Prevalence of aminoglycoside resistance genes in Pseudomonas aeruginosa isolated from a tertiary care hospital in Makkah, KSA

Aminoglycosides are the most frequently prescribed antimicrobial agents in Saudi Arabia; they are routinely used for the treatment of gram-negative bacillary infections. The aim of this study was to detect the resistance patterns against different aminoglycoside antibiotics and the prevalence of the genes encoding for resistance in Pseudomonas aeruginosa isolates from Hera General Hospital, Makkah, KSA. All isolates that were resistant to one or more aminoglycoside antibiotic were subjected to antibiotic susceptibility test and PCR analysis to detect the presence of the resistance genes: aac (6')-Ib, aac(3)-Ia, aac(3)-II, ant(2'')-Ia, rmtB, rmtC, armA, rmtA, rmtD, rmtE, and npmA. The results showed that 46.1% of the isolates were resistant to one or more aminoglycoside antibiotics, but only 43.3% of these aminoglycoside-resistant isolates harbored resistance genes. In addition, 43% of the isolates were resistant to ciprofloxacin, amikacin, and ceftazidime. The resistance genes most frequently observed in these isolates were rmtB (7.6%) followed by aac (6')-Ib (6.1%), rmtC (4.6%), and armA (1.5%). Taken together, these results indicate that the aminoglycoside-resistance genes are highly prevalent and could easily spread among P. aeruginosa strains. Coordinated efforts and further research works are needed to control antibiotic resistance to aminoglycosides before to be a threatening crisis.

Keywords: aminoglycoside, resistance genes, P. aeruginosa, phosphotransferase, modifying enzyme

Introduction
Aminoglycosides are highly potent, broad-spectrum antibiotics with many desirable properties used for the treatment of life-threatening infections [1]. Aminoglycosides are the most frequently prescribed antimicrobial agents in Saudi Arabia; they have been definitively established for the treatment of gram-negative bacillary infections. Aminoglycosides include many different agents such as gentamicin, tobramycin, amikacin, streptomycin, neomycin, and paromomycin; gentamicin, tobramycin, and amikacin are the most frequently prescribed. Aminoglycosides act primarily by binding to the aminoaoyl site of the 16S ribosomal RNA within the 30S ribosomal subunit, leading to misreading of the genetic code and inhibition of translocation [2,3]. Aminoglycosides initially penetrate the organism by disrupting the magnesium and calcium bridges between lipopolysaccharide moieties. They are then transported across the cytoplasmic membrane in an energy-dependent manner. This step can be inhibited in vitro by divalent cations, increased osmolality, acidic pH, and an anaerobic environment [3]. Aminoglycoside resistance can occur through the acquisition or upregulation of genes that encode inactivating enzymes or efflux systems. Bacterial production of inactivating enzymes is the most common aminoglycoside resistance mechanism in gram-negative organisms [3]; resistance is due to the inactivation of aminoglycoside modifying enzymes (AMEs; aminoglycoside phosphotransferases, acetyl-transferases, and nucleotidyl-transferases) by the products of genes in plasmids or transposons. For example, the enzyme encoded by the rmtA gene has been associated with high-level resistance against all parenteral aminoglycosides currently in use [4,5]. The emergence of resistant strains has somewhat reduced the potential of aminoglycosides in empiric therapies [6]. Multidrug-resistant Pseudomonas aeruginosa have been emerging worldwide. The most common aminoglycoside-modifying enzyme gene types in P. aeruginosa are aac(6')-I, aac(6')-II, ant(2'')-I, and aph(3')-[7,8] and their substrates are the most important antipseudomonal aminoglycosides [9]. To date, no studies have been conducted in Saudi Arabia.

Atif H Asghar & Omar B Ahmed*
Department of Environmental and Health Research, The Custodian of the Two Holy Mosques Institute for Haj and Umrah, Umm Al-Qura University, Makkah, Saudi Arabia
*Author for correspondence: abuaglah1@hotmail.com

RESEARCH
to determine which genes confer resistance to aminoglycosides in *P. aeruginosa*. This study aimed to detect the resistance patterns against different aminoglycoside antibiotics and the prevalence of the genes encoding for resistance in *P. aeruginosa* isolates from Makkah hospitals.

**Material and methods**

This study involved the use of previously collected *P. aeruginosa* isolates by the authors and published [10] from Hera General Hospital (HGH)-Makkah; 263 beds). A total of 65 non-duplicated *P. aeruginosa* clinical isolates were identified from HGH. The frequency of isolates according to the wards as follows; male ward (n=13), female ward (n=10), Intensive care unit (n=11), surgery ward (n=10), obstetrics and Gynaecology (n=7), newborn intensive care unit (n=4), nursery (n=5) and pediatrics (n=3). The frequencies of isolates were: abscess (n=20), axillary (n=11), high vaginal swabs (n=10), pleural fluid (n=5), pus swab (n=8) and sputum sample (n=9). The study was approved by Ethics Committee in The Custodian of the Two Holy Mosques Institute for Hajj and Umrah, Umm Al-Qura University and a written informed consent has been taken from the subjects participating in the study.

