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Detection of bla $_{TEM}$ β-lactamase genes from clinical isolates of Pseudomonas aeruginosa in Chennai, India

Merina Paul Das*

Department of Industrial Biotechnology, Bharath University, Chennai- 600073, India

ABSTRACT

Existence of metallo-β-lactamases (MBLs) genes plays an important role in spreading β-lactam antibiotic resistance in the producing strains of these enzymes. The resistance of gram-negetive bacteria, such as Pseudomonas aeruginosa, to different antimicrobial agent, especially β-lactam and carbapenem, has been reported with increasing frequency worldwide. The aim of this study was to investigate the prevalence of bla_{TEM} gene encoding metallo-β-lactamases among a collection of Pseudomonas aeruginosa strains isolated from patients at different hospitals and health centers in Chennai, India through duplex PCR method. Susceptibility of 65 P. aeruginosa to 4 different antibiotics was determined using disc diffusion method. Isolates showing resistance to cefotaxime, ceftizoxime, amikacin and imipenem were subjected to micro broth dilution assay to determine their MIC values. Isolates containing bla_{TEM} were analyzed by Agarose Gel Electrophoresis (AGE). No other MBL genes were detected in this study. Six patterns were found for isolates containing bla_{TEM} gene by AGE. The bla_{TEM} was the main gene encoding MBLs among the isolates of P. aeruginosa in our study.

Keywords: Pseudomonas aeruginosa, Metallo-β-lactamases, TEM, PCR

Corresponding author

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INTRODUCTION

Pseudomonas aeruginosa is a leading cause of nosocomial infections, giving rise to a wide range of life-threatening conditions. Its intrinsic resistance to many antimicrobial agents and its ability to develop multidrug resistance imposes a serious therapeutic problem [1]. Various antimicrobial agents such as carbapenems, including meropenem and imipenem, are the most effective antibiotics [2] used for the treatment of infections caused by P. aeruginosa; however, increasing use of these compounds has resulted in the development of carbapenem-resistant P. aeruginosa [3].

Mechanisms of resistance to carbapenems in P. aeruginosa are associated with reduced uptake of the agent resulting from the loss or reduced expression of the OprD porin, combined with derepression of the chromosomal ampC β lactamase gene [4]; overexpression of an efflux pump system [5, 6]; and production of a Metallo-β-lactamases (MBLs) [7]. MBLs are the rapidly evolving group of β-lactamase enzymes (eg. TEM, SHV, CTX etc.) produced by these gram-negative bacteria, which have the ability to hydrolyse a broad range of anibiotics including penicillins, cephalosporins, cephamycins, oxacephamycins and carbapenems [8]. MBL use zinc ions at their active sites to catalyze the hydrolysis of all classes of beta lactam antibiotics, including carbapenems [9]. Some of carbapenem-resistant clinical isolates were found to produce a new MBL, TEM-1, which efficiently hydrolyzes carbapenems as well as other β-lactams. Moreover, TEM-1 is notable for its special character, in that it is hardly blocked by β-lactamase inhibitors such as clavulanate, sulbactam and tazobactam [10]. Therefore, strains producing TEM-1 are difficult to control with β-lactams antibiotic and related drugs in combination. MBLs are generally mutants of classical TEM genes.

Though presence of MBLs amongst antibiotic-resistant gram-negative aerobic rods P. aeruginosa have been reported from India. The present study was carried out to determine the prevalence of mainly TEM genes responsible for MBL production amongst the MBL positive P. aeruginosa species isolated from the patients admitted to different health care and hospitals in Chennai, India and we report analysis of the β-lactamase content and genetic support of P. aeruginosa were subjected to a PCR-aided rapid detection method with the bla-TEM-specific primers.

MATERIALS AND METHODS

Clinical isolates and identification

A total ninety of clinical isolates were collected from hospitals and health centres in south India during this period of study. The specimens included urine (25%), blood (5%), wound swab (32%), throat swab (25%), stool (5%) and other sites (9%). All isolates were identified by conventional bacteriological tests.

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Antimicrobial agents and susceptibility test

Antibiotic susceptibility test was performed by the disc diffusion method on Mueller Hinton agar media (Hi-Media, Mumbai) following the zone size criteria recommended by the National Committee for Clinical Laboratory Standards (NCCLS) [11]. Antibiotic discs such as cefotaxime (CTX:30 µg), ceftizoxime (ZOX:30 µg), amikacin (AN:30 µg) and imipenem (IMP:10 µg) have been used to identify the presence of MBLs in this study.

