037), PCR-grade H₂O, and 1 µL of template DNA. The total volume was 50 µL. The amplicons, which had undergone electrophoresis on a 1.5% agarose gel, were exposed to ultraviolet radiation in the presence of 2 µL of ethidium bromide. A DNA ladder of 100 bp (Thermo Scientific, Fermentas, USA, Cat. No. SM0241) was used to determine the size of the DNA fragments. For single PCR, the mixture included 12.5 mL of 2 × Master Mix Red (amplicon), 0.2 mM/mL of each primer (1 µL) (Pishgam, Iran), 2 mL (approximately 100 ng/mL) of genomic DNA, and 9.5 mL of ddH2O. Amplification was performed under the following conditions: 94 °C for 5 minutes, 35 cycles of 94 °C for 45 seconds, 55 °C for 60 seconds, and 72 °C for 120 seconds, with a final elongation at 72 °C for 10 minutes using a Mastercycler® pro Gradient (Eppendorf,
Hamburg, Germany). The PCR products, which had undergone electrophoresis on a 1.5% agarose gel, were exposed to ultraviolet radiation in the presence of 2 µL of ethidium bromide. A DNA ladder of 100 bp (Thermo Scientific, Fermentas, USA, Cat. No. SM0241) was used to determine the size of the DNA fragments.

**Statistical Analysis**

Statistical analysis was carried out using SPSS version 21 (SPSS, Inc. USA). The data was reported as a mean ± standard deviation. A P value of less than 0.05 was considered significant, and the confidence interval was 95%.

**Results**

In this investigation, a total of 60 *P. aeruginosa* isolates obtained from the patients at teaching hospitals of Sistan, Iran, from July 2020 to May 2021 were employed. The resistance patterns of the *P. aeruginosa* isolated showed that 85% (51/60) were resistant to gentamicin, 65% (39/60) were resistant to amikacin, and 23.33% (14/60) were resistant to tobramycin. Additionally, 94.11% (48/51) of the aminoglycoside-resistant isolates harbored one or more resistance genes, and none of the susceptible isolates harbored the tested resistance genes. Of 60 *P. aeruginosa* isolated from patients at hospitals in Sistan, 55 were positive (91.66%) and 5 were negative (8.33%) for any of the genes under investigation (*P*<0.05), as shown in Figure 1. The AME genes and 16S rRNA methylase genes were screened using the multiplex PCR technique. The results showed that the most prevalent AME gene was *aadB*, which was present in 48.33% of the isolates (29/60) (*P*<0.05), while a 16S rRNA methylase encoding gene (*rmtA*) was observed in 11.66% of the isolates. The least prevalent genes were *aac*, *aadA*, and *Aph(3′)-Ib* with prevalence rates of 10, 10, and 11.66%, respectively (*P*>0.005) (Figure 1).

**Discussion**

*Pseudomonas aeruginosa* is a prevalent pathogenic bacterium that poses a significant hazard to human health. In 2017, the World Health Organization (WHO) included *P. aeruginosa* in the short list of antibiotic-resistant organisms known as the ESKAPE group of pathogens.25 Besides the compromised skin barrier, two other variables contribute to the increased incidence of *P. aeruginosa* infections among burn victims. Initially, it was evident that the presence of *Pseudomonas* in the gastrointestinal microbiome significantly increased after the burn damage. Furthermore, sera derived from burn isolates harbored the tested resistance genes. Of 60 *P. aeruginosa* isolated from patients at hospitals in Sistan, 55 were positive (91.66%) and 5 were negative (8.33%) for any of the genes under investigation (*P*<0.05), as shown in Figure 1. The AME genes and 16S rRNA methylase genes were screened using the multiplex PCR technique. The results showed that the most prevalent AME gene was *aadB*, which was present in 48.33% of the isolates (29/60) (*P*<0.05), while a 16S rRNA methylase encoding gene (*rmtA*) was observed in 11.66% of the isolates. The least prevalent genes were *aac*, *aadA*, and *Aph(3′)-Ib* with prevalence rates of 10, 10, and 11.66%, respectively (*P*>0.005) (Figure 1).

**Table 1. Specific Primers and PCR Conditions**

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence (5′→3′)</th>
<th>Predenaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Elongation</th>
<th>Final elongation</th>
<th>Size (bp)</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>armA-F</td>
<td>TGGGAAGTIAACGACGAC</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>212</td>
<td>20</td>
</tr>
<tr>
<td>armA-R</td>
<td>CAACTCCCTTCTCCCTTCCA</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>20</td>
<td>20</td>
</tr>
<tr>
<td>rmtA-F</td>
<td>CTAGGGTTACATCTTTCCT</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>635</td>
<td>21</td>
</tr>
<tr>
<td>rmtA-R</td>
<td>TTTGCTTCCATGGCTTCGCC</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>21</td>
<td>21</td>
</tr>
<tr>
<td>Aph(3′)-Ib-F</td>
<td>CTTGGTGATAACGGCAATTCC</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>548</td>
<td>22</td>
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<tr>
<td>Aph(3′)-Ib-R</td>
<td>CCAATCGCAGATAGAAGGCAA</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>22</td>
<td>22</td>
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<tr>
<td>Aph(6′)-IVF</td>
<td>AGCGAAAATGTTGAGTGGCT</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>399</td>
<td>23</td>
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<tr>
<td>Aph(6′)-IVR</td>
<td>TCCGTTGATATCCGATAGA</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>23</td>
<td>23</td>
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<tr>
<td>aadA1-F</td>
<td>ATAGGGGAAGCAGGGATCG</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>792</td>
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<tr>
<td>aadA1-R</td>
<td>TTATTTGCGTACCTCTTGGT</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>aadB-F</td>
<td>ATGACACAACGGAGTCGCC</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>534</td>
<td>23</td>
</tr>
<tr>
<td>aadB-R</td>
<td>TTAGCCCGCATATCGCGACC</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>72°C, 1 min</td>
<td>72°C, 2 min</td>
<td>72°C, 10 min</td>
<td>23</td>
<td>23</td>
</tr>
<tr>
<td>aac-F</td>
<td>CCAATACTCTTGCCTCATTG</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>55°C, 60 s</td>
<td>35°C</td>
<td>35°C</td>
<td>72°C, 10 min</td>
<td>542</td>
</tr>
<tr>
<td>aac-R</td>
<td>GAGTTGTAGGCAACACCCG</td>
<td>94°C, 5 min</td>
<td>94°C, 45 s</td>
<td>55°C, 60 s</td>
<td>35°C</td>
<td>35°C</td>
<td>72°C, 10 min</td>
<td>24</td>
</tr>
</tbody>
</table>

