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RESEARCH ARTICLE

Transcriptional expression of *PmrB* and *arn*A within polymyxin-resistant nosocomial isolates of *Pseudomonas aeruginosa* from India

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ABSTRACT

Objective: The polymyxin group of antibiotics is considered to be one of the most effective antimicrobial agents against many serious pathogenic bacteria, but the excessive use of these antibiotics has led to the development of drug resistance among bacteria. This study was designed to characterize polymyxin-resistant *P. aeruginosa* and to explore the role of *PmrB* and *arn*A in resistant phenotype.

Methods: mRNA and cDNA of five selected polymyxin-resistant strains representing different MIC range; isolated under normal condition of strain growth, after treating sample/media with FeCl3 and MgCl2 alone, or after treating with FeCl3 and polymyxin antibiotic. The transcriptional expression was observed for *PmrB* and *arn*A by quantitative real time RT-PCR in reference to *P. aeruginosa* PAO1. The presence of plasmid mediated colistin resistance determinants *mcr-1* was screened by PCR. Susceptibility of the strains was determined by disc-diffusion method and DNA fingerprinting was carried out by performing REP-PCR.

Results: A down regulated expression of *PmrB* and *arn*A was observed even after the unique induction with FeCl3 and MgCl2. All the isolates were found to be resistant against cefepime and different clonal patterns of resistance were found among the isolates.

Conclusion: This study has drawn a new insight into polymyxin resistance which will help in the detection and control of infections caused by multidrug resistant *P. aeruginosa*. The low susceptibility rate to aminoglycoside, piperacllin-tazobactam and ciprofloxacin was found and in addition, detection of *PmrB* and *arn*A as molecular markers in the follow up of infections caused by multidrug resistant *P. aeruginosa*. *J Microbiol Infect Dis 2018; 8(2):61-68.*

Keywords: *arn*A, *mcr-1*, *PmrB*, Polymyxin, *Pseudomonas aeruginosa*, Multidrug resistance

INTRODUCTION

Polymyxins, essentially polymyxin B and colistin (polymyxin-E), are the bacterial membrane inhibitors produced from *Bacillus spp* [1] and are mostly active against Gram negative bacteria [2]. It is an effective drug for the treatment of many serious infections like pseudomonal lung infection, ear, and eye infections [3]. Polymyxins are excreted primarily by the kidneys and are poorly absorbed from the gastrointestinal tract. However, these antibiotics were gradually abandoned in most parts of the word around 1980 because of the reports of their serious toxic effects, mainly on the kidney and nervous system [5-6]. But polymyxins have re-emerged in clinical practice owing to the worldwide increasing prevalence of nosocomial infections caused by multidrug-resistant (MDR) *Pseudomonas aeruginosa* and *Acinetobacter baumannii* [7-8]. *P. aeruginosa* is an opportunistic Gram-negative pathogen and is the third leading cause of nosocomial acute infections and fatal chronic lung diseases in patients with cystic fibrosis and immunocompromised individuals [9-10]. Polymyxin-resistant organisms were also reported from many parts of the world [11].

Polymyxin resistance in *P. aeruginosa* occurs mainly through the alteration in the LPS moiety by the addition of 4-amino-4-deoxy-L-arabinose

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(LAra4N) to a phosphate group of lipid A [12]. This addition causes an absolute positive increase in lipidA charge, thus lowering the affinity of positively charged polymyxins. PmrA-*PmrB* and PhoP-PhoQ are the main two-component regulatory systems for the biosynthesis of LAra4N that confer the polymyxin resistance on the organisms. Recently, *mcr-1*-mediated acquired colistin resistance has been described in *Enterobacteriaceae* family [13]. Since polymyxins (polymyxin B and colistin) are considered as last-choice treatment for non-fermenting gram negative bacilli. Increasing resistance pattern of the organisms against this group of antibiotics was reported [8].This study was designed to screen the polymyxin-resistant *P. aeruginosa* and to investigate the role of *PmrB* and *arn*A in conferring polymyxin resistance in a tertiary referral hospital of northeast India.

