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Original Article

ESBL, MBL AND AMP C-B LACTAMASES PRODUCED BY SUPERBUGS: AN EMERGING THREAT TO CLINICAL THERAPEUTICS

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ABSTRACT

Objectives: The present study was undertaken to determine the prevalence of multi drug resistant (MDR) and multiple β-lactamase producing *Pseudomonas aeruginosa* isolates in lower respiratory tract infection (LRTI) patients at a tertiary care hospital in India.

Methods: A total of 80 consecutive, non-duplicate isolates of *P. aeruginosa* were studied for the presence of class A or B β-lactamase. Antibiotic susceptibility tests and PCR amplification of genes encoding class A (PER-1 and CTX-M 1, 2, 9) and class B β-lactamases (*bla*VIM-2, *bla*IMP-1 and *bla*SIM-1) were performed.

Results: Out of 80 *P. aeruginosa* isolates, 65% (52/80) of the isolates were MDR with 34 being Metallo-β-lactamase (MBL) producers, 23 were extended spectrum β-lactamase (ESBL) producers and 21 were positive for AmpC production. The cross-class resistance rates to other antibiotics was significantly higher in class A and B β-lactamase producers than in non-producers ($P<0.05$ for fluoroquinolone, aztreonam, ceftazidime and meropenem). Combined disk test (CDT) for MBL highest sensitivity and specificity compared to PCR. Combined disk method (CDM) for ESBL co-related well with PCR (sensitivity and specificity).

Conclusion: This study reports the validation of a simple and accurate MBL and ESBL detection method which can be easily integrated into the daily routine of a clinical laboratory.

Keywords: *Pseudomonas aeruginosa,* Beta-lactamase genes, MDR.

INTRODUCTION

Pseudomonas aeruginosa is considered as the most commonly isolated Gram-negative organism in the blood stream, wound infections, pneumonia, abdominal and urogenital sepsis. It also infects immune compromised patients, these poses a serious health issue [1].

P. aeruginosa shows high antibiotic resistance which can be due to several factors working synergistically i.e., over expression of efflux pumps as well as chromosomal or plasmid encoded beta lactamases.

Metallo-beta-lactamases (MBLs) are called carbapenemases which includes the VIM and IMP and their variants such as SPM-1, GIM-1, NDM-1, AIM-1 and SIM-1 enzymes.

The VIM and IMP enzymes are by far the most common MBLs found in carbapenem-resistant bacteria, including carbapenem-resistant *P. aeruginosa* [2].

Extended-spectrum ß-lactamases (ESBLs) are encoded by plasmid genes TEM, SHV and CTX-M-genes, which show resistance to penicillins, 3rd generation cephalosporins and also to later generation cephalosporins such as Cefepime, cefotaxime and ceftazidime [3].

Thus, the present study was conducted with an objective to know the anti biogram and to detect the presence of ESBL and MBL producing *P. aeruginosa*, so as to help in formulating an effective antibiotic and hospital infection strategy to prevent the spread of these strains.

MATERIALS AND METHODS

Study site and subjects

LRTI patients attending outpatients ward or admitted in the ward in department Pulmonary medicine at a tertiary care hospital in Lucknow, India during September 2010 to August 2012 were enrolled. The inclusion criteria for patient enrollment was ≥ 18-years of age with symptoms suggestive of LRTI (i.e., two or more of the following symptoms: cough, sputum production, shortness of breath, wheeze, fever during this illness, chest pain) and who gave written consent for participation.

Sample processing

Samples obtained were cultured on MacConkey and Pseudomonas isolation agar plates (Hi-media) and incubated at 37 °C for 16-18 hrs. Identification of *P. aeruginosa* isolates was done by standard biochemical procedure [4].

Antibiotic susceptibility testing

Susceptibility of the isolates was tested by Kirby Bauer disk diffusion method as per Clinical and Laboratory Standards Institute (CLSI) 2010guidelines [5].

P. aeruginosa ATCC 27853 was used as quality control. Antimicrobial susceptibility profiles were compared for class A and B β-lactamase producers and non-producers. Strains resistant to all of the agents in 2 or more of the following antimicrobial categories were defined as multiresistant: β-lactam antibiotics, including imipenem, amino-glycosides, and the fluoroquinolones ciprofloxacin [6].

MIC of meropenem and ceftazidime by agar dilution method

Minimum inhibitory concentration of meropenem and ceftazidime resistant strains was determined by the agar dilution method [5]. Dilutions of meropenem and ceftazidime ranging from 2 µg/ml to128 µg/ml were prepared in doubles. MIC of ≥ 16 µg/ml was interpreted as resistant [5]. Both antibiotic powders were obtained from HIMEDIA, Mumbai, India.

Phenotypic detection of MBL, ESBL and Amp-C-β-lactamase

Detection of the ESBLs

All the isolates of *P. aeruginosa, which* showed resistance to ceftazidime were evaluated for ESBL production by using combination disk method (CDM) [7] and double-disk approximation test (DDAT) [8].

