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Phenotypic Detection of Multidrug-Resistance Pseudomonas aeruginosa Isolated from Various Clinical Samples

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***Abstract*---**Introduction: β-Lactamase production is considered one of the most important resistance mechanisms among virulent Pseudomonas aeruginosa isolates. The aim of the study was to detect Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) in Pseudomonas aeruginosa obtained from various clinical samples. Materials and Methods: The study was conducted in the Department of Microbiology, Govt. Medical College, Shahdol. All clinical specimens were processed and P. aeruginosa isolates were identified by standard protocols. Antibiotic sensitivity testing for all isolates was done using Kirby-Bauer disc diffusion method. Detection of ESBL and MBL was done by Disc potentiation test. Results: Among 347(27.2%) isolates of P. aeruginosa, 284(88.2%) were MDR, 139(40%) and 73(21%) were ESBL and MBL producers respectively where 39(11.2%) isolates produced both ESBL and MBL. Conclusion: A high prevalence of MDR, ESBL, MBL positive isolates among P. aeruginosa is increasing at an alarming rate. Thus, proper antibiotic policy and measures to restrict the indiscriminative use of cephalosporins and carbapenems should be taken to minimize the emergence of this MDRPA.

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Introduction

Pseudomonas aeruginosa is a gram negative rod bacterium, which is reported to be ubiquitous in humans, animals, and the natural environment. The widespread habitat of *P. aeruginosa* makes it very difficult to control the organism in a hospital setting. It is an important opportunistic clinical pathogen, causing a variety of healthcare-associated infections, such as pneumonia, sepsis, wound infections, and urinary tract infections (1, 2). This organism is an important cause of septic mortality in burn patients (3). *P. aeruginosa* is a major cause of chronic lung infections in children and young adults with cystic fibrosis and can be especially severe in neutropenic or cancer patients (4). Infections caused by *P. aeruginosa* are often difficult to treat because of its intrinsic and acquired resistance too many commonly prescribed antimicrobial agents, eventually leading to the emergence of multidrug-resistant *P. aeruginosa* (MDRPA) strain (5). These bacteria are known to produce detect Extended Spectrum β-Lactamase (ESBL) and Metallo β-Lactamase (MBL) (6).

The large scale use of the third generation cephalosporins like cefotaxime, ceftriaxone, and ceftazidime has led to the evolution of newer betalactamases such as the ESBLs. ESBLs are plasmid mediated enzymes that hydrolyze the oxyimino *β* lactams but have no effect on the cephamycins (cefoxitin, cefotetan) and the carbapenems (imipenem). Being plasmid mediated, they can be easily transferred from one organism to another (7).Due to frequent resistance to aminoglycosides, fluoroquinolones, ureidopenicillins, and third-generation cephalosporins, carbapenems are important agents for managing such infections. Carbapenem resistance in *P. aeruginosa* is attributed to various causes such as reduced expression of outermembrane proteins and carbapenamases (8).MBL producing *P.aeruginosa* have become a growing therapeutic concern worldwide. The rapid detection of MBL positive isolates is necessary to control infection and to prevent their dissemination (9).

The methods for detection of ESBLs and MBL can be broadly divided into 2 groups: phenotypic methods that use non molecular techniques, which detect the ability of the ESBL and MBL enzymes. In genotypic methods, which use molecular techniques to detect the gene responsible for the production of the ESβL and MBL enzymes (10). Clinical diagnostic Laboratories use mostly phenotypic methods because these tests are easy to perform and are also cost effective. The aim of this study was to detection of ESBL and MBL production among strains of *P. aeruginosa* obtained from various clinical samples.

Material and Methods

Study Design and samples

A study was conducted during the period of January 2021 to May 2022 in the Department of Microbiology, Govt. Medical College, Shahdol,M.P. Clinical history of the patient was taken regarding age, sex, date of admission, immune status, antibiotic treatment, date of discharge, etc and recorded in the patient pro forma. A total of 1276 clinical samples were collected from outdoor patients and indoor patients of various wards and ICU of the Hospital depending upon the clinical diagnosis of respective patients. These included pus, urine, blood, Throat swabs, sputum, endotracheal secretions and various body fluids. All clinical samples were collected as per standard microbiological guidelines (11).

