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PHENOTYPIC DETECTION OF ESBL AND AmpC β-LACTAMASES AMONG CLINICAL ISOLATES OF *PSEUDOMONAS AERUGINOSA* IN A TERTIARY CARE HOSPITAL IN CHENNAI

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ABSTRACT

The aim of this study was to carry out phenotypic detection of ESBL and AmpC producing *Pseudomonas aeruginosa* by carrying out the following *in vitro* antimicrobial assays-screening of isolates for ESBL production, Confirmation of ESBL production by Double Disc Synergy Test (DDST), Modified Double Disc Synergy Test (MDDST), Screening of isolates for AmpC production, Disc antagonism test and ESBL and AmpC E – test .The results of the study showed that (50%) isolates were ESBL positive by (DDST) and (52.4%) by (MDDST). (61.9%) isolates were positive for AmpC production by screening method. (61.9%) and (57.1%) isolates were positive for AmpC by disc antagonism method and (37.3%) were found positive for both ESBL and AmpC production by ESBL and AmpC E-test. Hence the present investigation was carried out to find the frequency of ESBL and AmpC production in clinical samples of *Pseudomonas aeruginosa* isolates.

Keywords: Double Disc Synergy Test (DDST), Modified Double Disc Synergy Test (MDDST), Disc antagonism test, ESBL, AmpC, E – test, Multidrug resistance, *Pseudomonas aeruginosa*

INTRODUCTION

The misuse and overuse of antibiotics has increased the frequency of antimicrobial resistance globally and multi-drug resistant (MDR) bacteria are now recognized as a major cause of hospital-acquired infections (HAI) **[1]**. *Pseudomonas aeruginosa* (*P. aeruginosa*) is an aerobic, gram-negative bacilli that can be found ubiquitously in soil, plants, hospital, reservoirs of water, including showers, sinks, and toilet water **[2]**. A recent report from the National Healthcare Safety Network, summarizing the health care-associated infections from 4515 US hospitals from 2011 to 2014, reported to be the sixth most common nosocomial pathogen overall and second most common pathogen in ventilator-associated pneumonia (VAP) in US hospitals**[3]**. *Pseudomonas aeruginosa* is leading cause of opportunistic nosocomial infections. It is responsible for 10% of all hospital-acquired infections **[4]**. Infections caused by *P. aeruginosa* are often severe, life-threatening and are difficult to treat because of limited susceptibility to antimicrobial agents and high frequency of emergence of antibiotic resistance during therapy **[5]**. ESBL-producing gram-negative bacteria become new alarm for emerging infectious pathogens to human and animals. ESBL-bacteria are capable of efficiently hydrolyzing many beta-lactam antibiotics, including cephalosporins and monobactams **[6]**, **[7]**. The antibiotic resistance mechanisms include the acquisition of extended-spectrum β-lactamases, carbapenemases, aminoglycoside-modifying enzymes and 16S ribosomal ribonucleic acid methylases. Mutational changes causing the up-regulation of multidrug efflux pumps, repression of ampC, modification of antimicrobial targets and changes in the outer membrane permeability barrier **[8]**. An increase in strains resistant to the third- and fourth-generation cephalosporins and carbapenems has become a serious clinical problem worldwide. The primary cause of cephalosporin resistance in *P. aeruginosa* isolates is the overexpression of the chromosomal AmpC enzyme (mainly resistant to ceftazidime) and the production of the metallo-β-lactamases, MBLs (resistant to cephalosporins and carbapenems) **[9]**, **[10]**. Therefore, there is a need of area-specific monitoring studies based on *P. aeruginosa* resistance patterns and the data generated would help clinicians and policy makers to provide correct treatment for patients. Hence, the present study was carried out to find the prevalence of ESBL and AmpC β-Lactamases among clinical isolates of *Pseudomonas aeruginosa* in a tertiary care hospital in Chennai.

