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Isolation and Determination of Multidrug Resistant *Pseudomonas Aeruginosa* from Clinical Samples

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Abstract: *Pseudomonas aeruginosa* emerged as an important human opportunistic pathogen as it continues to develop resistance to the antibiotics. It is also one of the most common and lethal pathogens responsible for ventilator-associated pneumonia in incubated patients, with directly attributable deaths reaching 38%. Multidrug-resistant *P. aeruginosa* are becoming more prevalent, and current antimicrobial treatments for cystic fibrosis are unable to eradicate *P. aeruginosa* infections. Therefore, alternative mechanisms for targeting *P. aeruginosa* have been the focus of much research. Thus in this current work, *P. aeruginosa* strains were isolated from the clinical samples and identified as *P. aeruginosa* using standard characterization methods. Out of 95 isolated strains, 25 Multi drug resistant strains were screened by assessing their antibiogram pattern. *P. aeruginosa* employ a cell-cell signalling system referred as quorum sensing (QS) to control the expression of several virulent genes. As *Pseudomonas aeruginosa* continues to develop resistance to the antibiotics, the quorum sensing enhanced transcriptional regulator QscR might serve as an alternate target. Thus the present study was carried to explore the prevalence of MDR strains of *Pseudomonas aeruginosa*.

Keywords: *Pseudomonas aeruginosa*, multi drug resistance, antibiotics, quorum sensing, antibiogram

I. INTRODUCTION

The bacterium *Pseudomonas aeruginosa* is an increasingly prevalent human pathogen, responsible for 12% of hospital-acquired urinary tract infections, 10% of blood stream infections, and 8% of surgical wound infections [1]. It is also one of the most common and lethal pathogens responsible for ventilator-associated pneumonia in intubated patients, with directly attributable deaths reaching 38% [2]. Cystic fibrosis patients are characteristically susceptible to chronic infection by *P. aeruginosa*, which is responsible for high rates of illness and death in this population. The ability of *P. aeruginosa* to invade a potential host relies largely on its ability to control many of its virulence factors by a mechanism that monitors cell density and allows communication between bacteria ([3]-[5]).

Multidrug-resistant *P. aeruginosa* are becoming more prevalent, and current antimicrobial treatments for cystic fibrosis are unable to eradicate *P. aeruginosa* infections. Therefore, alternative mechanisms for targeting *P. aeruginosa* have been the focus of much research [6]. Therapeutics that target and inhibit QS in *P. aeruginosa* would attenuate the virulence of the bacterium and thus potentially assist the host immune response in clearing the infection. The two QS networks, LasR and RhIR represent an ideal target for potential therapeutics [7]. The las system consists of the LasR transcriptional regulator and the LasI synthase protein. LasI is essential for the production of the AHL signal molecule N-(3-oxododecanoyl)-L-homoserine lactone (3O-C12-HSL) ([8]-[10]). LasR requires 3O-C12-HSL in order to become an active transcription factor. It was recently demonstrated that, in the presence of 3O-C12-HSL, LasR forms multimers, and that only the multimeric form of this protein is able to bind DNA and regulate the transcription of multiple genes. A second QS system in *P. aeruginosa* consists of the RhII and RhIR proteins ([11]-[13]).

The RhII synthase produces the AHL N-butyryl-L-homoserine lactone (C4-HSL), and RhIR is the transcriptional regulator. Only when RhIR is complexed with C4-HSL does it regulate the expression of several genes. Both 3O-C12-HSL and C4-HSL have been shown to freely diffuse out of bacterial cells; however, 3O-C12-HSL diffusion is significantly slower than that of C4-HSL. In addition to LasR and RhIR, there is a third, orphan LasR-RhIR homolog, QscR, for which there is no cognate acyl-HSL synthase gene [14]. A qscR mutant is hypervirulent. The influence of QscR on the expression of a few genes controlled by the LasR-I and RhIR-I systems has been examined. These genes are prematurely activated in a qscR mutant and include genes in the phz1 and phz2 phenazine synthesis operons; hcnAB, the hydrogen cyanide synthesis operon; lasB, which codes for elastase; rhII; and lasI. The mechanism for transient repression of these genes by QscR is not clear ([15]-[18]). At low acyl-HSL concentrations, QscR can form heterodimers with LasR and RhIR. This might inactivate LasR and RhIR.

