Antibiotic Resistant Clinical Isolates of Pseudomonas aeruginosa harbor lasA Gene

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Abstract

Pseudomonas aeruginosa a leading causative organism of nosocomial infections, periodically develop multidrug-resistance and causes increased mortality rate among hospitalized patients. This creates a major concern in the selection of appropriate antibiotics and periodic assessment of antibiotic resistant pattern is highly essential for efficient treatment of *P. aeruginosa* infections. Here, we attempted to understand the current scenario of antibiotic resistance of clinical isolates of *P. aeruginosa*. Twenty commonly used antibiotic were tested for antibiotic resistance/susceptibility among isolates. Virulence gene distributions in antibiotic sensitive and resistant isolates were studied by PCR. Here we report that recent clinical isolates of *P. aeruginosa* are developing resistance to commonly used antibiotics. Further molecular characterization of clinical isolates revealed that highly resistant strains hold *lasA* gene suggesting the importance of lasA towards antibiotic resistance.

INTRODUCTION

Nosocomial infections that cause critical public health problem have been considered a great challenge for effective treatment in recent years. *Pseudomonas aeruginosa* is one of the leading causative organisms of nosocomial infection in hospitals and healthcare centers (Lyczak. J. B et al., 2000 and Driscoll. J. A et al., 2007). *P. aeruginosa* causes broad range of acute and chronic nosocomial infections which includes ventilator-associated pneumonia, postoperative wound infection, burn wound infection, urinary tract infection, keratitis and otitis in immunocompromised patients (Branski. L. K et al., 2009 and Engel. J et al., 2009). Mortality rate by *P. aeruginosa* infection has been reported to be relatively high (above 70%) in patients admitted in intensive care units (Sadikot. R. T et al., 2005).

Treatment of *P. aeruginosa* infections has been a major challenge due to developed resistance to antibiotics by intrinsic mechanisms such as hydrolytic enzyme production, efflux system, porin loss, targeted mutations and excessive antibiotic administration during treatment (Haghi. F et al., 2018). These development leads to the emergence of multidrug-resistant (MDR) forms of *P. aeruginosa*. Infections caused by MDR *P. aeruginosa* is considered as serious threat in healthcare settings with higher mortality rate of 40% in spite of treatment using conventional antibiotics (Porras-Gomez. M et al., 2012). Studies have reported that MDR *P. aeruginosa* strains are currently resistant to third generation antibiotics like cephalosporins, fluoroquinolones, aminoglycosides and carbapenems and are gaining resistance against many other antibiotic classes, making it difficult to select appropriate antibiotics for treatment (Yayan. J et al., 2015).

Several bacterial components and extracellular virulence factors contribute to the pathogenesis of MDR *P. aeruginosa*. These include pili, flagella, Type III system effector proteins (ExoS, ExoT, ExoY and ExoU), Alkaline protease, Exotoxin A, Elastase, LasA protease and hemolytic Phospholipase-C which help in colonization and dissemination into host system (Cullen. L et al., 2015, Galle. M et al., 2012 and Barker. A. P et al., 2004). These secretory toxins are translocated directly into the host cell with the help of specific translocation apparatus such as Type II and Type III secretion systems present on the bacterial cell wall. Once translocated into the host system, these toxins inactivate host immunity proteins such as immunoglobulins, cytokines complement proteins and disrupt host epithelial matrix which leads to apoptosis (Ben Haj Khalifa. A et al., 2011, Casilag. F et al., 2016 and Sabharwal. N et al., 2014).

Development of intrinsic antibiotic resistance and expression

of virulence factors that invade and kill the host cells causes *P. aeruginosa* more persistent and difficult to treat using available antibiotics (Sadikot. R. T et al., 2005). Hence, it is important to study the dynamic nature of antibiotic resistance and its correlation with virulence factors of the strain for developing better therapeutic strategies. In this study, we investigated the current antibiotic response pattern and distribution of virulence genes among clinical isolates of *Pseudomonas aeruginosa* recovered from non-CF patients.

MATERIALS AND METHODS

Ethical statement:

This study was approved by Institute Biosafety Committee (IBSC) of Pondicherry University (PU/IBSC/1/2016/4/891 dated 01/03/2017).

Collection and Identification of Clinical isolates:

Clinical strains of *P. aeruginosa* were isolated from different sources (wound swabs, pus aspirates, endotracheal aspirates, tracheal aspirate, infected tissue biopsies and infected ear canal) of non-Cystic Fibrosis (CF) in-patients from Jawaharlal Institute of Postgraduate Medical Education and Research (JIPMER), Puducherry, India. The clinical isolates were characterized based on their colony morphology on Cetrimide agar, hemolytic test, oxidase and catalase reaction and ability to grow at 42º C (Driscoll. J. A et al., 2007). *P. aeruginosa* MTCC 2453 (MTCC, India) was used as reference strain for the phenotypic and biochemical characterization of clinical isolates. The clinical isolates of *P. aeruginosa* were designated as PAJ 1 to 11.