METHODS

Sample collection

A total of 290 clinical specimens were collected from the patients who attended or were admitted in different wards and outpatient department of Silchar Medical College and Hospital during September 2013 to August 2014. Duplicate isolates, excluding *P. aeruginosa*, from the same clinical specimen were not included in the study. *P. aeruginosa* strains selected for this study, were characterized and identified by standard biochemical testing, pigment production, growth on cetrimide agar, and 16s rDNA sequence analysis [14].

Screening of polymyxin-resistant P. aeruginosa

All identified *P. aeruginosa* stains were screened by Kirby-Bauer disc diffusion method using the antibiotics polymyxin-B (300 units) and colistin (colistin sulphate; 10 µg) on Muller-Hinton medium (Hi-media, Mumbai, India), in order to select the polymyxin-resistant *P. aeruginosa* strains. The zone dimension was measured and the results were interpreted as per Clinical Laboratory Standard Institute (CLSI) methods [15]. The isolates that showed resistance to either of these two antibiotics were considered as positive. Minimum inhibitory concentration (MIC) was determined for the screened positive *P. aeruginosa* by agar dilution method against the antibiotics polymyxin-B (Samarth Life Sciences, Mumbai, India) and colistin (Wallace, HP, India) with a range from 2->256 µg/ml and the results were interpreted according to Clinical and Laboratory Standards Institute methods (CLSI 2013) [15].

Genotypic characterization for polymyxin resistance targeting pmrA, *PmrB* and *arn*A was performed by PCR assay for all the screened positive isolates. The whole pmrA, *PmrB* and *arn*A was amplified and sequenced for all the colistin resistant study isolates The amplified products were purified using MinElute PCR Purification Kit (Qiagen, Germany) then ligated into pGEM-T Vector (Promega, Madison, USA) and sequenced using Sanger sequencing method.

Screening of plasmid mediated colistin resistance (mcr-1)

The presence of *mcr-1* gene was screened by PCR assay for all polymyxin-resistant isolates using the primers CLR5-F (5′-CGGTCAGTCCGTTTGTTC-3′) and CLR5-R (5′-CTTGGTCGGTCTGTAGGG-3′) [16].

Transcriptional expression analysis by quantitative real time PCR

The transcriptional response of *PmrB* and *arn*A was observed by performing quantitative Real Time PCR (Applied Biosystem, USA). The primers used in this study are mentioned in Table 1. Five polymyxin-resistant isolates representing different MIC range were selected and *P. aeruginosa* PAO1was used as a reference strain. The isolates were grown in three different conditions. First, all the six strains (including the control) were inoculated in Luria Bertani broth (Hi-media, Mumbai, India) without any chemical or antibiotic pressure. Second, all these six strains were inoculated in Luria Bertani broth containing both 1 mM $FeCl_3$ and $MgCl_2$ whereas in the third condition only the five polymyxin-resistant strains were inoculated in Luria Bertani broth with 1mM $FeCl_3$ and 0.50 µg/ml of polymyxin B. All these freshly inoculated cultures (10ml LB broth in 50 ml of falcon tube) were incubated with shaking (160 rpm) at 37 ^{0}C for 12-16 hours followed by mRNA isolation. Total mRNA was isolated by using RNeasy mini kit (Qiagen,Hilden, Germany) and

immediately reverse-transcribed into cDNA using Quantiscript Reverse Transcription kit (Qiagen, Hilden, Germany). The cDNA was quantified by Picodrop (Pico 200, Cambridge, UK). Quantitative real time PCR was performed using Power Sybr Green Master Mix (Applied Biosystem, Warrington, UK) using Step One Plus real time detection system (Applied Biosystem, USA). The relative expression of *arn*A and *PmrB* in three different conditions were determined by ΔCt method [17]. The fold change of *arn*A and *PmrB* for each sample was normalized against a housekeeping gene rpsL of *P. aeruginosa* [18] which was used as an internal standard. Real time PCR was performed and the primers for each target used in separate reaction and each single reaction mixture (20µl) contained 100ng of template cDNA, 10 picomole each for forward and reverse primer (Hysel, New Delhi, India), 10 µl of Power SYBER green PCR master mix (Applied Biosystem, Warrington, UK) and 4 µl nuclease free water. The reactions were carried out under the following conditions; 95 ^{0}C for 2 min, 40 cycles of 95 ^{0}C for 20 s, 52 ^{0}C for 40 s, 72 ^{0}C for 30 s.

