Genomic Analysis to Elucidate the Antibiotic Resistance Mechanism of Extremely Drug-Resistant *Pseudomonas aeruginosa* Strains Isolated from Bangladesh

Bioresearch Communications
Volume 9, Issue 1, January 2023

DOI:
doi.org/10.3329/brc.v9i1.63601

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ABSTRACT: Multidrug-resistant *P. aeruginosa* has potential to cause nosocomial infections. In this study, whole-genome sequencing was performed of two extremely drug-resistant novel strains SRS1 and SRS4 isolated from Bangladesh. The size of draft genome of SRS1 is 6.8 Mbp, and 7.0 Mbp for SRS4. *In silico* analysis predicted that the genome of SRS1 has 82 and SRS4 has 75 antibiotic-resistant genes (ARGs). Antibiogram results revealed that both SRS1 and SRS4 were resistant to multiple members of the antibiotic groups of β–lactam, quinolones, and aminoglycosides families. In addition, the genomes of both SRS1 and SRS4 were predicted to have multiple mobile elements like prophages and plasmids. Comparative genome analysis with wildtype PAO1 and another drug-resistant *P. aeruginosa* strain JNQH-PA57 revealed that SRS1 and SRS4 contain more antibiotic resistance genes *like AAC (6´)-II, ANT (2´´)-Ia, ANT (3´´)-IIa, OXA-395, PME-1, qacEΔ1, tet(A), tet(D), VEB-9* than PAO1 and JNQH-PA57. This study shows the importance of the genomic study to understand the distribution of ARGs in Bangladeshi *P. aeruginosa* strains to demonstrate the mechanisms responsible for multi drug resistance.

KEYWORDS: *Pseudomonas aeruginosa,* whole-genome sequencing, multidrug-resistant, Bangladesh

Received: 14 August 2022, Accepted: 25 November 2022

Type: Original Research

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Introduction

Pseudomonas aeruginosa is a Gram-negative, opportunistic bacillus that causes acute or chronic infections in patients with burn wounds, immunodeficiency, cancer, and various respiratory tract illnesses (Kerr and Snelling, 2009; Mittal *et al.*, 2009; Mulcahy, Isabella and Lewis, 2010; Qin *et al.*, 2022). Multidrug-resistant *P. aeruginosa* causes a severe public health problem worldwide (Carmeli *et al.*, 1999; Huang *et al.*, 2020). The genome of *P. aeruginosa* is made up of a single circular chromosome (GC content of 65–67%, size 5.5–7 Mbp) and many accessory elements like plasmids, prophages, integrative and conjugative elements, insertion sequences, and transposons (Kerr and Snelling, 2009; Klockgether *et al.*, 2011; Lu *et al.*, 2015; Huang *et al.*, 2020).
In this study, the whole-genome sequencing (WGS) method was used to identify the antibiotic resistance genes (ARG) of two novel drug-resistant *P. aeruginosa* strains named SRS1 and SRS4 that have been isolated. Comparative genome analysis of SRS1 and SRS4 with two fully sequenced and closely related *P. aeruginosa* strains based on average nucleotide identity (ANI) was conducted to identify the unique ARGs in the studied strains isolated from Bangladesh.

Materials and Methods

Isolation of the bacterial strains and antibiogram

The bacterial strain SRS1 was isolated from a clinical sample, and strain SRS4 was isolated from a sewage outlet sample from the same hospital. The antibiotic susceptibility test was performed according to Kirby-Bauer disc Diffusion Method as stated in (Sarkar *et al.*, 2019), using the antibiotics amikacin, amoxiclav, aztreonam, ceftazidime, ciprofloxacin, cefepime, carbenicillin, colistin, gentamycin, meropenem, tazobactam-piperacillin (Oxoid, UK). The sensitivity to antibiotics was determined according to CLSI guideline using the (Table 1) (CLSI, 2021).

Assembly of DNA sequencing and annotation

Draft genome sequence assembly and annotation of *P. aeruginosa* SRS1 and SRS4 strains were performed following the process described in (Haque *et al.*, 2022). BioProject numbers of the studied strains in the National Center for Biotechnology Information (NCBI) were PRJNA664096 and PRJNA848280, respectively.

Identification of antibiotic resistance, mobile elements, and comparative genomic analysis

Identification of the antibiotic resistance genes was performed by using the Resistance Gene Identifier (RGI) from the Comprehensive Antibiotic Resistance Database (CARD) (Alcock *et al.*, 2020). Phage Search Tool Enhanced Release (PHASTER) server was used to identify prophage sequences in the genome. Multilocus sequence typing (MLST) was performed in MLST 2 (Liu *et al.*, 2018). MOB-Recon version

3.0.3 in the Galaxy server was used to get the plasmid sequences present in the contigs (Goecks *et al.*, 2010; Afgan *et al.*, 2018).

