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Research Article

Evaluation of Plasmid And Resistance Profile Of Pseudomonas Aeruginosa From Clinical Isolates In Pravara Rural Tertiary Care Hospital Of Western Maharashtra

Meghna R. Choudhari[1], Namita A. Raytekar[2]

Center for Biotechnology, Pravara Institute of Medical Sciences (DU), Loni, Dist. Ahmednagar, Maharashtra, India

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ABSTRACT

A species of considerable medical importance, *Pseudomonas aeruginosa (P. aeruginosa)* is an opportunistic pathogen, serious infection is often superimposed upon acute or chronic morbidity, thus it can be said that Pseudomonal infections are complicated and can be life-threatening. *P. aeruginosa* is a facultative anaerobe as it is well adapted to proliferate in conditions of partial or total oxygen depletion and categorized as ubiquitous pathogen. Its occurrence and rapid spread is a part of major concern making antibiotic therapy difficult for hospitalized patients. Plasmid (mobile genetic elements) provides additional characteristics, such as resistant to antibiotics resulting in rapid spread to other bacteria.

Methods: A total of 126 *P.aeruginosa* was obtained from bacteriology section of Department of Microbiology, Rural Medical College, Pravara Institute of Medical Sciences, Loni, Maharashtra, India. The antibiogram of 126 clinical isolates of *P.aeruginosa* were determined by disk diffusion followed by plasmid isolation using Alkaline lysis method and confirmed by Agarose gel electrophoresis.

Results: Among total clinical isolates 78 (61.90%) were classified as MDR strains with 48 (38.10%) as Non- MDR. The antibiogram study of MDR strains revealed that resistance was observed for Cifazolin (88.46%), Co-Trimoxazole (85.90%) followed by Meropenem (64.10%) Amikacin (53.85%), Ciprofloxacin (48.72%), Tobramycin (24.35%).Plasmids varied in size from 400 bp to 7 kbp mol. wt. Plasmid profile for the MDR isolates revealed 56 of the strains had one plasmid each, 8 strains possessed 2 plasmids, other 8 strains had 3 plasmids, 4 strains had 4 plasmids and 2 strains showed 5 plasmids.

Conclusions: Isolates of *P. aeruginosa* expressing plasmids in form of multiple resistance mechanisms has challenged the health care centers in various ways. Thus focusing such threat and exposing problem on drug overuse needs to be covered up instantaneously to avoid future inconvenience with MDR strains.

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INTRODUCTION:

Pseudomonas aeruginosa develops resistance to most of antibiotics thereby complicating the selection of appropriate treatment [1]. Antibiotic resistance genes in most bacteria are usually present on extra chromosomal elements such as plasmids; and these genetic materials aid in the transfer of resistance genes amongst bacterial population via horizontal transfer. Chemotherapy gets difficult as *Pseudomonas aeruginosa* are resistant wide variety of antibiotics [2, 3]. Due to the ubiquitous nature of *P. aeruginosa* is found in water, soil, on plants and it has been implicated in wound infection [4].Antibiotic resistant bacteria are ubiquitous; and several antibiotic resistant genes are usually transferred by genetic elements like plasmids–transfer these genes to susceptible bacteria [6]. Infections with antibiotic resistant bacteria make the therapeutic regimen options extremely difficult [5].Multi Drug Resistant *P. aeruginosa* (MDRPA) is a condition that bacteria resistant to three or more classes of antibiotics such as penicillins, cephalosporins, monobactam, carbapenem, aminoglycosides and fluoroquinolones. Inappropriate antibiotics administration can cause *P. aeruginosa* resistant to several classes of antibiotics [25]. The prevalence of multidrug-resistant (MDR) isolates has been increasing worldwide and possesses a serious problem in hospital settings; with significant rise in patient's morbidity and mortality [7].There are fatal infections in association with *Pseudomonas aeruginosa* acquired in hospital, as it is called as a stubborn MDR pathogen. Genome based resistance mechanism is evolving due to nonspecific antibiotic use [8].They are extremely resistant to disinfectants and can contaminate certain compounds and solutions [9].The excessive use of antibiotics is usually associated with massive development of resistant bacterial strains. Owing to its intrinsic and acquired antimicrobial resistance, it gets difficult to treat *P. aeruginosa* infections. Despite improvements in antibiotic therapy *Pseudomonas aeruginosa* is intrinsically resistant to a number of antimicrobial agents frequently including multiple classes of antibiotics [10]. Whereas resistance gene rapid spread is suspected to be a cause of increased antibiotic resistance cases in it [11].Various mode of gene transfer method such as conjugation, transformation, or transduction of plasmid add towards bacterial resistance for different classes of antibiotics. Plasmids are extra chromosomal element carry resistance gene for antibiotics with potential to circulate between various strains.

