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Evaluation of High Multiple Antibiotic Resistant Index and Virulent Factors among Clinical Strains of *Pseudomonas Aeruginosa*

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Abstract: ***Pseudomonas aeruginosa is one of the leading causes of hospital-acquired as well as community-acquired infections globally. The present study evaluated the prevalence of High multiple antibiotic resistant P.aeruginosa in the clinical environment and also determine the virulence factors which aids the organism for its multi drug resistant characteristics. The study examined 90 clinical samples in which 21(23.3%) isolates of P. aeruginosa were recovered from the total clinical samples. Among them 3(14.2%) were from burns, 9(42.8%) from Pus, 6(28.57%) from wound swabs and 3(14.2%) from sputum were isolated, identified and further confirmed by both phenotypic and 16srRNA method. Antibiogram, MAR index and the virulence factors contributes its part in the pathogenicity of the organism were also determined. There should be surveillance programs for the detection of MDR organisms in every locality. Infection control programs need to be implemented with quality control in every clinical setting.***

Keywords: *Pseudomonas aeruginosa*, MAR index, Protease, Slime activity, lecithinase

1. Introduction

Pseudomonas aeruginosa is a gram negative(1.5 x 3 - 4 µm), aerobic, rod-shaped, capsulated, non sporing bacteria and was first reported in 1862 by Luke, who observed rod-shaped particles in blue-green pus of human infections.(1).

In 1960s, *Pseudomonas aeruginosa* emerged as an important human pathogen recognized as an emerging opportunistic pathogen of clinical relevance. It has higher prevalence and mortality rate in hospital environment, especially among patients, particularly those with burns, wounds and cancer and in the critically ill admitted in intensive care unit. Typically it infects the pulmonary tract, urinary tract, burns, wounds and also causes other blood infections.(2)

Pseudomonas has several virulence factors that oppose host defenses which depend mainly on impair phagocytosis. The virulence factors include capsule which protect bacteria from phagocytosis, fimbraie responsible for adhesive factors which help adhering in to epithelial host cell and exoenzyme live phospholipase the enzyme usually act on the animal cell membrane by forming a pore in cell and cleared phospholipids. Also *pseudomonas* is producing protease which causes bleeding and tissue necrosis. (3). It is characteristically resistant to many antimicrobial agents owing to permeability, multi-drug efflux and a chromosomal AmpC β-lactamase. One in ten hospital acquired infections is from *Pseudomonas* (4)

In fact, multidrug resistance and biofilm formation are frequent problems with the treatment of *Pseudomonas aeruginosa* infections, which requires more investigations and a search for alternative targets for therapeutics against this infection (5).So, it is urged to record the prevalence of multi drug resistant *Pseudomonas aeruginosa* and its related virulent factor in the human environment for an effective treatment.

2. Materials and Methods

2.1 Isolation source and identification of *P.aeruginosa*

Totally 90 clinical samples (Burns, Pus, Wound swabs, Urine, Sputum etc) were collected from various hospitals in and around Puducherry, India during Jan, 2019 to Feb, 2019. All the clinical samples had been inoculated in Brain heart infusion agar (HiMedia, India) for enriching the bacterial growth and further cultured on Cetrimide agar with an incubation time of 24 h at 37°C for obtaining selective growth of *Pseudomonas* sp. All the presumptive *Pseudomonas* isolates were been identified to species level by using standard microbiological methods

2.2 Genotypic identification of *P.aeruginosa* isolates

The colony PCR protocol been adopted for identifying the pure cultured isolates of presumed *P.aeruginosa*. Colonies have been picked up with a sterilized toothpick, and suspended in 0.5 ml of sterilizes saline in a 1.5 ml centrifuge tube. Centrifuged at 10,000 rpm for 10 min. After removal of supernatant, the pellet has been suspended in 0.5 ml of Insta Gene Matrix (Bio-Rad, USA). Incubated 56°C for 30 min and then heated 100°C for 10 min. After heating, supernatant can be used for PCR. For starting up the PCR, Add 1 µl of template DNA in 20 µl of PCR reaction solution. Use 518F/800R primers for bacteria, and then perform 35 amplification cycles at 94°C for 45 sec,

55°C for 60 sec, and 72°C for 60 sec. DNA fragments have been amplified about 1,400 bp in the case of bacteria. Amplified genes were electrophoresed out on 1% agarose gel with a size marker (ladder 100, Wako, Japan) Included a positive control (*E. coli* genomic DNA) and a negative control in the PCR.