Comparative genomic analysis was performed against wildtype PAO1, and one closely related and drug-resistant *P. aeruginosa* JNQH-PA57 strain (Table 3). Average nucleotide identity (ANI) (Table 2) was performed using JSpeciesWS (Robocop GmbH - Version: 3.9.3) (Richter *et al.*, 2015). All the genomes were analysed in CARD and compared against the antibiotic resistance genes that are predicted to be present in *P. aeruginosa* SRS1 and SRS4. The heat map was generated in Morpheus from the Broad Institute (https://software.broadinstitute.org/morpheus).

Results and Discussion

P. aeruginosa is listed among the "critical" group of pathogens by the World Health Organization (WHO) (Pang et al., 2019). This manuscript briefly focuses on the phenotypic and genotypic characterization of these two newly isolated *P. aeruginosa* strains, SRS1 and SRS4, from Dhaka, Bangladesh. Furthermore, a comparative genome analysis was performed based on the ANI (Table 2). SRS1 and SRS4 are more closely related to the reference strain JNQH-PA57 (98.77 and 98.64 % similarity) than the wildtype strain PAO1 (98.61 and 98.58, respectively).

P. aeruginosa SRS1 and SRS4 calculated genome sizes have been reported as 6.8 and 7.0 Mbp and contain 6687 and 7054 coding genes, respectively. Both strains contain multiple mobile genetic elements and a nearly similar GC content of 66% (Table 3). *P. aeruginosa* SRS1 and SRS4 were resistant to multiple antibiotics (Table 1). ARGs in *P. aeruginosa* SRS1 and SRS4, PAO1, and JNQH-PA57 were identified using CARD, and a heat map was generated. This analysis revealed the presence of a total of 82 and 75 different ARGs in the genomes of *P. aeruginosa* strains SRS1 and SRS4, respectively (Figure 1).

The strains SRS1 and SRS4 were resistant to carbapenem, the 3rd or 4th generation of cephalosporins. The genomes of strains SRS1 and SRS4 contained several β-lactam tolerating resistance-nodulation division (RND) efflux pump proteins MexAB-OprM, MexCD-OprJ, and OXA-class β-lactamase enzymes (Figure 1). According to previous studies (Poole, 2000; Drawz and Bonomo, 2010; Wang et al., 2020; Yu et al., 2021), those proteins were involved in the extensive β-lactam group of antibiotic resistance. Both SRS1 and SRS4 were deficient in the MuxA protein-coding gene, a membrane-fusion component of the efflux pump, involved in increased susceptibility to β-lactam and monobactam drugs (Yang et al., 2011; Sung et al., 2021). This particular protein is present in the PAO1 and JNQH-PA57 genome, whereas the absence of this specific gene may contribute to the higher resistance of β-lactam antibiotics in SRS1 and SRS4. Also, both SRS1 and SRS4 genome sequences have VEB-9 β-lactamase, one of the subtypes of Vietnam Extended Spectrum Beta-lactamase (VEB), a member of the ESBL family. The VEB family of beta-lactamase are prevalent in clinical strains of *P. aeruginosa* and confer resistance to ceftazidime, aztreonam, and cefepime (Laudy et al., 2017). Interestingly these two strains also have the genes for a Guiana-Extended-Spectrum β-lactamase, GES-9, which confers resistance against aztreonam and the activity of GES-9 is inhibited by imipenem and piperacillin (Poirel et al., 2005). Tawfik et al. reported the co-existence of bla_{oxa-10} with bla_{GES-9} in MDR *P. aeruginosa* strains from nosocomial infections, which is also in line with the finding that the strain SRS1 had a higher antibiotic resistance profile than strain SRS4 which did not harbour any gene for OXA-10 (Tawfik et al., 2012). These findings correlated with the phenotypic characterization of the strains as they were resistant to all broad-range β-lactam and monobactam antibiotics used in the study.

