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Mutational and Phylogenetic Analysis of *nfxB* Gene in Multidrug-Resistant Clinical Isolates of *Pseudomonas aeruginosa* Hyperexpressing MexCD-OprJ Efflux Pump

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Abstract

The present study focused on MexCD-OprJ efflux pump and its regulatory gene *nfxB* in multidrug resistant (MDR) clinical isolates of *Pseudomonas aeruginosa* collected from Kerala, South India. Semi-quantitative reverse transcription-PCR technique was employed to detect hyperexpression of the efflux pump gene, *mexD*. Amplicons from *nfxB* gene of isolates hyperexpressing the efflux pump were sequenced for mutational and phylogenetic analysis. Among 29 isolates of MDR *P. aeruginosa*, increased *mexD* transcription was detected in 10.3% of the isolates when compared with *P. aeruginosa* reference strain, PAO (MTCC-3541). Various synonymous and non-synonymous mutations in *nfxB* regulatory gene sequences were detected. Notably, mutations detected in the strains designate Pa6 and Pa7 have been found to be novel and are hitherto unreported in GenBank data base. The genetic divergence and homogeneity of the *nfxB* regulatory gene sequences of *mexCD-oprJ* operon were clearly apparent in the phylogram generated employing similar sequences retrieved from the public database.

Keywords

Multidrug-Resistant, *Pseudomonas aeruginosa*, Efflux Pump, Regulatory Gene, Mutational Variations, Phylogenetic Analysis

1. Introduction

MexCD-OprJ efflux pump of resistance-nodulation-cell division superfamily,

normally quiescent in wild-type strain of *P. aeruginosa*, does not contribute to intrinsic antimicrobial resistance. This pump is capable of extruding a variety of antimicrobial agents, biocides, organic solvents, dyes and detergents. The *mexCD-oprJ* operon is regulated by repressors such as NfxB and EsrC. The *nfxB* gene encoding repressor protein, NfxB, is located upstream of the structural genes *mexCD-oprJ* with an intergenic regulatory region facilitating divergent transcription. NfxB shows similarity to proteins of the LacI-GalR family. It binds to a site composed of two 39 bp repeats within the aforementioned intergenic sequences between *nfxB* and *mexC* which negatively regulates *mexCD-oprJ* as well as its own expression. Mutations within *nfxB gene* negatively impact NfxB repressor activity leading to hyperexpression of *mexCD-oprJ*, and strains harbouring such defects are known as *nfxB*-type mutants [1] [2]. EsrC, a second regulator of MexCD-OprJ, encoded by a gene *PA*4596, is located downstream of *mexCD-oprJ* operon. EsrC represses transcription of *mexCD-oprJ* only under "envelope stress" (membrane-damaged) condition and is dependent on NfxB [3]. The present study was undertaken to analyze mutational and phylogenetic relatedness of *nfxB* regulatory gene in MDR isolates of *P. aeruginosa* hyperexpressing MexCD-OprJ efflux pump.

2. Materials and Methods

2.1. Bacterial Isolates

Of a total of 144 MDR gram-negative bacteria, collected during the period 2012-2016 from various clinical laboratories in Kerala, 29 isolates of *P. aeruginosa* (designated as Pa1 - Pa29) were included in this study along with a reference *P. aeruginosa* MTCC-3541 (Microbial Type Culture Collection) strain, PAO.

2.2. Random Amplified Polymorphic DNA (RAPD)

Random Amplified Polymorphic DNA (RAPD) typing was performed with RBa-D5 primer (GenBank accession. no. AM911680) according to manufacturer's instructions. Images of DNA banding patterns obtained after agarose gel electrophoresis were analyzed using the PyElph software [4] to prepare dendrograms using unweighed pair-group method arithmetic mean (UPGMA) method.

