Molecular Detection of Extended Spectrum Beta-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa*

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Abstract

*Pseudomonas aeruginosa* producing extended spectrum beta lactamases (ESBL) is a major concern in the hospital settings. It is usually reported in *Enterobacteriaceae* and is less frequently observed in *P. aeruginosa*. There is no recommended test for ESBL detection in *P. aeruginosa*. Therefore, we determined the occurrence of ESBL in clinical isolates of *P. aeruginosa* by both phenotypic and genotypic methods. Antimicrobial susceptibility tests were done on two hundred and thirteen isolates of *P. aeruginosa*. Phenotypic detection of ESBL was performed using combined disk method and ESBL encoding genes such as *blaVEB, blaPER, blaPSE, blaGES, blaTEM, blaSHV, blaCTX-M, blaBEL, blaOXA1, blaOXA10, blaOXA2* were studied by simplex PCR. Of the 213 isolates, 85 were identified as resistant to ceftazidime and 27/85 isolates were confirmed to be ESBL producers by phenotypic method. The presence of genes encoding ESBLs comprising of *blaTEM* (n=44), *blaOXA-10* (n=19) isolates, *blaOXA-1* (n=5), *blaOXA-2* (n=3) were found. All OXA gene positive isolates exhibited the ESBL phenotype. The *blaGES* gene were identified in 4/85 (5%) isolates. This study shows the prevalence of ESBL among clinical isolates of *P. aeruginosa* and in particular, the presence of GES β lactamases.

Keyword: Extended Spectrum β-lactamases, Class D β-lactamases, *Pseudomonas aeruginosa*, PCR

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INTRODUCTION

Pseudomonas aeruginosa is a multidrug resistant bacterium which has intrinsic resistant mechanisms and is responsible for hospital acquired infections. The production of beta-lactamases is the most common mechanism of bacterial antimicrobial resistance, which is primarily mediated by plasmids. Extended spectrum beta lactamases (ESBL) are a group of beta-lactamases which hydrolyze penicillins and cephalosporins including oxyimino beta-lactamases and aztreonam, but are inhibited by beta-lactamase inhibitors. Co-resistance to many additional antibiotic classes is common in organisms that produce ESBLs, thereby limiting therapeutic options. Penicillinases belonging to the molecular class A serine beta lactamases (CTX-M families, TEM and SHV) and class D (OXA-type) beta lactamases are the commonly reported beta-lactamases; VEB, PSE, CARB, PER and GES are less frequently reported. Insertion sequence (IS) elements are closely linked to blaESBL genes. Insertion Sequence 26 (IS26) is located upstream of blaTEM gene and is frequently reported to reside in a resistance plasmid which has the feature of transposition and target site duplication (TSD). IS26 is significant in the acquisition and dissemination of antibiotic resistance gene. The overexpression of inducible chromosomal AmpC beta-lactamases in P. aeruginosa confers resistance to broad spectrum antibiotics which may be difficult to detect by phenotypic methods. Increased membrane permeability and the existence of several efflux systems adds to the difficulties in detection of antibiotic resistance. There is no reliable test for phenotypic detection of ESBLs in clinical isolates of P. aeruginosa. Molecular methods are used to detect antibiotic resistance genes for monitoring the emergence of drug resistance in the clinical setting. The detection of these genes could help to establish standards for hospital infection control measures. Hence the study was aimed to detect ESBL production by both phenotypic and genotypic methods.

METHODOLOGY

During the period of one year (2013-2014), 213 Pseudomonas aeruginosa clinical isolates were collected from two tertiary care hospitals in South India. The ethical clearance was obtained from the institutional human ethics committee (IHEC No.: UM/IHEC/02-2014-I). Kirby Bauer disc diffusion method was used to evaluate all clinical isolates for antibiotic susceptibility. For the phenotypic detection of ESBL, combined disk method was used. Briefly, the test isolate was swabbed on Mueller Hinton agar plates after adjusting the opacity to 0.5 McFarland standard. Ceftazidime (30 μg) and ceftazidime/clavulanic acid (30/10 μg) discs was kept adjacent to each other at a distance of 20mm. After incubation at 37°C for 24 hrs, a zone size greater than 5mm with the ceftazidime/clavulanic acid disc when compared to the ceftazidime disc was considered as positive for ESBL production. The identification of P. aeruginosa by biochemical tests was confirmed with PCR. The boiling lysis method was used to extract DNA. An overnight culture was centrifuged at 10,000rpm for 10 minutes and to the supernatant 300μl of nuclease free water was added, boiled at 100°C for 10 minutes and stored at -20°C for at least 6 hours. The supernatant was collected after centrifugation at 10,000 rpm for 10 minutes and stored at 20°C. For PCR amplification, 2μl was used as a template and kept at -20°C for further use. PCR was performed for the confirmation of species using a species-specific primer which targets the 16S rRNA gene (Primer F: GGGGGATCTTCGGACCTCA and R: TCCTTAGAGTGCCCACCG) and the amplicon size was 956bp. The presence of ESBL-encoding genes was determined using primer specific for blaVEB, blaPER, blaPSE, blaGEX, blaTEM, blaSHV, blaCTX-M, blaBEL, blaOAX1, blaOAX2, blaOXA2 by simplex PCR (Table 1). For blaTEM positive isolates, IS26 transposon was detected by PCR.
Results

