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PCR Detection of *bla*IMP Gene in Metallo-β-Lactamase Resistant *E. coli* Isolated from Clinical Samples

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A B S T R A C T

PCR detection of *bla*IMP gene in Imipenem resistant *E. coli* is the aim ot this study. Total of n=66 of n=23 Imipenem resistant *E. coli* were selected for the present study based on the drug resistance pattern and their MIC to Imipenem. Phenotypic detection of MBL production was carried out and subsequently, PCR amplification of *bla*IMP was carried out. Among selected (n=23) Imipenem resistant *E. coli* (IREC), 100% (n=23) strains were positive for Imipenem mediated MBL production. Twenty-three MBL positive IREC isolates were shown presence of Plasmid DNA, with size measured about approx. 6kb. PCR amplification of *bla*IMP gene codes for Imipenem mediated MBL production yielded amplified *blaIMP* gene product with amplicon size of 189 bp. The presence *bla*IMP gene also contributes for MBL production which makes organisms to show reduced susceptibility against Imipenem antibiotic

Introduction

β-lactam antibiotics are among the most often used antimicrobial agents and an increasing incidence of resistance to these drugs is a public health concern. β-lactam antibiotics as a class have a broad spectrum of antibacterial activity, including important Gram-positive and Gram-negative pathogens. Because of their favourable characteristics, β-lactam are the most broadly used antibiotics worldwide (Livermoore, 2006). These antibiotics act by inhibiting a set of transpeptidase enzymes (also called penicillin binding proteins or PBPs) that are essential for the synthesis of the peptidoglycan layer of the bacterial cell wall (Sauvage *et al.*, 2008). The inhibition of peptidoglycan synthesis results in the death of growing bacteria and accounts for the antimicrobial effect of β-lactam antibiotics. In response, bacteria have evolved defence mechanisms to resist the lethal effects of these drugs (Bush *et al.*, 2011). Due to widespread β-lactam antimicrobial use, bacterial resistance has been increasing and now represents a serious threat to the continued use of antibiotic therapy (Babic *et al.*, 2006).

Site directed mutagenesis studies of the active site histidines in MBLs have been performed

and the results are consistent with an important role for these residues in zinc binding and hydrolytic activity (Haruta *et al.,* 2000). Saturation mutagenesis and directed evolution studies of MBLs have also yielded insights into the sequence requirements for enzyme function. Materon *et al.,* analysed the residues in and near the active site of the subclass B1 IMP-1 enzyme using a randomization and genetic selection strategy (Materon *et al.,* 2004). For these studies, the codons for 29 residue positions in IMP-1 were individually randomized by oligonucleotide mutagenesis to create 29 random libraries. Each random library was then introduced into *E. coli* and clones expressing functional β-lactamase mutants were identified by selection for growth on agar plates containing β-lactam antibiotic

The recently discovered NDM-1 β-lactamase provides an example of the potential for dissemination of MBLs. NDM-1 was first detected in 2008 in *K. pneumonia* and *E. coli* in a patient returning to Sweden from India. NDM-1 has been shown to be present at significant frequency within *Enterobacteriaceae* in India and has subsequently been shown to be present in bacterial isolates in a number of countries worldwide. The bla_{NDM-1} gene has been found on several plasmid types, including IncA/C, IncF, IncL/M, and it can be transferred among gram negative bacteria by conjugation. However, in contrast to the situation with the genes encoding IMP- and VIM-type MBLs, the bla_{NDM-1} gene has not been found in integrons structures. Nevertheless, NDM-1 has spread broadly and rapidly (Nordmann *et al.,* 2011). The ISAba125 insertion element has been associated with the bla_{NDM-1} gene suggesting insertion sequences may contribute transfer of NDM-1. Therefore, the principal objective of this study was to investigate the presence of *bla*IMP gene and associated plasmids from these *E. coli* clinical *isolates*.

Materials and Methods

Bacterial isolates

The total of n=23 Imipenem resistant *E. coli* were selected for the present study. Identification was done based on culture characteristics. The antimicrobial susceptibility test was carried out as per CLSI standards for determination of drug resistance, further MIC of imipenem was also carried out. Based on that the isolates were selected for further study. All strains were cultured in Luria-Bertani (LB) broth and stored in cryovials with 30% glycerol at −20°C.

