Molecular characterization of strong biofilm producing MDR, XDR and PDR *Pseudomonas aeruginosa* isolated from a tertiary care hospital in South India

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Abstract

Background : *Pseudomonas aeruginosa* is an opportunistic, gram negative bacterium that causes serious infections, especially among immunocompromised patients. An unusually high incidence of nosocomial *P. aeruginosa* infections was observed among patients hospitalized at Sri Sathya Sai Institute of Higher Medical Sciences, Puttaparthi, India, between 2014 November and 2015 February. Some of the patients being treated for a variety of cardiac and urological disorders in the Cardiothoracic and Vascular Surgery - Intensive Care Unit (CTVS-ICU) and Urology ward were found infected with *P. aeruginosa*. Active surveillance and environmental sampling revealed the presence of two additional MDR *P. aeruginosa* in the tap waters of CTVS-ICU. Based on the Antibiotic sensitivity pattern, fourteen *P. aeruginosa* MDR (n=7), XDR (n=6) and PDR (n=1) isolates (inclusive of the two tap water isolates) were shortlisted for additional investigations. These isolates were characterized for antibiotic sensitivity, biofilm production, minimum biofilm inhibitory concentration (MBIC), presence of antibiotic resistance genes, efflux pumps, and integrons. Results : Mutations in *gyrA*, *gyrB*, *parC*, *mexR*, *nfxB*, *mexB* and *mexF* genes were correlated to enhanced antibiotic resistance. Notably, the isolates were also found to harbor integrons and blaNDM-1. Pulsed Field Gel Electrophoresis (PFGE) and Random Amplified Polymorphic DNA (RAPD) based phylogenetic analyses grouped the clinical isolates into three distinct ward specific clusters, while the tap water isolates were grouped into a separate cluster. Detection of nosocomial *P. aeruginosa* in the CTVS-ICU and urology ward triggered the activation of enhanced surveillance and infection control measures to contain and eliminate *P. aeruginosa* infections. Conclusions : Multiple clones of *P. aeruginosa* are prevalent in the study center. Hence, continuous screening and identification of potential reservoirs is absolutely essential to control the spread of drug resistant *P. aeruginosa* infections.

Background

Nosocomial infections by *Pseudomonas aeruginosa* have become a critical clinical challenge globally, and are associated with significant morbidity and mortality particularly in developing countries (1, 2). The organism is a ubiquitous, opportunistic human pathogen that causes bacteremia, ventilator-associated pneumonia (VAP), abdominal, genito-urinary, skin, post-operative infections and sepsis (3, 4). *P. aeruginosa* is the most frequently encountered pathogen among Intensive Care Units (ICUs) (5) and is included among the list of "ESKAPE" pathogens (6) and "Meet WHO's dirty dozen" by Infectious Diseases Society of America (IDSA) and World Health Organization (WHO) respectively. *P. aeruginosa* infections account for more than 19.8% of all ICU infections globally. In India, the situation is even more stark (13.2 to 38%) (7). The bacterium possesses several intrinsic resistance mechanisms which include active efflux pumps, low outer membrane permeability, production of antibiotic degrading enzymes and biofilm forming capacity (8). Recalcitrance due to robust biofilm formation by *P. aeruginosa* is believed to be a major contributing factor to its colonization and persistence on many biotic and abiotic surfaces including water bodies and pipes. These biofilms are highly tolerant against traditional antibiotics (9). Mutations in DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV (*parC* and *parE*) genes confer resistance to fluoroquinolones (10, 11). Further, presence of multiple efflux pump-associated regulatory genes (*mexR*, *mexZ* and *nfxB*) have been reported to confer resistance to tetracycline, chloramphenicol and fluoroquinolones albeit at a lower level (12-14). With the capacity to acquire additional resistance determinants through Horizontal Gene Transfer (HGT) (15-17), emergence of nosocomial extensively drug resistant (XDR) and pan drug resistant (PDR) *P. aeruginosa* has now become a new normal. Integrons, transposons, and conjugative plasmids are the most frequently associated mobile genetic elements (MGEs) among antimicrobial resistant (AMR) *Pseudomonas* infections. Class I integrons, prevalent among resistant isolates are generally found to possess gene cargo conferring resistance to β -lactams, aminoglycosides, macrolides and sulfonamides antibiotics (18, 19). Scant information is available on the molecular analyses of nosocomial XDR and PDR *P. aeruginosa* infections in the Indian context.

