

Detection of Antibiotic Resistance Genes in *Pseudomonas aeruginosa* by Whole Genome Sequencing

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Background: Multidrug-resistant *Pseudomonas aeruginosa* has become a hazard to public health, making medical treatment challenging and ineffective. Whole-genome sequencing for antibiotic susceptibility testing offers a powerful replacement for conventional microbiological methods.

Objective: The present study evaluated the presence of antibiotic resistance genes in selected clinical strains of *P. aeruginosa* using whole-genome sequencing for antibiotic susceptibility testing.

Results: Whole-genome sequencing of *P. aeruginosa* susceptible to common antibiotics showed the presence of 4 antibiotic resistance gene types, *fosA*, *catB7*, *blaPAO*, and *blaOXA-50*. Whole genome sequencing of resistant or multidrug-resistant *P. aeruginosa* showed the presence of multiple ARGs, such as *sul1*, *aac(3)-Ic*, *blaPAO*, *blaGES-1*, *blaGES-5 aph (3')-XV*, *blaOXA-50*, *aacA4*, *catB7*, *aph (3')-IIb*, *aadA6*, *fosA*, *tet(G)*, *cmlA1*, *aac(6')Ib-cr*, and *rmtF*.

Conclusion: The acquisition of antibiotic resistance genes was found to depend on the resistance of *Pseudomonas* to antibiotics. The strain with the highest resistance to antibiotics had the highest acquisition of antibiotic resistance genes. MDR-*P. aeruginosa* produces antibiotic resistance genes against aminoglycoside, β -lactam, fluoroquinolones, sulfonamides, phenicol, and fosfomycin antibiotics.

Keywords: antibiotic resistance, genes, *Pseudomonas aeruginosa*, whole genome, sequencing

Introduction

Pseudomonas aeruginosa (*P. aeruginosa*) is a gram-negative rod bacterium that is one of the causative agents of nosocomial infections. It is the third most prevalent bacterium identified from infections contracted in intensive care units and is the main cause of morbidity and death in people with cystic fibrosis (CF), chronic obstructive pulmonary disease (COPD), diabetes, severe kidney and liver failure. Due to its inherent resistance to multiple antimicrobial drug classes as well as its potential to quickly develop resistance to other medications during chemotherapy, multidrug-resistant *Pseudomonas aeruginosa* (MDR-*P. aeruginosa*) has become a hazard to public health, making medical treatment challenging and ineffective.^{1,2} The infections caused by MDR-*P. aeruginosa* are challenging to treat because of its potent intrinsic and acquired resistance mechanisms to many classes of antibiotics.^{3,4} Inherent resistance to several antibiotics exists in *P. aeruginosa*, and adaptive resistance develops as a result of the selection of point mutations that may result in resistance to cephalosporins, carbapenems, fluoroquinolones, and polymyxins.⁵

Acquisition of drug-modifying enzymes in *P. aeruginosa*, such as extended-spectrum-lactamases, carbapenemases, and aminoglycoside-modifying enzymes, can be aided via horizontal gene transfer. These resistance mechanisms are frequently passed on through the same genetic components, leading to an MDR-*P. aeruginosa* phenotype.⁶ To assess antimicrobial susceptibility profiles in medical microbiology, bacteria is routinely cultured with antimicrobial drugs, but now whole-genome sequencing for antibiotic susceptibility testing (WGS-AST) offers a powerful replacement for conventional methods. WGS-AST essentially aims to forecast the phenotype that would have been identified if the strain

had been examined using the reliable culture-based test for antibiotic resistance. The literature on molecular genetic research that links genes with indications of antibiotic resistance has mostly been used to curate databases.⁷ The Comprehensive Antibiotic Resistance Database (CARD) (<https://card.mcmaster.ca/>) is a periodically updated biological database of the collection of references on the genes, proteins, and phenotypes of antibiotic resistance.^{8,9} The antibiotic resistance ontology serves as a unique organizing concept for the CARD, which combines diverse molecular and sequence data. The CARD can also swiftly discover probable antibiotic resistance genes in fresh, unannotated genome sequences. This special website offers an informational tool that connects issues about antibiotic resistance in medicine and many other fields, such as agriculture, food security and the environment. Furthermore, it helps users search newly sequenced genomes for possible antibiotic resistance gene prediction.¹⁰ The present study evaluated the presence of antibiotic resistance genes in selected clinical strains of *P. aeruginosa* using whole-genome sequencing.