518F 5' CCAGCAGCCGCGGTAATACG 3'
800R 5' TACCAGGGTATCTAATCC 3'

Purification of PCR products removes unincorporated PCR primers and dNTPs from PCR products by using Montage PCR Clean up kit (Millipore). Sequencing the purified PCR products of approximately 1,400 bp were sequenced by using the primers (785F 5' GGA TTA GAT ACC CTG GTA 3' and 907R 5' CCG TCA ATT CCT TTR AGT TT 3') (6). Sequencing were performed by using Big Dye terminator cycle sequencing kit (Applied BioSystems, USA) and the sequenced products had been resolved on an Applied Biosystems model 3730XL automated DNA sequencing system (Applied BioSystems, USA).

2.3 Determination of Multiple Antibiotic Resistances

Antimicrobial susceptibility testing of the recovered *P.aeruginosa* isolates were performed by Kirby-Bauer agar diffusion method(7). Commercially available (Hi-Media) antimicrobial discs of Amikacin (Ak 30µg), Gentamicin (G 10 µg), Meropenem (Mr 10µg), Ceftriaxone (Ci 30µg), Ampicillin (A 10µg), Carbenicillin(Cb 100U), Cefoxitin (Cx 30 µg),Cefadroxil (Cfr30 µg),Kanamycin (K 30µg), Nalidixic acid(Na 30 µg), Netillin (Net 30 µg), Norfloxacin (Nx10 µg), Nitrofurantoin (Nit 300U), Neomycin (N 30µg), Penicillin G (P10 µg),Ciprofloxacin (Cf 5µg), Tetracycline (T30µg), Chloramphenicol (C 30µg), Azithromycin (At 15µg), Clindamycin (Cd 2µg), Polymyxin B (Pb 300U) were used on Mueller Hinton agar(MHA, Hi-Media) to test the susceptibility. Antibiogram (sensitive or resistance) profile was obtained as per the protocols designed by Clinical and Laboratory Standards Institute Guidelines. (2011/M100S21; http://clsi.org; Clinical and Laboratory Standards Institute 2011).

2.4 MAR index of the isolates

The MAR index to a single isolate is defined as a/b, where 'a' represents the number of antibiotics to which the isolate was resistant and 'b' represents the number of antibiotics to which the isolate was exposed. MAR index value higher than 0.2 is considered to have originated from high-risk sources of contamination like human, commercial poultry farms, swine and dairy cattle where antibiotics are very often used. MAR index value of less than or equal to 0.2 considered the origination of strain from animals in which antibiotics are seldom or never used(8).

2.5 Protease activity

Commercially prepared sterile evaporated skim milk with two fold concentration was utilized. A 30% skim milk-agar medium was prepared by adding 15 mL of the skim milk to 85 mL of sterile Nutrient agar (NA) held at 42° C; it was then poured into petri dishes. Protease activity was assayed by spreading *Pseudomonas* isolates on Skim milk agar medium. After incubation for up to 72 h at 37°C, the production of protease was shown by the formation of a clear zone caused by casein degradation.(9)

2.6 Lecithinase Activity

For lecithinase enzyme, 10 mL of the 50% egg yolk was added to 90 mL of sterilized Tryptic soy agar. The formation of a white precipitate around or beneath the inoculums spot revealed lecithinase formation(10).

2.7 Lipase Activity

Lipase activity was observed by the appearance of a turbid halo around the inocula on Tryptic soy agar plates supplemented with 1% Tween 80 as explained by Rollof *et al*(11).

2.8 Slime Test

BHI agar plates were prepared containing 0.8g/L Congo red(12). *Pseudomonas* isolates were inoculated onto the surface of the medium and the plates were incubated at 37ºC for 24h. Slime producing bacteria appeared as black colonies, whereas non - slime producers remained none pigmented.

