Mohanam & Menon | Article 7673
J Pure Appl Microbiol. 2022;16(3):1736-1742. doi: 10.22207/JPAM.16.3.14
Received: 10 March 2022 | Accepted: 25 May 2022
Published Online: 16 July 2022

P-ISSN: 0973-7510; E-ISSN: 2581-690X

RESEARCH ARTICLE OPEN ACCESS

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

Lavanya Mohanam[1,3] and Thangam Menon[2]*

[1]Department of Microbiology, Chettinad Hospital and Research Institute, Chettinad Academy of Research and Education, Kelambakkam, Chennai - 603 103, Tamil Nadu, India.
[2]Department of Microbiology, Saveetha Dental College and Hospitals, Saveetha Institute Medical and Technical Sciences, 162 PH Road, Chennai - 600 077, Tamil Nadu, India.
[3]Department of Microbiology, Dr. ALMPGIBMS, Taramani, University of Madras, Chennai - 600 113, Tamil Nadu, India.

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

***Correspondence:** thangam56@gmail.com

Citation: Mohanam L, Menon T. Molecular Detection of Extended Spectrum Beta-Lactamases in Clinical Isolates of *Pseudomonas aeruginosa*. *J Pure Appl Microbiol.* 2022;16(3):1736-1742. doi: 10.22207/JPAM.16.3.14

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.[1] Extended spectrum beta lactamases (ESBL) are a group of β lactamases which hydrolyze penicillins and cephalosporins including oxyimino β-lactamases and aztreonam, but are inhibited by β-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 β lactamases (CTX-M families, TEM and SHV) and class D (OXA-type) β lactamases are the commonly reported β-lactamases; VEB, PSE, CARB, PER and GES are less frequently reported.[2] In addition, Insertion sequence (IS) elements are closely linked to blaESBL genes. Insertion Sequence 26 (IS26) is located upstream of bla_{TEM} gene and is frequently reported to reside in a resistance plasmid which has the feature of transposition and target site duplication (TSD).[3] IS26 is significant in the acquisition and dissemination of antibiotic resistance gene. The overexpression of inducible chromosomal AmpC β-lactamases in *P. aeruginosa* confers resistance to broad spectrum antibiotics which may be difficult to detect by phenotypic methods.[4] 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.[5] The following antibiotics (HiMedia Labs Mumbai, India) were used: piperacillin (100 µg), piperacillin/tazobactum (100µg/10 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), ofloxacin (5 µg), aztreonam (30 µg), cefepime (30 µg), ceftazidime (30 µg), amikacin (30 µg) gentamicin (10 µg), imipenem (10 µg) and meropenem (10 µg). Clinical and Laboratory Standards Institute (CLSI) 2013 guidelines were used for interpretation.[6]

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.[7]

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.[8] PCR was performed for the confirmation of species using a species-specific primer which targets the 16S rRNA gene (Primer F: GGGGGATCTTCGGACCTCA and R: TCCTTAGAGTGCCCACCCG) and the amplicon size was 956bp.[9] The presence of ESBL-encoding genes was determined using primer specific for bla_{VEB}, bla_{PER}, bla_{PSE}, bla_{GES}, bla_{TEM}, bla_{SHV}, bla_{CTX-M}, bla_{BEL}, bla_{OXA1}, bla_{OXA10}, bla_{OXA2} by simplex PCR (Table 1).[2,10-11] For bla_{TEM} 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, broncho alveolar 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 bla_{TEM} in 44/85(52%) isolates and bla_{SHV} gene was not identified in any of the isolates tested. Forty-four bla_{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 bla_{TEM} (Figure 2).