Isolation and identification of bacterial isolates

The clinical samples were inoculated on Blood Agar (BA), MacConkey Agar (MA), Nutrient Agar(NA) and was incubated at 37°C for 24 hours. Plates were observed for colony morphology and characteristic smell. Gram staining was performed for the identification of the bacteria according to standard technique using. Typical colonies of bacterial isolates were sub-cultured on peptone water and incubated at 37ºc for 4 hours. After incubation, fresh culture of test organism was inoculated into different biochemical media. *P. aeruginosa* were identified on the basis of various characteristics such as non-lactose fermenting colonies on MacConkey agar, positive catalase and oxidase test, pigment production, growth on Cetrimide agar and growth at 42° c (12).

Antibiotic Susceptibility test

Antibiotic Susceptibility testing of the isolates was performed on Muller Hinton Agar (MHA) by Kirby-Bauer disc diffusion method as per the Clinical and Laboratory Standards Institute guidelines (13). The test organism was picked up with a sterile loop, suspended in peptone water and incubated at 37ºC for 2h. The turbidity of the suspension was adjusted to 0.5 McFarland's standard (1.5 x 10^8 CFU/ml). It was then spread on the surface of Muller Hinton Agar (MHA) plate using cotton swab.The following standard antibiotic discs were placed on MHA plate: ampicillin (10 µg), amoxicillin + clav (20+10 µg), imipenem (10 µg), cefepime (30 µg), cefuroxime (30 µg), ceftazidime (30 µg), ceftazidime + clav (30+10 µg), piperacillin (100 µg), , amikacin (30 µg), gentamicin (10 µg), tobramycin (10 µg), netilimicin(10 µg), ofloxacin (10 µg), levofloxacin (5 µg), colistin (10 µg): The diameter of the zone of inhibition produced by each antibiotic disc was measured by antibiotic zone scale, recorded and the isolate were classified as resistant and sensitive on the basis of guidelines published by the CLSI. *Pseudomonas aeruginosa* isolates that showed resistance to at least one agent in three or more classes of antibiotics was titled as Multi Drug Resistant (MDR) (14) and further preserved for other analysis. *E. coli* (ATCC 25922) and *P. aeruginosa* (ATCC 27853) were used for control strains.

Phenotypic Detection of ESBL Positive Isolate

Isolates resistant to ceftazidime and/or cefepime were tested for ESBL production by disc potentiation test**.** A disc of ceftazidime (30μg) and ceftazidime + clavulanic acid (30+10 μg) was placed 20mm apart, centre to centre on Mueller-Hinton agar plate, and was incubated overnight at 37°C. A zone difference greater than or equal to 5mm around ceftazidime and ceftazidime + clavulanic acid was interpreted as ESBL positive isolate (15).

Phenotypic Detection of MBL Positive Isolates

Isolates resistant to imipenem was tested for MBL production by disc potentiation test. A disc of imipenem (10μg) and imipenem + EDTA (10μg/750 μg) was placed 20mm apart, centre to centre on Mueller Hinton agar plate, and was incubated overnight at 37°c. A zone difference greater than or equal to 7mm around imipenem and imipenem + EDTA disc was interpreted as MBL positive isolates (16). This is also called Imipenem - EDTA Combined disk synergy test. Disc potentiation test performed to check ESBL production and MBL production is shown in Figures 1 and 2.

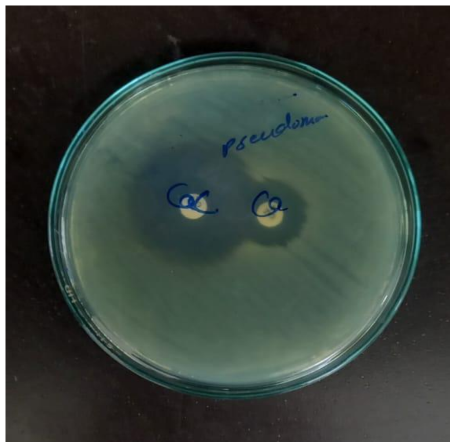

Figure 1. Disc potentiation test for detection of ESBL by using ceftazidime and cefta + clav

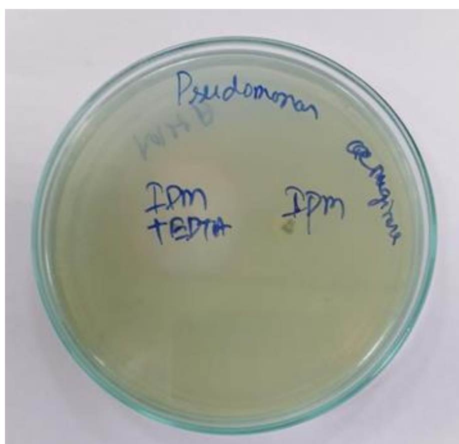

Figure 2. Disc potentiation test for Detection of MBL by using Imipenem and EDTA+imipenem

Data Management and Analysis

All the results analysed by descriptive statistics using ratios and percentages and bar graphs were prepared in Microsoft excel to represent antibiotic susceptibility, ESBL and MBL production.