A greater public health concern has raised an alarming situation due to the emergence and spread of multidrug resistant bacteria. In view of therapeutic options, the treatment of MDR strains has apparently exhibited the severe limitations and led the urgency for identification of novel antimicrobial targets and development of new antimicrobials [19]. It is well known that many gram negative bacterial pathogen communicate with each other by means of a small chemical signal molecules and tune the expression of several genes and coordinate a virulence determinates that contribute the pathogenesis to host [20]. This mechanism of chemical communication system among bacteria, termed as Quorum sensing represents as an alternative and novel promising therapeutic target to inhibit virulence system and also the expression of many genes that facilitates the interactions between the pathogen and the host.

It is also possible that QscR sequesters acyl-HSL signals and thereby delays the expression of LasR- and RhIR-dependent genes [21]. To develop a better view of the role of QscR in *P. aeruginosa* gene regulation, we focused on QscR. In view of the above, we believe that QscR can directly influence specific genes in response to the LasI-generated signal 3OC12-HSL. Thus in the present study, *Pseudomonas aeruginosa* was isolated from the clinical samples and the Multi drug resistant strains were assessed by antibiogram pattern.

II. MATERIAL AND METHODS

A. Collection and Processing of Samples

The Pus samples from medical laboratories were collected and each sample was taken carefully from the site of infection and placed in tubes and then transferred to the laboratory. Further the samples were streaked on nutrient agar plates and the plates were incubated at 37 °C for 24 hours. Then the characteristic suspected single colonies were subjected to Gram's staining and then sub-cultured in Mac Conkey agar [22]. The pure isolates of *Pseudomonas aeruginosa* were transferred to 1% nutrient agar slant and stored in the refrigerator at 4 °C for further use.

B. Morphological and Biochemical Identification

The sub-cultured isolates in selective and differential media were subjected to identification based on Bergey's manual of determinative bacteriology [23], morphological characterization, viz., Gram staining, motility, catalase and oxidase. Further identifications were made with biochemical tests such as sugar fermentation tests for glucose, sucrose, lactose, maltose and mannitol, Triple Sugar Iron agar test, Indole production test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test and Nitrate reduction test.

C. Antibiotic Sensitivity

Antibiotic sensitivity testing (AST) is used to determine the susceptibility of the isolates of *P. aeruginosa* to a range of potential therapeutic agents. The test was performed using disc diffusion or Kirby-Bauer test method [24]. Commercially available antibiotic discs (Himedia) were used for the test to determine the Antibiotics sensitivity pattern. Around 4-5 bacterial colonies were selected and inoculated into Nutrient broth and incubated at 35°C for 2-5 hours and then adjusted to 0.5 McFarland turbidity standard to get 1×10^8 CFU/mL as total count. While, sterilized Muller Hinton agar was prepared and dispensed into sterile petridishes and were allowed to solidify. The plates were allowed to dry in incubator for 30 min to remove the excess moisture from the surface and inoculated by swabbing.

Each isolate was examined by disk diffusion method for its susceptibility to the antimicrobial agents viz., Ampicillin (10µg), Chloramphenicol (30µg), Streptomycin (10µg), Tetracycline (30 µg), Cefuroxime (30µg), Ceftriaxone (30 µg), Ofloxacin (10µg) and Ciprofloxacin (10µg).

The antibiotic discs were carefully placed on the plates under sterile conditions and allowed to stand for 30 minutes (Pre-diffusion time) at refrigeration condition and incubated at 37°C for 24hours. The diameter of the zones of inhibition was measured by using zone measuring ruler (Hi-Media) without opening the lid at the end of incubation period.

