Original Article

DOI: 10.7860/JCDR/2022/55865.16717

Microbiology Section

Diagnostic Characterisation of Various Phenotypic Methods for Class-A Extended Spectrum of β-Lactamase among Multidrug Resistant *Pseudomonas aeruginosa* Isolated from Diabetic Patients

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ABSTRACT

Introduction: *Pseudomonas aeruginosa* often causes nosocomial infection, especially in high risk group patients like diabetics. It shows a high degree of resistance to broad spectrum of antibiotics due to its high adoptability in hospital settings, so their infections are difficult to treat. Extended Spectrum β-Lactamase (ESBL) enzymes confer resistance to most of the β-lactam antibiotics, including penicillin, cephalosporins and monobactams.

Aim: To identify ESBL producing strains among Multidrug Resistant (MDR) *Pseudomonas aeruginosa* isolated from diabetes patients using various phenotypic methods with their performance characteristics.

Materials and Methods: An observational descriptive cross-sectional study was conducted in the Department of Microbiology at Sawai Man Singh Medical College, Jaipur, Rajasthan, India, from April 2017 to March 2019. Various clinical samples received from diabetic patients were cultured and *P. aeruginosa* were identified as per standard protocol. Antimicrobial susceptibility testing was done according to Clinical and Laboratory Standards Institute (CLSI) guidelines. ESBL producing MDR *P. aeruginosa* was detected by using standard Epsilometer test (E test), Phenotypic Confirmatory Disc Diffusion Test (PCDDT) and Double Disc Synergy Test (DDST). Test characteristics sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and accuracy were calculated. Kappa coefficient was used to show diagnostic agreement between the tests.

Results: A total 430 clinical samples were received from diabetic patients, out of 430 samples, 72 (16.7%) *P. aeruginosa* were recovered. Multidrug resistance was exhibited by 34 isolates out of 72 *P. aeruginosa* strains. Out of 34 MDR strains, 10 (29.4%) were found ESBL producers by PCDDT, 9 (26.5%) by DDST while 10 (29.4%) were found positive by E test. Sensitivity, specificity and accuracy for PCDDT was found 90%, 95.8% and 94.1% respectively and 'almost perfect agreement' was observed with E test.

Conclusion: Magnitude of multidrug resistant strains was found 47.22% among *P. aeruginosa* isolated from diabetic patients which is an alarming sign. The ESBLs were found in 29.4% isolates. So, screening of ESBLs with the use of simple test like PCDDT in *Pseudomonas aeruginosa* will direct us for treatment option of suitable antibiotic regimens in diabetic patients and to prevent the spread of drug resistant organisms in hospitals.

Keywords: Antibiotic resistance, Diabetes mellitus, Diagnostic agreement, Sensitivity, Specificity

INTRODUCTION

Diabetes mellitus is a chronic disorder that is a major public health problem. According to the International Diabetes Federation, in India 77 million adults have diabetes in 2019 and this number is expected to almost double to 134 million by 2045 [1]. Diabetics are more susceptible to infections due to increased glucose levels and suppressed immune response as well as the neuropathy and decreased blood flow to extremities that lead to slow-healing wounds [2]. Foot ulceration frequently develops during the course of diabetes [3]. A quarter of diabetic patients will develop diabetic foot ulcers, of which 50% become infected which have need of hospitalisation whereas 20% necessitate amputation of their foot or leg [4].

Among diabetic patients, *Pseudomonas aeruginosa* is frequently associated with infections [5]. The pathogenesis of this organism is based on its ability to produce a variety of toxins and proteases to resist phagocytosis [6]. Previous injudicious use of antibiotics frequently leads to multidrug resistant *Pseudomonas aeruginosa* which makes it clinically more important as they are difficult to treat. It has an intrinsic resistance to different classes of antimicrobial agents, along with the ability to acquire resistance. β-lactam group of antibiotics (penicillins, cephalosporins, monobactam and carbapenems) are used for treatment of *P. aeruginosa* infections. Development of resistance to β-lactam antibiotics is mainly due to β-lactamase enzyme production which is either plasmid or chromosomally mediated. Extended Spectrum β-Lactamase (ESBL) enzymes confer resistance to most of the β-lactam antibiotics, including penicillins, cephalosporins and monobactam [7]. These ESBLs were found among members of *Enterobacteriaceae* group but now they have also been found in *Pseudomonas aeruginosa* and other strains due to horizontal gene spread [8].

