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ORIGINAL PAPER

Mutational analyses of regulatory genes, *mexR, nalC, nalD* and *mexZ* of *mexAB-oprM* and *mexXY* operons, in efflux pump hyperexpressing multidrug-resistant clinical isolates of *Pseudomonas aeruginosa*

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Abstract
The present study deals with membrane-bound efflux pumps, MexAB-OprM and MexXY and their respective regulatory genes *mexR, nalC, nalD* and *mexZ* in multidrug resistant (MDR) *Pseudomonas aeruginosa*. Following antibiotic sensitivity testing and detection of various beta-lactamases, hyperexpression of efflux pump genes, *mexB* and *mexY* in the isolates was investigated using semi-quantitative and real-time reverse transcription-PCR. Amplicons from regulatory genes were sequenced and subjected to mutational and phylogenetic analysis. Twenty-nine clinical isolates of *P. aeruginosa* were obtained from a total of 144 MDR gram-negative bacteria collected from Kerala State, South India. All strains were found to be resistant to ampicillin and nalidixic acid with 13.8, 44.8 and 31% testing positive for extended-spectrum beta-lactamases, metallo-beta-lactamases and AmpC producers respectively. Increased *mexB* and *mexY* transcription was detected respectively in 10.3 and 20.7% of the isolates in comparison with *P. aeruginosa* reference strain, PAO (MTCC). Co-expression of MexY was also observed in MexB overproducers. Various synonymous/and non-synonymous mutations in regulatory gene sequences of efflux pump operons were detected. In the strain designate Pa16, *mexR* was found to harbour four novel point mutations with one transversion and three transitions which included a substitution of an *ochre* codon with that for serine. The gene also displayed a novel mutation involving insertion of a cysteine at the 444th base position, followed by an *opal* codon. The genetic divergence and homogeneity of the concatenated (*mexR, nalC* and *nalD)* regulatory gene sequences of *mexAB-oprM* operon was apparent in the phylogram generated with similar sequences retrieved from public database.

Keywords Efflux pumps · Multidrug-resistant · Mutational analysis · Phylogram · *Pseudomonas aeruginosa* · Regulatory genes

Introduction

Pseudomonas aeruginosa, a 'priority pathogen' has now been included in a list of 12 families of bacteria which pose a serious health threat to man (WHO 2017). This opportunistic microbe is also held responsible for nosocomial infections worldwide. Known to possess a large number of virulence factors, it causes severe infections with high morbidity and mortality rate, particularly in immune-compromised patients or those with underlying disease (Poole 2001; Strateva and Yordanov 2009; Askoura et al. 2011; Porras-Gómez et al. 2012; Chatterjee et al. 2016). *P. aeruginosa* continues to display multidrug-resistant phenotypes through (i) intrinsic resistance mechanisms such as those mediated by constitutive expression of AmpC beta-lactamase, efflux pumps and porin down-regulation and (ii) acquired resistance caused by mutational changes or acquisition of resistance mechanisms via horizontal gene transfer. Together, these processes contribute toward development of overwhelming resistance against a variety of structurally unrelated antibiotics leading to difficulties or failure in therapy (Poole 2011).

Efflux pumps of clinical relevance in *P. aeruginosa* belong to the Resistance-Nodulation-Division (RND) family, of which MexAB-OprM, MexCD-OprJ, MexEF-OprN, and

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MexXY represent the predominant sets of efflux systems, with a broad range of drug specificities. RND efflux pump is essentially a tripartite system composed of a cytoplasmic membrane transporter, a membrane fusion protein and an outer membrane channel, that ensures transportation of antimicrobial agents from cytoplasm via periplasmic space to the extracellular environment (Poole 2004; Piddock 2006; Lister et al. 2009). Among the RND pumps, MexAB-OprM and MexXY are constitutively expressed in wild-type strains at a basal level and account for innate resistance. However, MexCD-OprJ and MexEF-OprN efflux pumps are induced by their substrates (Zavascki et al. 2010).

Expression of the MexAB-OprM is governed mainly by regulatory loci such as *mexR, nalC* and *nalD* (Fig. 1a) amongst several others. The *mexR* gene, encoding a repressor protein of MarR family, is located upstream of the *mexAB-oprM* operon. An intergenic region with divergently oriented genes on either sides, *mexR* and *mexAB-oprM*, controls their transcription. MexR binds to this intergenic region as a stable homodimer and represses transcription from the *mexAB-oprM* operon. Mutations in *mexR* (*nalB* mutants), resulting in loss of dimerization and binding capacity of MexR protein, lead to hyperexpression of MexAB-OprM. MexR protein is also endowed with oxidation-sensing mechanism which regulates virulence and antibiotic resistance in *P. aeruginosa* (Chen et al. 2008; Lister et al. 2009; Choudhury et al. 2016). The *nalC* encodes a protein, NalC, of TetR family which acts as a repressor of a divergent two-gene operon comprising of *PA3720* and *PA3719* (renamed *armR*). ArmR acts as an anti-repressor by allosterically inhibiting the dimeric MexR repressor resulting in derepression of *mexAB-oprM* (Cao et al. 2004; Braz et al. 2016). Another member of the TetR family of transcriptional regulators, NalD, acts as a secondary repressor of the tripartite MexAB-OprM multidrug efflux system by binding to a sequence between *mexAB-oprM* and the *mexR*