Profiling of plasmids deals with total plasmid content of bacteria or plasmid digested with restriction endonucleases and subject cleaved plasmid DNA to electrophoresis for analysis. Thus plasmid profiling is a powerful tool in assessing the blowout of antibiotic resistance, as resistance is carried between bacteria by means of plasmids [12].It is also termed as intrinsic resistance. Therefore, the determination of antimicrobial susceptibility patterns of clinical isolates is often crucial for optimum treatment of infected patients because such protocols will guide physicians on the proper type of drugs to administer to a given patient [15].It has been perceived that antibiotic susceptibility of bacterial isolates is not constant but dynamic and varies with time and environment [17]. As the biofilm forming ability of *P.aeruginosa* is a major hurdle in drug therapy enhancing resistance profile of this notorious and dreaded pathogen.

This study was designed to screen the antibiotic resistant patterns of *P. aeruginosa* from clinical sample. An emphasis was laid on genetically distinguish clinical isolates of *P.aeruginosa* by plasmid profiling and focusing their antibiogram analysis - a better understanding on resistance pattern in Western region of Maharashtra.

MATERIALS AND METHODS:

Bacteriology:

A total of 126 isolates of *P. aeruginosa* were received from Department of Microbiology, Rural Medical College, from various wards of Pravara Rural Hospital, Loni of Western Maharashtra. The culture plates were processed using standard microbiological procedures. Characterization and identification of *P. aeruginosa* was carried out in Department Microbiology using a combination of colonial morphology, Gram stain characteristics.

Antimicrobial Sensitivity Testing:

Antimicrobial susceptibility testing was performed and interpreted according to the CLSI Guidelines [13].In brief, selective colonies from the culture plate were inoculated into 2ml of peptone water and Incubated at 37^0C for 2 hr. Turbidity was compared to that of 0.5 McFarland standards. A cotton swab was immersed and rotated in this inoculum, the swab was then pressed to the inner surface

of the tube so as to remove excess inoculum. It was then used for carpet streaking on Muller Hinton agar plate. The required antibiotic discs were then placed aseptically on this medium with sterile forcep. The plate was then incubated for 24 hr at 37^0C. Next day the zone size was recorded and reported as sensitive or resistant by comparing the zone size to the Kirby-Bauer chart. Depending on the isolate *Pseudomonas aeruginosa*, antibiotic discs were selected from among the following to determine antibiotic sensitivity pattern of *P.aeruginosa* isolates:Amikacin (10mcg/disc), Tobramycin (10mcg/disc), Meropenem (10 mcg/disc), Ciprofloxacin (5mcg/disc),Cefazolin (30 mcg/disc), Co-trimoxazole (25 mcg/disc) were tested (HIMEDIA, MUMBAI, INDIA).

Plasmid Isolation and Profiling:

Plasmid isolation was done using Alkaline lysis with SDS: Minipreparation method [14, 18]. The plasmid extracted was then electrophoresed to analyze molecular weight with marker DNA. Grouping of the strains was done as per the size and number of the plasmid DNA bands. Plasmid isolation and profiling was done for both MDR and non-MDR strains among 126 isolates of *P. aeruginosa*.

