

Phenotypic Diversity of MDR Strains of *Pseudomonas Aeruginosa* in Burn Patients and their Multiple Antibiotic Resistance (MAR) Index

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Abstract: *Pseudomonas aeruginosa* was isolated from patients with burn infections. Phenotypic diversity of the isolates were observed by studying the morphological and physiological characteristics of the isolates. Pigmentation and auxotrophic nature of the isolates were observed along with the sensitivity pattern and drug resistance. Multiple Antibiotic Resistance (MAR) index was calculated. Out of the 52 isolates obtained, 16 isolates (28%) showed a MAR index higher than 0.2 suggesting their origin from a high risk source of contamination where antibiotics are often used.

Keywords: *Pseudomonas aeruginosa*, Phenotypic diversity, Multiple Antibiotic Resistance (MAR) index

1. Introduction

Pseudomonas Aeruginosa is an opportunistic pathogen capable of causing nosocomial infections. Infections with *Pseudomonas aeruginosa* in particular by a multidrug resistant (MDR) strain is wide spread in hospitals. Infection at times can be fatal in burn patients and immune compromised patients. The minimal nutritional requirements of *Pseudomonas aeruginosa*, its tolerance to antimicrobial agents contribute to its ecological success and to its role as an effective opportunistic pathogen.

Pseudomonas aeruginosa virulence is defined by several factors and it has been shown that environmental conditions such as oxygen tension, iron availability and the presence of antibiotics modulate the synthesis and function of those factors. *Pseudomonas aeruginosa* produces two different ADP- ribosyl transferase toxins: ETA and exoenzyme S. Exoenzyme S causes significant tissue damage in lungs, burn, and wound infection produced by the majority of *Pseudomonas aeruginosa* strains. Although Multi drug-resistant *Pseudomonas aeruginosa* (MDRPA) infections have been described in patients with cystic fibrosis or immunocompromised conditions and in isolated outbreaks in intensive care units with burn infected patients and certain recent reports in critically ill patients in non outbreak settings have raised concerns because of the scarcity of novel agents to effectively treat MDRPA infections. The more the bacteria come in contact with antibiotics, the greater are the chances of becoming resistant.

The frequent use of multiple antibiotics in the most severely ill patients could lead to the acquisition of, or alternatively the transformation to, highly virulent strains of *Pseudomonas aeruginosa* that pose a significant threat to the patients. The ability of multi drug resistant strains to persist for prolonged periods in such patients may allow for the development of extremely virulent phenotypes. As bacteria are continuously overcoming the tools with which humans have to fight there is a need for search for new antibiotics. This study evaluates the occurrence of *Pseudomonas*

aeruginosa infections in burn infected patients and it also observes the phenotypic diversity of Multi drug resistant strains by studying the Morphological, Physiological, Pigmentation characteristics and the Auxotrophic nature of the strain. Sensitivity pattern, Percentage of Drug resistance & Multiple Antibiotic resistance (MAR) index of the MDR strains are also studied.

2. Materials and Methods

Sample Collection

The Pus samples were obtained from the burn infected patients admitted in hospitals in and around Chennai from June 2016-Dec 16. About 52 clinical isolates were obtained and the isolates were screened for the presence of *Pseudomonas aeruginosa* and the cultures were stored for further use. A chart was prepared to categorise the burn infected patients according to their age and sex.

Morphological study

The isolated strains were purified and subjected to gram staining and their motility was checked by hanging drop method. The isolates were streaked on Nutrient Agar and Cetrimide Agar plates. The plates were incubated at 37°C for 24 hours and the colony morphology was observed and noted. Biochemical tests were performed and the results were compared and confirmed with the standard reactions of *Pseudomonas aeruginosa*.^[1]

Antimicrobial Sensitivity Testing

The sensitivity pattern of *Pseudomonas aeruginosa* strain to drugs like Ampicillin (30µg), Amikacin (30µg), Amoxicillin (30µg), Carbenicillin (100µg), Ciprofloxacin (10µg), Ceftazidime (30µg), Cefotaxime (30µg), Gentamicin (10µg), Imipenem (30µg), Kanamycin (10µg), Norfloxacin (30µg), Netilmycin (30µg), Ofloxacin (30µg), Piperacillin (100µg), Tobramycin (30µg), and Trimethoprim (30µg) was checked by disc diffusion test