MATERIALS AND METHODS

Clinical isolates

This study was conducted in a tertiary care hospital in Chennai from April 2017 to April 2018. The bacteria *Pseudomonas aeruginosa* was isolated from various clinical samples. Totally, 126 isolates were evaluated for the ESBL and AmpC β-Lactamases production. The isolates were identified by standard laboratory techniques. Subculturing was done on regular basis in order to maintain the fresh cultures for the experiment. *Pseudomonas aeruginosa* ATCC 27853 was included as quality control strain **[11]**. The isolates were confirmed by Grams staining, biochemical tests, pigment production and growth at 42°C **[11]**. Antibiotic susceptibility testing was done on Muller–Hinton agar by Kirby–Bauer disc-diffusion method and the results interpreted as per the CLSI **[12]**. Screening of ESBL **[13]**, Confirmation of ESBL production by Double Disk Synergy Test (DDST) **[14]**, Modified Double Disc Synergy Test (MDDST) **[13]**, **[15]**, Screening of isolates for AmpC production **[16]**, Disc antagonism test **[17]** ESBL and AmpC E-test **[18]** were carried out.

Detection of ESBLs

Screening of ESBLs: All the strains which showed a diameter of less than 27mm for cefotaxime and less than 25mm for ceftriaxone, were selected for checking the ESBL production **[13]**.

Confirmation of ESBL production by Double Disk Synergy Test (DDST)

ESBL production was confirmed by the double disk synergy test (DDST) method. DDST was performed as a standard disk diffusion assay on muller hinton agar plates. Standardized bacteria suspension was aseptically swabbed on the muller hinton agar plates. Amoxycillin-clavulanic acid disc (20/10 µg) was placed at the centre of the plate, and cefotaxime (30 µg) and ceftazidime (30 µg) discs were each placed at a distance of 15 mm (centre to centre) from the amoxycillin-clavulanic acid disc. The plates were incubated at 37 ^{0}C for 18-24 hrs. ESBL production was confirmed phenotypically when a difference of ≥5 mm increase in the inhibition zone diameter for the zones of inhibition of the cephalosporins (cefotaxime and ceftazidime) tested alone and in combination with amoxicillin-clavulanic acid or key hole formation was observed **[14]**.

Modified Double Disc Synergy Test (MDDST)

The ESBL production was tested by the Modified Double Disc Synergy Test (MDDST) by using a disc of Amoxicillin-clavulanate (20/10 µg) along with cefpodoxime, ceftazidime, aztreonam, cefotaxime and ceftriaxone. A lawn culture of the organisms was made on a Mueller-Hinton agar plate, as was recommended by

CLSI **[13]**, **[15]**. A disc which contained Amoxicillin-clavulanate (20/10 µg) was placed in the centre of the plate. The other discs were placed 15mm apart respectively, centre to centre to that of the amoxicillin-clavulanate disc **[10]**. Any distortion or increase in the zone towards the disc of amoxicillin-clavulanate was considered as positive for the ESBL production. *Klebsiella pneumoniae* 700603 was used as a control strain for a positive ESBL production and *Escherichia coli* 25922 was used as a negative control for the ESBL production.

Screening of isolates for AmpC production: *P. aeruginosa* isolates were screened for AmpC β-lactamases by standard disc diffusion breakpoint for cefoxitin. Isolates with zone diameter less than 18 mm for cefoxitin were considered as probable AmpC producers which were further confirmed by other methods **[16]**.

Confirmatory methods

Disc Antagonism Test: Ceftazidime disc (30µg) or cefotaxime disc (30µg) was placed at a distance of 20mm from cefoxitin disc (30 µg) on a MHA plate inoculated with test organism as per CLSI guidelines. Isolates showing blunting of ceftazidime or cefotaxime zone of inhibition adjacent to cefoxitin disc were screened positive for AmpC β-lactamase production. Also strains showing reduced susceptibility to ceftazidime or cefotaxime and cefoxitin disc were considered positive for AmpC β-lactamase production **[17]**.

ESBL and AmpC E-test: ESBL and AmpC detection Ezy MIC Strip (MIX+/MIX)- The test strains were inoculated on to mullerhinton agar. The E-test strip is coated with mixture of 4 different antibiotics with and without clavulanic acid in a concentration gradient manner. The upper half has ceftazidime, cefotaxime, cefepime and cloxacillin (Mixture) + clavulanic acid with highest concentration tapering downwards, whereas lower half is similarly coated with ceftazidime, cefotaxime, cefepime and cloxacillin (Mixture) in a concentration gradient in reverse direction. The results were interpreted as per recommendations of the manufacturers as follows: if the value of the ratio between MIC for MIX and MIC for MIX+ was equal to or more than 8, the isolate was considered ESBL and AmpC producer **[18]**.