MPI: multiple PCR-I; MPII: multiple PCR-II; SPCR: single PCR.
patients can potentially enhance biofilm production by *P. aeruginosa* while suppressing the growth of other microbial species. Prompt and accurate diagnosis is essential for *P. aeruginosa* infections since it can positively alter clinical outcomes. The prevalence of aminoglycoside resistance in *Enterobacteriaceae* has changed over the last decades. Bacterial multidrug resistance (MDR) is an important problem in *P. aeruginosa* due to its ability to mix several mechanisms, transposons, plasmids, and chromosomally encoded genes such as methyltransferases or pumps. Methyltransferase genes are distributed throughout the bacterial genome, poised to initiate antibiotic resistance. 16S rRNA methyltransferases (16S RMTases) provide strong resistance to all medically important aminoglycosides with just one gene. This differs from other aminoglycoside resistance mechanisms, which require multiple genes to reach the same resistance level. There have been global reports of 16S RMTase genes such as *armA*, *rmtA*, *rmtH*, and *npmA*. Their appearance is a public health concern because 16S RMTase genes are linked to other genes that make bacteria resistant to antibiotics. These genes include *carbapenemases* and extended-spectrum β-lactamas, found in bacteria resistant to more than one drug. Furthermore, 16S RMTase genes are linked to mobile genetic elements such as transposons and plasmids, which makes it easier for them to spread through horizontal gene transfer. In the present study, for the first time after the COVID-19 pandemic, the frequency of AME genes in clinical isolates of *P. aeruginosa* from southeastern Iran was evaluated. The resistance patterns of *P. aeruginosa* isolated showed that 85% (51/60) were resistant to gentamicin, 65% (39/60) were resistant to amikacin, and 23.33% (14/60) were resistant to tobramycin. Moreover, 94.11% (48/51) of the aminoglycoside-resistant isolates harbored one or more resistance genes, and none of the susceptible isolates harbored the tested resistance genes. The results showed that the most prevalent AME gene was *aadB*, which was present in 48.33% of the isolates. The least prevalent genes were *aac, aadA*, and *Aph(3′)-Ib*, with prevalence rates of 10, 10, and 11.66%, respectively. Our results do not agree with the results of the study by Salimzadeh et al., who reported that the *aadB* gene had the lowest frequency of AME genes in *Pseudomonas aeruginosa* isolated from hospitals in Tehran, Iran. Notably, our results do not agree with those of the study by Ghotaslou et al either, who identified *ant (3′)-Ia* and *aph (3′)-Ib* as the most prevalent AME-encoding genes in *Enterobacteriaceae* isolates in Northwest Iran. Diverse investigations have shown that the occurrence of AME-encoding genes in *P. aeruginosa* strains might vary across various countries. Possible causes include different lengths of hospital stays and different therapy approaches. An analysis conducted in France on 120 *P. aeruginosa* bacteremia isolates collected from 1999 to 2004 revealed that 25 samples (21%) carried at least one of the AME genes, namely *ant (2′)-I, aac (6′)-I*, and *aac (3)-I*. Research conducted in northern Poland analyzed 25 samples of *P. aeruginosa* obtained from two critical care units over seven years (2002-2009). The study found that *ant (2′)-Ia* was present in 36% of the isolates, *aac (6′)-Ib* in 28% of the isolates, and *aph (3′)-Ib* in 8% of the isolates. This significant difference may also be due to geographical factors. It is noteworthy that *P. aeruginosa* strains can acquire mutations that make them resistant to aminoglycosides. This resistance may occur in addition to the horizontal transfer of AME genes. However, our findings are in line with those of the study by Teixeira et al., who reported that the *aadB* gene was the most frequently detected gene in *P. aeruginosa* isolates from Cumana, Venezuela. Jones et al. discovered a link between having *aadB* and being resistant to kanamycin, gentamicin, and tobramycin in all 33 strains with this gene cassette. Our limitation in this study is that we did not conduct transcriptional or functional investigations to ascertain whether mutations specifically impeded protein function and influenced gene expression. Considering these supplementary parameters in future investigations may enhance the precision of predicting aminoglycoside resistance phenotypes.

**Conclusion**

Taken together, our findings suggest that the presence of the *aadB* gene in 29 out of 55 positive isolates of *P. aeruginosa* in Sistan, Iran, might result in heightened biological resistance and an enhanced probability of resistant fluctuations in this bacterium.

**Acknowledgments**

The authors extend their appreciation and acknowledgment to the faculty of Science and Veterinary Medicine at the University of Zabol, Iran.

**Authors’ Contribution**

References


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