Results

Out of total 1276 clinical samples processed between the time period, 347(27.2%) *Pseudomonas aeruginosa* were isolated. *P. aeruginosa* isolates were 100% sensitive to colistin, and 69.1% sensitivity was reported towards imipenem whereas piperacillin was recorded intermediate sensitivity 42.6%. Aminoglycosides antibiotics i.e gentamicin, tobramycin, netilimicin and amikacin were not given satisfactory results and their resistance were 69.5%, 62.3%, 67.8% and 64.3%. Likewise, much less sensitivity was recorded towards cephalosporins: cefuroxime, ceftazidime, ceftazidime+ clavulanic acid and cefipime i.e, 11.8%, 17.5%, 21.9% and 25.3% respectively. Antibiogram obtained for *P. aeruginosa* isolates is shown in Table 1. Out of 347 isolates of *P. aeruginosa* , 284 isolates were observed MDR which is 81.8% of total isolates. ESBL production was seen in 139 (40%) isolates, MBL production was observed 73 (21%) and both ESBL and MBL producers was 39(11.2%) as shown in Table 2. MDR *P. aeruginosa* were mainly isolated from pus (38%) followed by endotracheal aspirates (21%), sputum (20%), urine (7%), Blood (3%) and various body fluid (11%). This is shown figure 3.

Table 1
Antibiotic susceptibility pattern of *Pseudomonas aeruginosa*

Antibiotic disc(in mcg)	*Pseudomonas aeruginosa* (347)			
	Sensitive		Resistance	
	Number	Percentage	Number	Percentage
Ampicillin (10)	39	11.2%	308	88.8%
Amoxycillin+clav	58	16.7%	289	83.3%

(20+10)				
Gentamicin(10)	106	30.5%	241	69.5%
Tobramicin(10)	131	37.7%	216	62.3%
Netilmicin(10)	112	32.2%	235	67.8%
Amikacin(30)	124	35.7%	223	64.3%
Ofloxacin(10)	91	26.2%	256	73.8%
Levofloxacin(5)	114	32.8%	233	67.2%
Cefepime(30)	88	25.3%	259	74.7%
Ceftazidime(30)	61	17.5%	286	82.5%
Ceftazidime+clav (30+10)	76	21.9%	271	78.1%
Cefuroxime(30)	41	11.8%	306	88.2%
Piperacillin(100)	148	42.6%	199	57.4%
Imipenem(10)	240	69.1%	107	30.9%
Colistin(10)	347	100%	0	0.0%

Table 2
Distribution of MDR, ESBL and MBL producers

MDR	ESBL	MBL	Both ESBL & MBL
284	139	73	39

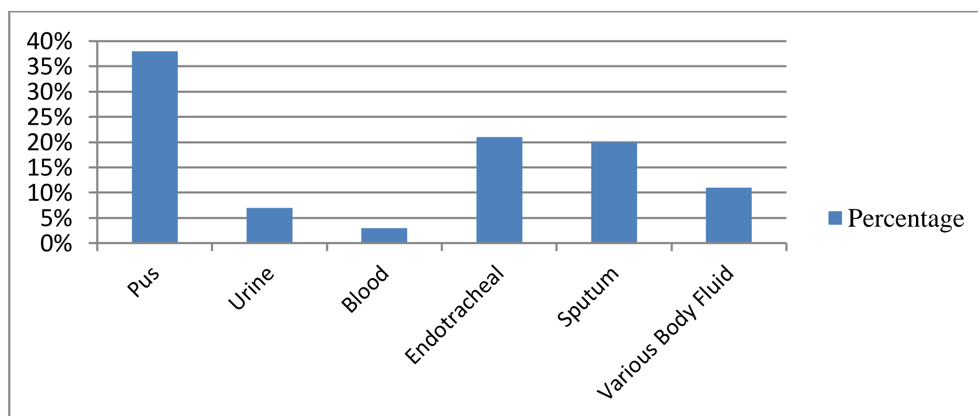

Figure 3. Percentage of MDRPA isolated from various clinical samples

Discussion

P.aeruginosa has been emerged as a significant pathogen and is the most common dreadful gram negative bacilli found in various health care associated infections all over the world due to its virulence, well known ability to resist killing by various antibiotics and disinfectants. The bacterial resistance has been increasing and this has both clinical and financial implication in therapy of infected patients. So, its identification and selection of appropriate antibiotic to initiate therapy is essential to optimizing the clinical outcome (17). The main objective of the study was to determine the antibiotic resistance mechanisms developed in *P. aeruginosa* species and its epidemiological importance. In our study, from 1276 clinical isolates, 347 *P. aeruginosa* isolates were obtained, with a prevalence rate of 27.2%. Similar prevalence rate of 32.1% and 20.3% was reported by Rajat *et al.* and Javiya *et al.* in Gujarat, India respectively(18,19). Low