### Determination of antibiotic susceptibility for *P. aeruginosa* isolates

A standardized inoculum (1 × 10^8 CFU/mL) of all isolates was inoculated on the surface of a large (150 mm diameter) Mueller-Hinton agar plate at 35°C. The antimicrobial susceptibility of all clinical isolates was examined using the disc diffusion method with various antibiotics (TABLE 1). The tested antibiotics were ceftazidime (CAZ) (30 µg), cefotaxime (CTX) (30 µg), Ciprofloxacin (CIP) (10 µg) amikacin (AK) (30 µg), piperacillin/tazobactam (TZP) (100/10) (CRO) (30 µg), ceftepime (FEB) (30), imipenem (IPM) (10), colistin (CL) (10 µg), gentamicin (GN) (10 µg) and tobramycin (TOB) (10 µg).

Antibiotic disks were placed on the inoculated agar surface. Plates were incubated for 16-24 h at 35°C. The zones of growth inhibition around each of the antibiotic disks were measured to the nearest millimeter. The diameter of the zone is related to the susceptibility of the isolate and to the diffusion rate of the drug through the agar medium. The zone diameters of each drug are interpreted using the using Clinical and Laboratory Standards Institute (CLSI) method [11]. CLSI provides for three categories of identification: susceptible, intermediate and resistant. Susceptible defines a level of antimicrobial activity associated with a high likelihood of therapeutic success. Intermediate defines a level of antimicrobial activity associated with uncertain therapeutic effect. Resistant defines a level of antimicrobial activity associated with a high likelihood of therapeutic failure [11].

### DNA extraction

DNA was prepared by guanidinium thiocyanate extraction, as previously described [12]. Two bacterial colonies (3 mm in diameter) were collected from the nutrient agar plates and dispersed in 100 mL of 10 mM Tris-HCl (pH 8.0) and 1 mM EDTA. The cells were lysed with 500 mL GES reagent [5 M guanidinium thiocyanate (Sigma, city, state (abbrev), country), 0.1 M EDTA, and 0.5% (w/v) sarcosyl (Sigma)]. Following the addition of 250 mL 7.5 M ammonium acetate, the suspension was kept on ice for 10 minutes. For deproteination, 500 mL chloroform:isoamyl alcohol (24:1) was added and the mixture was centrifuged at 13,000 g for 10 minutes. The DNA was precipitated from the upper phase with 100% ethanol at -20°C for 1 hour. The extracted DNA was used as a template for PCR amplification.

### PCR analysis

All isolates that were resistant to one or more aminoglycoside antibiotic were subjected to PCR analysis to detect the presence of the following resistance genes: aac(6’)-Ib, aac(3)-Ia, aac(3)-II , ant(2”)-Ia, rmtB, rmtC, armA, rmtA, rmtD, rmtE, and npmA (TABLE 2).
PCR was performed in a final volume of 25 μL. The primers used for PCR amplification are listed in Table 2. Each reaction contained 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 0.2 mM of each deoxynucleoside triphosphate, 1.5 mM MgCl₂, 1.5 μL of each primer, 1.25 U of Taq DNA polymerase, and 2 μL of template DNA. Two multiplex reactions were performed. The first reaction included the aac(6’)-Ib, aac(3)-II genes using the following conditions: pre-denaturation at 94°C for 4 minutes; 35 amplification cycles of 94°C for 1 minute, 55°C for 1 minute, and 72°C for 1.5 minutes; and a final extension step of 72°C for 5 minutes. The second reaction included the armA, rmtB, rmtC, and rmtD genes using the following conditions: pre-denaturation at 94°C for 4 minutes; 35 amplification cycles of 94°C for 1 minute, 50°C for 1 minute, and 72°C for 1.5 minutes; and a final extension step of 72°C for 5 minutes. Amplified PCR products were detected by agarose gel electrophoresis. A DNA marker (Promega, USA) was run with each gel and the genotype was determined by the size of the amplified product.

**Statistical analysis**

Statistical analysis was carried out using Statistical Package for Social Sciences (SPSS) software (version 21.0) for Windows (x²-test). A p value of >=0.05 was considered significant.