Antibiotic Susceptibility Test:

The susceptibility profile of all clinical isolates against twenty commonly prescribed antibiotics was assessed by Standard disk diffusion assay (Bauer. A. W et al., 1966), as recommended by the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2016). Antibiotics tested against the clinical isolates were Amikacin (30 µg), Pipercillin-Tazobactam (100/10 µg), Cefaperazone (75 µg), Levofloxacin (5 µg), Meropenem (10 µg), Ceftazidime (30 µg), Gentamicin (10 µg), Pipercillin (100 µg), Cefotaxime (30 µg), Aztreonam (30 µg), Ceftriaxone (30 µg), Imipenem (10 µg), Ticarcillin-Clavulanate (75/10 µg), Carbenicillin (100 µg), Norfloxacin (10 µg), Lomefloxacin (10 µg), Polymixin B (300 units), Tobramycin (10 µg), Linezolid (30 µg) and Ciprofloxacin (5 µg). All the antibiotics were procured from Himedia, India. All the isolates were scored susceptible, intermediate or resistant by comparing their inhibition values with the standard CLSI values. *P. aeruginosa* MTCC 2453 was used as the reference strain for susceptibility profiling. Multiple antibiotic resistance (MAR) index was determined for all the strains based on the formula a/b, where (a) represents the number of antibiotics to which the test isolate was resistant and (b) represents the total number of antibiotics test (Krumperman. P. H, 1983 and Paul. S et al., 1997).

Detection of virulence genes by PCR:

Clinical isolates of P. aeruginosa were tested for the presence of Exoenzyme S (exoS), hemolytic-Phospholipase C (plcH), Elastase (lasB) and Serine protease (lasA). Amplification of exoS, plcH and lasB virulence genes was carried out as reported earlier (Wolska. K et al., 2009, Morales-Espinosa. R et al., 2012 and Lanotte. P et al., 2004). Amplification of lasA gene was performed using primer sequence mentioned in Table 1 and with following cycling condition. Initial denaturation at 94ºC for 5 minutes, followed by 30 cycles of denaturation at 94ºC for 30 seconds, annealing at 54ºC for 30 seconds, extension at 72ºC for 1 minute and a final extension at 72ºC for 5 minutes. P. aeruginosa PAO1 was used as positive control in all the PCR.

RESULTS AND DISCUSSION

Isolation and characterization of clinical isolates of P. aeruginosa:

Bacterial strains isolated from non-CF patients were phenotypically and biochemically characterized according to Bergey's Manual of Systematic Bacteriology (Palleroni. N. J et al., 1984). The clinical isolates producing greenish-yellow pigmented colonies on Cetrimide agar were selected for identification. All the isolates were positive for -hemolysis activity, catalase and oxidase production and survived at 42°C (Data not shown). Based on the biochemical typing, the clinical isolates were identified as *Pseudomonas aeruginosa* and were designated as PAJ1, 2, 3, 4, 5, 6, 7, 8, 9, 10 and 11. The clinical isolates were further tested for antibiotic resistance and virulence profiling pattern.

Antibiotic resistant profiling among clinical isolates of P. aeruginosa:

Though bacterial infections are treated using antibiotics, most of the bacterial strains develop resistance in course of time. Several studies had reported that *P. aeruginosa* is

evolving resistance against most of the commonly prescribed antibiotics through intrinsic mutational changes, production of specific hydrolyzing enzymes and intensive use of antibiotics (Ben Haj Khalifa. A et al., 2011, Hurley. M. N et al., 2012, Lister. P. D et al., 2009 and Morales-Espinosa. R et al., 2012). This creates a major concern in the selection of appropriate antibiotics and hence periodical understanding of antibiotic resistant pattern among clinical isolates of *P. aeruginosa* is highly essential for efficient treatment.

In this study we attempted to understand the current scenario of response of antibiotics among clinical isolates of *P. aeruginosa*. Antibiotic resistant profiling among clinical isolates of *P. aeruginosa* was studied against 20 commonly used antibiotics. We observed that most of the clinical isolates were resistant to maximum number of antibiotics tested with high MAR index (Data not shown). Almost all the clinical isolates were resistant to Linezolid (100%). In terms of sensitivity, the isolates were highly sensitive to Gentamicin (91%) and Polymixin B (100%). In addition we also observed that the clinical isolates were developing resistance against Cefotaxime (64%) and Ceftriaxone (46%) (Fig.1).

Antibiotic resistant strains harbor lasA gene:

Presence and expression of virulence gene among isolates plays a major contribution in development of antibiotic resistance (Porras-Gomez. M et al., 2012). Understanding the distribution of such virulence genes in clinical isolates would reveal the molecular mechanism behind the development of antibiotic resistance. In this study we analyzed the distribution of virulence genes among antibiotic resistant and sensitive clinical isolates of *P. aeruginosa*. Virulence genes tested in this study are exoS, plcH, lasB and lasA that encode for Exoenzyme S, hemolytic Phospholipase C, Elastase and Las A protease respectively (Table 2A and B). We observed maximum distribution of all four virulence genes in Resistant/Intermediate strains compared to Sensitive strains. Interestingly we observed presence of lasA gene in all the resistant/Intermediate strains of *P. aeruginosa*, whereas the antibiotic sensitive strains lack lasA gene suggesting the importance of lasA gene towards development of antibiotic resistance (Fig.2).