Materials and Methods

Bacterial Sample

Three strains (p-5, p-7, and p-73) were selected from 108 *Pseudomonas* species that were published previously by the author.¹¹ Table 1 shows that strain p-5 is susceptible to all tested antibiotics, while strain p-7 was found to be nonsusceptible to ceftazidime, ciprofloxacin, and cefepime. The p-73 strain was nonsusceptible to all tested antibiotics except colistin.

DNA Extraction

For DNA extraction, bacterial colonies were taken from an overnight culture, washed with alkaline TE buffer in 2 mL tubes and then resuspended in 0.5 mL TE buffer. Bacterial cell walls were removed by 0.1 mm glass beads for 5 minutes in the BioSpec Mini-Beadbeater-16 (BioSpec Inc., USA) and then left for 5 minutes in a refrigerator. DNA-containing aqueous layers were isolated from proteins and cell debris using phenol/chloroform (1:24 pH 8.0). DNA was precipitated using isopropanol, washed with 70% ethanol, air dried and resuspended in 40 μ L TE (pH 8.0). The quantity and quality of DNA were checked using Qubit[®] (Invitrogen, Applied Biosystems, USA) and an Agilent Bio analyser 2100 using 1000 DNA Chip (Agilent Inc., USA).

PCR

The three strains were identified by polymerase chain reaction (PCR) using specific primers L lipoprotein (OprL) (OprL-F ATGGAATGCTGAAATTCGGC, OprL-R CTTCTTCAGCTCGACGCGACG)¹² for the detection of *P. aeruginosa* species. The extracted DNA was submitted to PCR for confirmation as *P. aeruginosa*. PCR was performed with a final volume of 25 μ L. The primers used for PCR amplification are listed in Table 2. Each reaction contained 20 mM Tris-HCl (pH 8.4);

Table 1 Antimicrobial Susceptibility Patterns of the Three Selected *P. aeruginosa* Strains

Antibiotic	Sample (p-5)	Sample (p-7)	Sample (p-73)
Amikacin	Sensitive	Intermediate	Resistant
Imipenem	Sensitive	Sensitive	Resistant
Piperacillin/Tazobactam	Sensitive	Sensitive	Resistant
Ceftazidime	Sensitive	Resistant	Resistant
Ciprofloxacin	Sensitive	Resistant	Resistant
Cefepime	Sensitive	Resistant	Resistant
Colistin	Sensitive	Sensitive	Sensitive
Cefotaxime	Sensitive	Sensitive	Resistant

Table 2 ARG Database of the P-5 (Susceptible) Strain After Whole Genome Sequencing

Resistance Gene	Identity	Query/HSP	Contig	Position in Contig	Phenotype	Accession No.
fosA	99.02	408/408	NODE_1_length_486363_cov_13.5115_ID_1	153,008.153415	Fosfomycin resistance	NZ_ACVU01000146
catB7	99.37	639/639	NODE_6_length_234299_cov_15.8583_ID_11	182,526.183164	Phenicol resistance	AF036933
blaPAO	99.5	1194/1194	NODE_18_length_118379_cov_11.8332_ID_35	89,688.90881	Beta-lactam resistance	FJ666065
blaOXA-50	99.87	789/789	NODE_7_length_212028_cov_16.8625_ID_13	98,838.99626	Beta-lactam resistance	AY306133

50 mM KCl; 0.2 mM each deoxynucleoside triphosphate; 1.5 mM MgCl₂; 1.5 µL each primer; 1.25 U of Taq DNA polymerase; and 2 µL template DNA. 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.