Gel Electrophoresis:

Plasmid isolated was then confirmed by Agarose gel electrophoresis (AGE). The plasmid DNA extracted was then electrophoresed to analyze molecular weight with marker DNA. Electrophoresis was done on horizontal bed apparatus for 3 hours at 60 mA using 0.8% agarose gel and 500 mLTris base- acetic acid - EDTA buffer(1XTAE) to maintain pH - 8.3 as per Bikandi *et al* procedure. Plasmid DNA was observed using ethidium bromide and UV trans-illuminator. By UV exposer Plasmid DNA band illuminates and molecular weight was compared with standard marker.

Non-MDR isolates, out of 48 isolates 9 were plasmid bearing.

RESULTS:

A total of 126 *Pseudomonas aeruginosa* isolate were received from Department of Microbiology for a period of six month from September 2016- February 2017. *P. aeruginosa* was isolated from various clinical specimen constituting 126 isolates studied **[Table1]**. In accordance to the resistance pattern of (n = 126) *P. aeruginosa* from isolates were categorized as MDR **78 (61.90%)** (resistant to > 3 antimicrobial categories) and Non - MDR **48 (38.10%)** strains **(Table -2)**. Hence are categorized under multi-drug-resistant *Pseudomonas aeruginosa* (MDRPA) strains in clinical isolates.

Antibiogram of *Pseudomonas aeruginosa* for 78 MDR isolates presented in **(Table -3)** shows highest resistance for Cifazolin (88.46%), Co-Trimoxazole (85.90%) followed by Meropenem (64.10%) Amikacin (53.85%), Ciprofloxacin (48.72%), Tobramycin (24.35%).Thus highest sensitivity was observed for Tobramycin (75.65%) followed by Ciprofloxacin (51.28%).Among all clinical specimen pus sample showed highest MDR strains. Thus **(Table 4)** describes the Antibiotic resistance pattern (MDR) *P. aeruginosa*. The most prevalent pattern with 12 isolates each was AK^r,CZ^r,COT^r and CZ^r,MRP^r,COT^r. **Figure-1** represents the gel image of plasmid DNA profile of *P.aeruginosa***.** Plasmid profiling data for MDR and Non- MDR isolates revealed that plasmid was found in both type and size ranged from 400 bp to 7 Kb mol. wt. (**Table 5 and 6)**. As per the data for Non-MDR isolates, out of 48 isolates 9 were plasmid bearing.

Table 1: Distribution of P. aeruginosa among various clinical samples.

Sr.No.	Specimen type	No. of isolates
1	Pus (P)	81 (64.28%)
2	Urine (U)	18 (14.28%)
3	Miscellaneous (M)	14 (11.11%)
4	Opthal (O)	7 (5.55%)
5	Sputum (Sp)	6(4.76%)
	Total	126

Table 2: Distribution of P. aeruginosa as MDR & Non-MDR isolates

S.No.	Type of specimen	No.(%) of isolates	No. of MDR	No. of Non-MDR
			Isolates (%)	Isolates (%)
1	Pus (P)	81	53(65.43%)	28 (34.56)
2	Urine (U)	18	10 (55.55)	8(44.44%)
3	Miscellaneous (M)	14	8(57.14%)	6(42.85%)
4	Opthal (O)	7	4(57.14%)	3(42.85%)
5	Sputum (Sp)	6	3(50%)	3(50%)
	Total clinical isolates	126	78 (61.90%)	48 (38.10%)

As per the resistance pattern the isolates (n= 126) of *P. aeruginosa* from numerous sources were categorized as MDR (resistant to ≥ 3 antimicrobial categories) and non MDR strains **(Table 2)**.

Table 3: Antibiogram of P. aeruginosa MDR isolates

S.No.	Type of antibiotic	Standard antibiotic concentration (mcg/disc)	No.(%) of MDR isolates N=78	
			No. (%) of Resistance	No. (%) of Susceptible
1	Cifazolin (CZ)	30	69 (88.46%)	9 (11.54%)
2	Co-Trimoxazole (COT)	25	67 (85.90%)	11 (14.10%)
3	Meropenem (MRP)	10	50 (64.10%)	28 (35.90%)
4	Amikacin (AK)	10	42 (53.85%)	36 (46.15%)
5	Ciprofloxacin (CIP)	5	38 (48.72%)	40 (51.28%)
6	Tobramycin (ToB)	10	19 (24.35%)	59 (75.65%)

Table 4: Antibiotic resistance pattern (MDR) Pseudomonas aeruginosa.