RESULTS

Clinical isolates: A total of 126 *Pseudomonas aeruginosa* clinical isolates were collected from tertiary care hospital in Chennai and were screened for the ESBL and AmpC β-Lactamases production.

Detection of ESBLs

Confirmation of ESBL production by Double Disc Synergy Test (DDST) and Modified Double Disc Synergy Test (MDDST): The results of Double Disc

Synergy Test (DDST) and Modified Double Disc Synergy Test (MDDST) are presented in **(Table 1)**. Total of 63 (50%) and 66 (52.4%) isolates out of 126 *Pseudomonas aeruginosa* isolates were found positive for ESBL production by (DDST) and (MDDST) respectively **(Table 1, Figure 1 & 2)**.

Screening of isolates for AmpC production: The results of screening of isolates for AmpC production are presented in **(Table 2)**. 78 (61.9%) isolates were found to be cefoxitin resistant which was considered as probable AmpC producers **(Table 2, Figure 3)**.

Confirmatory method: Disc Antagonism Test- The results of disc antagonism test by ceftazidime- cefoxitin and cefotaxime-cefoxitin disc for confirmation of AmpC production are presented in **(Table 2)**. 78 (61.9%) and 72 (57.1%) isolates showed blunting of ceftazidime and cefotaxime zone of inhibition adjacent to cefoxitin disc which were screened positive for AmpC β-lactamase production **(Table 2, Figure 3 & 4)**.

ESBL and AmpC E-test: The results of ESBL and AmpC E-test are presented in **(Table 3)**. Total 47 (37.3%) isolates out of 126 isolates were found to be positive for ESBL and AmpC production by E-test **(Table 3, Figure 5)**.

Table 1: Phenotypic detection ESBL producing *P. aeruginosa* isolates

S. No.	Detection of ESBLs	Positive isolates (n=126)
1.	Double Disc Synergy Test (DDST)	63 (50%)
2.	Modified Double Disc Synergy Test (MDDST)	66 (52.4%)

Table 2: Phenotypic detection AmpC producing *P. aeruginosa* isolates

S. No.	Detection of AmpC	Positive isolates (n=126)
1.	Screening of isolates for AmpC production	78 (61.9%)
2.	Disc Antagonism Test: ceftazidime- cefoxitin	78 (61.9%)
3.	Disc Antagonism Test: cefotaxime-cefoxitin	72 (57.1%)

Table 3: ESBL and AmpC E-test

S. No.	Detection of ESBL and AmpC	Positive isolates (n=126)
1.	ESBL and AmpC detection Ezy MIC Strip (MIX+/MIX)	47 (37.3%)