D. Identification of Multidrug Resistant Strains

The multidrug resistant *Pseudomonas aeruginosa* was identified by measuring the zone of inhibition in accordance with CLSI standard chart for Enterobacteriaceae [25]. The isolates that showed resistance against three or more antibiotics were considered as MDR *Pseudomonas aeruginosa*.

III. RESULTS AND DISCUSSION

Pseudomonas aeruginosa is an important opportunistic human pathogen that infects immune-compromised individuals and people with cystic fibrosis. The increase in multidrug resistant strains of *P. aeruginosa* attributes the significant increase in severity of the

disease and also difficulty in treating the infection that probably leads to death [26]. Thus, the prevalence study and inhibition of multidrug resistance of *P.aeruginosa* by inhibiting the QS enhanced transcriptional regulator are necessary to control *P.aeruginosa* infection in public health sector.

A. Collection, Processing of Samples and Identification of *Pseudomonas Aeruginosa*

A total of 394 samples were collected from medical laboratories from salem region during the year 2015-2016. Based on the growth characteristics on enrichment media (MacConkey Agar), 152 samples (47.5 %) were observed for turbid growth. Among these 152 samples, 95 samples (62.5 %) were conformed as *P.aeruginosa* based on their growth characteristics (Figure.1) and various biochemical tests were noted in Table I and Figure.2.

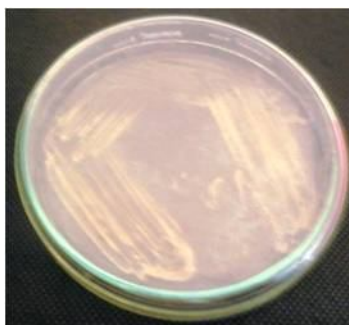


Fig. 1 Characterization of *Pseudomonas aeruginosa* isolated from samples. *P. aeruginosa* was observed as a medium-sized, light white/almost clear colored colony. The primary staining resulted gram-negative, pink colored, medium rod shaped appearance.

TABLE I CHARACTERIZATION OF *PSEUDOMONAS AERUGINOSA* BY BIOCHEMICAL TEST

S.No.	Name of the test	Observations
1	Gram's staining	G-ve rod
2	Motility test	Positive
3	Catalase test	Positive
4	Oxidase test	Positive
5	Glucose fermentation	Negative
6	Sucrose fermentation	Negative
7	Lactose fermentation	Negative
8	Maltose fermentation	Negative
9	Mannitol fermentation	Positive
10	Indole production test	Negative
11	Methyl red test	Negative
12	Voges - Proskauer test	Negative
13	Citrate utilization test	Positive
14	Urea hydrolysis test	Negative
15	Nitrate reduction test	Positive

The studies of [27], reported 56 *P. aeruginosa* isolates from 525 samples (blood and wound swabs) from 60 patients admitted at Vardhman Mahavir Medical College and Safdarjang hospital, New Delhi, India. Similarly in 2002, [28] reported 495 *P. aeruginosa* isolates from 1548 clinical samples from Mangalore. In 2012,[29] isolated 290 *P. aeruginosa* from various clinical samples from a from healthcare associated infections at a tertiary care hospital from Andhra Pradesh. Reference [7] reported a significant number of *P.aeruginosa* isolates during 2007 in Bangalore. The studies of [30] reported *P.aeruginosa* among 85 patients in New Delhi, India.

B. Antibiotic Susceptibility and Multidrug Resistance Determination of *P. Aeruginosa*

All the 95 *P. aeruginosa* isolates were subjected to the antimicrobial susceptibility testing with different types of antibiotics such as penicillin, phenicols, aminoglycosides, tetracyclins, cephams and fluoroquinolones. The diameter of zone of inhibition against the antibiotics in accordance with NCCL standard chart for enterobacteriaceae was considered to determine the isolate as resistant or intermediate or sensitive towards each antibiotics (Table II). If the diameter of the zone of inhibition of $\leq 13\text{mm}$ for Ampicillin, $\leq 12\text{mm}$ for Chloramphenicol, $\leq 11\text{mm}$ for Streptomycin, $\leq 11\text{mm}$ for Tetracycline, $\leq 14\text{mm}$ for Cefuroxime, $\leq 13\text{mm}$ for Ceftriaxone, $\leq 12\text{mm}$ for Ofloxacin and $\leq 15\text{mm}$ for Ciprofloxacin, then the isolate was considered to be resistant.