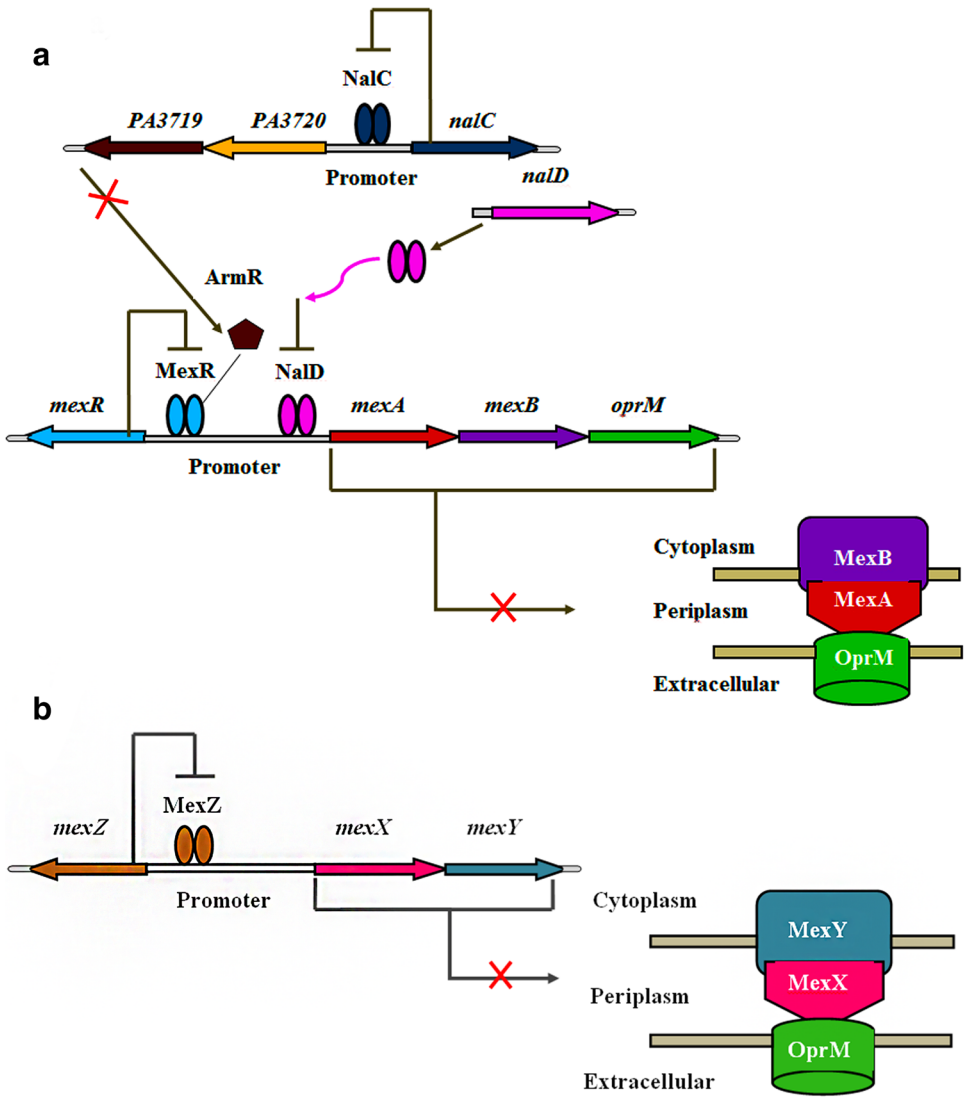

Fig. 1 Schematic diagram of transcriptional regulatory mechanisms controlling MexAB-OprM and MexXY-OprM efflux pumps in *P. aeruginosa*. **a** Transcriptional repression of the *mexAB-oprM* operon is mediated directly by MexR and NalD proteins and indirectly by NalC, which represses ArmR protein, an anti-repressor of MexR. **b** Transcriptional repression of the *mexXY* operon is mediated directly by MexZ

binding site proximal to the *mexA* promoter. Hence, impairment of NalD in nalD-type mutants, leads to MexAB-OprM overexpression (Morita et al. 2006; Chen et al. 2016).