Because of increased incidence of ESBL producing strains among clinical isolates, there are limited treatment options. So, this is important to isolate and identify the resistant strains therefore appropriate antibiotic therapy can be given and further development of antibiotic resistance can be hindered.

Knowledge of the prevalence of ESBL among *P. aeruginosa* isolates is limited as compared to *Enterobacteriaceae* [9]. European Committee on Antimicrobial Susceptibility Testing (EUCAST) had recommended the phenotypic tests Phenotypic Confirmatory Disc Diffusion Test (PCDDT) and Double Disc Synergy Test (DDST) for ESBL detection in *Enterobacteriaceae* and not recommended any

test for *P. aeruginosa* strains [9]. Besides this limited data are available on performance parameter of various phenotypic tests for ESBLs, has led to the search for a correct and cost efficient test to detect the presence of ESBL in *P. aeruginosa* [10].

This study was aimed to determine the antimicrobial resistance patterns among *P. aeruginosa* isolated from diabetes patients and to identify ESBL producing strains among Multidrug Resistant (MDR) *Pseudomonas aeruginosa* using various phenotypic methods with their performance parameters. So that it will help to identify accurate and less cumbersome method for ESBL detection in *P. aeruginosa* and to prevent complications in diabetic patients.

MATERIALS AND METHODS

This was an observational descriptive, cross-sectional study conducted from April 2017 to March 2019, in the Department of Microbiology at Sawai Man Singh Medical College, Jaipur, Rajasthan, India. Ethical approval was taken from the Ethical Committee of SMS Medical College and attached Hospitals, Jaipur (No.2266 MC/EC/2016 dated 29.03.2016). Informed consent was taken from patients before collecting samples.

Sample size: Prevalence of MDR *P. aeruginosa* among diabetic patients was found to be 6.7% [11]. Considering 95% confidence interval, 80% power of the study and 2.5% absolute precision, the sample size was calculated as 385. Including 10% wastage factor, the sample size was 424. Hence final sample size considered for the study was 430.

Inclusion criteria: Samples received from diabetic patients were included in the study.

Exclusion criteria: Duplicate clinical samples and leaked samples were excluded from the study.

A total of 430 clinical samples such as pus, urine, blood, burn swab, tracheal swab, sputum received from diabetic patients were cultured on MacConkey agar and *P. aeruginosa* isolates were identified by colony morphology, pyocyanin pigment, gram staining, catalase test and oxidase test [12]. Antimicrobial sensitivity testing was done by the Kirby-Bauer disc diffusion method as per Clinical and Laboratory Standards Institute (CLSI) guidelines [13]. Isolates which were found resistant to atleast one agent in three or more antimicrobial group were identified as multidrug resistant *P. aeruginosa* [14]. The ESBL detection was done on these isolates by different phenotypic tests (PCDDT, DDST) and compared with standard E test.

Phenotypic Methods for Detection of ESBL

1. Phenotypic Confirmatory Disc Diffusion Test (PCDDT): This test was performed as per standard guidelines. Two antibiotic discs were placed at a distance of 20 mm on Muller Hinton Agar inoculated with the test organism, one disc containing ceftazidime (30 µg) alone and other disc containing ceftazidime clavulanic acid in combination (30/10 µg). After overnight incubation at 37°C, increase of 5 mm or more in the zone of inhibition of the combination discs in comparison to the ceftazidime disc alone was considered to be an ESBL producer [15].