Muller Hinton nutrient broth was used as growth medium before testing the sensitivity of strains. Muller Hinton plates were inoculated with the culture in sterile swabs and streaked over the entire surface of the plate. The plates were incubated at 37°C for 24 hours. All antibiotic discs were obtained from HiMedia and a control strain ATCC 27853 was used for identification. Multi Drug Resistant strains were screened by comparing the sensitivity pattern obtained. The strains were characterized as sensitive, intermediate and Resistant. According to the available standards the resistant isolates obtained were labelled as Multidrug Resistant and they were used for the following studies

Auxotrophic Studies

Multidrug resistant strains of *Pseudomonas aeruginosa* isolates from burn patients were tested for auxotrophy. The inoculum was adjusted to 0.5 Mc Farland and diluted to have the final concentration of 100 cells/μl. One microliter of the inoculum was spotted on Mueller Hinton agar (MHA) and Minimal Agar Medium (MAM) where the MHA served as the complex medium and MAM as nutrient deficient medium. The plates were incubated at 37°C for 48 hrs. Those isolates, which did not grow on Minimal Agar Plates (MAM) plates but grew on Mueller Hinton agar (MHA) were considered as auxotrophs. Those isolates, which were able to grow on both MHA and MAM plates were considered prototrophs.

Pigment Production

Pigment production is not a characteristic feature of all the *Pseudomonas aeruginosa* isolates and if present it plays a significant role in the classification of the *Pseudomonas aeruginosa*. All the clinical isolates obtained from burn patients were observed for pigment production. The isolates were streaked on Cetrinide agar and were incubated at 37°C for 24 hours. The plates were observed for the presence of pigments visibly. Pyomelanin, Pyorubrin, and pyocyanin are some of the common pigments observed in *Pseudomonas aeruginosa*.

Calculation of Multiple Antibiotic Resistance Index (MAR):

The Multiple Antibiotic Resistance of the isolates were calculated by

$$\text{MAR Index} = \frac{\text{No. of antibiotics to which the isolate was resistant}}{\text{No. of antibiotics tested}}$$

The drug resistance percentage was also calculated by dividing the completely resistant strains by the isolates to the antibiotics by the total number of strains [2]. The MAR indexes of hospital isolates of *Pseudomonas aeruginosa* were determined with reference strain

3. Results and Discussion

The age and sex distribution of the patients were studied and the results are represented in Figure 1. The isolates were observed morphologically and biochemically and was compared with the standard strains.

Table 1: Results of Auxotrophic Study

Sl. No.	Isolates Name	MHA	MAM
1.	PA1.	+	+
2.	PA2.	+	+
3.	PA3.	+	—
4.	PA4.	+	—
5.	PA5.	+	+
6.	PA6.	+	+
7.	PA7.	+	+
8.	PA8.	+	+
9.	PA9.	+	—
10.	PA10.	+	+
11.	PA11.	+	—
12.	PA12.	+	—
13.	PA13.	+	+
14.	PA14.	+	—
15.	PA15.	+	+
16.	PA16.	+	+

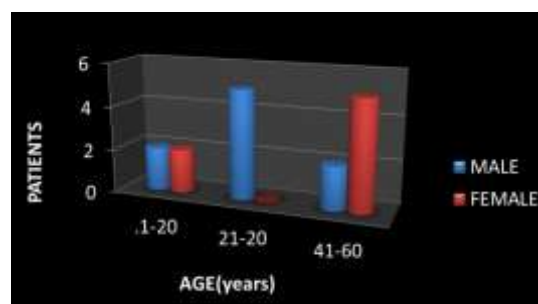


Figure 1: Age and Sex Distribution of Patients

Table 2: Rate of Drug Resistance

Sl.No.	Antibiotic	Drug Concentration	Resistance In Percentage
1.	Amikacin	30μg	37%
2.	Gentamicin	10μg	43%
3.	Piperacillin	100μg	62%
4.	Ciprofloxacin	10μg	100%
5.	Ampicillin	10μg	100%
6.	Clotrimazole	10μg	100%
7.	Optochin	30μg	100%
8.	Nalidexic acid	30μg	100%
9.	Norfloxacin	10μg	31%
10.	Ofloxacin	20μg	18%
11.	Methicillin	30μg	100%
12.	Erythromycin	30μg	100%
13.	Ceftazidime	30μg	100%
14.	Bacitracin	30μg	100%



Figure 2: Multiple Antibiotic Index (MAR)

All the isolates were tested for its auxotrophic nature. The isolates were streaked in Muller Hinton Agar (Complex medium) and Minimal Agar Medium (nutrient deficient).