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

Table 1. Primers used for the detection of ESBL

Target gene	Sequence (5' to 3')	Expected amplicon size	Tm
VEB	F: ACGACTTCCATTTCCCATGC		
	R: GGACTCTGCAACAAATACGC	643bp	58
PER	F: AATGAATGTCATTATAAAAGC		
	R: AATTTGGGCTTAGGGCAGAA	925bp	52
GES	F: ATGCGCTTCATTCACGCAC		
	R: CTATTTGTCCGTGCTCAGG	846bp	55
OXA-1	F: CAACGGATTAACAGAAGCATGGCTCG		
	R: GCTGTRAATCCTGCACCAGTTTTCCC	198bp	60
OXA-2	F: GACCAAGATTTGCGATCAGCAATGCG		
	R: CYTTGACCAAGCGCTGATGTTCYACC	256bp	
OXA-10	F: CGCCAGAGAAGTTGGCGAAGTAAG		
	R: GAAACTCCACTTGATTAACTGCGG	156bp	
CTX M-U	F: ATGTGCAGYACCAGTAARGT		
	R: TGGGTRAARTARGTSACCAGA	593bp	50
TEM	F: CATTTCCGTGTCGCCCTTATTC		
	R: CGTTCATCCATAGTTGCCTGAC	800bp	60
SHV	F: AAAGATCCACTATCGCCAGCAG		
	R: ATTCAGTTCCGTTTCCCAGCGG	231bp	
BEL	F: CGACAATGCCGCAGCTAACC		
	R: CAGAAGCAATTAATAACGCCC	600bp	55
PSE	F: AATGGCAATCAGCGCTTC		
	R: GCGCGACTGTGATGTATA	699bp	

this study all the isolates were negative for bla_{VEB} and *blaPER*. The bla_{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 bla_{GES} positive isolates were sequenced and submitted to GenBank and accession numbers were obtained (Table 3). Among the *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 bla_{VEB}, bla_{PER} and the minor ESBLs such as bla_{PSE} and bla_{BEL} were not found in any of the tested isolates.

Table 2. Distribution of ESBL genes among *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%)

DISCUSSION

Phenotypic detection of ESBL in *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.[12] Current ESBL detection methods employed for *Enterobacteriaceae* are not recommended to be used for *P. aeruginosa*. Since there were no CLSI guidelines for *P. aeruginosa* in the year that this study was conducted, the interpretation criteria for *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 *P. aeruginosa* isolates, the spread of metallo beta lactamases may be another reason for the non-detection by phenotypic tests.[4]

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

Table 3. Sequencing of *blaGES* gene

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

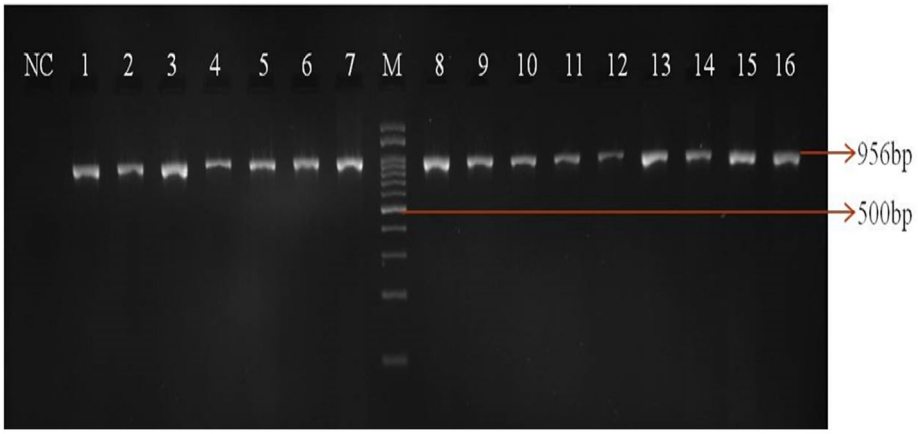

Figure 1. Simplex PCR for detection of 16SrDNA

β-lactamases. Previously, there have not been many reports of bla_{TEM} and bla_{SHV} genes in *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.[2] In this study, bla_{TEM} was the most prevalent followed by bla_{OXA-10} whereas another study showed that