2. Double Disk Synergy Test (DDST): Muller Hinton Agar plate was inoculated with test organism. One disc of Amoxicillin clavulanate (20/10 µg) was placed in the centre of the plate and other disc containing ceftazidime 30 µg was placed at a distance of 30 mm from amoxicillin clavulanate disc. After overnight incubation at 37°C, enhancement in zone of inhibition of ceftazidime toward the augmentin disc was interpreted as ESBL producing organism [16].

3. Epsilometer (E test): An overnight broth culture of the test organism of 0.5 McFarland standardised was inoculated on a Mueller Hinton agar plate then E test strip was placed on the dried surface of the agar plate. One end of the E strip carries a stable concentration gradient of ceftazidime, while other end carries gradient of ceftazidime+clavulanic acid (4 µg/mL). After overnight incubation at 37°C, Minimum Inhibitory Concentrations (MICs) was interpreted as the point of intersection of the inhibition ellipse with E test strip edge. Ratio of minimum inhibitory concentration of ceftazidime and ceftazidime clavulanic acid ≥8 was considered positive result [15].

STATISTICAL ANALYSIS

Data was entered in Microsoft Excel (2010) and appropriate statistical calculations were done. Test characteristics sensitivity, specificity, Positive Predictive Value (PPV), Negative Predictive Value (NPV) and accuracy were calculated. Kappa coefficient was used to show diagnostic agreement between the tests. As described by Landis JR and Koch GG, in 1977, according to value of kappa coefficients, agreement was interpreted as almost perfect (κ value 0.81-1.00), substantial (κ value 0.61-0.80), moderate (κ value 0.61-0.80), fair (κ value 0.21-0.40) and slight agreement (κ value 0.01-0.20) [17].

RESULTS

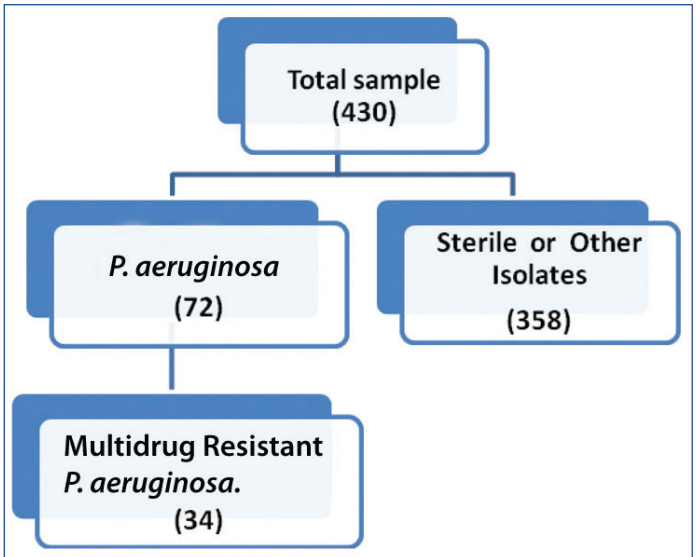
A total of 430 clinical samples received from diabetic patients were included in this study with 297 (69.1%) male and 133 (30.9%) female patients. Majority of the patients (43.7%) were from age group 41-60 years followed by above 60 years (32.8%), 21-40 years (19.3%) and below 20 years (4.2%). Total 80.7% diabetic patients were from Inpatient Departments (IPD) while 19.3% were from Outpatient Department (OPD) [Table/Fig-1].

Variable	Diabetic patients (n, %)
Gender	
Male	297 (69.1%)
Female	133 (30.9%)
Age (years)	
Below 20	18 (4.2%)
21-40	83 (19.3%)
41-60	188 (43.7%)
Above 60 years	141 (32.8%)
Department wise distribution	
IPD patients	347 (80.7%)
OPD patients	83 (19.3%)

[Table/Fig-1]: Age, gender and department wise distribution of diabetic patients (N=430).