Fig. 2 Biochemical characterization of *Pseudomonas aeruginosa*. (a) Sugar fermentation tests for glucose, sucrose, lactose, maltose and mannitol, Triple Sugar Iron agar test, Indole production test, methyl red test, Voges-Proskauer test, citrate utilization test, urease test and Nitrate reduction test (b) Catalase test (c) Oxidase test.

TABLE II

NCCL GUIDE LINES TABLE FOR DETERMINING THE *PSEUDOMONAS AERUGINOSA* AS RESISTANT/ INTERMEDIATE/SENSITIVE AGAINST ANTIBIOTICS

Type	Antibiotics	Zone of inhibition in mm		
		Resistant	Intermediate	Sensitive
Pencillin	Ampicillin	≤ 13	14 – 16	≥ 17
Phenicols	Chloremphenicol	≤ 12	13 – 17	≥ 18
Aminoglycosides	Streptomycin	≤ 11	12 – 14	≥ 15
Tetracyclins	Tetracycline	≤ 11	12 – 14	≥ 15
CEPHEMS	Cefuroxime	≤ 14	15 – 22	≥ 23
	Ceftriaxone	≤ 13	14 – 20	≥ 21
Fluoroquinolones	Ofloxacin	≤ 12	13 – 15	≥ 16
	Ciprofloxacin	≤ 15	16 – 20	≥ 21

The isolates that exhibited resistance against three or more antibiotics were considered as multidrug resistant strains. Among these MDR isolates, resistance of 100 % was observed against Ampicillin and 72 % of sensitivity is observed against Ofloxacin. The pattern of resistance, intermediate and sensitivity of MDR isolates were reported in Table.III and Figure.3. The isolates that exhibited resistance to more than 3 antibiotics and their pattern of zone of inhibition were shown in Figure 4.

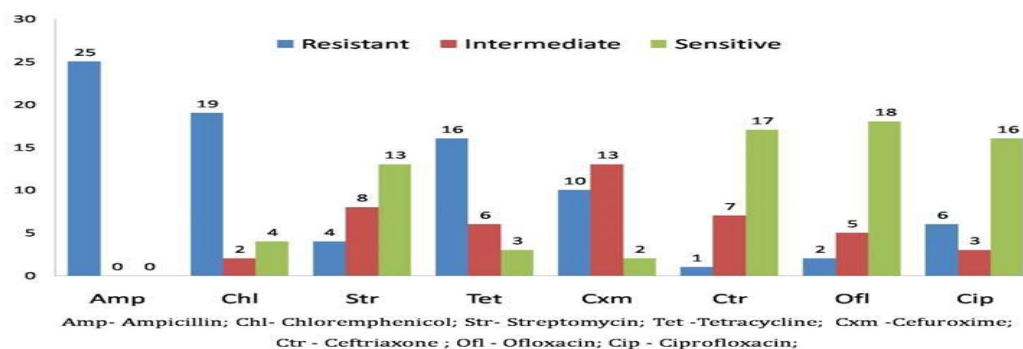


Fig. 3 The sensitivity pattern of 25 determined *Pseudomonas aeruginosa* against eight antibiotics.

The studies of Ramana et al (2012) [29] showed the highest number of *Pseudomonas* infections was found in urinary catheters, followed by endotracheal tips and central venous catheters. Maximum resistance was seen to cefotaxime and gentamycin (40%)

followed by ciprofloxacin (39%), amikacin (26%), cefoperazone- sulbactam (22%), piperacillin-tazobactam (16%), and imipenem (14%).