Whole Genome DNA Sequencing

Libraries for whole genome DNA sequencing were prepared using the Illumina NexteraXT Library Preparation Kit, and samples were barcoded using the NexteraXT Index Kit (Illumina Inc., USA). An Agilent Bio analyser 2100 1000 DNA Chip (Agilent Inc., USA) was used to confirm and quantify DNA sequencing libraries that had been prepared using 1 ng of input genomic DNA. Sequencing of *P. aeruginosa* genomes was performed in an Illumina MiSeq using a pair ends protocol and a version-2500 cycles nano kit. FastQC (BaseSpace Labs, Illumine Inc., USA) was used to check the quality of paired-end sequence reads. SPAdes Genome Assembler 3.0 (Algorithmic Biology Lab, St. Petersburg, Russia) was used to perform de novo assembly of *P. aeruginosa* genomes. Assembled contigs were used for 16S rRNA-based species identification using Species Finder 1.0 Server from the Center for Genomics Epidemiology (<http://www.genomicepidemiology.org/>). In this study, antibiotic resistance mechanisms of the strains were predicted by mapping assembled contigs and paired-end sequence reads against The Comprehensive Antibiotic Resistance Database (CARD) (<http://arpcard.mcmaster.ca/>). Sequence data were mapped against the CARD database using DNASTAR SeqMan NGen version 12.2 (DNASTAR, Madison, USA). The minimum match percentage for mapping used was 99%, and a minimum template coverage of 90% was used as the cut-off. In addition to DNASTAR, antibiotic resistance genes were also predicted using SRSRT2 (BaseSpace Labs, Illumine Inc., USA, <https://www.illumina.com/products/by-type/informatics-products/base-space-sequence-hub/apps.html>), which is a program designed to take Illumina sequence data and search for matching sequencing in the Multilocus sequence typing (MLST) database and/or a database of gene sequences (eg, resistance genes or virulence genes). MLST is the “gold standard” of typing for many species, and when used with WGS, it is more affordable, making it more accessible to regular research and diagnostic labs and enabling comparison with earlier data.

Results

The OprL amplicon genes were detected in the three *P. aeruginosa* isolates (Figure 1). Whole genome sequencing of *P. aeruginosa* (p-5) showed the presence of 4 ARG types with 99–100% identity. These genes included fosA, catB7, blaPAO, and blaOXA-50. The most frequently detected ARG class was β-lactam resistance 2/4 (50% of ARGs), followed by phenicol resistance 1/4 (25%) and fosfomycin resistance 1/4 (25%) (Table 2). Whole genome sequencing of *P. aeruginosa* (p-7) showed the presence of 12 ARG types with 99–100% identity. These genes included sul1, blaPAO, blaGES-1, aph(3′)-XV, blaOXA-50, aacA4, catB7, aph(3′)-IIb, aadA6, fosA, tet(G), and aac(6′)Ib-cr. The most frequently detected ARG class was aminoglycoside resistance 5/12 (41.7% of ARGs), followed by β-lactam resistance 3/12 (25%), fluoroquinolone resistance 1/12 (8.3%), sulfonamide resistance 1/12 (8.3%), tetracycline resistance 1/12 (8.3%), phenicol resistance 1/12 (8.3%), and fosfomycin resistance 1/12 (8.3%) (Table 3). Whole genome sequencing of *P. aeruginosa* (p-73) showed the presence of 12 ARG types with 99–100% identity. These genes included sul1, aac(3)-Ic, aadA6, blaOXA-50, aacA4, blaGES-5, aph(3′)-IIb,