Out of 430 clinical samples, 72 (16.7%) isolates were recovered as *P. aeruginosa*. On antimicrobial susceptibility testing, 34 (47.2%) isolates were identified as MDR *P. aeruginosa* with 24 (70.6%) males and 10 (29.4%) females and male to female ratio was 2.4:1 [Table/Fig-2]. Maximum 16 (47.1%) MDR *P. aeruginosa* were isolated from pus samples of diabetic patients followed by urine 9 (26.5%), burn swab 4 (11.8%), throat swab 2 (5.9%), sputum 2 (5.9%) and blood 1 (2.9%). Total 29 (85.3%) MDR *P. aeruginosa* were isolated from IPD diabetic patients while 5 (14.7%) were from OPD [Table/Fig-3]. Highest resistance was observed towards piperacillin 29 (85.3%) followed by aztreonam 27 (79.4%), tobramycin 25 (73.5%), ciprofloxacin 23 (67.6%), gentamycin 22 (64.7%), ceftazidime 21 (61.8%), piperacillin/tazobactam 16 (47.1%), imipenem 14 (41.2%) and meropenem 9 (26.5%). Polymyxin and colistin was observed 100% sensitive to MDR *P. aeruginosa* in diabetic patients [Table/Fig-4].

All MDR *P. aeruginosa* isolates were further evaluated for detection of ESBL resistance mechanism. Out of 34 isolates, 10 (29.4%) isolates were detected as confirmed ESBL producers by Epsilometer test. Total 10 (29.4%) isolates were found ESBL



[Table/Fig-2]: MDR *Pseudomonas aeruginosa* isolated from clinical samples received from diabetic patients (N=430).

Variables	MDR *P. aeruginosa* (n, %)
Clinical sample wise distribution	
Pus	16 (47.1%)
Urine	9 (26.5%)
Burn swab	4 (11.8%)
Throat swab	2 (5.9%)
Sputum	2 (5.9%)
Blood	1 (2.9%)
Department wise distribution	
IPD patients	29 (85.3%)
OPD patients	5 (14.7%)

[Table/Fig-3]: Clinical sample and department wise distribution of MDR *P. aeruginosa* isolates (N=34).

Antibiotic group	Antibiotic agent	MDR *P. aeruginosa* (n, %)
Penicillin	Piperacillin (100 µg)	29 (85.3%)
β-Lactamase inhibiter	Piperacillin/Tazobactam (100/10 µg)	16 (47.1%)
Aminoglycosides	Gentamicin (10 µg)	22 (64.7%)
	Tobramycin (10 µg)	25 (73.5%)
Quinolones	Ciprofloxacin (5 µg)	23 (67.6%)
	Norfloxacin (10 µg) (tested only for Urine N=9)	4 (44.4%)
Monobactam	Aztreonam (30 µg)	27 (79.4%)
Cephalosporins	Ceftazidime (30 µg)	21 (61.8%)
Carbapenems	Imipenem (10 µg)	14 (41.2%)
	Meropenem (10 µg)	9 (26.5%)
Non ribosomal peptides	Polymyxin (300 U)	0
	Colistin (10 µg)	0

[Table/Fig-4]: Antibiotic resistance pattern of MDR *P. aeruginosa* isolates (N=34).

producer by PCDDT among them nine were found true positive and one was false positive in concordance with standard E test while by DDST 9 (26.5%) isolates were found ESBL producer among them, seven isolates were true positive and two were false positive [Table/Fig-5]. Sensitivity, specificity and accuracy for PCDDT was found 90%, 95.8% and 94.1% respectively while for DDST sensitivity 70%, specificity 91.7% and accuracy 85.3% was observed [Table/Fig-6].

When the diagnostic agreements were evaluated between the phenotypic tests, 'almost perfect agreement' was observed between PCDDT and E test with kappa coefficient of 0.858 while there was 'substantial agreement' between DDST and E test (κ=0.635) and PCDDT and DDST (κ=0.781) [Table/Fig-7].