In this study, we characterized drug-resistant *P. aeruginosa* (14 nos) isolated from clinical and environmental sources at Sri Sathya Sai Institute of Higher Medical Sciences, Prasanthigram (SSSIHMS-PG), a 330 bedded, tertiary care hospital in South India from November 2014 to February 2015. They were analyzed for their clonal similarity, biofilm forming capacity, presence of antibiotic resistance determinants like efflux pumps, NDM-1, integron gene cassettes and mutations in the *gyrA*, *gyrB*, *parC*, *mexR* and *nfxB* genes.

Methods

Clinical isolation

Due to the unusually high incidence of *P. aeruginosa* infections (50 nos) among the patients of the CTVS-ICU and urology wards at Sri Sathya Sai Institute of Higher Medical Sciences – Prasanthigram (SSSIHMS-PG), Puttaparthi, India; enhanced surveillance in and around the hospital was initiated to identify and eliminate any potential sources of infections. Bedside rails, dressing trolleys, blood pressure monitoring machines and other biomedical equipment located in the CTVS-ICU and Urology ward were sampled. Hand swabs from nurses and attending physicians who came in contact with these patients were also collected for testing. Further, samples of hand sanitizers, hand washing solutions, and tap water from these units were investigated. All *P. aeruginosa* isolates were analyzed for their antibiotic resistance profiles. Fourteen drug resistant isolates (MDR, XDR and PDR) which include the two MDR CTVS –ICU tap water isolates were characterized further. The remaining isolates (36 nos) which were found to be sensitive to the tested antibiotics were excluded from the study.

Bacterial identification and antibiotic susceptibility testing (AST)

Initial identification of clinical and environmental isolates was performed by Vitek-2 (BioMérieux) as per manufacturer's instructions. Further, identity of these isolates was confirmed by 16s rDNA sequencing (20). Antimicrobial susceptibility testing (AST) was carried out using Kirby-Bauer disk diffusion method (Hi-media) for all the 50 *P. aeruginosa* isolates. Reconfirmation of the resistance profile for the fourteen drug resistant *P. aeruginosa* isolates was done by Vitek-2 (AST-N281 cards) and the isolates were categorized as MDR, XDR and PDR. To determine the minimum biofilm inhibitory concentration (MBIC) of the study isolates, imipenem, levofloxacin, piperacillin-tazobactam, and ceftazidime (Sigma-Aldrich, St. Louis, MO) prepared in cation-adjusted Mueller-Hinton broth (Hi-media) were used. Results were interpreted as per Clinical and Laboratory Standards Institute (CLSI) guidelines (21).

Random amplified polymorphic DNA (RAPD)

RAPD was performed to understand the genetic diversity among the isolates. Genomic DNA was isolated from overnight cultures using NucleoSpin® tissue kits (Macherey-Nagel). Genomic DNA from each isolate was subjected to RAPD-PCR (Sapphire Amp Fast PCR Master Mix) using the primer Kt5: 5'-AGCAAGCCGG-3'. The PCR conditions used for the amplification were initial denaturation at 94°C – 4 min followed by 40 cycles of 94°C – 1 min, 35°C – 1 min, 72°C – 2 min and a final extension of 72°C – 5 min. A phylogenetic tree was constructed from the RAPD finger print data using Neighbor Joining method by NTSYS software. Inter node branches with bootstrap value more than 50% were inferred as distinct clusters in the tree.