The MexXY multidrug efflux system is considered as a significant determinant of aminoglycoside resistance in clinical strains of *P. aeruginosa*, particularly those isolated from the patients with cystic fibrosis (Morita et al. 2012). Unlike other operons encoding the MexAB-OprM, MexCD-OprJ and MexEF-OprN systems, *mexXY* operon lacks the gene encoding outermembrane protein. Instead, the MexXY system primarily utilizes OprM as its outer membrane component and possibly other outer membrane proteins, such as OpmB, OpmG, OpmH, and OpmI to form a functional tripartite. Expression of the *mexXY* operon is negatively regulated by the *mexZ* gene product, located upstream of the operon and transcribed divergently (Fig. 1b). Yet another member of TetR family of proteins, MexZ contains a DNA-binding helix-turn-helix motif at its N-terminal. Three types of MexXY-overproducing mutants have been described and they are classified as *agrZ, agrW1* and *agrW2*. The *agrZ* mutants harbour mutations inactivating *mexZ* gene including those caused by single amino acid substitutions in the DNA-binding, dimerization or other structural domains of the encoded repressor. In *agrW1* mutants, impaired protein synthesis occurs due to a variety of defects in ribosomal proteins whilst *agrW2* mutants hyperexpress MexXY with alterations in the sensor ParS or the response regulator ParR of the two-component ParRS system known to play critical roles in multidrug resistance (Lister et al. 2009; Morita et al. 2012; Li et al. 2015).

The present study focused on MexAB-OprM and MexXY efflux systems in multidrug resistant (MDR) clinical isolates of *P. aeruginosa* collected from Kerala, the southernmost State in India. Both semi-quantitative and real-time reverse transcription-PCR techniques were employed to detect hyperexpression of the efflux pump genes, *mexB and mexY*. Amplicons from regulatory genes were sequenced to determine effects of the observed mutations on the functioning of the efflux pumps and their role in multidrug resistance. A phylogenetic analysis, based on the regulatory sequences obtained in this study, in combination with similar sequences retrieved from public databases, was also carried out to understand their genetic relatedness.

Materials and methods

Bacterial strains

A total of 144 MDR gram-negative clinical bacterial isolates were collected from various clinical laboratories in Kerala State, India, during the period from December 2012 to January 2016. From these, 29 isolates of *P. aeruginosa* were included in this study employing the reference strain, *P. aeruginosa*—PAO MTCC (Microbial Type Culture Collection) and a control strain MTCC *E. coli* 41, obtained from Institute of Microbial Technology (IMTECH), Chandigarh, India. Identification of isolates was based on colony morphology and standard biochemical tests (Baron et al. 1994).

Antimicrobial susceptibility testing

Antibiotic sensitivity test was done by Kirby–Bauer disc diffusion method according to the Clinical and Laboratory Standards Institute recommendations (CLSI 2012) using a total of 16 commercially available antibiotic discs (HiMedia Mumbai, India): amikacin-30 mcg (AK), ampicillin-10 mcg (AMP), aztreonam-30 mcg (AT), cefotaxime-30 mcg (CTX), ceftazidime-30 mcg (CAZ), cefepime-30 mcg (CPM), chloramphenicol-30 mcg (C), ciprofloxacin-5 mcg (CIP), colistin-10 mcg (CL), gentamicin-10 mcg (GEN), meropenem-10 mcg (MRP), nalidixic acid-30 mcg (NA), ofloxacin-5 mcg (OF), piperacillin/tazobactam-100/10 mcg (PIT), polymyxin-B-300 units (PB) and tetracycline-30 mcg (TE).

Detection of extended-spectrum beta-lactamase (ESBL)

ESBL status was phenotypically established by combined disc diffusion method using ceftazidime (30 mcg) disc alone and in combination with clavulanic acid (ceftazidime + clavulanic acid, 30/10 mcg discs). The test organism was considered to produce ESBL, if the zone of inhibition of ceftazidime + clavulanic acid disc was ≥ 5 mm in comparison to that produced by ceftazidime disc alone (Easwaran et al. 2016).

Detection of metallo-beta-lactamase (MBL)

Phenotypic MBL production was detected by imipenem-EDTA combined disc method as described by Yong et al. (2002). Two 10 µg imipenem discs were placed on the plate inoculated with the test organism and 10 µl of 0.5M EDTA solution was added to one of them to obtain a concentration of 750 µg. A zone of inhibition ≥ 7 mm around imipenem + EDTA disc when compared to that of imipenem disc alone indicated MBL production.

Detection of AmpC beta-lactamase production

AmpC-producing phenotypes were detected by AmpC disc test as described by Black et al. (2005). A lawn of *E. coli* MTCC 41 was made on Mueller–Hinton Agar plate and a cefoxitin (30 mcg) disc was placed. A sterile disc was rehydrated with 20 µl of sterile saline and inoculated with several colonies of the test organism and placed adjacent to

cefoxitin disc. Flattening or indentation of the cefoxitin zone of inhibition in the vicinity of the test organism disc was taken as positive and the undistorted zone was considered as negative.

Quantification of *mexB* and *mexY*

Total RNA was extracted from mid-exponential phase ($OD_{600} = 2.0$) bacterial cultures grown in Luria Bertani medium using TRI Reagent®(Sigma Aldrich, USA) and residual DNA was removed by adding DNase I (Promega, USA) according to the manufacturer's instructions. The cDNAs synthesis was carried out essentially as described by Dumas et al. (2006) with some modifications. Briefly, a 5 µl reaction containing 0.5 µl of random hexamers (100 ng/µl) and 1.0 µl of 0.5 µg/µl RNA was incubated at 65 °C for 10 min and placed on ice. To this, added 1.0 µl of 10 mM dNTP mix, 2.0 µl of 10× M-MLV reverse transcriptase buffer, 1.0 µl of M-MLV reverse transcriptase, 0.5 µl of RNasin, 0.5 µl DTT (20 mM) and made upto 10 µl with sterile nuclease-free water.