The isolates which don't grow on Minimal Agar Medium but grew on Muller Hinton Agar were known as Auxotrophs. The isolates which do not grow in both are Prototrophs. 37% of the isolates were found to be auxotrophic (6 isolates). The reproducible results were obtained on repeating the experiment thrice. Table 1 illustrates the auxotrophic nature of the isolates.

According to Stevenson^[3] among 350 *P.aeruginosa* isolates, 56 were auxotrophs, which did not grow on minimal agar medium (MAM) and required Muller Hinton agar medium (MHA) for their growth. Of the 56 auxotrophs, four were isolated from the chronically infected patient who was diagnosed as CF six years ago. One auxotroph was isolated. The respiratory samples collected during their subsequent follow-ups were found negative for the presence of auxotrophs. These auxotrophs were recovered when these patients presented with pulmonary exacerbation.

According to Poole^[4] The resistance against different antibiotics was compared between auxotrophs and prototrophs. Although auxotrophs were small in number, they showed significantly higher resistance against ceftazidime (60%) as compared to prototrophs ($p < 0.05$). Three auxotrophs (isolate no. 1-3) were multidrug resistant (A6 antibiogram). reproducible results were obtained on repeating the experiment thrice. Isolated strains were plated on Cetrimide agar and the colonies were observed for pigmentation. Out of the 16 isolates, 12 (75%) showed pyocyanin production and they were bluish green in colour. 4 isolates (25%) showed pyoverdine as they appeared greenish yellow in colour. Pigment production is a contributory phenotype characteristic in the classification of *P. aeruginosa* such as pyocyanin or PCN (blue-green) pyoverdine or fluorescein (greenish-yellow), pyomelanin (red-brown) and pyrroline (red).

Pyoverdine (Fluorescein), is another virulence factor produced by *Pseudomonas sp.*, especially *P.aeruginosa*. Pyoverdine, encoded by the *pvd* genes acts as a siderophore, involves in a complex iron acquisition system tightly binding and transporting soluble iron (Fe III) from the environment under iron-deficient condition, which has been determined to be an essential component in biofilm formation^[5].

All the isolates were subjected to antibiotic sensitivity testing. Isolates showed different sensitivity pattern. Antibiotic Susceptibility Pattern of *Pseudomonas aeruginosa* was tabulated in table 2. MAR Index was calculated and the results were shown in Figure 2.

Opportunistic pathogens presenting broad spectrum antibiotic resistance have emerged extensively in hospital environments, causing serious infections in immunocompromised hosts. Organisms resistance to antibiotic treatment; such as MRSA (Methicillin resistant *Staphylococcus aureus*), VRE (Vancomycin resistant *Enterococcus*) and amino glycoside. *Pseudomonas aeruginosa* are now endemic, causing serious nosocomial infections, especially in neutropenic patients^[6].

Recently, increased resistance has been observed against 3rd generation cephalosporins for gram negative bacilli, especially *Pseudomonas aeruginosa*. Cefepime and Ceftazidime are the commonest 3rd generation antibiotics in ICU protocols. Resistance to Ceftazidime (100%) are significant in our study. Previous studies suggest that the selective pressure from the use of antimicrobial agents is a major determinant for the emergence of resistant strains^[7].

The evolution of multi resistant *Pseudomonas aeruginosa* and its mechanisms of antibiotic resistance have been examined. Primary mechanisms include reduced cell permeability, efflux pumps, changes in the target enzymes and inactivation of the antibiotics^[8].

The values for all the Multi drug resistant strains were higher than 0.2 suggesting their origin from a high risk source of contamination where antibiotics are often used. The low Multiple Antibiotic Resistance (MAR) Index value (0.11-0.68) suggested that isolated *P. aeruginosa* were unlikely to have a predisposition to develop resistance under conditions of antibiotic selection. Multiple antibiotic resistances can occur even in the absence of plasmid or transposon. It is suggested that under laboratory conditions, the absence of antibiotics in the culture media probably enhance plasmid instability^[9]. In conclusion it is understood that multidrug resistant strains are emerging drastically and it is essential to combat different strategies medically, to overcome them.

4. Acknowledgement

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5. Competing Interests

We have no conflict of interest

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