Table Iii

Antibiogram Pattern For Isolated 25 Mdr Pseudomonas Aeruginosa Against Eight Antibiotics And With Their Zone Of Inhibition In Mm Ampicillin (A); Chloremphenicol(C); Streptomycin (S); Tetracycline (T); Cefuroxime (Cxm); Ceftriaxone (Ctr); Ofloxacin (Of); Ciprofloxacin (Cip)

Isolate	Antibiotics -Zone of inhibition (mm)								No. Of Resistance
	A 13mm	C 12mm	S 11mm	T 11mm	CXM 14mm	CTR 13mm	Of 12mm	CIP 15mm	
Pae4	R (8mm)	R (10mm)	I (14mm)	R (10mm)	I (16mm)	R (13mm)	S (19mm)	S (26mm)	3
Pae7	R (9mm)	R (11mm)	S (18mm)	S (16mm)	I (15mm)	S (30mm)	S (18mm)	R (14mm)	3
Pae9	R (9mm)	R (10mm)	R (11mm)	I (14mm)	S (23mm)	S (23mm)	S (21mm)	I (19mm)	3
Pae11	R (9mm)	R (9mm)	R (11mm)	R (10mm)	S (25mm)	I (14mm)	I (14mm)	R (13mm)	5
Pae17	R (10mm)	R (10mm)	I (13mm)	R (11mm)	R (14mm)	S (23mm)	S (16mm)	S (24mm)	4
Pae23	R (11mm)	S (21mm)	S (16mm)	R (11mm)	R (14mm)	I (18mm)	I (15mm)	I (18mm)	3
Pae28	R (12mm)	R (11mm)	S (18mm)	S (16mm)	R (14mm)	I (19mm)	S (25mm)	S (24mm)	3
Pae32	R (10mm)	R (12mm)	I (15mm)	R (11mm)	I (21mm)	S (23mm)	S (19mm)	S (25mm)	3
Pae36	R (9mm)	R (10mm)	I (13mm)	R (10mm)	I (22mm)	S (26mm)	I (13mm)	S (24mm)	3
Pae41	R (12mm)	S (19mm)	R (11mm)	R (11mm)	I (22mm)	I (20mm)	S (19mm)	S (22mm)	3
Pae45	R (9mm)	R (9mm)	S (16mm)	R (10mm)	R (12mm)	S (22mm)	S (18mm)	S (22mm)	4
Pae46	R (9mm)	R (10mm)	S (17mm)	R (11mm)	R (14mm)	S (24mm)	S (21mm)	S (24mm)	4
Pae50	R (8mm)	R (11mm)	S (15mm)	I (13mm)	I (19mm)	S (25mm)	S (16mm)	R (15mm)	3
Pae55	R (11mm)	R (9mm)	S (18mm)	R (11mm)	I (20mm)	S (24mm)	I (15mm)	S (26mm)	3
Pae59	R (11mm)	R (10mm)	S (18mm)	I (14mm)	R (13mm)	I (20mm)	S (21mm)	S (25mm)	3
Pae62	R (10mm)	R (11mm)	I (15mm)	R (11mm)	I (15mm)	S (22mm)	S (18mm)	S (21mm)	3
Pae63	R (11mm)	S (19mm)	I (13mm)	R (10mm)	I (16mm)	I (18mm)	S (19mm)	R (12mm)	3
Pae66	R (10mm)	I (15mm)	R (11mm)	R (11mm)	I (19mm)	S (25mm)	S (16mm)	I (20mm)	3
Pae71	R (11mm)	R (10mm)	S (19mm)	I (14mm)	R (14mm)	S (24mm)	I (15mm)	S (24mm)	3
Pae73	R (10mm)	I (13mm)	S (18mm)	I (13mm)	R (14mm)	S (22mm)	R (12mm)	R (15mm)	4
Pae74	R (9mm)	S (22mm)	S (18mm)	R (11mm)	I (21mm)	I (18mm)	S (18mm)	R (15mm)	3
Pae77	R (9mm)	R (12mm)	S (18mm)	S (15mm)	R (13mm)	S (22mm)	S (21mm)	S (24mm)	3
Pae81	R (12mm)	R (10mm)	I (13mm)	R (11mm)	I (15mm)	S (22mm)	S (17mm)	S (21mm)	3
Pae83	R (8mm)	R (12mm)	S (15mm)	I (13mm)	I (19mm)	S (23mm)	R (10mm)	S (29mm)	3
Pae87	R (9mm)	R (10mm)	I (13mm)	R (11mm)	R (13mm)	S (26mm)	S (17mm)	S (27mm)	4