Antimicrobial susceptibility testing

Antibiotic susceptibility pattern of polymyxin-resistant *P. aeruginosa* was determined by Kirby-Bauer disc diffusion method in order to investigate the therapeutic option for the infections caused by this organism. Susceptibility testing was done against the antibiotics cefepime (30 µg), amikacin (30 µg), gentamicin (10 µg), ciprofloxacin (5 µg), piperacillin-tazobactam (100/10 µg) and imipenem (10 µg) (Hi-media, Mumbai, India) and the results were interpreted as per CLSI guidelines (CLSI 2013) [15].

DNA fingerprinting of polymyxin-resistant P. aeruginosa

The heterogeneity in the isolates was determined by repetitive extragenic palindromic (REP) PCR (Applied Biosystem, USA) using primers REP F (5'-XCGXCTTATCXGGCCTAC-3') and REP R (5'-NCGNCTTATCNGGCCTAC-3') (Hysel, New Delhi, India). PCR was performed according to the following reaction condition; initial denaturation at 95 ^{0}C for 3mins, 30 cycles of denaturation at 95 ^{0}C for 20 s, annealing at 46°C for 40 s and extension at 72 ^{0}C for 3mins; and final extension at 72 ^{0}C for 10 mins [19-20].

Statistical analysis

The statistical power and the sample size of the study were calculated as described previously 21. The sample size and power of the study was determined to verify whether the less number of polymyxin resistance isolates is due to lack of statistical power or have no relation with the sample.

Ethical approval

The work was approved by Institutional Ethical committee of Assam University, Silchar vide Reference Number: IEC/AUS/C/2014-001. The authors confirm that participants provided their written informed consent to participate in this study.

RESULTS

Screening for polymyxin-resistant Pseudomonas aeruginosa

Out of 290 clinical specimens collected between September 2013 and August 2014 from male/female (1:3) in the age range 8-79 years, a total of 163 consecutive, non-duplicate clinical isolates of *Pseudomonas aeruginosa* were obtained and among them only 21 (12.9%) *P. aeruginosa* isolates were found to be resistant to colistin (polymyxin E); but only 20 isolates (12.3%) showed resistance against polymyxin B, however none of them carried *mcr-1*.The clinical details of these resistant isolates are shown in Table 2. Polymyxin-resistant *P. aeruginosa* showed high MIC pattern against polymyxin B and colistin and most of the strains showed an MIC value of >256 µg/ml (Table 3). No mutation was obtained in the pmrA, *PmrB* or *arn*A sequences based on whole gene sequence analysis. At 5% level of significance, the power of the study was found to be significant, so the less percentage of polymyxin-resistant *Pseudomonas aeruginosa* isolates obtained in this study does not have any alliance with the sample size of the study.

Transcriptional analysis

Transcriptional analysis revealed that the expression levels of *PmrB* gene in wild strains of polymyxin-resistant *P. aeruginosa* (RQ=0.604-0.639) was lower than the expression level of control strain *Pseudomonas aeruginosa* PAO1

(RQ=1) (Fig 1, Table 4). A down regulated expression of *PmrB* (Fig 1) was observed when the cells were treated with Fecl3 (RQ=0.295-0.605) only or with both polymyxin and Fecl3 (RQ=0.293-0.467) when compared with the contron strain of PAO1 (RQ=1). (Fig 1) (Table 4).Under the exposure of MgCl2, except one strain PAP-4 (RQ=1.605), all the isolates were found to have reduced expression (RQ=0.439-0.667) than the control strain *P. aeruginosa* PAO1.