2.3. Semi-Quantitative RT-PCR

Semi-quantitative RT-PCR using primers (Eurofins Genomics India Pvt. Ltd., Bangalore) for genes such as *rpsL* and *mexD* [5] [6] were performed as described by us in a related study [7]. The RT-PCR reactions were performed using a minicycler (MJ Research, USA) in a reaction volume of 25 µl containing 12.5 µl of 2x Emerald GT master mix (TaKaRa Inc., Japan), 0.25 µM of each primer and 0.5 µl of cDNA. Amplification products were analyzed on 1% (w/v) agarose gels to detect the presence of the expected amplicons and compare the band intensities with those from the reference strain *P. aeruginosa* MTCC-PAO.

2.4. PCR Amplification and Sequencing of the *nfxB* Gene and Its Phylogenetic Analysis

The regulatory *nfxB* gene primer [8] was used for DNA amplifications and sequencing [7]. The ampliconic sequences were analyzed with NCBI BLAST tool (https://www.ncbi.nlm.nih.gov/) by comparison with sequences of the reference strain, *P. aeruginosa* PAO1, retrieved from the GenBank database. The phylogram construction, employing *nfxB* gene sequences obtained in this study in combination with similar sequences retrieved from public databases, was carried out by Bayesian inference using Markov Chain Monte Carlo method [9]. The jModelTest software [10] used for the analysis of nucleotide sequence evolution with respect to each gene was based on Akaike Information Criterion (AIC).

2.5. Nucleotide Sequence Accession Numbers

The *nfxB* gene nucleotide sequences obtained in the present study were deposited in the GenBank database under the following accession numbers MH346508-MH346510.

3. Result and Discussion

The RAPD profiles generated in our study were found to be distinctive and reproducible (**Figure 1**). The dendrogram showed two major clusters—A and B, in which major cluster A was found to be subdivided into two sub-clusters A1 and A2 (**Figure 2**). The *mexD* was significantly overexpressed in three isolates such as Pa6, Pa7 and Pa13 (**Figure 3**). Isolates with efflux pump activity were found to be included in the sub-cluster A1 of RAPD dendrogram. Of them, Pa7 and Pa13 belonged to a single clade, whilst strain Pa6 was found grouped into another clade. Mutation analysis of *nfxB* gene revealed point mutations, T → C at 239th position in Pa6, T → C at 349th position in Pa7 and C → T at 14th position in Pa13 (**Table 1**). Notably, mutations detected in Pa6 and Pa7 isolates have been found to be novel and are hitherto unreported in GenBank data base, whilst the mutation detected in Pa13 has already been reported earlier [11]. Phylogram (**Figure 4**) was constructed using three *nfxB* gene sequences of MexCD-OprJ overproducers—Pa6, Pa7 and Pa13 and nine other selected sequences of *P. aeruginosa* isolate retrieved from the GenBank database including the reference strain, *P. aeruginosa*—PAO1 (GenBank accession no. AE004091. 2). For this analysis, *Pseudomonas chlororaphis* (GenBank accession no. CP011110.1) was taken as

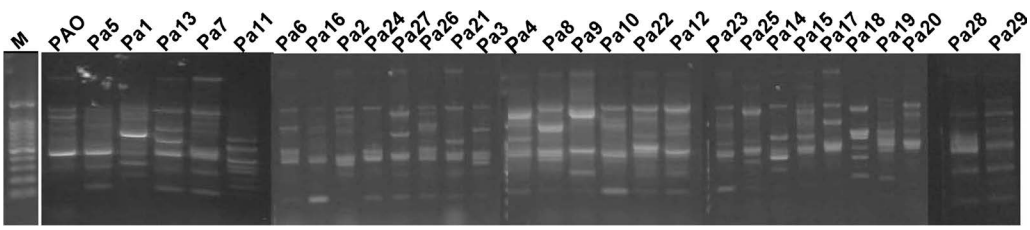

Figure 1. RAPD profiles of reference strain PAO (MTCC) and MDR *P. aeruginosa* clinical isolates. Lane denoted M represents 100 bp DNA ladder.