3. Result and Discussion

The colony morphology and cultural characteristics of the isolated organisms was presumptively identified as *Pseudomonas aeruginosa.* Gram staining and motility showed gram negative rods with active motility of the organism. Pigment production was accomplished after overnight incubation. The pyocyanin pigment produced by the organisms was indicated by color change in the solid media. 21(23.3%) isolates of *Pseudomonas* were recovered from the total 90 clinical samples screened in the study. Among these isolates 3(14.2%) were from burns, 9(42.8%) from Pus, 6(28.57%) from wound swabs and 3(14.2%) from sputum were isolated during the investigation. (Fig.1)

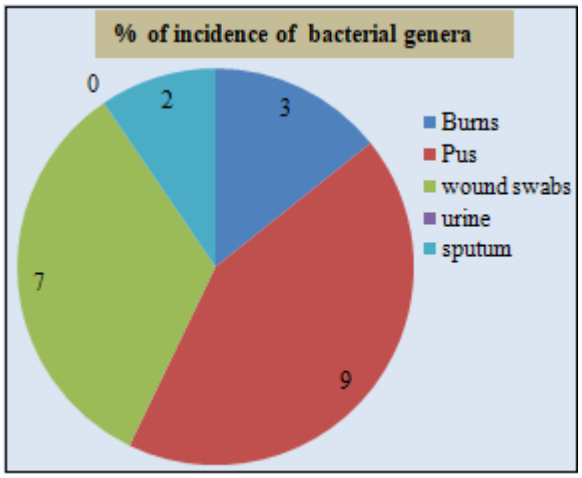

Figure 1: Distribution of Pseudomonas isolates on clinical samples

3.1. Molecular identification of *Pseudomonas* strains

Based on the traditional phenotypic identification methods the strains recovered by enrichment methods were presumably identified as *P.aeruginosa*. *P. aeruginosa* was gram negative, motile and exhibits positive reaction on catalase and oxidase test. Hence confirmation was required for *Pseudomonas aeruginosa* at molecular level; 16S rRNA gene region was amplified and sequenced. PCR amplification of 16S rRNA gene region by using universal primer, the obtained PCR product resulted in 1501 bp. The sequences were submitted to NCBI database and the accession numbers were obtained MK598324, MK598327, MK598330, MK598331, MK598332, MK598333, MK598334 and MK598336(13). By using BLAST analysis, 98 sequences of NCBI data gave 99% similarity.

1 TTGTAAGTTT TTTGACACTG TTCAATTGAA CGCTGAGGGC AGGCCTAACA CCTGCAAGTT
61 GAGCGGATAA CGCGAGCTTG CTCCTGGATT CAAGGGGGGA CGGGTGAATA ATGCCTTGGA
121 ATCTGCCTGA TAGGGGGGGA TAACGTCCGG AAACGGGTGG TAATTCCGCA TACGTCCTGA
181 GGGAGAAAGG GAGGGATTCT CAGACCTCAC GATATCAGAT GAGCCTAGGT TGGATTAGGT
241 AGTTGGTGGG GTAAAGGCCT ACCAAGGGGG CGATCCGTAA GTGGTTTGAG AGGATGATCC
301 ATCACCATGG AAATGAGACA CGGTCCAGAA TTCTTCGGGA GGCAGCCATG GGGAATTTTG
361 GACAATGGGG GAAAGCCTGA TCCAGCCATT CCGGGTGTGT GAAGAAGGTC TTTGGATTGT
421 AAAACCCTTT AAGTCGGGAG GAAGAGCAGT AAGTTAATTC CCCGGTGTTT TGACGTTACC
481 AACAGAATAA GCACCGGGTA AATTCGTGCC AGCAGCCGCG GTAATACGAA GGGTGCCAGC
541 GTTAATTGGA ATTACTGGGC GTAAAGCGCG GGTAGGTGGT TCAGCAAATA GGATGTGAAA
601 TTCCCGGGCT CAACCTGGGA AATGCATCCA AAAATAATCA GGTTGAGTAC GGTAGAGGGT
661 GGTGGAATTT CCTGTGTAGC CATGAAATGG GTAGATATAG GAAGGAACCC CAGTGGGGAA
721 GGGGACCACC TGGAGAGATC ATGACAGTTA GGTCAGAAAG CGTGGGGAGC AAACCGGATT
781 AGATTCCCTG GTAGTTCCCG CCGTGAACGG AGATGTTGCC TAGCCGTTGG GATCCTTGAG
841 ATCCTGAGTG GCGCAGCTAA CGCTATAAGT TGACCGCTGG GGGAGTACGG CCGCACTGTT
901 AAAACTCAAA TGAATTGACG GGAGCCCGCC CAACCGGGGA AGCAGGTGAT TTAATTCAAA
961 CCACCCCGAA AAACCTTACC TGGCCTTGAC ATGCTGAAAA CTTTCCAAAA ATGGATTGGG
1021 GCCTTCGGGA ACTCAGACCC AGGTGCTGCA TGGCTGTCGT CCGCTCGTGT CCTGAGATGT
1081 TGGGTTAAGT CCCGTAACGA GCGCAACCCT TGTCCTTAGT TACCAACACC TCGGGTGGGC
1141 ACTCTACTGA GACTGCCGGT GACAAACCGG ATGAAGGTGG GGATGACGTC AAGTCATCAT
1201 GGCCCTTACG GCCAGGGCTA CACACGTGCT ACAATGGTCG GTACAAAGGG TTGCCAAGCC
1261 GGGGAGTGGA GCTAATCCCA TAAAACCGAT CGTAGACCGG ATCCTGCTCT GCAACTCGAC
1321 TGCGTGAAAT CGGAATCGCT AGTAATCGTG AATCAGAATG TCACGGTGAA TACCTTCCCG
1381 GGCCTTGTAC ACACCGCCCG TCACACCATG GGAGTGGGTT GCTCCAGAAG TAGCTAACTC
1441 TAACCGCAAG GGGGACGGTT ACCACGATTC TGATTCTGGA CTGGGGTGAA CCTAGAAACA
1501 AAAAACCAAA