Pulsed-Field Gel Electrophoresis (PFGE)

Genomic DNA from the 14 *P. aeruginosa* isolates was fingerprinted by PFGE following SpeI restriction enzyme digestion. SpeI digested genomic DNA fragments were separated in two step runs; Step1: 16h at 6V/cm with initial and final pulse times of 0.5 sec and 25 secs respectively; Step2: 2h at 6V/cm with initial and final pulse times of 30 secs and 60 secs respectively using the CHEF- DR II system (Bio-Rad, Melville, NY, USA). DNA from *Salmonella* serotype Braenderup (H9812) digested with XbaI was used as a DNA size marker. The gel was photographed and profiles were visualized using the GelDoc® (Bio-Rad, Hercules, CA, USA) photo documentation system. Banding patterns were analyzed using BioNumerics Version 7.6 (Applied Maths, Austin, TX, USA). The resulting dendrograms were generated at 2.0% position tolerance and 1.5% optimization using the unweighted pair-group method with arithmetic mean (UPGMA) and the Sørensen–Dice similarity coefficient.

Biofilm formation and MBIC analysis

Biofilm production was determined using the method of O'Toole GA (22, 23) with minor modifications. Briefly, 250 µl of *P. aeruginosa* cultures grown in Trypticase Soy Broth (TSB) (OD650 = 0.1) were seeded in a 96 well microtitre plate (Nunc TSP System). Plates were covered with peg lids (TSP lids; Nunc International) and incubated without shaking at 37°C for 24 hrs. Biofilms that formed on the peg lids were stained using 200µl of crystal violet (0.1% w/v). Post washing, biofilms attached to the pegs were immersed in 250 µl of 70% ethanol for 10 min to solubilize the crystal violet. Absorbance was recorded at 650 nm using SpectraMaxM5 multi-mode microplate reader. The mean readings of four independent measurements of each strain were calculated (24). To identify the MBIC value (23) for each of the tested antibiotics, peg lids with biofilms were transferred into a 96-well plate containing 100µL of antibiotics (2 to 1024µg/ml) and incubated at 37°C for 24hrs without shaking. Based on the AST patterns of the isolates, antibiotics for MBIC analysis were chosen. Peg lids with biofilms were then transferred to a new sterile 96-well plate, containing 150µl LB broth and centrifuged at 2,000 rpm for 30 min to recover the biofilm. Initial mean absorbance was measured at 600nm. The plates were then re-incubated at 37°C for 12hrs and final mean absorbance was recorded (600nm). The difference between mean absorbance values, before and after incubation was calculated. All experiments were performed in triplicates. *P. aeruginosa* ATCC 27853 (quality control strain) was used as a negative control.

PCR and DNA sequencing

PCR amplification of *gyrA*, *gyrB*, *parC*, *nfxB*, *mexR*, *mexF*, *mexB*, *blaNDM-1*, integrase (*Int1*) and 5' (5'-CS) - 3' (3'-CS) conserved segment of class 1 integrons (Supplementary tables 1 and 2) was carried out using Sapphire Amp Fast PCR Master Mix (25µl), 10 pmol of each primer and 50 ng of the template DNA in a 50µl reaction volume. Amplified products were purified using GeneJET PCR Purification Kit (Thermo-Scientific) and subjected to DNA sequencing.

Nucleotide sequence accession numbers

The nucleotide sequence data reported in the current study has been submitted to the GenBank database and were assigned the following accession numbers: MF118882 - MF118895 (*gyrA*), MF118896 - MF118909 (*gyrB*), MF957174 - MF957187 (*parC*), MF356378 - MF356391 (*nfxB*), MF140258 - MF140271 (*mexR*), MF356392 - MF356396 (*NDM*), MF410358 - MF410371 (*mexB*), MF371395 - MF371408 (*mexF*), and MF957188 - MF957199 (*Int1* gene).

Results

Clinical characteristics and categorization of nosocomial isolates

Of the 14 drug-resistant study isolates, two were isolated from tap waters of the CTVS-ICU, while the remaining 12 were clinical isolates. Clinical characteristics of patients at the time of the isolation of the nosocomial strains include urological abnormalities (8 nos), Congenital Heart Disease (CHD) (3 nos) and Rheumatic Heart Disease (RHD) (1 no). (Table 1). Clinical and tap water isolates recovered in the study were identified as *P. aeruginosa* by Vitek-2 and re-confirmed by 16s rDNA sequencing. The study isolates were tested against Penicillins (carbenicillin, piperacillin+tazobactam and ticarcillin+clavulanic acid), cephalosporins (ceftazidime, cefipime and cefoperazone and sulbactam), monobactam (aztreonam), aminoglycosides (amikacin, gentamicin, netilmicin and tobramycin), carbapenems (imipenem, meropenem and doripenem), fluoroquinolones (ciprofloxacin, levofloxacin and ofloxacin), and polymyxins (colistin and polymyxin – B). Further, they were categorized as MDR, XDR and PDR based on the antibiotic sensitivity patterns (Table 2).