Sr.No.	Pattern No.	Antibiotic Resistant Pattern	Number of isolates (N= 78)	Isolates
1	3	CZ,MRP,CIP	3	M127,U376,M444
2	3	AK,CZ,MRP	3	P221,M253,U364
3	3	AK,CZ, COT	12	P23, Sp61, P117 ,P119 ,P261 ,P264 ,P294,P295,P656,P770,P874,P977,
4	3	CZ,COT,CIP	3	P60,P101,P289
5		CZ,COT,TOB	1	P285
6	3	CZ,MRP,COT	12	P76,P80,P85,P95,P144,P287,P449, ,P780,P781, P782, P791,P808
7	3	CZ,CIP,TOB	4	O49, ,P691,M439,U816
8	3	AK,MRP,COT	3	M195,P804,U985
9	3	MRP,COT,TOB	2	P293,P915
10	3	AK, CZ,MRP,TOB	1	M477
11	3	MRP,COT,CIP	2	P721,P776
12	4	AK, CZ,MRP,COT	6	P88, P245,P260,P409,P709,P659
13	4	AK, CZ,COT, CIP	4	Sp64,P63, P73,P281
14	4	CZ,MRP,CIP,COT	2	St 97,P464
15	4	CZ,COT,CIP,TOB	5	O76,O244,U376,P632,U923
16	4	AK,MRP,COT, CIP	1	M343
17	4	MRP,COT,CIP,TOB	2	U342,U550
18	5	CZ,MRP,COT,CIP,TOB	1	O52
19	5	AK,CZ,MRP,COT,CIP	5	P253,P263,P286,P716,P876
20	5	AK,CZ,MRP,COT,CIP,TOB	4	U165,P289, M457,U798

The antibiotics belonging to same group are considered under unit pattern as Amikacin , Tobramycin categorized as single pattern **(Table 4)**

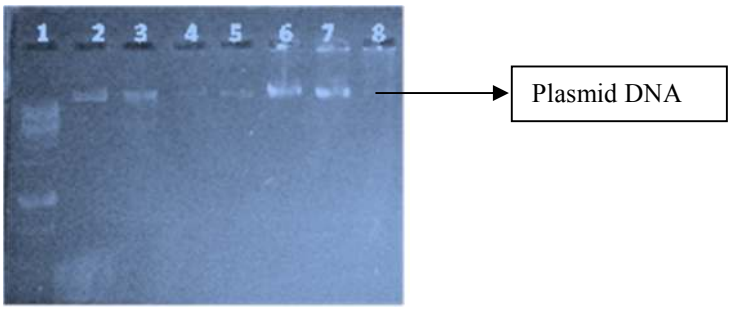

Figure-1: Plasmid DNA profile of P.Aeruginosa

Lane 1: 0.1 to 1.5 kb ladder,lane 2-8 : samples Sp61, Sp97, U165, U364 M444, M457, P770, Plasmid size 1.5 kb