MBL – Metallo - β-lactamases

Phenotypic detection of MBLs

The phenotypic detection method of MBL production was designed using a single agar plate and consist of two components (i) In the combined-disk test, two IPM disks (10 µg), one containing 10 µl of 0.1 M (292 µg) anhydrous EDTA (Sigma Chemicals, St. Louis, MO), were placed 25 mm apart. An increase in zone diameter of >4 mm around the IPM-EDTA disk compared to that of the IPM disk alone was considered positive for an MBL, (ii) In the DDST, an IPM (10 µg) disk was placed 20 mm from a blank disk containing 10 µl of 0.1 M (292 µg) EDTA. Enhancement of the zone of inhibition in the area between the two disks was considered positive for an MBL. Disks were applied to the surface of the inoculated agar as shown in and plates were incubated overnight at 37°C (Drieux *et al.,* 2008; Chauhan *et al.,* 2015).

DNA extraction

Plasmid DNA was extracted from bacterial isolates by alkaline lysis method. The DNA bands were observed on agarose gel

electrophoresis under UV-transilluminator. The extracted DNA was stored at -20^{0}C in TE buffer (Sambrook *et al.,* 2009).

PCR amplification of metallo-ß-lactamase coding genes

PCR amplification was performed to detect plasmid-encoded *bla*-IMP responsible for metallo-ß-lactamase production. Primers, sourced from Chromous Biotech Pvt Ltd, Bangalore, were for *bla*IMP-5' – GGAATAGAGTGGCTTAACTCTC 3' (Forward), *bla*IMP (reverse) 5' – CCAAACTACTAGGTTATCT – 3' (Fallah *et al.,* 2013). For PCR amplification, about 100 ng of DNA was added to 50 µl mixture containing 2.5 mM of dNTPs, 0.5 mM of each forward and reverse primer and 3 U of *Taq* polymerase in 10x PCR buffer containing 1.5 mM $MgCl_2$. Amplification was performed in a Corbett CGI-96 Thermocycler with cycling parameters comprising initial denaturation at 94^o C for 5 min each followed by 35 cycles of denaturation at 94^o C for 30 s, annealing at 55^o C for 30 s, extension at 72^o C for 30 sec and final extension at 72^o C for 7 min. A 100 bp DNA ladder and 500 bp DNA ladder (Bangalore Genei Pvt Limited, India) were used to measure the size of amplicons.

Results and Discussion

Selection of bacterial strains

The total of n=23; Metallo-β-Lactamase *producing E. coli* were selected for the present study after antimicrobial susceptibility test and MIC to Imipenem. Among selected (n=23) Imipenem resistant *E. coli (IREC).* The marginal increase in zone of inhibition by >5 mm of Imipenem/ EDTA combination in comparison with Imipenem alone (Fig 1). Among n=23 *E. coli* isolates, all of them were positive for metallo-ß-lactamase production. This was phenotypically confirmed by MBL phenotypic detection method. Twenty-three *E. coli* isolates were positive MBL producers.

PCR detection of *bla*IMP gene

All the n=23 MBL producing MDR *E. coli* strains have been selected for genotypic study, i.e., PCR amplification of MBL producing genes, *bla*IMP ((accession no. MF169878). The samples showed amplification of 189 bp (Fig. 2), with primers specific to *bla*IMP. The BLAST analysis of the respective sequences was matching 100% *bla*IMP.

The increasing incidence of MBL producing *Enterobacteriaceae*, particularly *E. coli* and *K. pneumoniae* posing a serious threat to public health across the world. The present study n=23 *E. coli* isolates, all of them were positive for metallo-ß-lactamase production. This was phenotypically confirmed by MBL phenotypic detection method. Twenty-three *E. coli* isolates were positive MBL producers.

Earlier studies indicated a high incidence of MBL producing *E. coli* (18.98%) in different clinical samples. A previous study from an another tertiary care hospital in Nepal reported comparatively lower incidence of MBL producing gram negative bacteria (1.3%) in lower respiratory tract specimens (Mishra *et al.,* 2012).

The majority of MBL producing isolates of *E. coli* (53.56%) were from patients admitted to ICU. The ICU has been described as a factory for creating, disseminating, and amplifying antimicrobial resistance (Bora *et al.,* 2014).

MBL producing bacterial isolates can confer resistance to carbapenems and all beta-lactam agents except aztreonam although coexistence of other resistance mechanisms such as AmpC type beta-lactamases or ESBLs render them resistant to aztreonam (Bora *et al.,*

2014). Likewise, all the isolates of *E. coli* and *K. pneumoniae* with MBL production in the present study were found to be resistant to carbapenems. These isolates also exhibited a high level of resistance to the penicillin's, the third and fourth generation cephalosporin as well as to the beta-lactam/beta-lactamase inhibitor combination tested in the study. These findings are similar with other reports (Johnson *et al*, 2010: Deshmukh *et al.*, 2014), whereas in few reports MBL producing *enterobacteriaceae* isolates were found to be susceptible to various carbapenems as well as to piperacillin/tazobactam by disc diffusion testing (Seema *et al.*, 2011).