The genomic sequence analysis of SRS1 and SRS4 also revealed the presence of all 3 classes of Aminoglycoside Modifying Enzymes (AME) i.e., aminoglycoside nucleotidyltransferase (ANT), acetyltransferases (AAC) and phosphotransferase (APHs). The AAC(6')-Ib-cr6 acetyltransferase acetylates not only Tobramycin and Amikacin but also Fluroquinolone antibiotics (Robicsek et al., 2006; Thacharodi and Lamont, 2022). Along with AAC (6')-Ib-4, it is the most abundant type of AAC found in Gram-negative bacteria. The other AAC enzyme AAC(6')-II which acetylates all types of Gentamicin but not of Amikacin was found in the strains included in this study but absent from both the wildtype and reference strains (Rather et al., 1992). The presence of ANT(2') enzymes has a considerable effect on the decreased susceptibility to Gentamicin and Tobramycin but does not affect Amikacin, whereas APH(3') enzymes confer resistance to all three aminoglycosides, i.e., Gentamicin, Tobramycin and Amikacin to a varying degree (Subedi D, Vijay AK et al., 2018; Thacharodi and Lamont, 2022). Among the two studied strains, *P. aeruginosa* SRS4 was sensitive to Amikacin (Table 1). Such different resistant profiles could not be clearly explained from the existing data. However, one possible reason might be the missing gene for the efflux pump protein MexY in the genome of the SRS4 strain as observed in this study. (Figure 1). Besides, the genomic presence of most of the reported RND efflux pump genes in strain SRS1 and SRS4 (Figure 1) contribute to resistance to broad-spectrum antibiotics penicillin/β-lactams, fluoroquinolones, and aminoglycosides (Chaudhary et al., 2017; Pang et al., 2019; Xu et al., 2020), making them MDR-strains and increasing their pathogenicity. In contrast, both the SRS1 and SRS4 strains were found sensitive to colistin-polymyxin, which might be due to the absence of *mcr-1* & *mcr-2* genes (Azimi and Lari, 2019; Ilbeigi et al., 2021).

Furthermore, strain SRS1 harboured the genes for TriABC-OpmH, the last RND-type efflux pump identified in *P. aeruginosa*, conferring resistance only to Triclosan (Mima et al., 2007). The strain SRS4 had gene sequences for membrane fusion proteins TriA and TriB but the efflux protein TriC was missing. The presence of Major Facilitator Superfamily efflux pump gene *cmlA9* (chloramphenicol), Small Multidrug Resistance family efflux pump gene *qacEΔ1* (quaternary ammonium compound) and *tetA* and *tetD* (Tetracycline) in both SRS1 and SRS4, strengthens their multidrug phenotypes (Subedi et al., 2018; Hoque et al., 2022).

In summary, the presence of ARGs in SRS1 and SRS4 confer their resistance to broad-spectrum antimicrobial drugs, including β-lactams, fluoroquinolones, and aminoglycosides. The results are consistent with the phenotypic characteristics obtained from the antibiotic susceptibility testing performed in our study (Table 1). Furthermore, the comparative genome analysis of SRS1 and SRS4 are more closely related to the

resistant strain JNQH-PA57 than that of the laboratory strain PAO1. Compared to these strains, SRS1 and SRS4 have more ARGS in their genomes. Thus, it may be more resistant to antimicrobial compounds than JNQH-PA57 and PAO1.

Conclusion

WGS analysis of MDR *P. aeruginosa* strains SRS1 and SRS4 identified many distinctive genes compared to PAO1 and a resistant strain JNQH-PA57. Such findings will provide further insights into understanding the pathogenicity and virulence factors involved in *P. aeruginosa* strains prevalent in Bangladesh. These findings may also provide useful information in the preventive approaches against adverse outcomes exerted by the novel *P. aeruginosa* SRS1 and SRS4 strains.

Table 1. Antibiogram test results of the isolated strains.

	Resistance profile (*R/S)	
Antibiotics (µg)	**SRS1**	**SRS4**
Amikacin (30)	R	S
Amoxiclav a. Amoxicillin (20) b. Clavulanic Acid (10)	R	R
Aztreonam (30)	R	R
Ceftazidime (30)	R	R
Ciprofloxacin (05)	R	R
Cefepime (30)	R	R
Carbenicillin (100)	R	R
Colistin (10)	S	S
Gentamicin (10)	R	R
Meropenem (10)	R	R
Tazobactam-piperacillin a. Tazobactam (10) b. Piperacillin (100)	R	R

*R=Resistance, S=sensitive

Table 2. Average nucleotide identity (ANI) of the studied strains

STRAINS	SRS1	SRS4	PAO1	JNQH-PA57
SRS1	100	99.89	98.61	98.77
SRS4	99.7	100	98.58	98.64
PAO1	98.75	98.7	100	98.77
JNQH-PA57	98.95	98.9	98.66	100

