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Molecular Detection of ESBL (CTX-M and SHV) genes in *Pseudomonas aeruginosa*

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Abstract

Phenotyping is commonly used for detection of extended spectrum *beta lactamase* (ESBL) production in Gram-negative isolates. ESBLs are mainly coded for b three important genes, namely bla_{TEM}, bla_{SHV} and bla_{CTX-M}. Multiplex PCR as a rapid method has been used to identify two common genes (bla_{CTX-M} and bla_{SHV}) responsible for extended spectrum *beta lactamase* production in members of *Pseudomonadaceae* family isolated from different clinical samples from a speciality hospital at Chennai. A total of 10 non repetitive clinical isolates of 24 patients from urine was selected for study. Phenotypic identification for ESBL production was confirmed by multiplex PCR for bla_{CTX-M} and bla_{SHV} performed for the ESBL positive isolates. Among the ESBL strains of *P.aeruginosa*, 8 isolates were found to be positive for bla_{SHV} gene and á isolates were found to be positive for bla_{CTX-M3} like gene. The present study demonstrates rapid detection of bla_{SHV} and bla_{CTX-M} in isolates belonged to *Pseudomonadaceae* using multiplex PCR. This genotypic method provided rapid and efficient differentiation of ESBLs in the laboratory.

Keywords : ESBL, *Pseudomonas sp*, SHV and CTX-M.

INTRODUCTION

Infection by extended spectrum β-lactamase (ESBL) producing Gram-negative pathogens has become an increasing problem.Production of extended spectrum β-lactamases (ESBLs) and AmpC β-lactamases is the most common mechanisms of antimicrobial resistance in Gram-negative bacilli. Infection by ESBL producing, Enterobacteriaceae has become a serious problem in India. It has been reported the prevalence of ESBLs to be in the range 6-88 per cent in various hospitals (Baby-Padmini and Appala raju, 2004), especially among *Pseudomonas* CTX-M 3 , a variant of CTX-M 15 has also been reported from India in one study (Bonnet, 2004). *Pseudomonas aeruginosa* is a common bacterium which can cause disease in animals and humans. It is found in soil, water, skin, flora and mostly in man-made environments throughout the world. It thrives not only in normal atmospheres, but also with little oxygen, and thus, has colonised many natural and artificial environments. It uses a wide range of organic material for food; in animals, the versatility enables the organism to infect damaged tissues or people with reduced immunity. The symptoms of such infections are generalized inflammation and sepsis. If such colonisations occur in critical body organs such as the lungs, the urinary tract, and kidneys, the results can be fatal (Balcht *et al.,* 1994). A prospective study was undertaken to know the occurrence of ESBL producing strains of *Pseudomonas aeruginosa* using molecular techniques (PCR and RAPD-PCR), and the antibiotic susceptibility to newer agents to guide empirical therapy for complicated nosocomial infections.

MATERIALS AND METHODS

Collection of Samples

Throat swabs, wound swabs and urine samples were collected from patients suspected to have the infections at the discretion of the provider. Swabs were also agitated or sequenzed with forceps in nutrient broth to release the organisms, if any. The samples of the broth were then plated on solid media. The urine sample was inoculated into broth using a sterile inoculation loop and organisms grown in pure culture were identified by standard biochemical test and antibiotic suspectibility test by disc diffusion method

Test For Beta Latamases

β-lactamases are enzymes possessed by many bacteria which break the β-lactam ring present in penicillins to detect the presence of β-lactamase in an organism. A large loopful of organism from pure culture was inoculated into the penicillin G buffer solution to give a very thick suspension and incubated at room temperature for one hour. A small drop of iodine solution was added and mixed and the results are recorded. Persistence of blue colour up to 10 minutes indicated negative and discolouration of blue colour within 10 minutes indicated positive result.

Detection of Extended Spectrum Beta-Lactamases (ESBLs)

ESBLs are enzymes that mediate resistance to extended-spectrum (third generation) cephalosporins (e.g.,

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ceftazidime, cefotaxime, and ceftriaxone) and mono lactams (e.g., aztreonam) but do not affect cephamycins (e.g., cefoxitin and cefotetan) or carbapenems (e.g., meropenem or imipenem). ESBLs are difficult to detect because they have different levels of activity against various cephalosporins. Thus, the choice of the antimicrobial agents to be tested is critical. For example, one enzyme may actively hydrolyze ceftazidime, resulting in ceftazidime minimum inhibitory concentrations (MICs) of 256 µg/ml, but have poor activity on cefotaxime, producing MICs of only 4 µg/ml. If an ESBL is detected, all penicillins, cephalosporins, and aztreonam should be reported as resistant, even if in vitro test results indicate susceptibility.

There are standard broth microdilution and disc diffusion screening tests using selected antimicrobial agents. Each *P.aeruginosa* isolate is considered a potential ESBL-producer and the expected test results are as follows:

S.NO.	ANTIBIOTICS	STRENGTH
1	cefpodoxime (CEP)	10mcg
2	ceftazidime(CA)	30mcg
3	aztreonam(AO)	30mcg
4	cefotaxime(CE)	10mcg
5	ceftriaxone(Cl)	10mcg

The sensitivity of screening for ESBLs in enteric organisms can vary depending on which antimicrobial agents are tested. The use of more than one antimicrobial agents has been suggested for screening and it improve the sensitivity of detection. Cefpodoxime and ceftazidime show the highest sensitivity for ESBL detection.