**Results**

The resistance profiles of the P. aeruginosa isolates showed that 46.10% (30/65) were resistant to one or more aminoglycoside antibiotics and that only 43.3% (13/30) of the aminoglycoside-resistant isolates harbored resistance genes; none of the susceptible isolates harbored the tested resistance genes. In addition, all of the isolates contained only a single aminoglycoside modifying gene - i.e. none of the isolates co-harbored more than one resistance gene. The results of the present

<table>
<thead>
<tr>
<th>Table 2. Primers used in the study.</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>No.</strong></td>
</tr>
<tr>
<td>1</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>2</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>3</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>4</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>5</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>6</td>
</tr>
<tr>
<td></td>
</tr>
<tr>
<td>7</td>
</tr>
<tr>
<td></td>
</tr>
</tbody>
</table>

**Figure 1. Antibiotic resistance among P. aeruginosa isolates.**

study showed that 43% of the isolates (28/65) were resistant to ciprofloxacin, amikacin, and ceftazidime while 35.4% (23/65) were resistant to imipenem and 32.3% (21/65) were resistant to piperacillin-tazobactam (FIGURES 1 and 4). In addition, 18.5% (12/65) and 17% (11/65) of the isolates exhibited resistance to gentamicin and tobramycin, respectively (FIGURE 1). The resistance genes observed most frequently in these isolates were rmtB (7.6%, 5/65), aac(6\prime)-Ib, (6.1%, 4/65), rmtC (4.6%, 3/65), and armA (1.5%, 1/65) (FIGURES 2 and 3). Moreover, none of the susceptible isolates harbored these resistance genes. The difference

**FIGURE 2.** Aminoglycoside resistance genes detected by PCR and 2% agarose gel electrophoresis. Lane 1: rmtC positive (711 bp), Lane 2: rmtB positive (173 bp), Lane 3: armA positive (315 bp), Lane M: 100-bp DNA ladder.

**FIGURE 3.** Frequency of aminoglycoside resistance genes in *P. aeruginosa.*

**FIGURE 4.** Antibiotic sensitivity test.
in the distribution of the resistance genes was statistically significant between the various aminoglycosides antibiotic resistance profiles (P value <0.05).

**Discussion**

Aminoglycosides are broad-spectrum antibiotics of high potency that have been traditionally used for the treatment of serious gram-negative bacteria such as Pseudomonas infections [13]. Aminoglycosides act by inhibiting protein synthesis via binding to the 16S rRNA and by disrupting bacterial cell membrane integrity [14]. *P. aeruginosa* is one of the most prevalent hospital acquired pathogens associated with higher mortality rates and antibiotic costs. In the present study, a total of 65 non-duplicated *P. aeruginosa* clinical isolates were identified in a tertiary hospital in Makkah. Treatment of *P. aeruginosa* is complicated by its ability to develop resistance to multiple classes of antibacterial agents, even during the course of infection treatment. In the present study, the antimicrobial susceptibility of *P. aeruginosa* strains isolated from HGH was tested. The resistance profiles showed that 46.1% were resistant to one or more antibiotics and 43% were resistant to amikacin, ciprofloxacin, and ceftazidime, as well as imipenem (35.4%), and piperacillin-tazobactam (32.3%). In addition, 18.5% and 17.0% of the isolates were resistant to amikacin, ciprofloxacin, and ceftazidime, respectively. Statistically, there is a significant difference in the frequency of resistance genes across the aminoglycosides resistance profiles (P value <0.05). These results highlight the importance of aminoglycoside-modification-related mechanisms in aminoglycoside resistance in *P. aeruginosa*. The resistance genes most frequently observed in these isolates were rmtB (7.6%) and aac(6’)-Ib (6.1%). Previous studies have demonstrated the existence of the rmtA and rmtB genes in *P. aeruginosa* isolates [29,30]. The results of this study are similar to other studies conducted in different countries [31-33]. None of the susceptible isolates harbored resistance genes, in agreement with several other studies conducted around the world [34]. The most common aminoglycoside resistance determinants found in *P. aeruginosa* are aac(6’)-II and ant(2’)-I in Europe; aph(3’)-VI, ant(2’)-I, and aac(6’)-I in Korea; and aac(6’)-31/aadA1 and aadA2 in Mexico and Brazil [35,36]. This difference in the distribution of aminoglycoside genes may be attributed to differences in aminoglycoside prescription patterns, selection of bacterial populations, or geographical differences in the occurrence of aminoglycoside resistance genes [9]. In conclusion, these aminoglycoside-resistance genes are highly prevalent and could easily spread among *P. aeruginosa* strains [34]. Coordinated efforts and new research are needed to control antibiotic resistance to aminoglycosides before to be a threatening crisis.

**Acknowledgements**

The authors are grateful to The Custodian of the Two Holy Mosques Institute for Hajj and Umrah, Umm Al-Qura University, Makkah, Saudi Arabia., for supporting this study.
RESEARCH

Omar B Ahmed

REFERENCES


Shaw KJ, Rather PN, Hare RS, Miller...