The isolates were collected from inpatients and outpatients of all ages with wound and ear infections (pus 66%; n=142), followed by respiratory tract infections (sputum 15%; n=32, bronchoalveolar lavage 2%; n=4, tracheal wash 1%; n=2), urinary tract infections (urine 10%; n=21), blood stream infections (blood 4%, n=8), other infections (semen 2%; n=4). Among the 213 P. aeruginosa isolates, highest antibiotic sensitivity was observed towards imipenem (90%) followed by cefepime (73%) and meropenem (72%). Highest resistance was observed towards ofloxacin (42%), gentamicin (38%) followed by piperacillin (37%) and ciprofloxacin (37%). Of the carbapenems tested, meropenem (25%) showed higher resistance than imipenem (8%) and a total of 22/213 (10%) isolates were resistant to both the carbapenems. Further, a total of 85/213 (40%) and 58/213 (27%) isolates were resistant to third (ceftazidime) and fourth (cefepime) generation cephalosporins, respectively.

Of the 213 isolates, 85(40%) isolates were found to be resistant to ceftazidime and 27 (32%) isolates were confirmed as ESBL producing strains by the combined disk method. The remaining 58 isolates (68%) were resistant to the clavulanate combination, and this may be due to the overproduction of Amp C β-lactamases. All the tested isolates were confirmed as P. aeruginosa by species specific 16S rRNA PCR (Figure 1). PCR detected \( \text{bla}_{\text{TEM}} \) in 44/85 (52%) isolates and \( \text{bla}_{\text{SHV}} \) gene was not identified in any of the isolates tested. Forty-four \( \text{bla}_{\text{TEM}} \) positive isolates were further tested for IS26 transposon gene by PCR. Of the 44 isolates, 37 (84%) were positive for the IS26 transposase gene indicating their possible role in acquisition and mobilization of \( \text{bla}_{\text{TEM}} \) (Figure 2).

Among 85 clinical isolates, \( \text{bla}_{\text{OXA-10}} \) was detected in 19 (23%) isolates; \( \text{bla}_{\text{OXA-1}}, \text{bla}_{\text{OXA-2}} \) were found in 5 (6%) and 3 (4%) isolates respectively (Table 2, Figure 3). All \( \text{bla}_{\text{OXA}} \) gene positive isolates exhibited the ESBL phenotype. \( \text{bla}_{\text{VEB}} \) and \( \text{bla}_{\text{PER}} \) types were found to be the most common ESBL in P. aeruginosa in several countries, whereas in