prevalence of prevalence rate of 9.3% was reported by Srinivas *et al.* in Andhra Pradesh, India (20). However, very low prevalence of 2.1% was obtained by Okon *et al.* in Northeastern Nigeria (21). This varied prevalence of *P. aeruginosa* in different places may attributed to the type of clinical specimens received for examination, studied population, type of hospitals and geographical locations.

Our study revealed, 284 (88.2%) *P. aeruginosa* isolates were MDR, Similar MDR rate of 84.7% was reported by Dash *et al* in South Odisha (22), 71% was reported by Mohanasoundaram *et al.* in Tamil Nadu, India(23) and Gill *et al.* in Rawalpindi, Pakistan had studied 180 *P. aeruginosa* isolates, which were obtained from different clinical specimens. Out of these, 22.7% were MDR (24). The high percentage of MDR strains were isolated from different clinical specimens is worrisome for the future. Accurate laboratory detection, control of patient-to-patient transmission and prudent use of antibiotics are cornerstones in containment of drug resistant. In our study, majority of MDR strains of *P. aeruginosa* isolated from pus samples (38%) followed by endotracheal aspirates (21%), sputum (20%), urine (7%), Blood (3%) and various body fluid (11%). This is due to long hospital stay of the patient.

In this present study, among the β lactam drugs; ceftazidime (82.5%), Cefuroxime (88.2%), Cefepime (74.7%) and Piperacillin (57.4%) showed the highest resistance which was similar to K.M Mohanasundaram et al., (84.6%) (25), Yapar et al., (84%) (26) and Ibukun et al., (79.4%) (27), reported more resistance against ceftazidime in their study. While Diwivedi et al., (63%) (28) & Arya et al., (55.4%) (29) were reported slightly lower ceftazidime resistance. Indiscriminate use of 3rd generation cephalosporin as broad spectrum empirical therapy and the secretion of ESBL enzymes mediate the resistance by hydrolysis of β-lactam ring of β-lactam antibiotics. Other mechanisms of drug resistance to β-lactam group of antibiotics are loss of outer membrane protein, production of class C AmpC β-lactamase and altered target sites.

ESBLs occur rarely in non-fermenters. Our study showed 139 (40%) isolates were ESBL producer. 42.30% ESBL producer were observed in the study of Varun Goel et al., (30). Lower ESBL producer were seen in the studies by Prashant et al., (31) and Agarwal et al., (32) which were 22.22% & 20.27% respectively. Whereas, Uma et al., observed high percentage of isolates (77.3%) to be ESBL producer.(33) Though imipenem was found unaffected by the action of the enzymes in many studies, MBL production in our study was 21% which is are similar to Upadhyay et al. (34)who had reported MBL production in *P. aeruginosa* as 20.8%. Sadhna et al. (16), Madhu et al. (35) and Behera et al. (36) have reported MBL production in *P. aeruginosa* as 41.0%, 61.5%, and 69.5%, respectively, which is higher as compared to this study. Aggarwal et al. (37) had reported MBL production to be 11.4% which is lesser as compared to this study. The percentage variation in the resistance mechanism could be due to the study environment where the study was done. These carbapenem agents may be of benefit in the treatment of ESBL infection; however, indiscriminate use of these agents may promote increased resistance to carbapenem.

Conclusion

The present study shows that ESBL and MBL production in *P. aeruginosa* is on the rise across the globe, thus making these infections difficult to treat. Early detection of ESBL and MBL production would be important for the reduction of mortality rate and spread of multidrug resistant organisms. These identification tests are simple, easy to perform and can be done along with antibiotic susceptibility testing. Moreover to avoid the resistance, antibiotic should be used judiciously and empirical antibiotic therapy should be determined for each hospital according to the antimicrobial surveillance of that center. Therefore, state and national level antimicrobial policy and guidelines should be introduced to preserve the effectiveness of antibiotics and for better management of the patient.

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