Figure 2: Amplified 16S rRNA gene region of *Pseduomonas aeruginosa* strain (Burns)

3.2. Multiple antibiotic resistance*s*

The main objective of this study was to investigate epidemiological data of *Pseudomonas aeruginosa* strains among clinical isolates and to determine the antimicrobial resistance pattern of bacteria against some commonly used antibiotics. (Table.1). The predominance of *Pseudomonas aeruginosa* resistance considered as serious problem in many countries (14),(15). It was also reported that *Pseudomonas aeruginosa* is one of the most common nosocomial pathogen and a leading cause of nosocomial infection(16),(17). In our present study complete resistance against Ampicillin, Carbenicillin, Cefoxitin, Clindamycin, Penicillin G and Polymyxin B were determined. High resistance displayed towards Penicillin G, Ampicillin, Clindamycin and Polymyxin B were reported in our previous studies.(13). All the strains were completely resistance to Carbenicillin and Cefoxitin the present investigation was justified with the earlier report(18),(19). High degree of Resistance to Cefadroxil and Gentamicin (90.9%), followed by Meropenem (72.7%) Kanamycin (63.6%), Amikacin, Ceftriaxone and Nitrofurantoin (54.5%) were recorded during the study were chronicled with previous report.(20). Moderate levels of resistance to Nitrofurantoin (54.5%), Nalidixic acid and Neomycin (45.4%), Netillin and Tetracycline(36.3%)were noticed during the study. High degree of resistance towards these drugs was documented in preceding investigation conducted at Nigeria. The present resistant pattern coincides with the yesteryear reports.(21)

Antibiotic resistance is a major problem in *P. aeruginosa*. The organism exhibits intrinsic resistance to several beta-lactam antibiotics and may also acquire additional resistance mechanism either due to mutational events or due to acquisition of transferable genetic elements.(22)

Upon analyzing the sensitivity rate is much lower than the resistant rate, Norfloxacin (90.9%) was the most susceptible drug followed by Ciprofloxacin (81.8%), Chloroamphenicol (63.6%) and Neomycin (54.5%) etc. Sharma *et al* reported 100% sensitivity to Ciprofloxacin (60%) in their study.(23). High degree of susceptibility towards Norfloxacin were documented in Egypt.(24). Similarly sensitivity against Neomycin also evidenced in earlier studies.(25). The antimicrobial agents are losing their efficacy because of the spread of resistant organisms due to indiscriminate use of antibiotics, lack of awareness, patient's noncompliance and unhygienic condition.(20)

Table 1: Resistance- Sensitivity rates of Pseudomonas aeruginosa to different antibiotics

S. No	Antibiotic used	% of Resistance	% of Sensitivity
01	Amikacin	54.5%	45.4%
02	Ampicillin	100%	Nil
03	Ciprofloxacin	18.1%	81.8%
04	Chloramphenicol	36.3%	63.6%
05	Ceftriaxone	54.5%	45.4%
06	Carbenicillin	100%	Nil
07	Cefoxitin	100%	Nil
08	Clindamycin	100%	Nil
09	Cefadroxil	90.9%	9.09%
10	Gentamicin	90.9%	9.09%
11	Kanamycin	36.3%	63.6%
12	Meropenem	72.7%	27.2%
13	Nalidixic Acid	45.4%	54.5%
14	Netillin	36.3%	63.6%
15	Norfloxacin	9.09%	90.9%
16	Nitrofurantoin	54.5%	45.4%
17	Neomycin	45.4%	54.5%
18	Penicillin	100%	Nil
19	Polymyxin-B	100%	Nil
20	Tetracycline	36.3%	63.6%