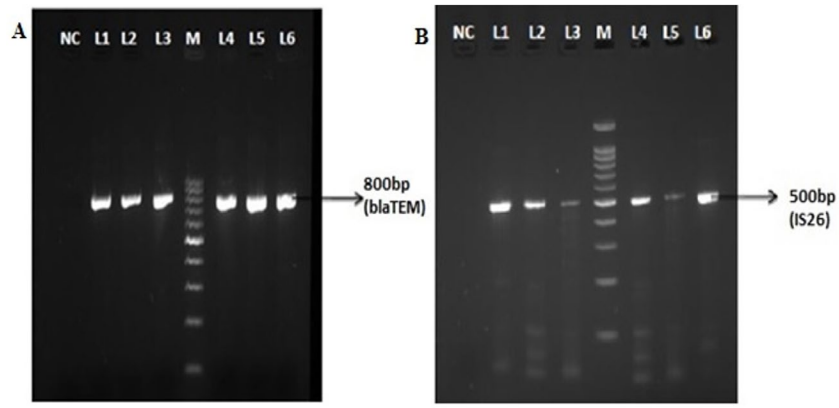

Figure 2. Simplex PCR for detection of blaTEM and IS26. NC-Negative control; M-100bp ladder; Lane L1-L6 in the figure A and B represents the clinical isolates of *P.aeruginosa* showing positive for *blaTEM* and *IS26* gene respectively

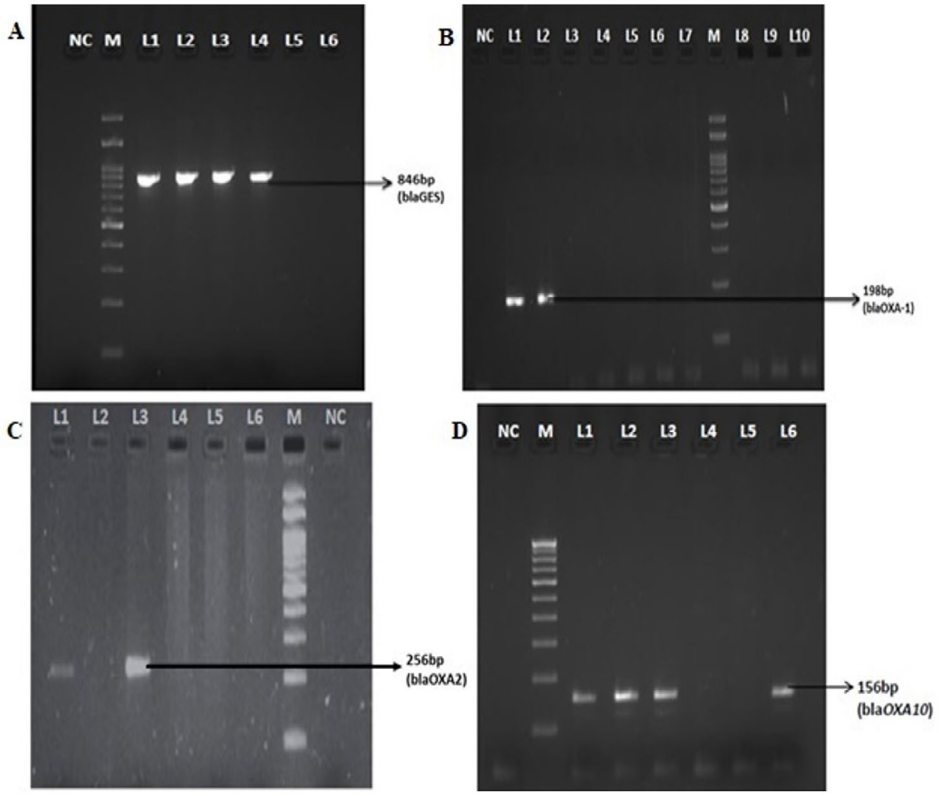

Figure 3. Simplex PCR for detection of blaGES, OXA-1, OXA-2 and OXA-10. NC-Negative control; M-100bp ladder; A: L1-L4 positive clinical isolates (blaGES), L5&L6 negative clinical isolates; B: L1&L2 positive clinical isolates (blaOXA-1), L3-L10 negative clinical isolates; C: L1 &L3 positive clinical isolates (blaOXA-2), L4-L6 negative clinical isolates; D: L1-L3 &L6 positive clinical isolates (blaOXA-10), L4 & L5 negative clinical isolates

the most prevalent ESBL gene was bla_{VEB} followed by bla_{TEM}, bla_{GES} and bla_{SHV}.[13] These may vary in different geographical regions.