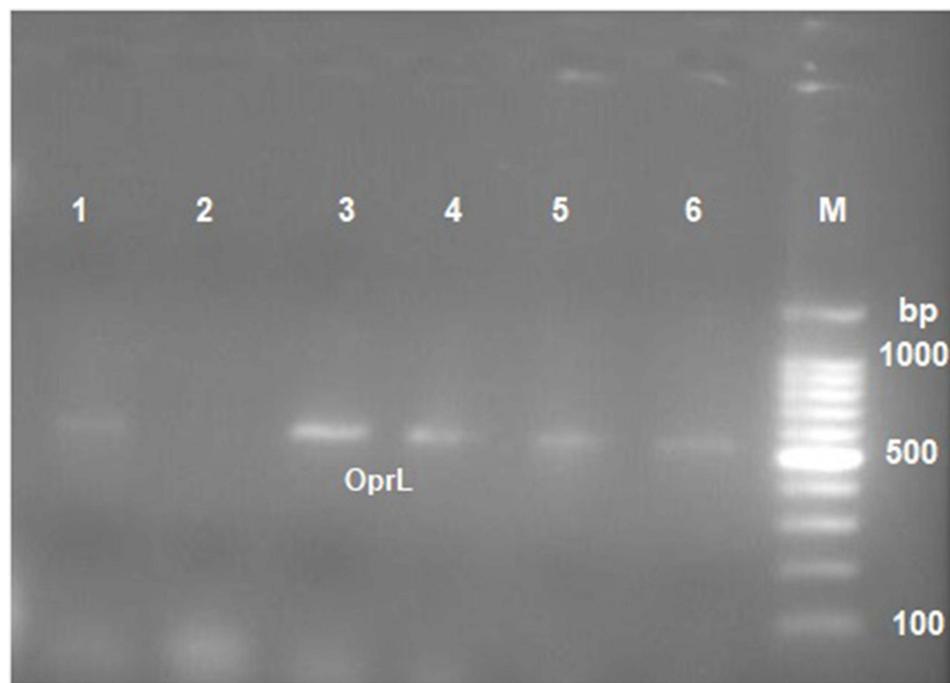


Figure 1 PCR results showing the *P. aeruginosa* Opr L gene, M: marker (100 bp), Line 1 and 3: positive control, Lines 2: negative control, lines 4,5,6: strains of *P. aeruginosa*.

blaPAO, cmlA1, fosA, rmtF, and aac(6')Ib-cr. The most frequently detected ARG class was aminoglycoside resistance 6/12 (50% of ARGs), followed by β -lactam 3/12 resistance (25%), fluoroquinolone resistance 1/12 (8.3%), sulfonamide resistance 1/12 (8.3%), phenicol resistance 1/12 (8.3%), and fosfomycin resistance 1/12 (8.3%) (Table 4).

Table 3 ARG Database of the P-7 (Resistant) Strain After Whole Genome Sequencing

Resistance Gene	Identity	Query/HSP	Contig	Position in Contig	Phenotype	Accession No.
blaPAO	99.25	1194/1194	NODE_22_length_106367_cov_17.0956_ID_43	13,725.14918	Beta-lactam resistance	FJ666065
blaOXA-50	99.87	789/789	NODE_40_length_61006_cov_22.582_ID_79	19,166.19954	Beta-lactam resistance	AY306132
blaGES-1	100	864/864	NODE_8_length_205688_cov_24.2091_ID_15	200,996.201859	Beta-lactam resistance	HQ170511
aacA4	99.46	555/555	NODE_8_length_205688_cov_24.2091_ID_15	201,998.202552	Aminoglycoside resistance	KM278199
aac(6')Ib-cr	99.04	519/519	NODE_8_length_205688_cov_24.2091_ID_15	202,034.202552	Fluoroquinolone and aminoglycoside resistance	EF636461
aph(3')-XV	100	795/795	NODE_8_length_205688_cov_24.2091_ID_15	202,885.203679	Aminoglycoside resistance	Y18050
fosA	99.02	408/408	NODE_7_length_206634_cov_19.8612_ID_13	25,420.25827	Fosfomycin resistance	NZ_ACWU01000146
sulI	100	837/526	NODE_113_length_527_cov_152.471_ID_225	2.527	Sulfonamide resistance	JN581942
catB7	98.75	639/639	NODE_37_length_85016_cov_22.0462_ID_73	32,630.33268	Phenicol resistance	AF036933
tet(G)	100	1176/1176	NODE_66_length_7571_cov_50.3563_ID_131	3509.4684	Tetracycline resistance	AF133140
aph(3')-Iib	98.76	807/807	NODE_22_length_106367_cov_17.0956_ID_43	563.1369	Aminoglycoside resistance	X90856
aadA6	100	846/846	NODE_82_length_1784_cov_63.3625_ID_163	859.1704	Aminoglycoside resistance	AF140629