| Target gene | Sequence (5’ to 3’) | Expected amplicon size | Tm |
|-------------|---------------------|------------------------|----|
| VEB         | F: ACAGCTTCCATTTCCATGC | 643bp | 58 |
|             | R: GACACTTCAAAACAAATAGGC |
| PER         | F: AATGAATGGATCTATTTAAAGC | 925bp | 52 |
|             | R: AATTGGGCTTAAAGCAGAA |
| GES         | F: ATGCGCTTCATTACGCGAC | 846bp | 55 |
|             | R: CTATTGCTCCGTCACGAGG |
| OXA-1       | F: CAACGGATTACAGAAGCAGCTGCG | 198bp | 60 |
|             | R: GCTGTAATCTCGACCGAGTCTTC |
| OXA-2       | F: GACCAAGATTCCGATCAGCAATGGG | 256bp |
|             | R: CTGTCACAGCGCTAGTGTGCTC |
| OXA-10      | F: CGCCAGAGAATTTGGCGAGAATAGAAG | 156bp |
|             | R: GAAACTCCACTTGATTAAGGCG |
| CTX M-U     | F: ATGTCGAGYACAGTAARGT | 593bp | 50 |
|             | R: TGGGTAARTARGTASACAGA |
| TEM         | F: CATTCCGTCGCCCTTATCC | 800bp | 60 |
|             | R: CGTTCAATCATTTTGCCGTCG |
| SHV         | F: AAAAGATGACTATCGAGGCCGGAGC | 231bp |
|             | R: ATTGCTTTCCGTTGCCAAC |
| BEL         | F: CGCCAATACGGCAGCAGTAC | 600bp | 55 |
|             | R: CAGAAACGCAATTAATAAGGCC |
| PSE         | F: AAATGCGAATCGACGCTTTC | 699bp |
|             | R: CGCGGACTGTTGATGTATA |
this study all the isolates were negative for \textit{bla}_\textit{VEB} and \textit{bla}_\textit{PER}. The \textit{bla}_\textit{GES} gene were identified in 4/82 (5%) isolates; two isolates showed resistant to both carbapenems tested; one isolate was susceptible to both the carbapenems, and another was resistant to meropenem and susceptible to imipenem. This shows varying extended activity of GES enzymes in hydrolyzing the carbapenem. All 4 \textit{bla}_\textit{GES} positive isolates were sequenced and submitted to GenBank and accession numbers were obtained (Table 3). Among the \textit{Enterobacteriaceae}, the most prevalent enzymes are the CTX-M group of enzymes; however, they were absent in this study. Other genes frequently seen in Pseudomonas such as \textit{bla}_\textit{VEB}, \textit{bla}_\textit{PER} and the minor ESBLs such as \textit{bla}_\textit{PSE} and \textit{bla}_\textit{BEL} were not found in any of the tested isolates.

**DISCUSSION**

Phenotypic detection of ESBL in \textit{P. aeruginosa} is difficult due to the presence of various resistance mechanisms such as over expression of inducible chromosomal AmpC β-lactamase and greater degree of impermeability or efflux-mediated resistance.\textsuperscript{12} Current ESBL detection methods employed for \textit{Enterobacteriaceae} are not recommended to be used for \textit{P. aeruginosa}. Since there were no CLSI guidelines for \textit{P. aeruginosa} in the year that this study was conducted, the interpretation criteria for \textit{Enterobacteriaceae} was followed. Resistance to the clavulanate combination was observed in 58% of isolates and this may be due to the co-existence of ampC production or the presence of inhibitor resistant ESBL variants. The level of AmpC production may obstruct or even obscure the phenotypic detection. In ESBL-producing \textit{P. aeruginosa} isolates, the spread of metallo beta lactamases may be another reason for the non-detection by phenotypic tests.\textsuperscript{4}

PCR cannot differentiate the broad spectrum and extended spectrum variants of TEM

| Strain id. | Accession number |
|-----------|-----------------|
| PA34      | MG696822        |
| PAE136    | MG589922        |
| PAE129    | MG742374        |
| PAE140    | MG755328        |

**Table 2. Distribution of ESBL genes among \textit{P. aeruginosa}**

| Gene screened | No. of positive isolates (%) |
|---------------|-------------------------------|
| VEB           | 0                             |
| PER           | 0                             |
| TEM           | 44 (52%)                      |
| SHV           | 0                             |
| CTX M         | 0                             |
| PSE           | 0                             |
| BEL           | 0                             |
| GES           | 4 (5%)                        |
| OXA - 1       | 5 (6%)                        |
| OXA - 2       | 3 (4%)                        |
| OXA -10       | 19 (22%)                      |

**Table 3. Sequencing of \textit{bla}_\textit{GES} gene**

| Strain id. | Accession number |
|-----------|-----------------|
| PA34      | MG696822        |
| PAE136    | MG589922        |
| PAE129    | MG742374        |
| PAE140    | MG755328        |

**Figure 1. Simplex PCR for detection of 16SrDNA**
β-lactamases. Previously, there have not been many reports of \textit{bla}_{TEM} and \textit{bla}_{SHV} genes in \textit{P. aeruginosa}, due to the high prevalence of oxacillinase and carbenicillinase genes, upregulation of chromosome-encoded cephalosporinase and the rarity of narrow-spectrum TEM type enzymes.\textsuperscript{2} In this study, \textit{bla}_{TEM} was the most prevalent followed by \textit{bla}_{OXA-10} whereas another study showed that