Seven of the 14 isolates were found to be MDR with resistance to more than three categories of the tested antibiotics. Six isolates which exhibited resistance to more than six categories of antibiotics were grouped as XDR. XDR strains were found to be sensitive to colistin. Notably, one isolate (208) was found to be PDR with resistance against all the eight categories of antibiotics tested and an MIC value of ≥ 16 µg/ml against colistin. Of note, the other 13 isolates had MIC values of 0.5 to 2 µg/ml against colistin (Table 2).

Phylogenetic analyses

Phylogenetic analyses using PFGE and RAPD finger print analyses unambiguously revealed the presence of four distinct clusters. Clinical isolates were grouped into three different clusters, while the two tap water isolates (T3, T4) grouped into an independent cluster. Strains 199, 200, 208, 217, 225, 239, 251

and 255 isolated from the patients of the urology ward were found clustered into a single group. The Second cluster comprised the isolates 236 and 237 isolated from the CTVS-ICU patients. Strain 227 (VAP- sputum) although isolated from a patient in CTVS-ICU, was found to be distinct and formed the third cluster. Based on the PFGE (Figure 1) and RAPD (Supplementary figure 1) analyses, it may be inferred that the clonality of the study isolates is ward and location specific.

Biofilm production and MBIC

In vitro, all the 14 study isolates were found to produce strong biofilms (mean absorbance: >2.224 @OD 600nm) (Figures 2a, 2b and supplementary table 3). In this experiment, *P. aeruginosa* ATCC 27853 was used as a negative control as it does not produce biofilms. As expected, the study isolates were found to exhibit higher MBIC values compared to their corresponding MICs (supplementary table 4). In comparison to their MIC values, the MBIC values of the tap water isolates was highest against levofloxacin, while the PDR isolate 208 exhibited the highest MBIC (1024 µg/ml) against imipenem. Five isolates (208, 225, 239, 251 and 255) showed maximum MBIC value (1024 µg/ml) against piperacillin-tazobactam. Similarly, three isolates (241, 251 and 255) exhibited higher MBIC values (1024 µg/ml) compared to their corresponding MIC values against ceftazidime.

Carbapenem and fluoroquinolone resistance

Our analysis revealed the presence of NDM-1 gene among five of the study isolates (199, 200, 208, 217, 225, 239, 251 and 255). Sequencing of the QRDR (Quinolone Resistance Determinant Regions) revealed that eleven isolates (199, 200, 208, 217, 225, 227, 236, 237, 239, 251 and 255) had mutations in both *gyrA* (83Thr-Ile) and *parC* (87Ser-Leu) genes. Mutations in the QRDR region of the *gyrB* were observed in twelve isolates. A total of five different mutations were identified in the *gyrB* gene among the study isolates. 468Glu-Asp mutation was found in seven isolates, 504Asp-His mutation in five isolates, 503Val-Leu and 533 Gln-His mutations in two isolates and 490Leu-Val mutation was seen in one isolate (Table 3).

Int1 gene and 5'-3' conserved segment of class 1 integrons were observed among twelve of the analyzed isolates (Table 3). Notably, isolate 208 which was resistant to all the tested antibiotics did not possess class 1 integrons raising the possibility that other mobile genetic elements may have played a role in its emergence into a PDR phenotype.