Fig.1 The increase in the zone of inhibition with an IPM (10 µg) disk plus 292 µg EDTA could increase the zone diameter by up to 4 mm compared to that of an IPM disk alone

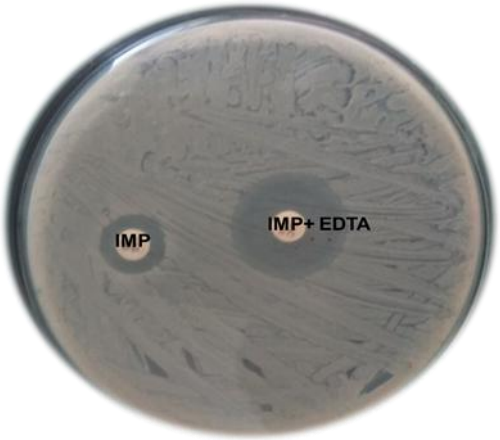
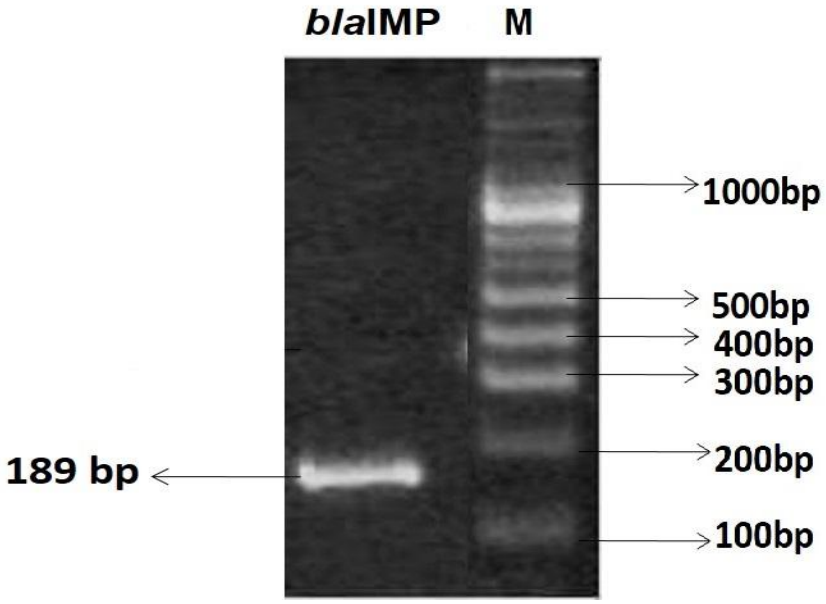


Fig.2 blaIMP gene with amplicon size of 189 bp



Lane 1 –M (100 bp ladder) ranging from 100bp – 1kb

All the 23 MBL producing MDR *E. coli* strains have been selected for genotypic study, i.e., PCR amplification of MBL producing gene *bla*IMP. The samples showed amplification of 189 bp (Fig. 2), with primers specific to *bla*IMP.

Since 1988, transferable carbapenem resistance has been found in several P. aeruginosa strains isolated in Toyama Prefecture, Japan (Lagatolla *et al.,* 2004). In 1991, an IMP-1 type MBL, initially characterized in a strain of *S. marcescens*, gave high-level resistance to various broad-spectrum β-lactams including imipenem **(**Ito *et al.,* 1995). This strain was isolated in a hospital in Aichi Prefecture and had the *intI1* gene just upstream of the bla$_{IMP-1}$ gene cassette on the chromosome.

It was confirmed that the IMP-1 type of MBL is the most common MBL in Japan at present, although IMP-3 **(**Iyobe *et al.,* 2000**)** and IMP-6 (Shigemoto *et al.,* 2010) have also been identified in Japan. Since very few amino acid substitutions exist among IMP-1, IMP-3, and IMP-6, the latter two MBLs are fundamentally variants of IMP-1. It is difficult to confirm whether all the *bla*$_{IMP-1}$-positive strains found in this study carry genuine *bla*$_{IMP-1}$, since the primer sets used in this study cannot distinguish *bla*$_{IMP-3}$ and *bla*$_{IMP-6}$ from *bla*$_{IMP-1}$

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