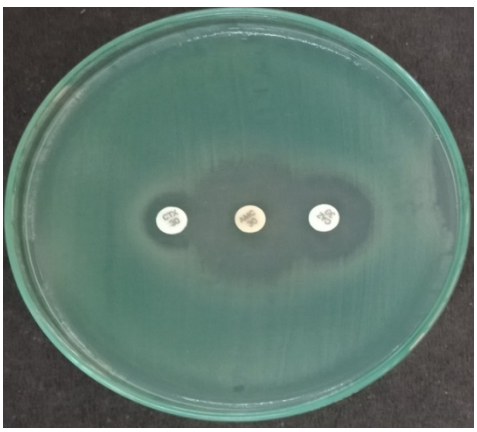

Figure 1: Double Disc Synergy Test (DDST) keyhole formation

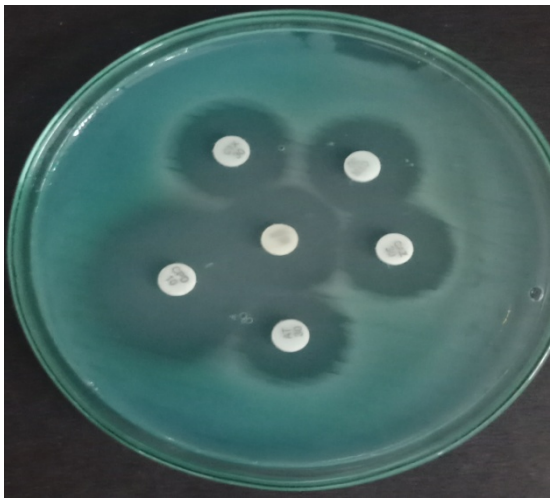

Figure 2: Modified Double Disc Synergy Test (MDDST)

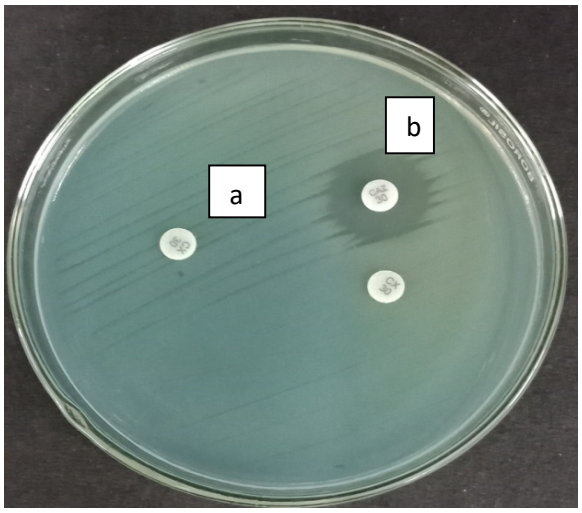

Figure 3: a- Screening of isolates for AmpC production- Isolates with zone diameter less than 18 mm for cefoxitin are considered as probable AmpC producers
b- Disc Antagonism Test: Ceftazidime- cefoxitin- Isolates showing blunting of zone towards ceftazidime disc

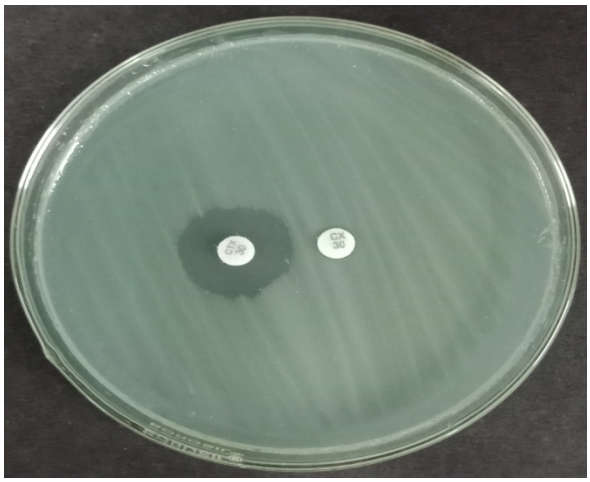

Figure 4: Disc Antagonism Test: cefotaxime-cefoxitin- Isolates showing blunting of zone towards cefotaxime disc

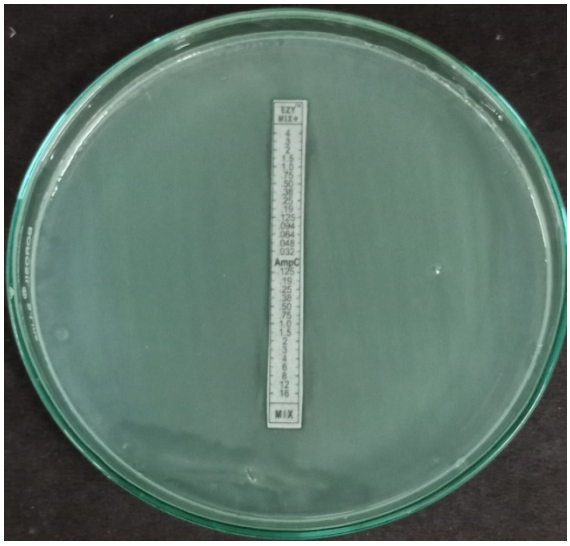

Figure 5- ESBL and AmpC E-test- isolates are positive for ESBL and AmpC production