Mutations in the efflux pump and efflux pump regulatory genes

Mutations in efflux pump genes were identified among some of the study isolates. In the *mexB* gene, isolate 208 was found to have 576Val-Gly and 578Thr-Asn mutations; isolate 227 had 607Ser-Thr, 611Thr-Ala and 612Val-Glu mutations; while isolate 237 had 615Phe-Leu mutation and isolate 239 possessed 589Gln-Pro mutation. *mexF* gene analysis revealed that isolate 217 had 792Glu-Ala and 816Lys-Met mutations while T3 and T4 isolates had 843Ala-Thr mutation (Table 3). Only the tap water isolate T4 was found to possess 93Leu-Val and 131Gln-His mutations in the *nfxB* gene. 126Val-Glu mutation was observed in the *mexR* gene among six of the isolates (227, 236, 237, 241, T3 and T4). Further, isolates 236 and 237 were found to possess an additional mutation (143Pro-Leu) in the *mexR* gene.

Interventions

Repeated chlorine treatment of water for one week was performed to eliminate the presence of the pathogens in the hospital water sources. Further, disinfection of all potential reservoirs (overhead water tanks, sumps and taps) was attempted and strict infection control measures were implemented. Additionally, plumbing (pipes and faucets) in the CTVS-ICU where *P. aeruginosa* spp. were isolated from tap water has been totally replaced. A new overhead water tank with hyper-chlorinated (2ppm) water was installed for the purpose of scrubbing in all the ICUs and operation theaters. Enhanced surveillance, awareness programs for the staff and continuous monitoring has been initiated by the infection control team. Follow up environmental surveillance in and around the hospital premises (repeat sampling over a period of three months) did not reveal the presence of *P. aeruginosa* and the incidence of *P. aeruginosa* infections subsided. However, regular screening of water samples in the hospital premises has been made mandatory to identify any potential source of infection.

Discussion

In this manuscript, we describe the microbiological and molecular characterization of drug-resistant clinical (n=12) and environmental (n=2) *P. aeruginosa* spp. from an outbreak at a tertiary care hospital in south India. Antibiotic susceptibility pattern of the isolates from patients from different wards and tap water sources indicated that *P. aeruginosa* isolates belonged to multiple genotypes. It was interesting to note that the two environmental *P. aeruginosa* isolates with similar AST profile clustered together in both PFGE and RAPD analyses and were distinct from the clinical isolates.

Biofilm forming capacity among *P. aeruginosa* spp. is a major antibiotic resistance factor and contributes to their ability to cause persistent infections. Even antibiotics that are effective against planktonic forms are unable to effectively penetrate biofilm, thus rendering them ineffective (20, 21). Although all 14 drug-resistant isolates were found to produce robust biofilms, the MBIC values indicate differential antibiotic response across the strains. As expected, MBIC values demonstrated enhanced antibiotic resistance against fluoroquinolones, carbapenems, piperacillin-tazobactam and cephalosporins compared to the *in vitro* susceptibility (MICs) of their corresponding planktonic forms.

Misuse of antibiotics and in particular, their availability over the counter in India (22) has led to their indiscriminate use and contributed greatly to the rise in antimicrobial resistance (23). Resistance to antimicrobials and disinfectants through the presence of efflux pumps, acquisition of multiple antibiotic resistant genes, altered membrane permeability and mutations in the target sites coupled with robust biofilm forming capacity contribute to the success of *P. aeruginosa* spp. as potent nosocomial pathogens, especially in elderly or immunocompromised patients and those with long hospitalizations.

Fluoroquinolone resistance in many of these isolates can be correlated to the presence of *gyrA* (83Thr-Iso) and *parC* (87Ser-leu) mutations. *gyrA* - *parC* mutations have been reported to be responsible for higher ciprofloxacin resistance than mutations in either one of the genes among *P. aeruginosa* isolates (24). To the best of our knowledge, this is the first report describing multiple mutations in the *gyrB* gene among fluoroquinolone resistant *P. aeruginosa* spp. It is intriguing to note that a single *gyrB* (533Gln-His) mutation in isolate 241 together with *mexR* (126Val-Glu) contributed to fluoroquinolone resistance while mutations in *gyrB* (503 Val-Leu and 504Glu-His) and *mexR* (126Val-Glu) genes in T3 isolate did not contribute to robust fluoroquinolone resistance. The presence of class 1 integrons and *bla*_{NDM-1} in some of the tested isolates further confounds the situation. Our findings suggest that there may be a correlation in the acquisition of *bla*_{NDM-1} gene and *gyrB* mutations. Consistent with previous reports, all the isolates that had *gyrA* mutation also possessed *parC* mutations (25).