Detection of the Ampc β-lactamases

In the initial screening test, a disc of cefoxitin (FOX-30 µg) was placed on a Mueller Hinton agar plate already inoculated with the test organism. Zones of inhibition around the cefoxitin disc were observed after overnight incubation. Isolates that yielded a zone diameter less than 18 mm were labeled as AmpC β-lactamases positive. All the strains were screened for the AmpC β-lactamase production by the disc antagonism test (DAT) [7]and boronic acid disk potentiation test (BADPT) [9].

MBL

The metallo-β-lactamase production was detected by three tests: double disk synergy tests (DDST) [10], combined disk test (CDT) [7] and modified Hodge test (MHT) [10].

PCR amplification of class A and class B β-lactamase genes

Multiplex PCR assay was performed to detect and differentiate three MBL encoding genes *bla*VIM-2*, bla*IMP-1 and *bla*SIM-1 families in a single reaction [11]. PCR for ESBL encoding genes *bla*PER-1 and *bla*CTX-M 1, 2, 9 was performed as described earlier [12].

Statistical methods

Data was analyzed using STATA version 11.1 (Stata Inc, College Station, TX, USA). To compare categorical variables, Fisher's exact test was used. Sensitivity, specificity, positive and negative predictive values (PPV and NPV), for phenotypic test and PCR against gold standard.

RESULTS

Isolates

During the study, a total of 270 samples was received from which 80 were confirmed as *P. aeruginosa* isolates obtained from the patients of LRTI.

Antimicrobial susceptibilities of clinical strains

Maximum sensitivity (90%) was seen with imipenem, followed by amikacin (86.2%) meropenem (72.5%) and piperacillin/tazobactam (71.2%).

The comparative antimicrobial resistances of β-lactamase producers and non-β-lactamase producers are summarized in (table 1).

Table 1: Comparison of the antimicrobial resistance (%) between the class A and/or class B β-lactamase producer and β-lactamase non producer *P. aeruginosa* isolates

Antimicrobial agent	β-lactamase producer (n=22)	Non β-lactamase producer (n=58)	*P-Value*
AMK	33.3	66.7	NS
ATM	40.0	60.0	0.012*
CIP	41.1	58.9	0.019*
CAZ	40.0	60.0	0.001*
FEP	39.6	60.4	0.009*
CRO	35.1	64.9	0.027*
IMP	25.0	75.0	NS
TZP	52.6	47.3	0.005*
TOB	47.8	52.1	0.010*
MEM	47.5	52.5	0.000*
A/C	29.3	70.7	NS
GEN	29.0	71.0	NS
AMP	28.2	78.8	NS
LVX	36.0	64.0	0.002*

*P≤0.05 is considered as being significant, *NS: Not significant

Abbreviations: AMK-amikacin, ATM-aztreonam,CIP-ciprofloxacin,CAZ-ceftazidime, FEP-cefepime,CRO-ceftriaxon,IPM-imipenem,TZP-piperacillin–tazobactam,TOB-tobramycin, FOX-cefoxitin, GEN-gentamicin, AMP-ampicillin, A/C-amoxicillin+clavulanic acid, LVX-levofloxacin.

MIC

The highest MIC for meropenem was 16µg/ml for 17 isolates. The highest MIC observed for ceftazidime was 128µg/ml for 12 *P. aeruginosa* isolates.

MDR

Among 80 (52) *P. aeruginosa* isolates were found to be MDR. Multidrug resistance was more prevalent in meropenem-resistant isolates than meropenem-susceptible isolates (92.5% vs 32.4%).

Screening for MBL

Out of 40 meropenem resistant isolates, 34 were MBL producers of which 22 (55%) were found to be positive in DDST and 12 (30%) in the CDT and none were positive in the MHT.

Screening for ESBL

Similarly, of 50 ceftazidime resistant isolates, 23 were ESBL producing; CDM gave a positive result in 13 (26%) isolates, whereas DDAT detected in 10 (20%) isolates.

Screening for AmpC

All *P. aeruginosa* isolates were tested for AmpC-β-lactamase production. Cefoxitin resistance was evident in 30 isolates, while 21 isolates were confirmed to be AmpC β-lactamase producers. Among the test isolates, 12 (40%) were detected by DAT as AmpC producers while 9 (30%) were confirmed by BADPT.

Genotype detection of β-lactamases genes

MBL genes

Multiplex PCR showed the presence of MBL genes in 59% (20/34) isolates; most common MBL subtype was *bla*VIM-2 (11/34; 32.3%) five isolates (14.7%) were positive for *bla*IMP-1 gene and only four (12%) were positive for *bla*SIM-1. Four isolates carried all three MBL genes.

ESBL genes

Genes encoding ESBL*s* were detected in 70% (16/23) of the isolates. CTXM-2 was detected in seven (30.4%) and PER-1gene was also detected in seven isolates (30.4%) where as, two (8.6%) isolates carried CTXM-1. None of the MDR *P. aeruginosa* carried CTXM-9 *genes* in our study.

None of the screening method showed a complete correlation when compared to PCR. The CDT had the highest sensitivity (92%) and specificity (99%) for detection of MBL (table 2).