Extraction of Genomic DNA

Template genomic DNA was prepared from overnight cultures grown on Luria-Bertani medium (LB media, Bangalore Genei, India) by inoculating a single colony into 100 ml of double distilled water, boiling at 95°C for 10 min. Equal volume of chloroform:isoamylalcohol (24:1 v/v) was added, centrifuged at 8000 g for 15 min [12]. The upper layer was used as crude DNA. The genomic DNA was purified by phenol extraction and ethanol precipitation method [12]. The DNA was stored at -20°C. The samples were run on 0.8 per cent agarose gel and stained with ethidium bromide. The stained gel was examined under the UV light [14].

Design of oligonucleotide primers

Amplification of TEM genes encoding β-lactamases was carried out by PCR. Table 1 shows a list of the different primers used in screening for genotypic expression of β-lactamase production by the isolates. It also shows their sequences and amplicon sizes in bp [15].

Table 1: Primers used for detection of *bla*$_{TEM}$ genes

Target	Primer	Sequence[5'-3']
TEM	757 *bla*$_{TEM}$ Forward	GCGGAACCCCTATTTG/964
	821 *bla*$_{TEM}$ Reverse	TCTAAAGTATATATGAGTAAACTTGGTCTGAC/964

PCR amplification of β-lactamase encoding TEM gene

PCR amplification was performed in a 50 µl volume with the GeneAmp PCR system 9600 (Perkin-Elmer Cetus, Norwalk, Conn.) thermal cycler. Reaction mixtures contained 1 µM (each) primer, 200 µM (each) deoxynucleoside triphosphate (Bangalore Genei, India), 1X reaction buffer containing 1.5 mM $MgCl_2$ (Bangalore Genei,India), 2.5 U of Taq DNA polymerase (Bangalore Genei, India), and approximately 25 ng of template DNA. After an initial denaturation step (2 min at 94ºC), 30 cycles of amplification were performed, as follows: denaturation at 94º C for 1 min, annealing at 55ºC for 1min, and DNA extension at 72ºC for 1.5 min for the amplification of bla$_{TEM}$ [16]. The amplicons were separated in 1.5 per cent agarose gel. The gel was visualized by staining with ethidium bromide (0.5 mg/ ml) in a dark room for 30 min. A 1000 bp ladder molecular weight marker (Bangalore Genei, India) was used to measure the molecular weights of amplified products. The images of ethidium bromide stained DNA bands were digitized using a gel documentation system (Alphaimager$_{TM}$ 3400, USA).

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RESULTS AND DISCUSSION

MBL detection by disc diffusion

A total of ninty clinical isolates from the different hospitals and health centres in Chennai, India, sixty five multi-resistant Pseudomonas aeruginosa strains were isolated Table 2. The clinical Pseudomonas isolates in this study exhibited high rates of resistance as well as multiple antibiotic resistance (MAR) to the different drugs were tested. Antibiotic susceptibility testing by disc diffusion suggested an uncommon mechanism of resistance, since the isolate was resistant to most β-lactams, including ureidopenicillins, ureidopenicillins–β-lactamase inhibitors, narrow-spectrum amikacin, cefotaxime, ceftizoxime and imipenem. The phenotypic expression of MBLs producing strains is resistant to β-lactamase inhibitors such as clavulanic acid can restore susceptibility to inactive cephalosporin. Overall, susceptibility rates to the most commonly used antipseudomonal drugs were as follows in Table 3.

Table 2: Isolation of *Pseudomonas aeruginosa* from different site

Site	Sample	*Pseudomonas* strain	Resistanat strain
Hospital 1	15	13	2 (P2 & P9)
Hospital 2	19	12	3 (P4, P5 & P8)
Health centre 1	25	19	4 (P6, P11, P16 & P22)
Health centre 2	31	21	3 (P3, P18 & P27)

Table 3: Drug resistant patterns of *Pseudomonas* sp

Strain No	Amikacin	Cefotaxime	Ceftizoxime	Imipenem
	Zone of Inhibition (mm)			
3	R	10	17	12
4	R	14	18	26
7	21	R	15	25
10	21	R	11	18
8	11	20	R	26
17	23	11	16	R
12	19	17	R	27
19	20	18	R	17
20	R	21	17	25
1	34	R	19	R
23	R	21	14	13
14	19	R	14	17

Antibiotic resistance is a major factor that derives change in the pattern of antibiotic prescribing and is the most important stimulus to the development of new antibiotics by pharmaceutical industry. Widespread antibiotic usage has lead to the development of antibiotic resistance by various pathogens. P. aeruginosa can pose a major threat to life, are often difficult and expensive to treat and can delay the discharge of patients from hospitals.

