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Detection of *bla*NDM-1 and Genetic Relatedness in Clinical Isolates of *Escherichia coli* Producing Extended Spectrum *β*-Lactamase from Tertiary Care Centres in South India

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Abstract

Background: Extended spectrum *β*-lactamases (ESBL) producing *E. coli* co-producing other *β*-lactamases and exhibiting co-resistance to different antibiotic classes continue to emerge as a threat to clinical field. This study aimed to analyze the co-production of New Delhi metallo-*β*-lactamase-1 (*bla*NDM-1) in ESBL producing plasmid-bearing clinical isolates collected from two tertiary care centres in Kerala, South India, and to understand their genetic relatedness. Methods: Antibiotic resistance phenotypes of 44 clinical isolates were determined by disc-diffusion method. Plasmid-bearing isolates, detected by the alkaline-lysis method, which also tested positive for ESBL production, were screened for the presence of *bla*NDM-1 by polymerase chain reaction. Plasmid, random amplified polymorphic DNA profiles and *bla*NDM-1 sequence-based phylogenetic tree were analyzed to understand the genotypic similarities among the isolates. Results: Beta-lactam antibiotics, quinolones, cephalosporins, used in this study, and AZM were found to be ineffective against the isolates as significantly high number of isolates were resistant to these antibiotics ($P < 0.01$). Plasmid bearing isolates constituted 57% (n = 25), all of which were found to be ESBL producers. *bla*NDM-1 amplicons were noticed in four (16%) isolates and these DNA sequences showed homology between them and with similar sequences reported from other countries like Japan and Korea. Plasmid and RAPD profiles demonstrated that most of the isolates, including those harbouring *bla*NDM-1 shared genetic similarities as well as an apparent geographical distinctiveness. Conclusion: The predominance of ESBL production and the occurrence of *bla*NDM-1 in plasmid-bearing isolates observed in our study corroborate the worldwide drug-resistance

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scenario. This study thus warrants the need for constant surveillance in the face of sparse information available in Kerala State on the emerging drug resistance in clinical bacteria.

Keywords

ESBL, *bla*NDM-1, Plasmids, *E. coli*

1. Introduction

Extended spectrum *β*-lactamases (ESBL) producing clinical bacterial isolates are emerging rapidly worldwide. These enzymes can hydrolyse broad spectrum cephalosporins and monobactams [1] [2]. ESBL production is a major characteristic of *Enterobacteriaceae* especially *E. coli* and *Klebsiella* spp. though other organisms also produce these enzymes less frequently [3]-[5]. A significant increase in the number of ESBL producing *E. coli* has been observed during the past decades which impacts clinical and community settings [6]-[8]. Since most of the ESBL genes are located on plasmids carrying resistance genes against other antibiotic classes, ESBL producers frequently exhibit resistance to multiple antibiotics posing a threat to healthcare management [3] [5] [9].

Carbapenems are the effective agents commonly used against ESBL producing bacteria whereas isolates resistant to these antibiotics and even the "last resort" agents like tigecyclins and polymyxins are now emerging in the clinical field [10]. Carbapenem resistance is mainly attributed to the production of various carbapenemases and one of these, the New Delhi metallo *β*-lactamase-1 (*bla*NDM-1), first identified in India, caused widespread alarm recently [11]. In addition, isolates co-producing various *β*-lactamases have also been frequently encountered in hospital settings from various parts of the world including India [12]-[15]. All these enzymes act as weapons to fight against the bactericidal and bacteriostatic effects of multiple antibiotics, facilitating evolution of deadly bacterial pathogens.

The aim of this study was to determine the prevalence and coproduction of ESBL and *bla*NDM-1 type carbapenemase in clinical isolates of *E. coli* collected from two tertiary care centers in Kerala. The closest relatives of the *bla*NDM-1 gene sequences have also been analyzed by constructing a phylogenetic tree. Plasmid profiles and Random Amplified Polymorphic DNA (RAPD) fingerprints were also analyzed to determine the genetic similarities and dissimilarities among the isolates.

2. Materials and Methods

2.1. Bacterial Isolates and Identity

Bacterial isolates were collected from two tertiary care centers in Kerala, South India. Isolate identity was confirmed by biochemical tests and ribotyping using Microbial Type Culture Collection (MTCC) *E. coli* strain 41 as reference. LPW57-5' AGTTTGATCCTGGCTCAG3' and LPW58-5' AGGCCCGGGAACGTATTCAC3' [16] were used as forward and reverse primers respectively for ribotyping. Isolates which were found to harbour plasmid DNA, detected by the alkaline lysis method, which also tested positive for ESBL production, were included in this study.