The cDNAs were subjected to semi-quantitative and quantitative PCR using primers (Table 1) as previously described (Yoneda et al. 2005; Dumas et al. 2006). The transcript levels of individual mRNA types were determined using SYBR Green PCR Master Mix (TaKaRa Inc., Japan) in an Illumina Eco™ Real-Time PCR system. The relative gene expressions were evaluated using the CT method (Pfaffl 2001) taking the constitutively expressed gene encoding ribosomal protein RpsL representing housekeeping function. *P. aeruginosa*—MTCC PAO was used as a reference for normalization of relative mRNA levels. The MexAB-OprM and MexXY were considered overexpressed when their transcriptional levels were at least threefold higher than those of the reference strain (Islam et al. 2004). The assays were performed in triplicate.

PCR amplification and sequencing of the *mexR, nalC, nalD and mexZ* genes

Genomic DNA was isolated by CTAB method (Ausubel et al. 1995). The primers used for amplification/sequencing are listed in Table 2. The PCR reactions were performed using a minicycler (MJ Research, USA) in a reaction volume of 25 µl containing 12.5 µl of 2× Emerald GT master mix (TaKaRa Inc., Japan), 0.25 µM of each primer and 100 ng template DNA. PCR products were cloned using CloneJET PCR cloning kit in accordance with the manufacturer's instructions and then transformed into *E. coli* DH5α strain. Recombinant plasmids were isolated using alkaline lysis method and sequenced at a commercial facility (Eurofins Genomics India Pvt. Ltd.). The nucleotide sequences of the amplicons were analyzed with BLAST tool of NCBI (http://www.ncbi.nlm.nih.gov) by comparison with sequences of the reference strain, PAO1 retrieved from the data bank.

Phylogenetic analysis

The DNA sequences of regulatory genes, *mexR, nalC, nalD* and *mexZ*, obtained in the present study were compared

Table 1 Primers used for semi-quantitative and quantitative PCR

Genes	Primers	Primer sequences (5′–3′)	Amplicon size (bp)	References
rpsL	F	GCAAGCGCATGGTCGACAAGA	201	Dumas et al. (2006)
	R	CGCTGTGCTCTTGCAGGTTGTGA		
mexB	F	GTGTTCGGCTCGCAGTACTC	244	Yoneda et al. (2005)
	R	AACCGTCGGGATTGACCTTG		
mexY	F	CCGCTACAACGGCTATCCCT	250	Yoneda et al. (2005)
	R	AGCGGGATCGACCAGCTTTC		

Table 2 Primers used for PCR amplification of genomic DNA and sequencing of amplicons

Genes	Primers	Primer sequences (5′–3′)	References
mexR	F	TGTTCTTAAATATCCTCAAGCGG	Quale et al. (2006)
	R	GTTGCATAGCGTTGTCCTCA	
nalC	F	TCAACCCTAACGAGAAACGCT	Quale et al. (2006)
	R	TCCACCTCACCGAACTGC	
nalD	F	GCGGCTAAAATCGGTACACT	Sobel et al. (2005)
	R	ACGTCCAGGTGGATCTTGG	
mexZ	F	ATTGGATGTGCATGGGTG	Sobel et al. (2003)
	R	TGGAGATCGAAGGCAGC	

with similar sequences of *P. aeruginosa* available in the NCBI database, taking *P. aeruginosa* PAO1 as the outgroup (Jochumsen et al. 2016). Minimal 'E' values, maximum query coverage, including geographical location of the source organisms, were the factors considered for sequence selection. All sequences were aligned using default configuration of multiple sequence comparison by clustalW embedded in MEGA6 software (http://www.megasoftware.net). To obtain greater accuracy in determining intraspecies phylogenetic relationships among the closely related strains of *P. aeruginosa*, the regulatory gene sequences from *mexR, nalC*, and *nalD* of *mexAB-oprM* were concatenated in a head to tail fashion to form a 'supergene' alignment (Gadagkar et al. 2005; Khan et al. 2008). Nucleotide sequence evolution with respect to each gene was carried out using the software, jModelTest (Posada 2008) based on Akaike Information Criterion (AIC). The phylogram was constructed by Bayesian inference using Markov Chain Monte Carlo method (Huelsenbeck and Ronquist 2001).

Statistical analysis

A significant difference between two proportions was checked by *Z*-test using MedCalc Statistical Software Version 17.9.7. A two tailed $p < 0.01$ was considered significant.

Nucleotide sequence accession numbers

The nucleotide sequences obtained in this study were deposited in the GenBank database under the following accession numbers: *mexR* gene sequences, accession numbers MG757454–MG757456; *nalC* gene sequences MG757451–MG757453; *nalD* gene sequences MG744560, MG757449, MG757450; *mexZ* gene sequences, accession numbers MG757457–MG757461.