3.3. Multiple Antibiotic Resistance Index

MAR index was calculated according to Kurumperman (8)for all the MDR *P.aeruginosa* strains and the graph illustrating the MAR index were shown in the Fig.3. In the present study none of the strains shown similar pattern of MAR index and the index were ranged between 0.45 to 0.8 which indicated that all the strains were Multi drug resistant and these isolated strains were repeatedly exposed to the currently used antibiotics. These figures were higher than the previous work done by Osundiya et al.(26) Who reported MAR index of *Pseudomonas* were 91.2%. High prevalence of multidrug resistance indicates serious need for antibiotics surveillance program. Multiple antibiotic resistances (MAR) analysis has been used to differentiate bacteria from different sources using antibiotics that are commonly used for human therapy. Compared to other methods bacteria source tracking such as genotypic characterization, the MAR indexing method is cost effective, rapid and easy to perform. It is also simple and does not require specialized training and expensive equipment.(27)

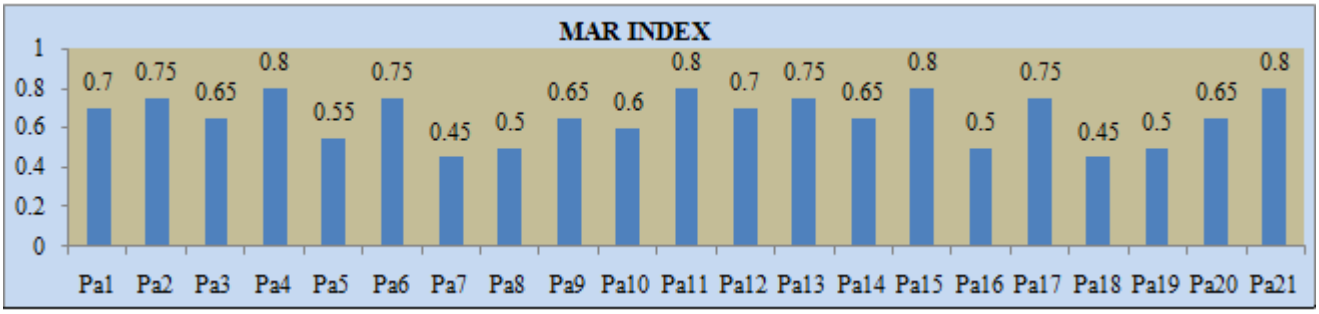

Figure 3: MAR index of *Pseudomonas aeruginosa* strains

3.4. Protease activity

63.6% of the strains were found positive for protease production activity. Attention is to be given to protease producing *P. aeruginosa* has slightly increased in the recent years as protease producing pathogen may cause tissue damage aids invasiveness and establishment of infection by overcoming host defenses and provide nutrients for bacterial proliferation.(28)