Our study result shows that bla_{TEM} was found in 52% of isolates and bla_{SHV} was not detected in any of the isolates. The study further demonstrated the presence of IS26 association with bla_{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 ISEcp1 which is frequently observed upstream of the bla_{CTX-M} genes. However, in this study, none of the isolates exhibited bla_{CTX-M} which differed from previous findings.[16-19]

Molecular class D β-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 bla_{OXA-10}, bla_{OXA-1} and bla_{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 β-lactamases; however, when expressed in *A. baumannii*, they can show carbapenemase activity.[21] In the present study, bla_{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 world-wide. Use of molecular methods for the detection of resistant genes is important to study their prevalence.

ACKNOWLEDGMENTS

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

FUNDING

This study was funded by University Grant Commission, New Delhi, India with reference No: F19-121/2014(BSR).

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.

REFERENCES

1. Potron A, Poirel L, Nordmann P. Emerging broad-spectrum resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii*: mechanisms and epidemiology. *Int J Antimicrob Agents.* 2015;45(6):568-585. doi: 10.1016/j.ijantimicag.2015.03.001
2. Weldhagen GF, Poirel L, Nordmann P. Ambler Class A Extended-Spectrum β-Lactamases in *Pseudomonas aeruginosa*: Novel Developments and Clinical Impact. *Antimicrob Agents Chemother.* 2003;47(8):2385-2392. doi: 10.1128/AAC.47.8.2385-2392.2003
3. He S, Hickman AB, Varani AM, et al. Insertion sequence IS26 reorganizes plasmids in clinically isolated multidrug-resistant bacteria by replicative transposition. *mBio.* 2015;6(3):e00762-15. doi:

10.1128/mBio.00762-15

4. Laudy AE, Rog P, Smolinska-Krol K, et al. Prevalence of ESBL-producing *Pseudomonas aeruginosa* isolates in Warsaw, Poland, detected by various phenotypic and genotypic methods. *PLoS One.* 2017;12(6):e0180121. doi: 10.1371/journal.pone.0180121
5. Bauer AW, Kirby WM, Sherris JC, Turck M. Antibiotic susceptibility testing by a standardized single disc method. *Am J Clin Pathol.* 1966;45(4):493-496. doi: 10.1093/ajcp/45.4_ts.493
6. CLSI. M100-S24 Performance Standards for Antimicrobial Susceptibility Testing; Twenty-Third Informational Supplement. 2013.
7. Taneja N, Sharma M. ESBLs detection in clinical microbiology: why & how? *Indian J Med Res*. 2008;127(4):297-300. https://pubmed.ncbi.nlm.nih.gov/18577783/.
8. Pitout JDD, Gregson DB, Poirel L, McClure JA, Le P, Church DL. Detection of *Pseudomonas aeruginosa* producing metallo-β-lactamases in a large centralized laboratory. *J Clin Microbiol.* 2005;43(7):3129-3135. doi: 10.1128/JCM.43.7.3129-3135.2005
9. Spilker T, Coenye T, Vandamme P, LiPuma JJ. PCR-based assay for differentiation of *Pseudomonas aeruginosa* from other Pseudomonas species recovered from cystic fibrosis patients. *J Clin Microbiol.* 2004;42(5):2074-2079. doi: 10.1128/JCM.42.5.2074-2079.2004
10. Strateva T, Ouzounova-Raykova V, Markova B, Todorova A, Marteva-Proevska Y, Mitov I. Problematic clinical isolates of *Pseudomonas aeruginosa* from the university hospitals in Sofia, Bulgaria: current status of antimicrobial resistance and prevailing resistance mechanisms. *J Med Microbiol.* 2007;56(7):956-963. doi: 10.1099/jmm.0.46986-0
11. Voets GM, Fluit AC, Scharringa J, Stuart JC, Leverstein-van Hall MA. A set of multiplex PCRs for genotypic detection of extended-spectrum β-lactamases, carbapenemases, plasmid-mediated AmpC β-lactamases and OXA β-lactamases. *Int J Antimicrob Agents.* 2011;37(4):356-359. doi: 10.1016/j.ijantimicag.2011.01.005
12. Jiang X, Yu T, Jiang X, Zhang W, Zhang L, Ma J. Emergence of plasmid-mediated quinolone resistance genes in clinical isolates of *Acinetobacter baumannii* and *Pseudomonas aeruginosa* in Henan, China. *Diagn Microbiol Infect Dis.* 2014;79(3):381-383. doi: 10.1016/j.diagmicrobio.2014.03.025
13. Pragasam AK, Vijayakumar S, Bakthavatchalam YD, et al. Molecular characterisation of antimicrobial resistance in *Pseudomonas aeruginosa* and *Acinetobacter baumannii* during 2014 and 2015 collected across India. *Indian J Med Microbiol.* 2016;34(4):433-441. doi: 10.4103/0255-0857.195376
14. Lerminiaux NA, Cameron AD. Horizontal transfer of antibiotic resistance genes in clinical environments. *Can J Infect Dis.* 2019;65(1):34-44. doi: 10.1139/cjm-2018-0275
15. Al Naiemi N, Duim B, Bart A. A CTX-M extended-spectrum β-lactamase in *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia. J Med Microbiol.* 2006;55(11):1607-1608. doi: 10.1099/jmm.0.46704-0
16. Picao RC, Poirel L, Gales AC, Nordmann P. Further identification of CTX-M-2 extended-spectrum β-lactamase in *Pseudomonas aeruginosa*. *Antimicrob Agents Chemother.* 2009;53(5):2225-2226. doi: 10.1128/AAC.01602-08
17. Polotto M, Casella T, de Lucca Oliveira MG, et al. Detection of *P. aeruginosa* harboring $bla_{CTX-M-2}$, bla_{GES-1} and bla_{GES-5}, bla_{IMP-1} and bla_{SPM-1} causing infections in Brazilian tertiary-care hospital. *BMC Infect Dis.* 2012;12(1):176. doi: 10.1186/1471-2334-12-176
18. Saxena S, Banerjee G, Garg R, Singh M. CTX-M and PER-1 group extended spectrum β-lactamases-producing *Pseudomonas aeruginosa* from the patients of lower respiratory tract infection. *Indian J Med Microbiol.* 2015;33(1):191-192. doi: 10.4103/0255-0857.148444
19. Gupta R, Malik A, Rizvi M, Ahmed M. Presence of metallo-beta-lactamases (MBL), extended-spectrum beta-lactamase (ESBL) & AmpC positive non-fermenting Gram-negative bacilli among Intensive Care Unit patients with special reference to molecular detection of bla_{CTX-M} & bla_{AmpC} genes. *Indian J Med Res.* 2016;144(2):271. doi: 10.4103/0971-5916.195043
20. Naas T, Poirel L, Nordmann P. Minor extended-spectrum β-lactamases. *Clin Microbiol Infect.* 2008;14:42-52. doi: 10.1111/j.1469-0691.2007.01861.x
21. Antunes NT, Lamoureaux TL, Toth M, Stewart NK, Frase H, Vakulenko SB. Class D β-lactamases: are they all carbapenemases? *Antimicrob Agents Chemother* 2014;58(4):2119-2125. doi: 10.1128/AAC.02522-13
22. Maurya AP, Choudhury D, Talukdar AD, Dhar A, Chakravarty A, Bhattacharjee A. A report on the presence of GES-5 extended spectrum beta-lactamase producing *Pseudomonas aeruginosa* associated with urinary tract infection from north-east India. *Indian J Med Res.* 2014;140(4):565-567. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC4277147/