Table 5: Plasmid distribution among the MDR isolates

Sr.No.	Isolates code No.	Total No. of Isolates	No of plasmid per isolate	Plasmid profile in kilobase pair (kb)/ base pairs (bp)	Specimen type of isolates
1	P144,P464	2	1	500 bp	Pus
2	O244,O76,P449,P632,P656,P791	6	1	600 bp	Opthal, Pus
3	P294,P295, P409,P261, P659, P721	6	1	700 bp	Pus
4	P781	1	1	1kb	Pus
5	Sp61,Sp97,U165,U364,M444 ,M457,P770,P780,P782,P776 ,U985 ,P876 ,P874, P977	14	1	1.5 kb	Sputum,urine, Miscellaneous
6	P60,P63,P73,P76,P80,P85,P221,M439,M477,P331,P441,P691,P965	13	1	2kb	Pus,Miscellaneous
7	O49,P285,M343,U550	4	1	4 kb	Opthal,Pus, Miscellaneous,urine
8	Sp64,P253	2	1	4.5 kb	Sputum,Pus
9	P779	1	1	5kb	Pus
10	U923,U816,P716,P915	4	1	6kb	Urine, Pus
11	M195,M253,U798	3	1	7 kb	Miscellaneous,Urine
12	P101	1	2	400,500 bp	Pus
13	P808	1	2	400,600 bp	Pus
14	P245	1	2	400Bp, 2kb	Pus
15	P804	1	2	600 Bp,2kb	Pus
16	U376	1	2	4kb,1.5 kb	Urine
17	U342	1	2	6kb,1.5kb	Urine
18	O52	1	2	6kb,4kb	Opthal
19	P293	1	2	7kb,4.5kb	Pus
20	U376	1	3	600,500,400 bp	Urine
21	P23	1	3	1.5kb,1kb,700 bp	Pus
22	P117,P119	2	3	400 bp,500 bp,2kb	Pus
23	P263	1	3	4kb,2kb,400bp	Pus
24	P287	1	3	4kb,2kb,600bp	Pus
25	P264	1	3	6kb,2kb,400bp	Pus
26	M127	1	3	7kb,4.5kb,1.5kb	Miscellaneous
27	P88,P95	2	4	400 bp,500 bp, 600 bp,2kb	Pus
28	P281	1	4	6.5kb,4.5kb,2kb,600 bp	Pus
29	P289	1	4	6kb,4kb,2kb,600 bp	Pus
30	P286	1	5	6.5kb,4.5kb,4kb,2kb,600 bp	Pus
31	P260	1	5	6.5kb,4.5kb,2kb, 1.5 kb,400 bp	Pus

Table 6: Plasmid distribution among the 9 isolates (Non-MDR)

Sr.No.	Isolates code No.	No of plasmid	Plasmid profile in kilobase pair (kb)/ base pairs (bp)	Specimen type of isolate
1	P412	1	400 bp	Pus
2	U358	1	400 bp	Urine
3	P41	1	500 bp	Pus
4	U368	1	500 bp	Urine
5	U381	1	500 bp	Urine
6	M31	1	500 bp	Miscellaneous
7	M248	1	1.5 kb	Miscellaneous
8	P246	1	2kb	Pus
9	P288	2	2kb,4kb	Pus

As per the distribution 9 Non-MDR isolates out of 48 showed plasmid **(Table 6)**

DISCUSSION:

To examine pathological consequences of Pravara rural hospital setting, study was conducted at Centre for Biotechnology, Pravara Institute of Medical Sciences-DU, Loni. *P.aeruginosa* is a notorious organism, is of matter of concern due its increased resistance to various antibiotics in hospitals. *P.aeruginosa* commonly isolated in hospital settings with high intrinsic drug resistance ability for structurally diverse antibiotics [19]. In the current study 126 non-duplicate isolates were received from Department of Microbiology, among the clinical isolates Pus was most predominant 81(64%) followed by Urine 18(14%),Miscellaneous 14 (11%), Opthal 7(6%),Sputum 6(5%).This above data for predominant pus is evidence regarding various clinical condition leads to pus accumulation, acting as a major source of infection as it provide moist environment for pathogens growth as well as spread infection [24].Antibiogram analysis of *Pseudomonas aeruginosa* was carried out for 78(61.90%) MDR isolates showing highest resistance for Cifazolin (88.46%) followed by Co-Trimoxazole (85.90%) and highest sensitivity was observed for Tobramycin (75.65%) followed by Ciprofloxacin (51.28%) in the existing study. Resistance pattern reported by Rustini *et al* was observed for Ciprofloxacin (28.42%) and low resistance was observed for Amikacin (8.42%) revealing high sensitivity for Amikacin that is different from present data [26].The antibiotic resistant pattern showed that *P. aeruginosa* had high resistant to Co-Trimoxazole 77.3% and had low resistant to Ciprofloxacin (35.5%) germane to present study [11].