DISCUSSION

Pseudomonas aeruginosa is an important human pathogen which causes a variety of infections and often difficult to treat due to its resistance to many antibiotics including the beta-lactams. The organism is notorious for being intrinsically resistant to many antimicrobial agents by exhibiting low permeability of its outer membrane, the constitutive expression of various efflux pumps and the naturally occurring chromosomal AmpC β lactamase. The organism can also acquire additional resistant genes from other resistant pathogens via genetic transfer mechanisms **[19]**. Community and hospital-acquired ESBL-producing Enterobacteriaceae and other related bacteria are prevalent worldwide **[20]**. The previous study reported that out of the total of 205 patients at the ICUs with nosocomial infections (NIs), ESBL-producing *P. aeruginosa* was responsible for 14.63% of NIs. The prevalence of ventilator-associated infection for ventilator-associated pneumonia (VAP) was (25 patients) 83.33%, also 16.6% (five patients) had sepsis due to VAP **[21]**. In the present study total of 63 (50%) and 66 (52.4%) isolates out of 126 *Pseudomonas aeruginosa* isolates were found positive for ESBL production by (DDST) and (MDDST) respectively **[Table 1, Fig 1 & 2]**. Based on another screening carried out previously, for ESBL detection 20.27% of ESBL production was reported among *P. aeruginosa* isolates. ESBL-producing bacteria are frequently resistant to many other classes of antibiotics, including aminoglycosides and fluoroquinolones. This is due to the coexistence of genes encoding drug resistance to other antibiotics on the plasmids which encode ESBL **[22]**. Mutation-dependent overproduction of intrinsic β-lactamase AmpC is considered the main cause of

resistance of clinical strains of *Pseudomonas aeruginosa* to anti-pseudomonal penicillins and cepha-losporins **[23]**. In the above study 78 (61.9%) isolates were found to be cefoxitin resistant and 78 (61.9%) and 72 (57.1%) isolates showed blunting of ceftazidime and cefotaxime by zone of inhibition adjacent to cefoxitin disc by disc antagonism method. Which was the positive indication of AmpC β-lactamase production **[Table 2, Figure 3 & 4]**. AmpC β-lactamases are clinically important cephalosporinases encoded on the chromosomes of many of the Enterobacteriaceae and a few other organisms, where they mediate resistance to cephalosporin, cefazolin, cefoxitin, most penicillins, and β-lactamase inhibitor-β-lactam combinations. In many bacteria, AmpC enzymes are inducible and can be expressed at high levels by mutation **[24]**. In another study 57 *P. aeruginosa* isolates, 35 (61.4%) isolates were PCR-positive for β-lactamase genes. 12 of 35 isolates were PCR-positive for combination of AmpC and ESBL genes, including TEM, GES, SHV, VEB and OXA-I genes. The sensitivity and specificity of AmpC and ESBL genes were 84.1% and 54.5%, respectively **[25]**. In previous screening of AmpC among the 128 Imipenem resistant isolates, 32 (25%) were capable of producing metallo β-lactamases of which, 4 (12.5%) produced extended spectrum and 26 (81%) produced AmpC β-lactamases. 4 isolates (12.5%) produced all 3 types **[26]**. In the present study 47 (37.3%) isolates out of 126 isolates were found to be positive for ESBL and AmpC production by E-test **[Table 2, Figure 5]**.

CONCLUSION

ESBL genes are very common in *P. aeruginosa* isolates. Phenotypic detection can enhance the sensitivity and has a potential role for detection of ESBL-producing *P. aeruginosa*. The prevalence of ESBL and chromosomal AmpC is commonly observed in *Pseudomonas aeruginosa*. The Routine screening for ESBL and AmpC production is needed to be done for all. As much data is not available for AmpC and ESBL detection of *Pseudomonas aeruginosa*. Hence the present study was carried out for early detection of these beta-lactamase-producing isolates in a laboratory by routine antibiotic susceptibility testing, especially for such nosocomial pathogens, which will help the hospitals to make policy for the limited use of antibiotics to minimize the emergence of this multidrug resistance.

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