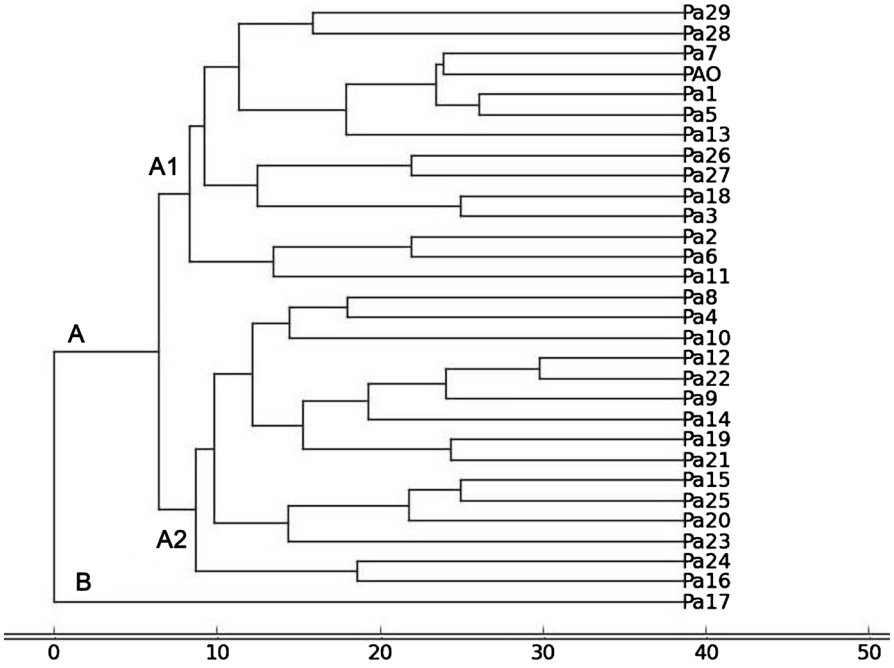

Figure 2. RAPD dendrogram showing clonal relatedness amongst *P. aeruginosa*.

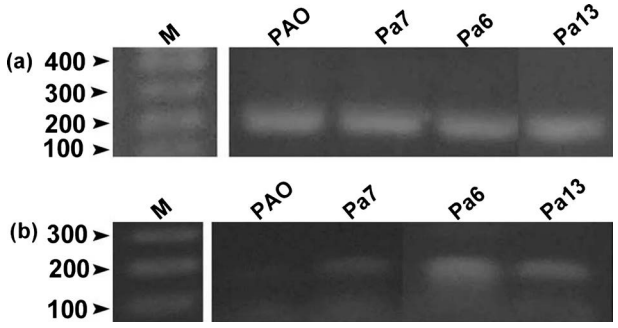

Figure 3. Semi-quantitative RT-PCR. Lane denoted M represents 100 bp DNA ladder. Other lanes show *P. aeruginosa* MTCC reference strain PAO and clinical isolates. (a) Expression of housekeeping gene, *rpsL* in PAO and three isolates, Pa7, Pa6 and Pa13. (b) Expression of *mexD* in PAO and three isolates, Pa7, Pa6 and Pa13.

Table 1. Summary of genetic analyses of mutations in *nfxB* gene of *P. aeruginosa* clinical isolates.

Isolates	*nfxB* mutation	
	Nucleotide	Aminoacid
Pa6	^{239}T → C	^{80}Leucine → Serine
	^{555}T → G	–
Pa7	^{39}G → A,^{183}A → G,^{423}G → A,^{480}T → C,^{486}A → T,^{537}T → C,^{543}C → T,^{555}T → G	–
	^{349}T → C	^{117}Serine → Proline
Pa13	^{14}C → T	^{5}Serine → Phenylalanine
	^{135}C → T,^{141}G → A,^{555}T → G	–

"–" represents no amino acid change.