Results

Antibiotic sensitivity

All of the 29 isolates of MDR, *P. aeruginosa*, were found to be resistant to ampicillin and nalidixic acid. Low resistance was observed against polymyxin-B antibiotic (48%). The Multiple Antibiotic Resistance (MAR) index value was calculated as described by Krumperman (1983). The MAR index of the clinical isolates tested against 16 antibiotics ranged from 0.5 to 1.0 (Table 3). Among them 27.6% (n = 8) exhibited a MAR index of 1.0, which indicated resistance to all the antibiotics tested.

ESBL, MBL and AmpC beta-lactamase production

ESBL, MBL and AmpC positive isolates amounted to 13.8% (n = 4), 44.8% (n = 13) and 31.0% (n = 9) respectively; 27.6% (n = 8) were co-producers of AmpC and MBL with only a single strain co-producing ESBL and MBL. About 41.4% (n = 12) of the isolates were found lacking in the expression of any of the three enzymes tested (Table 3).

Gene expression analysis

The relative expression levels of transcripts from *mexB* and *mexY* genes were determined by semi-quantitative and real-time PCR technique (data shown in Fig. 2 and Table 3 respectively). Gene expression analysis of *P. aeruginosa* revealed that 10.3% (n = 3) and 20.7% (n = 6) of isolates displayed increased transcription of *mexB* and *mexY* respectively. The overexpression ranged from 5.02 to 11.08 folds for MexB and 3.61 to 26.17 for MexY in comparison with those of PAO. Co-expression of MexY was also observed in MexB overproducers.

Mutational variations in efflux pump regulatory genes

Regulatory gene sequences of isolates with overexpressed Mex-efflux systems were analysed for mutations and all mutations found in individual amplicons are listed in Table 4. MexAB overproducers designated Pa13 and Pa16 were found to harbour mutations in their repressor genes—*mexR, nalC* and *nalD*. A combination of both transitional and transversional point mutations, either silent or leading to changes in amino acid substitutions were observed in *mexR* and *nalC* of Pa13 and Pa16 and in *nalD* of Pa16. However, *nalD* of Pa13 displayed only silent mutations, whilst Pa6 was conspicuous by the absence of mutations of either type. Interestingly, all point mutations observed in *mexR* of Pa16 strain were novel—hitherto unreported in NCBI database. Strikingly, *mexR* of Pa16 revealed yet another novel alteration, at the 443–444 position, of an *ochre* codon to that for serine, followed by a triplet insertion for cysteine and a stop codon of *opal* type. Sequencing results obtained from the MexXY overproducers showed a variety of silent/point mutations in *mexZ*. PCR amplification of *mexZ* in Pa6, which showed a 9.65 fold higher level of MexY compared to that of PAO strain, was unsuccessful.

Phylogenetic analysis

Sequences obtained from *mexR, nalC*, and *nalD* of three MexB overproducing isolates, Pa6, Pa13 and Pa16, were concatenated as a prelude to the phylogenetic study. The resultant composite sequence was then subjected to Bayesian

Table 3 MAR indices, beta-lactamase production and relative quantification of mRNA from *mexB* and *mexY* in 29 clinical isolates

Isolates	MAR indices	Betalactamases production			Relative expression[a]	
		ESBL	MBL	AmpC	*mexB*	*mexY*
Pa1	0.68	–	–	–	1.31	2.08
Pa2	0.75	–	–	+	0.14	1.36
Pa3	0.81	–	+	+	0.21	0.75
Pa4	0.5	+	–	–	0.35	1.65
Pa5	0.56	–	–	–	1.19	**24.42**
Pa6	0.5	–	–	–	**11.08**	**9.65**
Pa7	1.0	–	+	+	0.42	0.61
Pa8	0.81	–	+	+	0.12	2.04
Pa9	0.87	–	–	–	0.13	2.01
Pa10	0.87	–	+	–	0.22	1.97
Pa11	0.94	–	–	–	1.14	2.51
Pa12	0.94	+	–	–	0.12	1.34
Pa13	0.81	–	+	+	**5.02**	**26.17**
Pa14	0.5	–	+	–	0.72	0.89
Pa15	1.0	–	+	+	0.65	0.66
Pa16	1.0	–	+	+	**5.46**	**3.61**
Pa17	0.87	–	–	–	0.29	0.27
Pa18	0.94	–	+	–	ND	0.15
Pa19	0.94	+	+	–	2.11	2.17
Pa20	1.0	–	–	–	1.49	1.72
Pa21	0.75	–	+	–	0.11	1.58
Pa22	0.94	–	–	–	0.56	1.99
Pa23	1.0	+	–	–	0.82	0.8
Pa24	1.0	–	–	–	1.36	1.3
Pa25	1.0	–	+	+	0.28	**11.79**
Pa26	1.0	–	–	–	0.25	0.13
Pa27	0.68	–	–	–	0.56	1.49
Pa28	0.94	–	–	–	ND	0.21
Pa29	0.94	–	+	+	0.34	**4.08**