CDM had the highest sensitivity (92%) and specificity (97%) for detection of ESBL as compared to DDAT (table-3).

Table 2: Comparison of MBL phenotypic test against PCR

Phenotypic method	No. of PCR-confirmed MBL-carrying Organisms (*n* =12)	No. of PCR-confirmed non MBL-carrying Organisms (*n* =68)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CDT	11(92%)	1(1.4%)	92	99	92	99
DDST	11(92%)	11(16.1%)	92	84	50	98.2

Table 3: Comparison of ESBL phenotypic test against PCR

Phenotypic method	No. of PCR-confirmed ESBL-carrying Organisms (*n* =12)	No. of PCR-confirmed Non ESBL-carrying Organisms (*n* =68)	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)
CDM	11 (92%)	2(3%)	92	97	85	99
DDAT	12(100%)	2(3%)	83.3	100	100	97.1

DISCUSSION

P. aeruginosa has emerged as the most common dreadful gram negative bacilli found in various health care associated infections all over the world due to its virulence and ability to resist killing by various antibiotics. The bacterial resistance is on the rise, creating clinical as well as economical issues [13].

In India, the prevalence rate of *P. aeruginosa* infection varies from 10.5% to 30% [14]. Our study corroborated the same with a prevalence rate of 29.6%.

The Percentage of MDR *P. aeruginosa* in India ranges from 11.36% reported by Idris *et al.*,[15], 91.6% reported by Panranjothi *et al.*, [16],Our study showed 65% *P. aeruginosa* were as MDR, consistent with previous data.

Among the 3rd cephalosporins drugs, ceftazidime (62.5%) and ceftriaxone (67.5%), showed the highest resistance. The similar finding was reported by Diwivedi *et al.*,(63%) [17] and Senthamarai *et al.*, (65.38%) [13].

Out of 80 *P. aeruginosa* isolates, 22 were β-lactamase producers and 58 non β-lactamase producers. The β-lactamase producers were significantly resistant to piperacillin+tazobactam than non-producers (*P<0.005).

Our study showed that among the 80 *P. aeruginosa* isolates, only 46.0% *P. aeuginosa* isolates were ESBL producers, which was comparable to previous studies [18-21].

In *P. aeruginosa* production of ESBL is usually less because, because their resistance is mediated by various other mechanisms such as the production of MBL, porins mutation and the loss of certain outer membrane proteins and efflux pumps.

Out of 23 ESBL producers, 16 carried β-lactamase producing genes. *bla* PER-1 carried 30.4% and *bla*CTX-M-2 carried 30.4 %, followed by *bla*CTX-M-1 carried 8.6 %. Surprisingly, none of our isolates were positive for *bla*CTX-M-9 genes.

An earlier reported 87% prevalence of *bla*CTX-M enzyme amongst ESBL producers [22]. However, our study reported only 69.5% prevalence of ESBL genes.

The emergence of MBL mediated resistance in India is of serious concern. Carbapenems are effective therapeutic agents against highly resistant pathogen such as *P. aeruginosa*. In our study, the frequency of the MBL producing *P. aeruginosa* was 26.2%. Another recent study by Varaiya *et al.,*showed 20.8% of MDR *P. aeruginosa* were to be MBL producers [23] whereas, Upadhyay *et al.,* Reportedly 46.6% of MBL production among MDR *P. aeruginosa* isolates [7].

In our experience, out of the 40 meropenem resistant isolates, all the 34 were found to be positive for MBL by three different tests to detect MBL producers, in which MHT was unable to detect none. CLSI [5] recommends MHT for detection of carbapenemases activity in Enterobacteriaceae only. DDST detected 55% isolates to be MBL producers as opposed to 30% confirmed by CDT.

In total, 59% of MBL producers carried either *bla*IMP-1, *bla*VIM-2 and *bla*SIM-1 gene. The presence of *bla*VIM-2 gene appears to be more prevalent in our setup, wherein *P. aeruginosa* isolates were positive for *the bla*VIM-2 gene. Fortunately, in our setup we encountered very less prevalence of resistance genes among *P. aeruginosa* when compared to other studies. Amp-C production was quite high in our isolates compared to other studies. [7]

When compared statistical, results showed that the CDT had an excellent sensitivity and a specificity (sensitivity>92%, specificity>99%), then DDST. In comparison, ESBL results showed that the CDM had a sensitivity and specificity (sensitivity>92%, specificity>97%). Whereas, the DDAT had a sensitivity of 83.3% and specificity of 100%. Overall, the combined-disk test for MBL and CDM for ESBL better for detection. It is easy to perform, and the materials used are low-priced, safe, and easily available, making it highly applicable in routine clinical laboratories.

Our study validates a simple and highly sensitive phenotypes method for the detection of MBL and ESBL production in *P. aeruginosa* isolated from LRTI patients. Higher frequency of MDR *P. aeruginosa* along with production of beta lactamases enzyme is a worrying sign for the clinicians and microbiologists. We recommend the routine surveillance of antibiotic resistance in the hospital.

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CONFLICT OF INTERESTS

All authors have none to declare

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