Table 3. General features of the *Pseudomonas aeruginosa* strains studied

Accession number	Name of the strains	Place of isolation	Genome size (bp)	N50	GC (%)	Coding genes	Sub-systems	Total RNAs	MLST	No. of plasmids	No. of phage islands	References
PRJNA664096	*Pseudomonas aeruginosa* SRS1	Bangladesh	6,850,324	160204	66	6687	404	63	Nearest ST (3116, 2104)	4	4 (3 intact, 1 questionable)	This study
PRJNA848280	*Pseudomonas aeruginosa* SRS4	Bangladesh	7,070,620	90332	65.9	7054	405	62	Nearest ST (3116, 2104)	0	4 (4 intact, 0 questionable)	This study
PRJNA57945	*Pseudomonas aeruginosa* PAO1	Australia	6,264,404	Wildtype Strain	66.6	5858	390	76	549	No data	2 (2 intact, 0 questionable)	(Stover *et al.*, 2000; Jens *et al.*, 2010)
PRJNA655803	*Pseudomonas aeruginosa* JNQH-PA57	United Kingdom	6,747,067	Reference Strain	66.03	6374	399	77	1197	No data	4 (3 intact, 1 questionable)	(Hao *et al.*, 2021)

Figure 1. Heat map of the 89 antibiotic resistance genes. Dark black squares denote the presence of the genes (1), and grey squares denote the absence of the genes (0) listed.

arnA and *basRS*, LipidA modifying enzyme of Gram-negative bacteria contributing to cationic antibiotic resistance; AAC, Aminoglycoside acetyltransferase; ANT, Aminoglycoside nucleotidyltransferase; APH, Aminoglycoside phosphotransferase; *catB7*, chloramphenicol acetyltransferase (CAT); *cprR*, *cprS*, two component regulatory system conferring peptide antibiotic resistance; *dfrB2*, *dfrG*, trimethoprim resistant dihydrofolate reductase; ErmC, Erm 23S ribosomal RNA methyltransferase; FosA, fosfomycin thiol transferase; *fusD*, conferring resistance to Fusidic acid; *gyrA*, fluoroquinolone resistant gyrase A; OXA β-lactamase genes, OXA-10, 50, 395, 488, 677; Guiana-Extended-Spectrum β-lactamase GES-9, PDC β-lactamases, PDC-1, 12, 38, 98; PME β -lactamase, PME-1; Vietnamese Extended-Spectrum β-lactamase VEB-9; RND family of efflux systems MexAB-OprM, MexCD-OprJ, MexEF-OprN, MexJK-OpmH/OprM, MexMN-OprM, MexPQ-OpmE, MexVW-OprM, MexXY-OprM where the first protein (e.g., MexA) is the membrane fusion protein, second protein (e.g., MexB) is the inner membrane drug transporter, and the third protein (e.g., OprM) is the outer membrane porin. In case of MexGHI-OpmD, MuxABC-OpmB, TriABC-OpmH MexH, MuxA, TriA and TriB are the membrane fusion protein; MexG, MexI, MuxB, MuxC, and TriC are the RND family drug transporter whereas OpmD, OpmB and OpmH are the outer membrane porin respectively; RND efflux system Regulator: *nalC*, *nalD* & MexR, repressor of the MexAB-OprM operon; Type-A NfxB, mutant repressor of the efflux system MexCD-OprJ conferring antibiotic resistance; MexT, activator of MexEF-OprN and MexS, suppressor of MexT; MexL, repressor of the MexJK transcription, *rsmA*, negative regulator of MexEF-OprN overexpression; CpxR, RND antibiotic efflux pump, ParRS, RND efflux pump outer membrane porin; YajC, part of AcrAB-TolC efflux pump; *bcr-1*, *cmlA9*, *qacEΔ1*, *tetA*, *tetD*, major facilitator superfamily (MFS) antibiotic efflux gene; *emrE*, SMR antibiotic efflux pump; PmpM, multidrug and toxic compound extrusion (MATE) transporter; *soxR*, positive regulator of many efflux pumps; *rmtF*, 16S rRNA methyltransferase (G1405); *sul*1& 2, sulfonamide resistant dihydropteroate synthase.

Acknowledgments

This work was partially funded by the CTRGC 2020-2021 grant to SRS, North South University, Bangladesh.

Authors' Contributions

Conceptualization: SRS.
Data curation: MJU, NN.
Formal analysis: FAZ, IJ, SI, SRS.
Funding acquisition: SRS.
Methodology: SRS.
Writing – original draft: FAZ, IJ, SI, SRS.
Writing – review & editing: SRS, SI, FAZ, IJ, MJU.

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