The values shown in boldface in the table indicate efflux pump expressions which were found to be > 3.0 folds compared to the reference strain

ESBL extended-spectrum beta-lactamase, *MBL* metallo-beta-lactamase, *ND* not detected

'+ and –' represent beta-lactamase and non-beta-lactamase producers, respectively

[a]Relative to expression level in the reference strain PAO, assigned with a value of 1.0

phylogenetic analysis along with a select group of nine closely related sequences deposited in the NCBI database. The nine sequences were chosen on the basis of differences in geographical areas of their report, highest query coverage and minimum 'E' values, when subjected to BLASTN analysis with the sequences obtained from our PCR amplicons. Likewise, the *mexZ* sequences from five MexY overproducer strains—Pa5, Pa13, Pa16, Pa25 and Pa29—along with a set of nine other similar sequences retrieved from the public database were employed for the construction of the phylogram. *P. aeruginosa* strain, PAO1 (GenBank Accession No: AE004091.2), was taken as the outgroup for the purpose. The best fit model of sequence evolution based on AIC was detected as HKY for *mexR* and *nalC*, as HKY+I for *nalD* and GTR+I for *mexZ*. Potential scale reduction factor value assessed by MrBayes was observed to be 1.0 and the estimated sample size was above 100.

Phylogenetic analysis of three loci—*mexR, nalC* and *nalD*—concatenated composite sequence (Fig. 3) revealed that those from isolate Pa6 was found to form a distinct lineage. The sequence of Pa13 was genetically similar to that reported from Taiwan (GenBank Accession No: CP004061.1) as well as with a sequence (GenBank Accession No: CP008739.2) reported from the state of Tamil Nadu located in South India. Interestingly, the concatenated sequence of Pa16 isolate exhibited similarity with the sequence of *P. aeruginosa*, first reported with *bla*NDM-1 in North America (GenBank Accession No: CP012901.1)

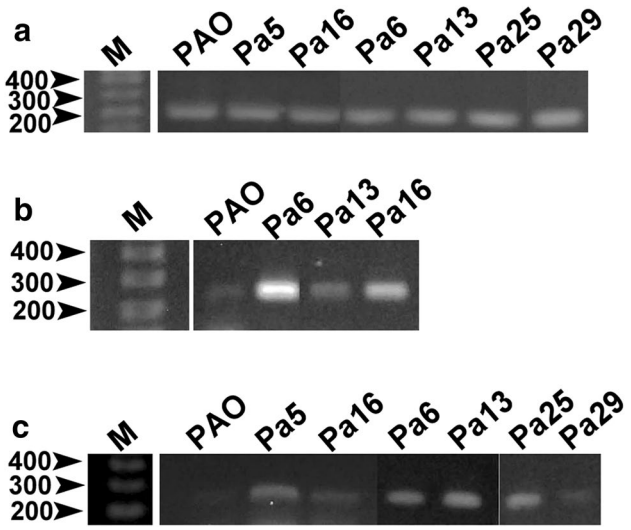

Fig. 2 Semi-quantitative RT-PCR. Lane M: 100 bp DNA ladder. Other lanes show *P. aeruginosa* MTCC strain PAO and clinical isolates. **a** Expression of *rpsL* in PAO and six isolates, Pa5, Pa16, Pa6, Pa13, Pa25 and Pa29. **b** Expression of *mexB* in PAO and three isolates, Pa6, Pa13 and Pa16. **c** Expression of *mexY* in PAO and six isolates, Pa5, Pa16, Pa6, Pa13, Pa25 and Pa29

which was colistin non-susceptible (Mataseje et al. 2016). The two sequences reported from Brazil (GenBank Accession No: CP021380.1) and Malaysia (GenBank Accession No: CP007147.1) were found to be clustered into a distinct clade. The phylogram generated employing mexZ sequence resulted in low clade credibility values (< 0.3) and hence was considered to be non-significant (data not shown).