Conclusions

Although the environmental isolates of *P. aeruginosa* recovered in the study could not be linked to the suspected clinical outbreak, the environmental isolates pose a critical challenge as they can act as reservoirs of antibiotic resistance genes. Of the three clusters, one cluster contained isolates of the urology ward, while the remaining two clusters belonged to the CTVS-ICU samples. This suggests that multiple clones may have been prevalent in the hospital. Hence, timely identification of potential reservoirs together with continuous screening by the hospital infection control teams is absolutely essential to control the spread of *P. aeruginosa* infections and to prevent the emergence of pan drug resistant variants of this opportunistic pathogen. These nosocomial pathogens may be lurking around in the clinics waiting for an opportune moment to initiate an outbreak if not contained.

Declarations

Ethics approval and consent to participate: The Institutional Ethics Committee of the Sri Sathya Sai Institute of Higher Learning approved the project (SSSIHL/IEC/PSN/BS/2014/03) and the requirement for written informed consent from patients was waived. The experiments in this study were approved by the Institutional Biosafety Committee of Sri Sathya Sai Institute of Higher Learning. All experiments were performed in accordance with relevant guidelines and regulations.

Consent for Publication

Not Applicable

Availability of data and materials

Data sharing is not applicable to this article as no datasets were generated or analysed during the current study

Competing Interests

The authors declare that they have no competing interests.

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Author Contributions

This project was designed by B.E.P. Bacterial isolation and AST by B.M.; 16s rDNA by P.C and M.L.; Biofilm assay and MBIC by B.M., P.C., C.D., and B.K.; PFGE and RAPD was by P.C, B.K, M.L. and B.M.; PCR optimization was by B.M; Infection control measures were initiated by S.S.R; and manuscript was written by B.E.P. All authors approve the final version of the manuscript submitted for publication.

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Author's information

Not Applicable

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Tables

Table 1: Clinical characteristics of patients with MDR, XDR and PDR *P. aeruginosa*.

Strain no.	Age	Gender	Clinical diagnosis	Specimen	Type of nosocomial infection
199	23	M	Ureteropelvic junction (UPJ) obstruction	Midstream urine	Non-CAUTI [#]
200	51	M	Retroperitoneal fibrosis	Midstream urine	Non-CAUTI
208	4	M	Bladder-outlet obstruction with Vesicoureteral reflux	Midstream urine	Non-CAUTI
217	42	M	Bilateral Renal Calculus	Midstream urine	Non-CAUTI
225	55	M	Stricture Urethra	Per urether catheter urine	CAUTI
227	26	M	Rheumatic heart disease	Sputum	VAT ^{\$}
236	17	M	Congenital acyanotic heart disease	Endotracheal tube	SSI
237	22	M	Congenital acyanotic heart disease	Pericardial fluid	SSI [¥]
239	57	M	Renal calculi	Midstream urine	Non-CAUTI
241	4	M	Congenital acyanotic heart disease	Blood	BSI [€]
251	48	M	Retroperitoneal fistula	Per urether catheter urine	CAUTI
255	4	M	Vesicoureteral reflux	Supra pubic catheter urine	CAUTI

[#]CAUTI – Catheter Associated Urinary Tract Infection; ^{\$} VAT – Ventilator Associated Pneumonia; [¥] SSI – Surgical Site Infections; [€] BSI – Blood Stream Infections

Table 2: Antibiotic susceptibility test report of the fourteen *P. aeruginosa* and categorization of nosocomial isolates as MDR, XDR and PDR