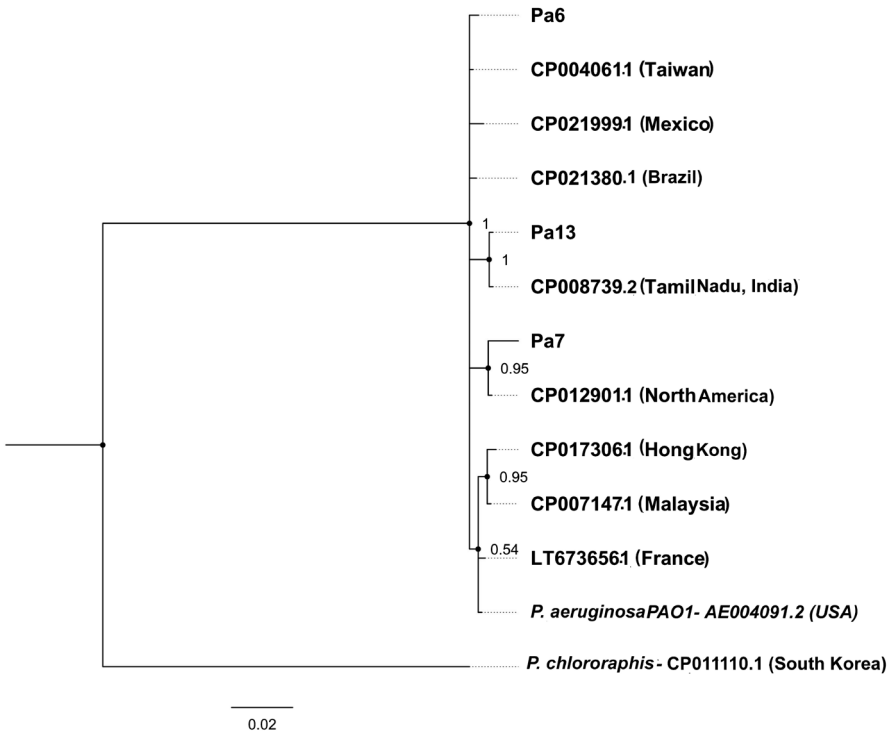

Figure 4. Bayesian phylogenetic tree reconstructed employing *nfxB* ampliconic sequences from *P. aeruginosa* isolates. Scale bar denotes probability of nucleotide change. Analysis was run for 1,000,000 generations. Clade credibility values are shown at each node. *P. chlororaphis* (GenBank accession no. CP011110.1) was used as the outgroup.

the outgroup as this sequence showed better sequence similarity with a higher query coverage. GTR was selected as the best fit model of sequence evolution on the basis of AIC. Potential scale reduction factor (PSRF) and estimated sample size (ESS) value were observed as 1.0 and above 100 respectively. Analysis was run for 1,000,000 generations and clade credibility values represented at each node were found to be >0.5. Bayesian phylogenetic analysis revealed that the sequence from Pa6 exhibited similarity with isolates reported from Taiwan (GenBank accession no. CP004061.1), Mexico (GenBank accession no. CP021999.1) and Brazil (GenBank accession no. CP021380.1). Pa13 isolate showed similarity with that reported from Tamil Nadu with GenBank accession no. CP008739.2 [11] whilst Pa7 was found to be similar to *P. aeruginosa* sequences deposited from North America with GenBank accession no. CP012901.1 [12].

4. Conclusion

Antibiotic resistance is a stark reality across the world including the Indian subcontinent. Antimicrobial selection pressure and spread of resistant organisms are the main leading factors for the emergence of resistance. The present study has been successful in unraveling vital information on the mutational variations of *nfxB* regulatory gene sequences of MexCD-OprJ efflux pump present in *P. aeruginosa*. Occurrence of genetic similarities of *nfxB* gene sequences among the isolates used in this study as well as with those previously reported from other

countries warrants the need for implementation of strict control measures to prevent spread of resistance genes.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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