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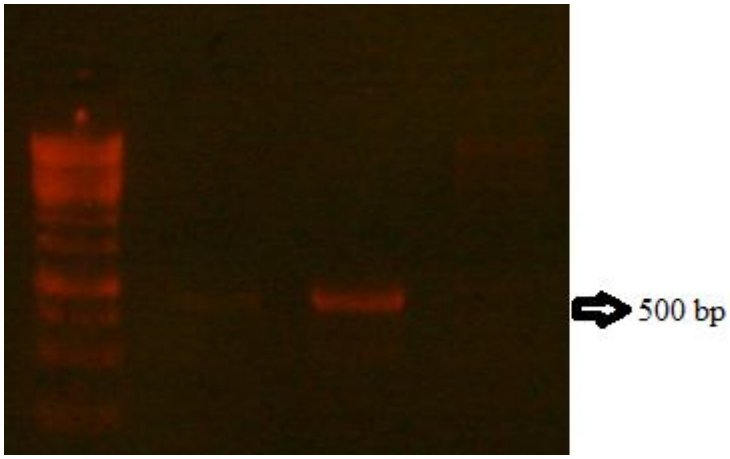
Prevalance of metallo-β-lactamase gene

On the basis of the resistance phenotype (resistance or intermediate susceptibility to various β-lactam substrate), Pseudomonas isolates were selected and studied for MBL production. In this study 12 isolates were found to be positive MBL producers among the 65 isolates of P. aeruginosa. The genomic DNA was isolated from the multi-resistant pseudomonas strains and run in agarose gel.

Identification of β-lactamase gene

PCR amplification of TEM was carried out on chromosomal DNA of P. aeruginosa. A total of 12 MBL positive Pseudomonas isolates were randomly selected to detect the presence of TEM genes. Among the 12 isolates 6 strains showed positive results for the presence of TEM gene. Amplified product of TEM gene in clinical P. aeruginosa isolates had a fragment size of 500 bp (Figure 1).

Figure 1: PCR detection of *bla*$_{TEM}$ gene



Pseudomonas species has rapidly become the most common MBLs producing organism, making it difficult to eradicate this organism from the high risk. Antimicrobial susceptibility testing showed that 48 per cent of isolates were resistant. This resistance is mainly associated with the production of enzymes called Temoniera (TEM) that are from chromosomal DNA.

Some MBLs confer high-level resistance to all oxyimino-cephalosporins, for other MBLs, resistance may only be slightly increased or selectively affected in certain β-lactams. This creates problem for the clinical laboratory. Some MBLs may fail to reach a level to be detectable by disc diffusion tests but result in treatment failure in the infected patient. The lack of correlation between MBL production and disc-diffusion susceptibility results was evident in the present study. These types of discrepancies between susceptibility data and disc diffusion results have increased the need for an improved method of MBL detection and to incorporate it into routine susceptibility procedures. The disc diffusion method does not allow routine

differentiation of strains producing these enzymes. Molecular methods, like PCR need to be used for the differentiation of β-lactamase-producing isolates.

In ordinary cases, it takes at least 3 days to receive the results of susceptibility tests from clinical laboratories, which might be too late to select appropriate antimicrobial agents for therapy. Thus, the PCR-aided rapid detection method described here would provide a clue for rigorous infectious disease control [16].

In this research we targeted to amplify the TEM genes from choromosomal DNA which encodes MBLs. Amplification of whole genomic DNA increased the positivity of detection, suggesting β-lactamase expression controlled by chromosomal DNA. Therefore, MBL positive strains had a common multidrug resistant phenotype which need of appropriate therapeutic protocols and surveillance studies to avoid the spread of these worrisome resistance genes. These studies could help us to design new drug targets harnessed with insilicobiology tools prospecting for a better future in clinical aspects.

CONCLUSION

In conclusion, the results obtained from this research indicated that TEM is the only MBLs enzyme to emerge among clinical isolates of P. aeruginosa to date. As patients with infections caused by MBL-producing organisms are at an increased risk of treatment failure, rapid identification of these organisms is essential. So, it is recommended that a simple test based on NCCLS recommendation should be adopted to confirm MBL-producing bacteria in clinical laboratories.

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