Phenotypic tests for ESBL		E test for ESBL		
		Positive	Negative	Total
PCDDT	Positive	9	1	10
	Negative	1	23	24
	Total	10	24	34
DDST	Positive	7	2	9
	Negative	3	22	25
	Total	10	24	34

[Table/Fig-5]: Comparison of phenotypic tests (PCDDT and DDST) with E Test for ESBL production (N=34).
PCDDT: Phenotypic confirmatory disc diffusion test; DDST: Double disc synergy test; ESBL: Extended spectrum β-lactamase

Test name	Sensitivity	Specificity	PPV	NPV	Accuracy
PCDDT vs E test	90%	95.8%	90%	95.8%	94.1%
DDST vs E test	70%	91.7%	77.8%	88%	85.3%

[Table/Fig-6]: Test characteristics of phenotypic tests (PCDDT and DDST) for ESBL production.

Test name	Kappa coefficient	95% Confidence interval	Agreement	Interpretation
PCDDT vs E test	0.858	0.668 to 1.000	94.1%	Almost perfect agreement
DDST vs E test	0.635	0.345 to 0.925	85.3%	Substantial agreement
PCDDT vs DDST	0.781	0.546 to 1.000	91.2%	Substantial agreement

[Table/Fig-7]: Diagnostic agreement between phenotypic tests PCDDT, DDST and E Test for ESBL detection.

DISCUSSION

Pseudomonas aeruginosa is one of the major causative agents of infections in diabetic patients due to weakening of immune status of host *P. aeruginosa* had a tendency for development of drug resistance due to its high adaptability in hospital environment. It shows intrinsic and acquired resistance to many antimicrobials. Currently, the effectiveness of many antibiotics has threatened due to emergence of antibiotic resistance. In the present study, magnitude of *P. aeruginosa* was found to be 16.7% in samples received from diabetic patients, which was in concordance to previous studies done by Dhanasekaran G et al., (18.79%) and Saha AK et al., (17.9%) [5,18]. Whereas Sivanmaliappan TS and Sevanan M, reported (70%) magnitude of Pseudomonas [11]. Most of infections with Pseudomonas species occur in immuno-compromised hosts hence diabetic patients are highly vulnerable to such type of infections.

On antibiotic sensitivity testing, multidrug resistance was exhibited by 47.2% (34/72) isolates of *P. aeruginosa* with predominance in males (male to female ratio of 2.4:1). Same findings (male to female ratio of 1.64:1) were reported by Saha AK et al., [18]. This may be because males are more exposed to the outer environment as compared to females.

The number of *P. aeruginosa* isolated differs from sample to sample in various studies. Diabetics are more prone to surgical site infection and foot ulceration with drug resistant organism, so, in the current study, 47.1% (16/34) MDR *P. aeruginosa* were isolated from pus samples of diabetic patients. Due to its high adaptability in hospital environment, it was isolated more from IPD patients (85.3%) in compare to outdoor patients.

In the present study, 29 (85.3%) MDR *Pseudomonas aeruginosa* showed resistance to piperacillin and 21 (61.8%) showed resistance to ceftazidime. Our findings were close to study of Sivanmaliappan TS and Sevanan M, who reported 83.3% resistance to piperacillin and 66.6% resistance to ceftazidime

[11]. Aztreonam has intermediate activity against aerobic gram negative infections and used as an alternative to aminoglycosides or third generation cephalosporin. It was found effective only in 20.6% isolates in the current study. Sachdeva R et al., (84.7%) and Andréa L et al., (95%) have shown high resistance for aztreonam against *P. aeruginosa* [19,20]. In carbapenem group of antibiotics imipenem was found more resistant 14 (41.2%) than meropenem 9 (26.5%). The current study findings were in concordance with Gladstone P et al., who reported 42.8% resistance to imipenem [21]. Concurrent administration of β-lactamase inhibitors expands the spectrum of activity of penicillin, it supports the efficacy of piperacillin/tazobactam, was found 56.9% effective in the present study. Polymyxin and colistin are the last line of drugs used against *P. aeruginosa* infections. In the current study those were found 100% effective. Possible reasons for different resistance rates in the different studies were not understood, but it may be dependent on prescription habits and the amount of antibiotics usage in different geographical settings.