Figure 1

Histogram showing antibiotic resistance profile among clinical isolates of Pseudomonas aeruginosa.

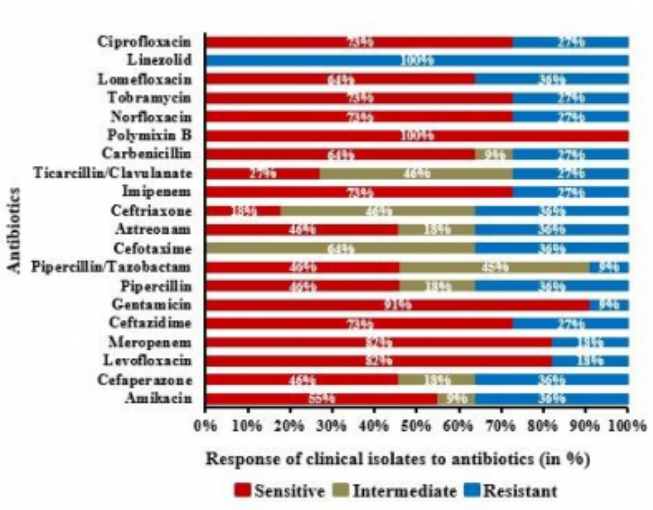


Table 1

Primer sequences used for genetic characterization of clinical isolates of Pseudomonas aeruginosa.

Virulence gene	Primers	Primer sequence (5'-3')	Tm	Size of PCR product	Reference
exoS	*exoS* – F	CGTCGTGTTCAAGCAGATGGTGCTG	55°C	444 bp	25
	exoS – R	CCGAACCGCTTCACCAGGC			
plcH	*plcH* – F	CGACGAGGGCGACGGCTTCTATGA	66°C	447 bp	26
	plcH – R	CCGGGCAGGCTCTTGGGCTCGTA			
lasB	*lasB*– F	GGAATGAACGAAGCGTTCTC	55°C	300 bp	27
	lasB – R	GGTCCAGTAGTAGCGGTTGG			
lasA	*lasA*– F	CGCTGAATGACGACCTGTT	54°C	316 bp	This study
	lasA– R	CGCAACTGGTATTCCTCGAA			

Table 2

Frequency of virulence genes in (A) Antibiotic sensitive clinical isolates and (B) Antibiotic Intermediate/Resistant clinical isolates of Pseudomonas aeruginosa.

(A)

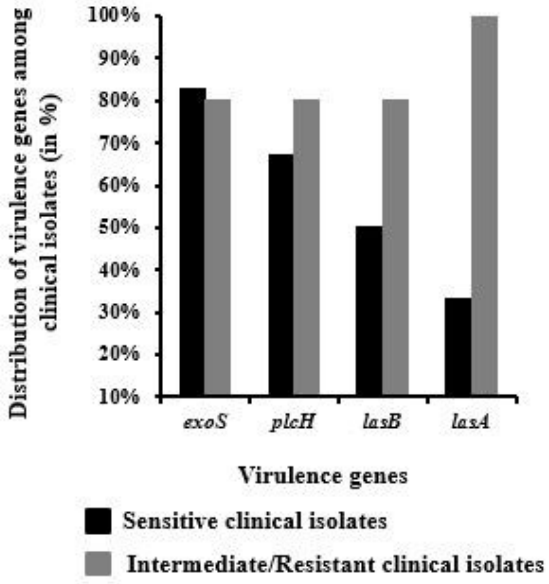
Sensitive clinical isolates	Virulence genes			
	exoS	*plcH*	*lasB*	*lasA*
PAJ-2	+	+	+	-
PAJ-4	-	+	-	-
PAJ-7	+	+	+	+
PAJ-8	+	-	-	-
PAJ-9	+	+	+	+
PAJ-11	+	-	-	-

(B)

Intermediate/ Resistant clinical isolates	Virulence genes			
	exoS	*plcH*	*lasB*	*lasA*
PAJ-1	+	+	+	+
PAJ-3	+	+	+	+
PAJ-5	+	+	+	+
PAJ-6	-	+	-	+
PAJ-10	+	-	+	+

Figure 2

Histogram showing distribution of virulence genes in antibiotic sensitive, intermediate and resistant clinical isolates of Pseudomonas aeruginosa.



CONCLUSION

Periodical emergence of multidrug-resistant strains of *P. aeruginosa* creates a major challenge for effective treatment of infections. Inheritance, mutations and distribution of virulence gene and their product contributes to emergence of these multidrug-resistant strains. In this study we reported that recent clinical isolates of *P. aeruginosa* are developing resistance to commonly used antibiotics. Further understanding of molecular signature among clinical isolates revealed that all highly resistant strains hold lasA gene. Further understanding of molecular mechanism of lasA towards antibiotics resistance will be helpful to develop effective therapeutic strategies against multidrug-resistant *P. aeruginosa* infection.

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