Strain no	Ticarcillin/ Clavulonic acid		Piperacillin/ tazobactam		Ceftazidime		Cefoperazone/ Sulbactam		Cefipime		Doripenem		Imipenem		Meropenem		Amikacin		Gentamicin		Ciprof
	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	MIC	Int	
199	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
200	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
208	>=128	R	>=128	R	>=64	R	32	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
217	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
225	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
227	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	8	I	>=16	R	>=64	R	>=16	R	>=4
236	>=128	R	64	S	>=64	R	32	R	>=64	R	2	S	2	I	4	S	>=64	R	>=16	R	>=4
237	>=128	R	32	S	>=64	R	16	I	>=64	R	2	S	2	S	1	S	>=64	R	>=16	R	>=4
239	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
241	>=128	R	32	S	>=64	R	16	I	>=64	R	2	S	2	S	1	S	>=64	R	>=16	R	>=4
251	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
255	>=128	R	>=128	R	>=64	R	>=64	R	>=64	R	>=8	R	>=16	R	>=16	R	>=64	R	>=16	R	>=4
T3	>=128	R	16	S	16	I	>=64	R	8	S	>=8	R	>=16	R	>=16	R	4	S	>=16	R	<=0.2
T4	>=128	R	32	S	16	I	>=64	R	16	I	>=8	R	>=16	R	>=16	R	4	S	>=16	R	<=0.2

MIC – Minimum inhibitory concentration (mg/ml); Int – Interpretation ; R- resistant; I- Intermediate Resistant; S- Sensitive: MDR – Multi Drug Resistant; XDR – Extensively drug resistant; PDR – Pan Drug resistant

Table 3: Comparative mutational analysis of genes involved in antibiotic resistance

Strain no	Fluoroquinolone Resistance			Regulatory genes		Efflux pump genes		MβL	Class1 integron
	GyrA	GyrB	ParC	<i>NfxB</i>	<i>MexR</i>	<i>MexB</i>	<i>MexF</i>	NDM	INT1/ (5'-CS 3'CS)
199	83T-I	468E-D	87 S-L	NIL	NIL	NIL	NIL	YES	Yes/Yes
200	83T-I	468E-D 504D-H 533Q-H	87 S-L	NIL	NIL	NIL	NIL	YES	Yes/Yes
208	83T-I	468E-D	87 S-L	NIL	NIL	576V-G 578T-N	NIL	YES	NIL
217	83T-I	468 E-D	87 S-L	NIL	NIL	NIL	792E-A 816K-M	YES	Yes/Yes
225	83T-I	468 E-D	87 S-L	NIL	NIL	607S-T 611T-A 612V-E	NIL	YES	Yes/Yes
227	83T-I	490 L-V	87 S-L	NIL	126 V-E	NIL	NIL	NIL	Yes/Yes
236	83T-I	NIL	87 S-L	NIL	126V-E 143P-L	615 F-L	NIL	NIL	Yes/Yes
237	83T-I	504 D-H	87 S-L	NIL	126V-E 143P-L	NIL	NIL	NIL	Yes/Yes
239	83T-I	468 E-D	87 S-L	NIL	NIL	589Q-P	NIL	YES	NIL
241	NIL	533Q-H	NIL	NIL	126V-E	NIL	NIL	NIL	Yes/Yes
251	83T-I	468E-D 503V-L 504D-H 533Q-H	87 S-L	NIL	NIL	NIL	NIL	YES	Yes/Yes
255	83T-I	468E-D 504 D-H	87 S-L	NIL	NIL	NIL	NIL	YES	Yes/Yes
T3	NIL	503V-L 504D-H	NIL	NIL	126V-E	NIL	843 A-T	NIL	Yes/Yes
T4	NIL	NIL	NIL	93L-V 131Q-H	126V-E	NIL	843 A-T	NIL	Yes/Yes

Figures

Figure 1

PFGE banding pattern and dendrogram for the 14 *P. aeruginosa* study isolates. Macro-restriction analysis by PFGE of the 14 SpeI-digested chromosomal DNA revealed the genetic diversity among the study isolates. Three distinct clusters were observed.

Figure 2

Determination of in vitro biofilm production among *P. aeruginosa*. a) All the 14 isolates were found to be strong biofilm producers upon crystal violet staining. b) Determination of biofilms was done by measuring the OD650 after dissolving the biofilms in 70% ethanol. All the experiments were performed in triplicates.

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