Phenotypic confirmation of potential ESBL-producing isolates of *K. pneumoniae*, *K. oxytoca*, or *E. coli* was done by testing both cefotaxime and ceftazidime, individually and in combination with clavulanic acid. Testing can be performed by the broth microdilution method or by disc diffusion method. For MIC testing, a decrease of > 3 doubling dilutions in an MIC for either cefotaxime or ceftazidime in combination with 4 mg / ml clavulanic acid, versus its MIC when tested alone, confirms an ESBL-producing organism. For disc diffusion testing, a > 5 mm increase in a zone diameter for either antimicrobial agent tested in combination with clavulanic acid versus its zone when tested alone confirms an ESBL-producing organism.

Genomic DNA Isolation

The bacterial cell wall is disrupted or ruptured by the addition of lysis buffer which contains 10% SDS an anionic detergent which denatures the proteins,

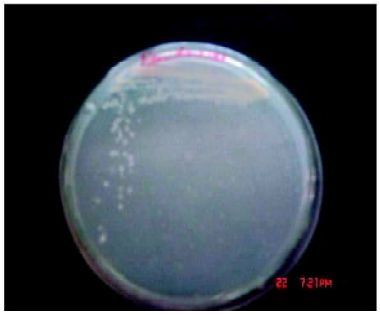

Fig1:*Pseudomonas* culture in mineral salt medium

Fig 2:Separation of Genomic DNA By Agarose Gel Electrophoresis

Fig 3 : Multiplex PCR for bla_{CTX-M} and bla_{SHV}

CTX-M GENE

SHV GENE

Tris- buffer is used to maintain the pH of the cells, EDTA chelates Mg++ and prevents the DNase activity. Proteinase K is used to digest protein and remove contamination. Addition of proteinase K to nucleic acid preparations rapidly inactivates nucleases that might otherwise degrade the DNA or RNA during purification. It is highly-suited to this application since the enzyme is active in the presence of chemicals that denature proteins, such as SDS chelating agents such as EDTA. Proteinase K is also stable over a wide pH range (4-12), with a pH optimum of pH 7.5-12. Phenol a strong denaturant denatures the proteinase k and leaves the DNA in the supernatant (Sambrook *et al.,* 1993).

Agarose Electrophoresis

For the majority of DNA samples, electrophoretic separation is carried out in agarose gels. This is because DNA molecules and their fragments are considerably larger than proteins; therefore larger size agarose gels are required. Under an electric field, any given fragment of DNA should move towards the anode with the same mobility. This is due to the charge per unit length owing to the phosphate groups. Separation on agarose gels is achieved because of resistance to their movement caused by the gel matrix. Thus the largest molecules will have difficulty moving, whereas the smallest molecules will be relatively unhindered. Consequently the mobility of DNA molecules during gel electrophoresis will depend on size. Gel concentrations must be chosen based on the molecules to be separated such as for plasmid molecules – 1%; genomic DNA – 0.8% and RNA – 1.5%, mitochondrial DNA – 0.8% (Sambrook *et al.,* 1993).

Polymerase Chain Reaction (PCR)

PCR is an *in vitro* method of enzymatic synthesis of specific DNA sequence. It is a very simple and inexpensive technology for characterizing, analyzing, synthesizing, a specific DNA or RNA from virtually, any living organism, plant, animal, virus or bacteria. It exploits the natural function of polymerase present in all living things to copy genetic material or to perform molecular photocopy.

RESULTS AND DISCUSSION

P. aeruginosa was isolated from different clinical samples adapting conventional methods based on morphological and biochemical characters. The clinical isolates were identified as *P. aeruginosa* (Table 1).The number of bacterial colonies was observed on the nutrient agar media. Among those, ten different colonies of *P.aeruginosa* were selected for ESBL detection (Fig 1).

Genomic DNA was isolated from the blood samples using standard proteinase k digestion and phenol : chloroform extraction method . The purified DNA was quantified, aliquated in small lots and stored at 20^0C for this study (Fig 2).

Phenotypic identification was done to all 10 isolates and to identify the production of ESBL by multiplex PCR. Out of 10 isolates, 8 showed positivity for ESBL production. The selected 10 bacterial isolates of *P. aeruginosa* isolates with multiple â-Lactamases could be demonstrated clearly by the multiplex PCR as shown in Fig.5. Among the several genes being responsible for the ESBL production, presence of two genes – bla_{SHV} (141 bp) and $bla_{CTX\text{-}M3}$ like (449 bp) were screened by PCR and multiplex PCR (Fig 3).

In the present study ESBL strains of *P. aeruginosa*, 8 isolates were found to be positive for bla_{SHV} gene and 2 isolates were found to be positive for blaCTX-M-3-like gene. More specifically the presence of bla_{SHV} and blaCTX-M-3-like is elucidated in their genomic DNA rather being mediated by the plasmids. This suggest that the antibiotic resistance could be mediated chromosomally among the nosocomial strains possessing further complications via genetic recombination, in turn demands newer drugs for an effective treatment. The results of this agreed with the findings of previous workers (Paterson and Bonomo, 2005; Matsumoto *et al.,* 1988; Pitout *et al.,* 2005; Nagano *et al.,* 2004; Al Naiemi *et al.,* 2005). From the above results, it can be concluded that there is high prevalence of ESBL producing strains of *P.aeruginosa* among the patients. According to the PCR and multiplex PCR assays, the most prevalent gene among ESBL producer was SHV whereas CTX-M-3-like was less prevalent.

Table 1: Biochemical Characters of *Pseudomonas aeruginosa*

BiochemicalTests	Characteristics
Catalase	Positive
Oxidase	Positive
Indole	Negative
Methyl red	Negative
Voges proskauer	Negative
Citrate	Negative
Nitrate reduction	Positive
Glucose	Negative
Sucrose	Negative
Lactose	Negative
Maltose	Negative

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