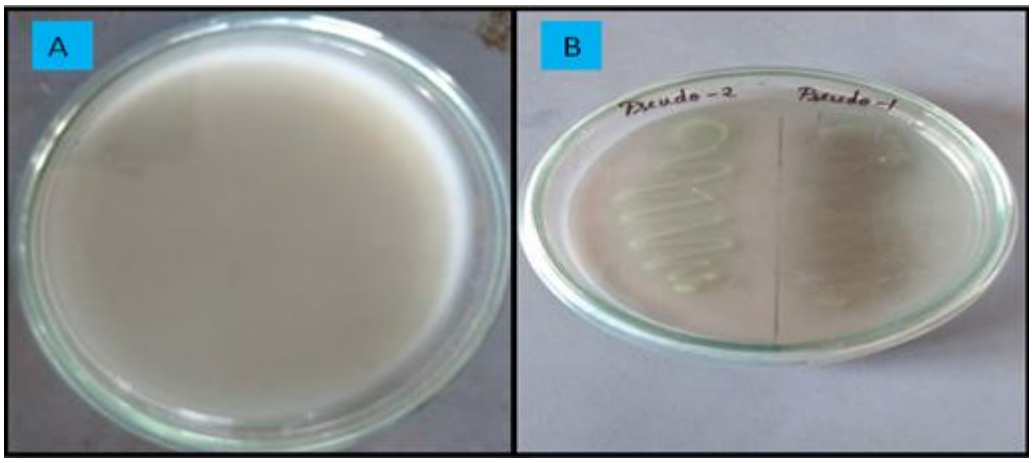

Figure 4: Protease production of *Pseudomonas aeruginosa*

3.5 Lecithinase/ Lipase Production

Microbial lipases have been characterized for their role in virulence and their potential application in biotechnology. 90% and 72.7 % of the strains were observed with Lipase and lecithinase production during the investigation. Lecithinase producing bacteria can act on lecithin and produce phosphorous and choline with precipitation of fat. *P.aeruginosa* found to produce two kinds of phospholipases or phosphor lecithinases such as PlcHR which is hemolytic and PlcN which is non-hemolytic. PlcN do not have any

pathogenic activity, whereas PlcHR act as an important virulent factor.(29),(30)

3.6 Slime Production

Slime production in bacteria play an important role in the adherence to mucous epithelia, important for colonization and virulence factor. The slime production was detected in 81.8% of the strains and it was well documented that slime productions are notoriously difficult to eradicate and are often resistant to systemic antibiotic therapy. Slime can reduce the immune response and opsonophagocytosis, thereby interfering with host defense mechanisms. The ability of an organism to produce slime is significantly associated with its capability to produce diverse illnesses. (31)

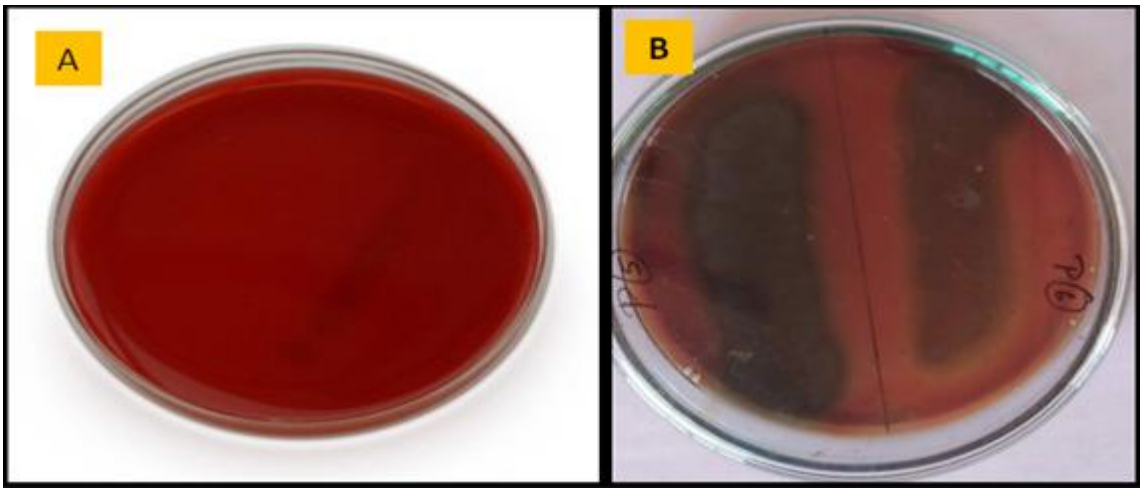

Figure 5: Slime production in *Pseudomonas aeruginosa*

4. Conclusion

On the basis of the present novel investigation it was clearly confirmed the dwelling of highly potential pathogenic multi drug resistant *Pseudomonas aeruginosa* in the clinical settings with several virulence factors in the human environment and in fact, the irrational and inappropriate use of antibiotics is responsible for the development of resistance of *Pseudomonas species* to antibiotic monotherapy. Hence, there is a need to emphasize the rational use of antimicrobials and strictly adhere to the concept of "reserve drugs" to minimize the misuse of available antimicrobials. To prevent the spread of the resistant bacteria, it is critically important to have strict antibiotic policies while surveillance programmes for multidrug resistant organisms and infection control procedures need to be implemented. It is desirable that the antibiotic susceptibility pattern of bacterial pathogens like *Pseudomonas. aeruginosa* in specialized clinical units to be continuously monitored and the results readily made available to clinicians so as to minimize the resistance and morbidity.

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