Table 4 ARG Database of the P-73 (Multiresistant) Strain After Whole Genome Sequencing

Resistance Gene	Identity	Query/HSP	Contig	Position in Contig	Phenotype	Accession no.
aph(3')-IIB	98.76	807/807	NODE_8_length_203585_cov_8.59024_ID_15	100,622.101428	Aminoglycoside resistance	X90856
blaPAO	99.25	1194/1194	NODE_8_length_203585_cov_8.59024_ID_15	113,784.114977	Beta-lactam resistance	FJ666065
cmlA1	99.13	1260/1260	NODE_112_length_3693_cov_25.7356_ID_223	1565.2824	Phenicol resistance	AB212941
fosA	99.02	408/408	NODE_10_length_182802_cov_9.72156_ID_19	161,034.161441	Fosfomycin resistance	NZ_ACWU01000146
blaOXA-50	99.87	789/789	NODE_42_length_59540_cov_10.9284_ID_83	17,700.18488	Beta-lactam resistance	AY306132
sulI	100	927/927	NODE_112_length_3693_cov_25.7356_ID_223	193.1119	Sulfonamide resistance	CP002151
rmtF	99.36	780/780	NODE_97_length_6574_cov_13.3382_ID_193	3129.3908	Aminoglycoside resistance	JQ955744
aac(3)-Ic	100	471/471	NODE_112_length_3693_cov_25.7356_ID_223	3131.3601	Aminoglycoside resistance	AJ511268
aadA6	100	846/846	NODE_149_length_1331_cov_44.1156_ID_297	404.1249	Aminoglycoside resistance	AF140629
aac(6')Ib-cr	99.23	519/519	NODE_97_length_6574_cov_13.3382_ID_193	4584.5102	Fluoroquinolone and aminoglycoside resistance	EF636461
aacA4	99.46	555/555	NODE_97_length_6574_cov_13.3382_ID_193	4584.5138	Aminoglycoside resistance	KM278199
blaGES-5	99.88	864/864	NODE_97_length_6574_cov_13.3382_ID_193	5276.6139	Beta-lactam resistance	DQ236171

Discussion

This paper investigated the prevalence of ARGs in three strains of *P. aeruginosa* using whole genome sequencing. The PCR technique confirmed that the three strains used in the study were *P. aeruginosa* species; hence, misidentification of *P. aeruginosa* was avoided. Due to the extraordinary ability of *P. aeruginosa* to develop resistance to a wide variety of antibiotics through diverse molecular pathways, the emergence of MDR-*P. aeruginosa* is in fact a worldwide health concern. In the present study, MDR-*P. aeruginosa* (p-73) showed resistance to different antibiotics, such as ceftazidime, cefotaxime, cefepime, piperacillin/tazobactam and imipenem. It was also resistant to aminoglycosides (amikacin) and fluoroquinolones (ciprofloxacin), but it remained susceptible to colistin. Recent studies have provided detailed descriptions of each resistance mechanism's prevalence and contribution to each class of antibiotics.^{13,14} It is known that some strains of *P. aeruginosa* have highly developed inherent and acquired resistance mechanisms that enable them to withstand the majority of antibiotics. Whole genome sequencing of susceptible *P. aeruginosa* (Table 2) showed the presence of 4 ARG types, fosA, catB7, blaPAO, and blaOXA-50, suggesting that *P. aeruginosa* is capable of natural transformation.¹⁵ Whole genome sequencing of resistant or MDR-*P. aeruginosa* showed the presence of multiple ARGs, such as sulI, aac(3)-Ic, blaPAO, blaGES-1, blaGES-5 aph(3')-XV, blaOXA-50, aacA4, catB7, aph(3')-IIB, aadA6, fosA, tet(G), cmlA1, aac(6')Ib-cr, and rmtF (Tables 3 and 4). Similar studies have shown the high incidence of antibiotic resistance genes in MDR-*P. aeruginosa*.^{16,17} Therefore, the acquisition of ARGs depends on the resistance of the strains to the antibiotics, ie, the least resistance to antibiotics indicates the least acquisition of ARGs against antibiotics. The p-5 strain had ARGs against a few antibiotics (β -lactam, phenicol, and fosfomycin) when compared to the resistant bacteria (p-7 strain), which had ARGs against β -lactams, aminoglycosides, fluoroquinolone, sulfonamide, tetracycline, phenicol, and fosfomycin. MDR-*P. aeruginosa* (p-73) had ARGs against aminoglycosides, β -lactams, fluoroquinolones, sulfonamides, phenicol, and fosfomycin. Decreased susceptibility of *P. aeruginosa* to commonly used antibiotics has also been shown in different studies.^{13,14,18} Antibiotic resistance is a major problem in dealing with *P. aeruginosa* infections. It was shown that *P. aeruginosa* isolates could be resistant to the commonly used