The studies of Shenoy et al (2002) [32] reported that the antibiotic sensitivity testing revealed that *Pseudomonas aeruginosa* strains were highly resistant to most anti-pseudomonal antibiotics; Amikacin was found to be most suitable for routine use with a sensitivity of 68.01% and the most resistant antibiotic was Netilmicin showing 70.04% resistance. Surprisingly, Gentamycin showed a relatively higher sensitivity of 55.87%. Sensitivity to Imipenem and Meropenem was 100%. The studies of Swetha et al. [31] during 2008 – 2009 reported the susceptibility of 39 *P.aeruginosa* isolates against 25 antibiotics. All the isolates exhibited multiple drug resistance varying from 5.40% to 100% with 16 of the 25 antibiotics tested. Resistance was observed against Clindamycin (94.59%), Ampicillin (86.49%), Cotrimoxazole (48.65%), Colistin (45.94%), Nalidixic acid (35.10%), Amoxyclave (18.90%), Cephalexin, Meropenem, Tobramycin, Nitrofurantoin, Tetracycline, Amoxicillin (8.10% each), and Streptomycin (5.40%).

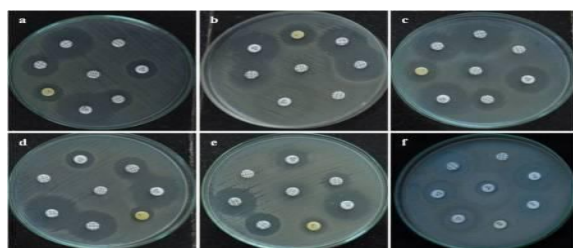


Fig. 4 Antibigram pattern of 6 *Pseudomonas aeruginosa* isolates that are determined as MDR strains. a. Isolate-17 b. Isolate-28 c. Isolate-32 d. Isolate-46 e. Isolate-62 f. Isolate-63

Emergence of drug resistant isolates is a greater public health concern. In this study, the occurrence of 25 isolates (26.31%) as multidrug resistant indicates the high level of MDR *P. aeruginosa* infections. Over the past few decades, the extensive use of antibiotics leads to the increase in different multi drug resistant bacterial strains. Among the determined MDR isolates of the present study, majority of strains exhibited resistance towards Ampicillin (100%), Chloremphenicol (76 %), tetracycline (64 %) and Cefuroxime (40 %) and the lowest resistance was observed towards Ceftriaxone, Ofloxacin, streptomycin and ciprofloxacin. Thus the antibiotics sensitivity patterns of the isolates were studied and the MDR strain of *Pseudomonas aeruginosa* was identified for further studies.

IV.CONCLUSIONS

Pseudomonas aeruginosa emerged as an important human opportunistic pathogen as it continues to develop resistance to the antibiotics. The pus samples were collected from Salem region and isolates of *P. aeruginosa* were determined based on their growth characteristics and various biochemical test. The antibiotics sensitivity patterns of these isolates were studied and the MDR strain of *P.aeruginosa* was identified. The surveillance of *P. aeruginosa* is studied by isolating 95 strains from the samples and characterized with the standard morphological, physiological and biochemical characterization methods. The antibiogram pattern revealed 25 MDR strains of *P. aeruginosa*. In which the majority of strains exhibited resistance towards Ampicillin (100%), Chloremphenicol (76 %), tetracycline (64 %) and Cefuroxime (40 %) . Thus the MDR *P. aeruginosa* isolated and reported in this study might serves as potential source to explore the QscR protein from these MDR strains of *P. aeruginosa*.

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