Discussion

MDR *P. aeruginosa* belongs to one of the most critical groups of bacteria responsible for difficult-to-cure and fatal infections in hospitals and nursing homes. This study deals with molecular investigations focused on MexAB-OprM and MexXY efflux pump-related gene sequences and their regulatory gene(s) based phylogenetic analysis in 29 MDR clinical isolates of *P. aeruginosa* from Kerala, the southernmost State in India. Isolates selected for our study were found to possess MAR indices which ranged from 0.5 to 1.0 against 16 antibiotics of different classes. Among them, 27.6% (n = 8) exhibited a MAR index of 1.0, with all strains showing resistance to both ampicillin and nalidixic acid. Of the isolates, 13.8% (n = 4), 44.8% (n = 13) and 31.0% (n = 9) were ESBL, MBL and AmpC producers respectively; 27.6% (n = 8) were co-producers of AmpC and MBL; only one strain was found to produce both ESBL and MBL. Beta-lactamases encoded by both chromosomal and plasmid genes hydrolyze and destroy broad-spectrum beta-lactam antibiotics and pathogens harbouring them are thus considered a serious health threat due to limited treatment options (Ansari et al. 2016). MexB overproduction was detected in three strains—Pa6, Pa13 and Pa16, whilst MexY overexpression was found in six isolates—Pa5, Pa6, Pa13, Pa16, Pa25 and Pa29. Notably, simultaneous expression of MexY was observed in MexB overproducers, a co-expression which has been reported previously (Llanes et al. 2004). However, no correlation was found to exist between expression levels of Mex efflux pumps and antibiotic resistance profile. Two isolates, Pa16—a co-producer of both MexB and MexY and Pa25—MexY overproducer, were found to display a MAR index of 1.0. All isolates hyperexpressing Mex efflux pump systems were found to be devoid of ESBL production. AmpC and MBL production were observed in Mex efflux pump overproducers—Pa13, Pa16, Pa25, and Pa29, whilst Pa6 (MexAB over producer) and Pa5 (MexXY overproducer) were devoid of betalactamase enzymes such as AmpC and MBL. Notably, Pa20, Pa24 and Pa26, all of which display a MAR index of 1.0 tested negative for beta-lactamases and efflux pump activity. This apparently could be due to the occurrence of other resistance mechanisms therein. For instance, we have obtained evidence of porin downregulation in these three isolates (unpublished observations).

Various synonymous/and non-synonymous mutations were identified in regulatory gene sequences—*mexR, nalC* and *nalD* of MexAB overproducers, Pa13 and Pa16. Pa13 isolate was found to possess a transversional point mutation, T→A at 377th position (^{126}Valine→Glutamic acid substitution) in *mexR* gene which has already been reported previously and was considered insignificant (Ziha-Zarifi et al. 1999; Quale et al. 2006; Choudhury et al. 2016). Two non-synonymous mutations, C→T at 20th position (^{7}Proline→Leucine substitution) and C→A at 427th position (^{143}Proline→Threonine substitution) were observed in *mexR* of Pa16 strain, which are novel and have not yet been reported in NCBI database. Also, we have encountered an interesting novel alteration, in *mexR* of Pa16 at the 443–444 position, of an *ochre* codon to that for serine, followed by a triplet insertion for cysteine and a stop codon of *opal* type. Mutation at 7th amino acid position of MexR in Pa16 was close to the N-terminal while other mutations were confined to the extreme end of the C-terminal region of the repressor protein (Lim et al. 2002; Suman et al. 2006; Wilke et al. 2008). The changes observed at 71st (Glycine→Glutamic acid), 209th (Serine→Arginine) amino acid positions of *nalC* gene were reported as nonsense mutations with no effect on MexAB-OprM pump expression by Pan et al. (2016); a similar mutation was detected by us in *nalC* of Pa13. A transition of G→A at 212th nucleotide position (^{71}Glycine→Glutamic acid) was also found in *nalC* of Pa16. The *nalD* of Pa16 was found to harbour mutation at 153th position of amino acid (Leucine→Glutamine). Mutation

Table 4 Summary of genetic analyses of mutations in *mexR, nalC, nalD* and *mexZ* in clinical isolates of *P. aeruginosa*

Isolates	*mexR* mutation	
	Nucleotide	Aminoacid
Pa13	^{15}G→C, ^{18}T→C, ^{33}C→T, ^{96}A→G, ^{264}C→T,^{327}G→A, ^{384}G→A	–
	^{377}T→A	^{126}Valine→ Glutamic acid
Pa16	^{20}C→T	^{7}Proline→Leucine
	^{201}G→A, ^{441}T→A	–
	^{427}C→A	^{143}Proline→Threonine
	^{443,444}AA→CT	^{148}Stop codon→Serine
	Insertion after 444 bp (TGCTGA)	Cysteine, stop codon
Isolates	*nalC* mutation	
	Nucleotide	Aminoacid
Pa13	^{212}G→A	^{71}Glycine→ Glutamic acid
	^{258}G→A, ^{417}G→A, ^{441}C→T, ^{447}T→C	–
	^{625}A→C	^{209}Serine→Arginine
Pa16	^{212}G→A	^{71}Glycine→Glutamic acid
	^{595}A→T	–
Isolates	*nalD* mutation	
	Nucleotide	Aminoacid
Pa13	^{78}G→A,^{168}C→T, ^{297}G→A, ^{555}T→C	–
Pa16	^{458}T→A	^{153}Leucine→Glutamine
	^{555}T→C	–
Isolates	*mexZ* mutation	
	Nucleotide	Aminoacid
Pa5	^{93}C→T, ^{210}C→T, ^{357}C→T, ^{367}T→C, ^{438}A→G	–
Pa13	^{93}C→T, ^{231}C→T	–
Pa16	^{4}G→A	^{2}Alanine→Threonine
Pa25	^{93}C→T, ^{438}A→G, ^{576}C→T, ^{585}C→A	–
Pa29	^{155}A→G	^{52}Tyrosine→Cysteine
	^{382}C→T, ^{438}A→G	–