Under normal condition, the expression of *arn*A in the control strain (RQ=1) was found to be higher when compared with the test sample, whereas under the treatment of FeCl3 and MgCl2, the expression was found induced in a single strain respectively such as PAF-9 (RQ=1.009) and PAP-4(RQ=1.159). However, the expression level of *arn*A was higher than the *PmrB* when induced with FeCl3 and the same result was observed even in case, where the cells were treated with both FeCl3 and polymyxin antibiotics (Fig 2) (Table 4).

Susceptibility pattern of Polymyxin-resistant strains

Antimicrobial susceptibility results of polymyxin-resistant *P. aeruginosa* strains showed low susceptibility rate to amikacin and gentamicin, β-lactam-β-lactamase inhibitor (Piperacillin-tazobactam) and quinolone group (ciprofloxacin) whereas a total resistance was observed in case of third generation cephalosporin (cefepime). Moderate susceptibility rate was observed against imipenem. The results of the susceptibility tests are given in Table 3.

Table 1: Oligonucleotides used in this study.

Primer pairs	Target	Sequence (5'-3')	Amplified product size (bp)	Reference
*arn*A F *arn*A R	*arnA*	CATGCCGACGACCCACGGGAA CAGTTGGCGGATACGCTCCAG	129	Our study
pmrA F pmrA R	*pmrA*	TCACTGAAACGAGGCTGCC GGACTCCGGTAGGCGGTT	704	Our study
PmrB F *PmrB* R	*PmrB*	AACCGCCTACCGGAGTCC AATGCGCAGGCTATCAGAT	1469	Our study
PmrB RT F *PmrB* RT R	*PmrB*	GCTGAGCGTGGCGGCGCTA CGGCCTGGTCTTCGGTGGCA	128	Our study
CLR5-F CLR5-R	mcr-1	CGGTCAGTCCGTTTGTTC CTTGGTCGGTCTGTAGGG	309	8

Table 2. Clinical details of patients and characteristics of polymixin-resistant *Pseudomonas aeruginosa.*

Serial number	Strain ID	Patient's Sex/Age	Clinical specimen	Ward	Clonal Type
1	PAS-2	M/35yrs	Pus	Surgery	Type 1
2	PAM-11	F/32yrs	Pus	Orthopaedics	Type 2
3	PAP-4	F/19 yrs	Urine	Paediatrics	Type 2
4	PAS-6	M/26 yrs	Stool	Surgery	Type 6
5	PAM-1	M/45 yrs	Pus	ENT	Type 7
6	PAS-1	F/20 yrs	Pus	Surgery	Type 3
7	PAM-4	M/21 yrs	Urine	Medicine	Type 9
8	PAF-9	F/45 yrs	Pus	ENT	Type 1
9	PAS-91	M/37 yrs	Pus	Surgery	Type 5
10	PAM-6	F/25 yrs	Urine	Medicine	Type 7
11	PAM-0	M/47 yrs	Stool	Medicine	Type 2
12	PAM-46	F/59 yrs	Urine	Medicine	Type 1
13	PAS-5	M/48 yrs	Pus	Surgery	Type 8
14	PAF-3	F/20 yrs	Pus	Paediatrics	Type 4
15	PAP-7	F/12days	Nasal secretion	Paediatrics	Type 4
16	PAO-7	M/40 yrs	Pus	Orthopaedics	Type 5
17	PAS-2	M/42 yrs	Urine	Surgery	Type 6
18	PAM-3	M/39 yrs	Sputum	Medicine	Type 6
19	PAS-9	M/53 yrs	Stool	Surgery	Type 9
20	PAM-1	F/1.5 yrs	Oral swab	Medicine	Type 8
21	PAO-4	F/29 yrs	Blood	Paediatrics	Type 3