antibiotics in admitted patients with a rate of more than 35%.¹⁹ Aminoglycosides are an essential part of the antipseudomonal chemotherapy used to treat a number of illnesses caused by *P. aeruginosa*.^{20,21} *P. aeruginosa* has multiple mechanisms of antibiotic resistance. One of these is the *rmtF* gene, which encodes a 16S rRNA methylase that confers resistance to aminoglycosides.²² Grandjean et al similarly provided the draft genome sequences of two multidrug-resistant strains, one from a patient with ventilator-associated pneumonia, where he discovered two aminoglycoside resistance genes, three beta-lactam resistance genes, the fosfomycin resistance gene *fosA*, and the sulfonamide resistance gene *sulI*. They discovered three aminoglycoside resistance genes, two beta-lactam resistance genes, the fosfomycin resistance gene *fosA*, the sulfonamide resistance gene *sulI*, the phenicol resistance gene *catB7*, and the trimethoprim resistance gene *dfrB1* in the other strain, which was derived from blood culture.²³ Additionally, Hussain et al reported the genome sequence of a multidrug-resistant *P. aeruginosa* strain isolated from a patient with a urinary tract infection.²⁴ This strain possessed a number of antibiotic resistance genes, including *blaVEB-1*, *blaPAO*, *blaOXA-50*, *catB7*, *fosA*, *tet(G)*, *aph(3')-via*, *aph(3')-IIb*, and *aadA6*.²⁴ The *aph(3')-IIb* variant has been reported in MDR-*P. aeruginosa* by Subedi et al.²⁵

The chromosomally encoded β -lactamase AmpC is the main source of antibiotic resistance to the beta-lactam class.²⁶ Many studies have reported the prevalence of *blaPAO* and *blaOXA50* in the *P. aeruginosa* genome.²² The *fosA* and *cmlA1* genes are responsible for fosfomycin and phenicol resistance, respectively, in the current genomes, suggesting that this strain is capable of expressing resistance to these antibiotic families.²⁷ The G+C content of the *blaOXA-50* gene suggests that it is a naturally occurring gene in the strain.²⁸ Dihydropteroate synthase, high-affinity sulfate permease, and sulfate transmembrane transporter activities are all regulated by the *Sul* gene.²⁹ The genes *sul1*, *sul2*, and *sul3* encode the dihydropteroate synthase enzyme, which is the most common mechanism of bacterial resistance to sulfonamides.^{30,31} It is very difficult to treat *P. aeruginosa* infections when a strain expressing *blaGES-5* is found, which raises the risk of nosocomial persistence transmission in hospital settings.³² In conclusion, this study confirmed the fact that the acquisition of ARGs depends on the resistance of *Pseudomonas* to antibiotics, ie, the least resistant strain to antibiotics had the lowest acquisition of ARGs, while the most resistant strain to antibiotics had the highest acquisition of ARGs. MDR-*P. aeruginosa* in this study produced ARGs against aminoglycoside, β -lactam, fluoroquinolones, sulfonamides, phenicol, and fosfomycin antibiotics.

Ethical Approval

Not applicable in this study as bacterial strains were collected from previous study mentioned in the text.

Disclosure

The author reports no conflicts of interest in this work.

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