'–' represents no amino acid change

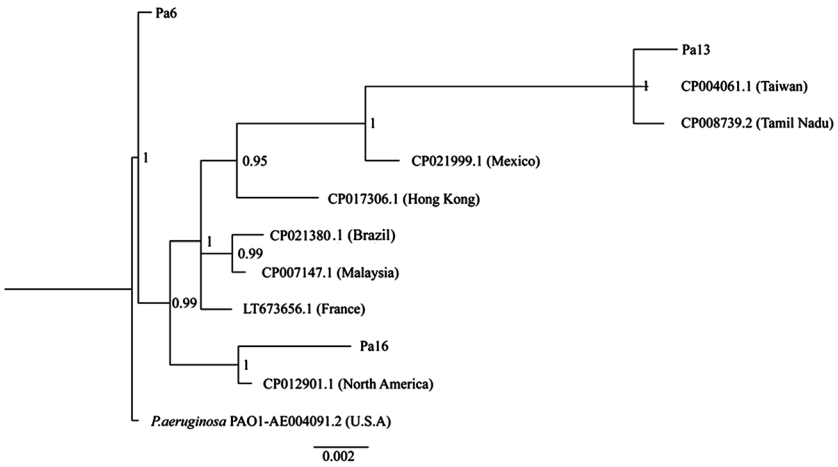

Fig. 3 Bayesian phylogenetic tree reconstructed from three loci of *P. aeruginosa*—*mexR,nalC* and *nalD*—concatenated alignment drawn to scale with the branch lengths representing evolutionary distance. Scale bar denotes probability of nucleotide change. Analysis was run for 1,000,000 generations. Clade credibility values are shown at each node. *P. aeruginosa*, PAO1, was used as the outgroup

was detected within the ligand binding domain of NalD (Chen et al. 2016). Pa6 was noticeable by the absence of mutations of either type. It would be pertinent to note that MexAB overexpression is reported to be regulated by various factors other than *mexR, nalC* and *nalD* (Maseda et al. 2004; Tian et al. 2016). However, two isolates, Pa16 and Pa29, were found to display point mutations in *mexZ* gene, leading to substitution of alanine by threonine at the 2nd and cysteine for tyrosine at the 52nd amino acid position respectively. Pa16 and Pa29 showed mutations in the DNA binding domain at the N-terminal of MexZ (Alguel et al. 2010; Jahandideh 2013). It may be noted that point mutations in *mexZ* gene of Pa16 and Pa29 observed in our study were found to be similar to mutations reported in GenBank accession nos. CP012901.1 (Mataseje et al. 2016) and WP_023123846.1 respectively, which perhaps might be responsible for upregulation of MexXY. Despite several attempts to amplify *mexZ* gene of Pa6 with different primer sets and cycling conditions, no specific PCR amplicon was obtained; a similar situation of amplification failure has also been reported by Poonsuk et al. (2014).

The genetic divergence and homogeneity of the concatenated (*mexR, nalC* and *nalD)* regulatory gene sequences was apparent in the phylogram. Interestingly, the sequence from Pa13 strain showed genetic similarity with MDR *P. aeruginosa*, VRFPA04 (GenBank Accession No: CP008739.2), obtained from a keratitis patient from a tertiary eye care center in Tamil Nadu, India (Murugan et al. 2016) and the homology was also evident with the sequences deposited from Taiwan (GenBank Accession No: CP004061.1). Notably, Pa16 was found to exhibit similarity with sequence from *P. aeruginosa* 15-1092 (GenBank Accession No: CP012901.1) isolated from an elderly Canadian patient who was directly transferred to Calgary hospital, Alberta, Canada, following a prolonged hospital stay in New Delhi, India (Mataseje et al. 2016). An estimated 10% of the 30 million Kerala population live overseas resulting in high mobility of people breaching geographical boundaries. Kerala State is also a well known tourist destination including that for medical tourism. High mobility of people thus provides ample scope for widespread dissemination of drug-resistant microbes with extensive phylogenetic divergence (Choudhury et al. 2012; Nithya et al. 2017).

To conclude, 10.3 and 20.7% of the clinical isolates of MDR *P. aeruginosa* investigated in this study displayed overexpression of *mexB* and *mexY* efflux pump genes respectively. Strikingly, all mutations observed in *mexR* of Pa16 strain were found to be novel and hitherto unreported. Phylogenetic analysis of efflux pump regulatory gene sequences revealed genetic similarities among the isolates used in this study as well as with those previously reported from other countries. The need for detailed molecular investigations on key genes governing multidrug resistance cannot be overemphasized. Similar studies should equip us with deeper insights into the working of such drug-efflux systems. This should pave the way for the development of strategic, therapeutic interventions to face the challenges posed by rapid evolution of microbial pathogens. The present global scenario demands effective implementation of control measures to prevent the spread of resistance genes and creation of greater awareness among the medical fraternity as well as the general public.

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Compliance with ethical standards

Conflict of interest All authors declare that they have no conflict of interest.

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