As antibiotics resistance is prime importance for MDR species hence a detail study conducted at MDR species. In present study (n = 126) *P. aeruginosa* from isolates were categorized as MDR 78 (61.90%) and Non MDR 48 (38.10%) strains with plasmid size ranged from 400 bp to 7 kb mol. wt. was observed for MDR and 400 bp to 4kb for Non MDR strains respectively. Where Among all clinical specimen pus sample showed highest MDR strains in present study.31.73% strains of *P. aeruginosa* were multidrug resistant with resistance most against cephalosporins [Cefepime (65.26%), cefotaxime (60.47%)], fluoroquinolones [Ciprofloxacin (46.1%), levofloxacin (31.87%)] aminoglycosides [Amikacin (37.72%), gentamicin (31.13%)] followed by ureidopenicillins and carbepenems. reported by Prakash V *et al* [8].100 % MDR with occurrence of amikacin resistance in 56.7% of isolates reported in Indian scenario by shahid *et al* with plasmid size belong to 48.5kb responsible for drug resistance much higher as compared with present study [22]. 30 (20.69%) of *P.aeruginosa* isolates were multi-drug resistant in report of chander *et al* [21].Rates of MDR *P. aeruginosa* (52%) and the sizes of plasmids varied from 1.6 to 140 kb [20].Whereas Okonko *et al* reports about 20% MDR, plasmid size ranged from 662bp to

830bp[11]. Another report of Odumosu *et al* reported plasmid size <1to ≥23kb for clinical isolates. Plasmid profile for the selected isolates revealed 6 of the strains had one plasmids each while 5 strains possessed 2–4 plasmids and 1 strain had 5 plasmids. All the isolates harbouring plasmids were resistant to this different range of antibiotics were studied such as, carbenicillin and ceftazidime but shows complete susceptibilities to cefepime, imipenem, and piperacillin–tazobactam antibiotics [23]. Other study from Rustini *et al* reported on 35.79% of *P. aeruginosa* isolates were resistant to three or more classes of antibiotics, plasmid analysis revealed that sixteen multidrug resistant *P. aeruginosa* isolates had a plasmid. An isolate with single plasmid band 300bp, twelve of the isolates had a single plasmid band >1kbp and two isolates had two plasmids band > 1kbp [26]. Whereas S. Rajan *et al* reported on highest resistance in *Escherichia coli* for Ciprofloxacin 88.9 % and Amikacin 89.8% where 60.6% of *E. coli* were MDR .The strain E3, E8 and E64 harbored 2 plasmids bands, and remaining isolates had one plasmid .The plasmid size ranges from 3530 bp to above 4973 bp [27] similarly our study plasmid size also showed a range around 4kb.Our result is in agreement with Chigor *et al* 22 (62.9%) isolates harbored plasmids all of which were no less than 2.1 kb in size and in present study much of the isolates carried plasmid ≥ 2 kb [28].

Plasmid-mediated resistance is the transfer of antibiotic resistance genes which are carried on plasmids. The plasmids can be transferred between bacteria within the same species or between different species via conjugation thus contributes in spread of antibiotic resistance gene. Even, rapid evolution of genome due to continuous selective pressure of antibiotics leads to development of resistance [8]. This study clearly evident about drug resistance and its association with plasmids. Hence above data suggests that surveillance study is mandatory to monitor circulation and resistance pattern of this notorious organism. The over use of broad-spectrum antibiotics for treatment regimen is probably responsible for the emergence of resistant strain.

CONCLUSION:

Pseudomonas aeruginosa considered as opportunistic pathogen which can be isolated from various kinds of infection. Its flexibility to adapt changing environment makes it a dreaded pathogen, especially in hospital settings. The widespread antibiotic use leads to emergence of resistance strain called intrinsic resistance associated with plasmid. Plasmid spread resistance rapidly eventually disturbing treatment stratagem, enhancing morbidity & mortality rates. Thus plasmid profiling is crucial step to study drug resistance property of notorious *P. aeruginosa*. Above study describes that surveillance study is mandatory to combat upcoming challenge for appropriate treatment regimen. This proves as a boon to physicians in prescribing effective combinations of antipseudomonal agents with less incite to emergence of MDR strains and its resistance profile in accordance to plasmid.

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ETHICAL APPROVAL:

The study was approved by the institutional ethics committee

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