Table 3: Susceptibility profile of Polymixin resistant *Pseudomonas aeruginosa.*

Serial number	Strain Code	Screening of polymixin resistance		Minimum inhibitory concentration (µg/ml)		Co-resistance profile
		Polymixin-B	Colistin	Polymixin-B	Colistin	
1	PAS-2	Resistant	Resistant	128	64	CEF,AMK,CIP,PIT
2	PAM-11	Resistant	Resistant	>256	>256	CIP, CEF, AMK,GEN
3	PAP-4	Resistant	Resistant	128	64	CEF,AMK,CIP,PIT
4	PAS-6	Resistant	Resistant	128	64	CIP,CEF, AMK,PIT
5	PAM-1	Resistant	Resistant	16	16	CEF,AMK,CIP,GEN
6	PAS-1	Sensitive	Resistant	256	128	CIP, CEF,AMK,PIT, IMP
7	PAM-4	Resistant	Resistant	>256	256	PIT, CIP, CEF, AMK
8	PAF-9	Resistant	Resistant	>256	>256	CIP, CEF, AMK, PIT
9	PAS-91	Resistant	Resistant	128	64	CIP, CEF, AMK, GEN
10	PAM-6	Resistant	Resistant	>256	>256	GEN,CIP,CEF, AMK
11	PAM-0	Resistant	Resistant	256	128	GEN,CIP,CEF,AMK
12	PAM-46	Resistant	Resistant	256	128	PIT, CIP, CEF, AMK
13	PAS-5	Resistant	Resistant	>256	>256	PIT, GEN, CEF, AMK
14	PAF-3	Resistant	Resistant	>256	>256	GEN, CIP, CEF, AMK
15	PAP-7	Resistant	Resistant	128	128	CIP,CEF,PIT
16	PAO-7	Resistant	Resistant	>256	>256	AMK, CEF, CIP, PIT
17	PAS-21	Resistant	Resistant	>256	>256	IMP, GEN, PIT, CIP, AMK
18	PAM-3	Resistant	Resistant	>256	>256	GEN, CIP, CEF, AMK
19	PAS-9	Resistant	Resistant	>256	>256	IMP, PIT, CIP, CEF
20	PAS-4	Resistant	Resistant	>256	128	AMK, GEN, CEF
21	PAM-1	Resistant	Resistant	256	256	PIT, GEN,AMK,IMP

AMK= Amikacin, CEF= Cefepime, CIP= Ciprofloxacin, GEN= Gentamicin, IMP= Imipenem, PIT= Piperacillin-Tazobactam

Table 4. mRNA expression details of clinical isolates of *Pseudomonas aeruginosa* and PAO1.

Sl. No	Strain Code	RQ values of *PmrB* gene in reference to PAO1 which is assigned to a value 1				RQ values of *arn*A gene in reference to PAO1 which is assigned to a value 1			
		Wild strains	Strains treated with $FeCl_3$	Strains treated with PB & $FeCl_3$	Strains treated with $MgCl_2$	Wild strains	Strains treated with $FeCl_3$	Strains treated with PB & $FeCl_3$	Strains treated with $MgCl_2$
1	PAO1	1 ± .066	1 ± .04	1 ± 0.079	1 ± 0.029	1 ± .052	1 ± .091	1 ± .07	1 ± 0.049
2	PAM-1	0.639 ± .072	0.378 ± .059	0.293 ± .065	0.439 ± .051	0.626 ± .071	611 ± .081	.645 ± .038	0.439 ± .051
3	PAP-4	0.604 ± .033	0.364 ± .054	0.439 ± .051	1.605 ± .031	0.631 ±. 40	.609 ± .034	.865 ± .075	1.159 ± .042
4	PAS-1	0.611 ± .083	0.605 ± .031	0.459 ± .022	0.667 ± .113	0.639 ± .35	.739 ± .129	.879 ± .043	0.605 ± .031
5	PAM-4	0.607 ± .033	0.311 ± .098	0.421 ± .015	0.439 ± .051	0.759 ± .083	.613 ± .039	.627 ± .051	.613 ± .039
6	PAF-9	0.628 ± .027	0.295 ± .046	0.467 ± .074	0.605 ± .031	0.667 ± .113	1.009 ± .042	.763 ± .048	0.439 ± .051

Typing of polymyxin-resistant P. aeruginosa strains

The REP PCR results showed that these polymyxin-resistant *P. aeruginosa* isolates were heterogeneous and revealed ten different clonal types of *P. aeruginosa* strains. Clonal type 1 was found to be the most prevalent type and was detected in 7 isolates (PAS-2, PAM-1, PAS-91, PAF-3, PAS-4, PAM-1, PAM-46), followed by type 2 (PAM-6, PAS-9), type 3 (PAS-1, PAS-3), type 4 (PAP-7, PAS-21), type 6 (PAM-11,PAF-9) and clonal type 10 (PAS-6, PAO-7) in two isolates whereas REP types 5(PAM-0), 7(PAS-5), 8 (PAP-4) and 9 (PAM-4) were detected in one isolate each (Table 3, Figure 3).