In the current study ESBL production in MDR *P. aeruginosa* was found 29.4% which was in accordance with the study done by Umadevi S et al., (19.4%) and Omer THS et al., (17.6%) [22,23]. While in contrast to the present study results, Bajpai V et al., and Sreeshma P et al., reported high rate of ESBL production among *P. aeruginosa* isolates [24,25]. ESBLs are rising in *P. aeruginosa* which was common among Enterobacteriaceae members. This may be due to horizontal transfer of gene responsible for ESBL production among Pseudomonas strains [8].

For ESBL detection, E test was used as a gold standard test which is a quantitative and sensitive test. This test is easy to perform but due to cost constraints it is not possible to perform practically. Other phenotypic tests PCDDT and DDST were also performed and compared with E test and found that PCDDT had higher sensitivity 90% and specificity 95.8% then DDST (sensitivity 70% and specificity 91.7%). These findings suggest that PCDDT was almost equally sensitive to E test. Diagnostic agreement showing 'almost perfect agreement' for PCDDT vs E test (Kappa coefficient 0.858 with confidence interval 0.668 to 1.000) which further shows that both test can be used interchangeably. DDST had shown lesser sensitivity and specificity and also shown comparatively weaker diagnostic agreement with both PCDDT and E test. The interpretation of results of DDST is also subjective.

Limitation(s)

This study was done in limited resources with time constraint, so smaller samples were included in the study. Although E test and others were used but there is no standard test for ESBL detection as it is less common in Pseudomonas. Genotypic characterisation of genes responsible for resistance was not done which may provide better picture of the situation. The present study used only ceftazidime/clavulanic acid antibiotic disc while antibiotic discs with other β-lactamase inhibitors such as piperacillin/tazobactam, cefoperazone/sulbactam may also be used for ESBL detection.

CONCLUSION(S)

In diabetic patients, high magnitude of MDR *Pseudomonas aeruginosa* as well as higher resistance for antipseudomonal antibiotics even for carbapenems were observed which is an alarming sign. Substantial amount of ESBL was detected which is not common among Pseudomonas. So, with routine antibiotic sensitivity testing is very important in order to provide the updated information about current activity of commonly used antipseudomonal drugs. Early detection of these β-lactamase has necessitates for preventing further spread of resistance. Phenotypic confirmatory disc diffusion test is found highly sensitive, specific test for screening of ESBLs, so it should be implemented in routine practice to guide therapeutic alternative of appropriate antibiotic regimen to the patient and to prevent further spread of ESBL positive organisms. A multicentre study with larger sample size and including genotypic characterisation of genes responsible for resistance is recommended to get a clear picture of the situation.

Acknowledgement

Authors are thankful to Head of the Department of Microbiology for permitting us to conduct the study and laboratory staff for their technical assistance.

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PLAGIARISM CHECKING METHODS: [Jain H et al.]
- Plagiarism X-checker: Mar 02, 2022
- Manual Googling: May 09, 2022
- iThenticate Software: May 10, 2022 (14%)

ETYMOLOGY: Author Origin

AUTHOR DECLARATION:
- Financial or Other Competing Interests: None
- Was Ethics Committee Approval obtained for this study? Yes
- Was informed consent obtained from the subjects involved in the study? Yes
- For any images presented appropriate consent has been obtained from the subjects. NA

Date of Submission: **Feb 24, 2022**
Date of Peer Review: **Apr 13, 2022**
Date of Acceptance: **May 14, 2022**
Date of Publishing: **Aug 01, 2022**