\textbf{Figure 2.} Simplex PCR for detection of \textit{blaTEM} and IS26. NC-Negative control; M-100bp ladder; Lane L1-L6 in the figure A and B represents the clinical isolates of \textit{P. aeruginosa} showing positive for \textit{blaTEM} and IS26 gene respectively

\textbf{Figure 3.} Simplex PCR for detection of \textit{blaGES}, OXA-1, OXA-2 and OXA-10. NC-Negative control; M-100bp ladder; A: L1-L4 positive clinical isolates (\textit{blaGES}), L5&L6 negative clinical isolates; B: L1&L2 positive clinical isolates (\textit{blaOXA-1}), L3-L10 negative clinical isolates; C: L1 &L3 positive clinical isolates (\textit{blaOXA-2}), L4-L6 negative clinical isolates; D: L1-L3 &L6 positive clinical isolates (\textit{blaOXA-10}), L4 & L5 negative clinical isolates
the most prevalent ESBL gene was \( \text{bla}_{\text{VEB}} \) followed by \( \text{bla}_{\text{TEM}}, \text{bla}_{\text{GES}} \) and \( \text{bla}_{\text{SHV}} \). These may vary in different geographical regions.

Our study result shows that \( \text{bla}_{\text{TEM}} \) was found in 52% of isolates and \( \text{bla}_{\text{SHV}} \) was not detected in any of the isolates. The study further demonstrated the presence of IS26 association with \( \text{bla}_{\text{TEM}} \) gene. Horizontal gene transfer mechanisms may have played a role in the dissemination of this kind of enzymes from the Enterobacteriaceae family. The selective pressure of antibiotics on the bacteria could have modified the resistant mechanisms.\(^{14}\) CTX-M variant was previously one of the most frequently reported enzymes in Enterobacteriaceae; later it was reported in \( P. \ aeruginosa \).\(^{15}\) Dissemination of the gene may be due to the insertion sequence \( \text{ISEcp1} \) which is frequently observed upstream of the \( \text{bla}_{\text{CTX-M}} \) genes. However, in this study, none of the isolates exhibited \( \text{bla}_{\text{CTX-M}} \) which differed from previous findings.\(^{16-19}\)

Molecular class D \( \beta \)-lactamases mainly OXA group exhibit greater diversity in their enzymatic activities and are the most frequent ESBLs found in Pseudomonas sp. The present study reports the presence of \( \text{bla}_{\text{OXA-10}}, \text{bla}_{\text{OXA-1}}, \text{and bla}_{\text{OXA-2}} \) in 19 isolates, 5 isolates and 3 isolates respectively. OXA-10 and to a lesser extent, OXA-2 are the origin of the majority of OXA-type ESBLs.\(^{20}\) OXA 10 and OXA 2 are classified as narrow-spectrum Class D \( \beta \)-lactamases; however, when expressed in A. baumannii, they can show carbapenemase activity.\(^{21}\) In the present study, \( \text{bla}_{\text{GES}} \) was identified in four isolates which showed both the ESBL phenotype and carbapenemase activities. This could be a stage in the transition between ESBLs and carbapenem-hydrolyzing enzymes of class A. GES has previously been observed in different places in India.\(^{22}\) The high frequency of ESBLs in the present study indicates the necessity for standardization of phenotypic method of detection and increasing its sensitivity. Further, PCR is essential for the characterization of ESBLs and monitoring the dissemination of resistant genes. Although the prevalence of ESBLs has been documented in numerous studies from around the world, geographical and institutional differences in their prevalence have been observed.

**CONCLUSION**

ESBL-producing P. aeruginosa strains are reported worldwide. Use of molecular methods for the detection of resistant genes is important to study their prevalence.

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None.

**CONFLICT OF INTEREST**

The authors declare that there is no conflict of interest.

**AUTHORS’ CONTRIBUTION**

Both the authors listed have made a substantial, direct and intellectual contribution to the work, and approved it for publication.

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**DATA AVAILABILITY**

All datasets generated or analyzed during this study are included in the manuscript.

**ETHICS STATEMENT**

This study was approved by the Institutional Human Ethics Committee, Dr. ALM PG IBMS, University of Madras, Chennai, India with reference number IHEC no:UM/IHEC/02-2014-I.

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