DISCUSSION

Polymyxin antibiotics are considered to be one of the oldest classes of antibiotics having 5 different types A, B, C, D and E but only polymyxin-B and polymyxin-E (colistin) have been used in clinical treatment [7]. Polymyxin antibiotics are most commonly used to treat the infections caused by *P. aeruginosa* and A. baumannii and carbapenemase producing Gram negative bacilli. There was a recent report [22] from India showing polymyxin resistance within non-fermenting Gram negative rods. In Asia, most of the studies reported the polymyxin resistance less than 10%, whereas the resistance rate in *P. aeruginosa* (>30%) is higherwhich is contradictory to our study [7]. However, their molecular basis is not described and information in this aspect is lacking in our country [8]. This current study focused on PmrA-*PmrB* component system where PmrA is a regulatory protein of *arn* operon which plays an important role in polymyxin resistance and *PmrB* is a sensor kinase protein in the cytoplasmic membrane that activates the transcriptional response of pmrA. In this study, we have observed a unique expression pattern of *PmrB* as well as *arn*A in polymyxin-resistant organisms. The q-PCR results showed a down regulated expression of both *PmrB* and *arn*A under normal condition of growth in polymyxin-resistant organism which disagrees with another study [11]. It is already established that exposure to high levels of Ferric iron in cytoplasm, activates *PmrB* protein which in turn phosphorylates pmrA. PmrA then regulates the expression of *arn* operon which is responsible for polymyxin resistance [18]. After exposing the cells with Fe3+, we failed to get any over expression of *PmrB*; although (PAF-9; Figure 1) showed some deviation from the other 4 isolates. In case of *arn* expression, only a single isolate (PAP-4; Figure 2) showed higher expression level of *PmrB* compared to *P. aeruginosa* PAO1.

Previous reports showed that in A. baumanii, the addition of ferric chloride can induce the expression level of *arn* operon in pmrAB system and thus can increase the MIC for colistin but there was no evidence of effect of Fe3+in the induction of polymyxin resistance in *P. aeruginosa* [23].Our study has described a down regulation due to low transcriptional expression. However, proteomic level expression analysis which wasnot be performed in this study would prove to be a better mean for understanding *PmrB* and *arnA* mediated resistance. Zavaski et al [24] in the year 2007 reported that the majority of *P. aeruginosa* strains used in their study showed the MIC of polymyxin B at 2 µg/ml whereas our study isolates showed an MIC range of 16 to >256 µg/ml against polymyxin B or colistin which is partly in agreement with the study conducted by Moskowitz et al. [5] where they have found that the P. *aeruginosa* strains isolated from cystic fibrosis patients exhibited MICs of colistin at a concentration greater than 512 µg/ml. It was found that most of the study isolates showed resistance to other antibiotics, limiting the treatment option. Typing of all the polymyxin-resistant strains was performed to establish the clonal relatedness among themselves and REP PCR results revealed10 different clonal types of *P. aeruginosa* which were responsible for the spread of polymyxin resistance and no dominant clonal type was responsible for any epidemic spread of this resistance determination this hospital setting.

This study revealed the molecular and genetic background of polymyxin-resistance in *P. aeruginosa* and the transcriptional response has displayed that low level transcription of *PmrB* and possibly *arnA* has a role in the polymyxin-resistance. Further, there could be some more factors which play a role in translational level of the above mentioned genes which leaves further scope for investigation. This study has drawn a new insight into polymyxin resistance, which with further study will help in devising a detection tool using *PmrB* and *arn*A as a molecular marker for detection of multidrug resistant *P. aeruginosa* and its control.

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