2.2. Antimicrobial Susceptibility Testing

Antibiotic sensitivity against 10 antibiotics, belonging to five different classes, was tested by Kirby-Bauer disc diffusion [17] method according to Clinical and Laboratory Standards Institute (CLSI) manual [18]. The antibiotic discs (Hi Media laboratories, Mumbai, India) used in this study were Ampicillin (AMP)—10 mcg, Ceftazidime (CAZ)—30 mcg, Cefotaxime (CTX)—30 mcg, Piperacillin/Tazobactam (PIT)—100/10 mcg, Azithromycin (AZM)—15 mcg, Gentamicin (GEN)—10 mcg, Nalidixic acid (NA)—30 mcg, Ciprofloxacin (CIP)—5 mcg, Meropenem (MRP)—10 mcg and Chloramphenicol (C)—30 mcg.

2.3. Plasmid and Genomic DNA Isolation

Plasmids and genomic DNA were isolated by alkaline lysis method [19] and the procedure described by Keller and Manak (1989) [20] respectively. The plasmid DNA was electrophoresced at 100 V for 1 h and 30 min in

0.5× Tris-Borate-EDTA (TBE), using 0.8% (w/v) agarose to obtain the plasmid profiles of different isolates. Molecular weights of plasmid DNA was determined using AlphaView (AV) version 3.2.2.0 software.

2.4. Screening and Confirmation of ESBL Production

Phenotypic screening and confirmation of ESBL production was performed as per CLSI manual. Disc diffusion technique was applied on Mueller-Hinton Agar (MHA) using ceftazidime (CAZ)—30 mcg, aztreonam (AT)—30 mcg and cefotaxime (CTX)—30 mcg, discs for screening and ceftazidime, ceftazidime-clavulanic acid (CAC)—30/10 mcg, and cefotaxime, cefotaxime-clavulanic acid (CEC)—30/10 mcg, discs for confirmation. Lawn culture of isolates was prepared on MHA and the culture with appropriate antibiotic discs was incubated at 37˚C for 18 h. The diameter of zone of inhibition was measured and the results were interpreted as per CLSI recommendations. *E. coli* MTCC41 served as the negative control.

2.5. PCR Amplification of *bla*NDM-1

A PCR-based screening was conducted to detect the presence of *bla*NDM-1 gene on plasmid DNA from the isolates, using primers NDM-F—5'GGTTTGGCGATCTGGTTTTC3', and NDM-R—5'CGGAATGGCTCAT-CACGATC 3' [21] [22]. The PCR reactions were performed in a minicycler (MJ Research, USA) in a volume of 25 µl containing 1× PCR buffer, 1.5 mM $MgCl_2$, 200 µM each of dNTPs, 2 U of Taq DNA polymerase, 0.5 µM primer and 100 ng template DNA. All reagents were purchased from Bangalore Genei Pvt. Ltd. HPLC purified primers were purchased from Sigma Aldrich Chemicals Pvt. Ltd. (Bangalore).

The PCR products were sequenced at a commercial facility (Xcelris Labs Limited, Ahmedabad). The nucleotide sequences and deduced protein sequences were analyzed with Basic Local Alignment Search Tool (BLAST) programs of National Center for Biotechnology Information (NCBI) (www.ncbi.nlm.nih.gov). Presence of open reading frames (ORFs) and conserved regions were detected by ORF finder (www.ncbi.nlm.nih.gov/gorf/gorf.html).

2.6. Phylogenetic Analysis

The DNA sequences of *bla*NDM-1 amplicons obtained in the present study were compared with those of *Klebsiella pnemoniae*, *Citrobacter freundii*, *E. coli*, *Serratia* sp. and *Acinetobacter soli* strains retrieved from the NCBI GenBank database. Nucleotide BLAST (BLASTN) was used for the sequence searches with default parameters and those DNA sequences, which showed hits with the study sequence, with minimum "E value" and maximum "query coverage" were considered for phylogenetic analysis. To determine the nearest phylogenetic neighbours, each sequence was subjected to the nucleotide sequence homology searches using BLAST homology search tool [23]. All the sequences were aligned using default configuration of multiple sequence comparison by log-expectation (MUSCLE) embedded in MEGA 5 (Molecular Evolutionary Genetics Analysis) software [24] [25]. The phylogenetic tree has been constructed by neighbour-joining method with 1000 heuristic bootstrap replicates and substitution model as "p distance".

2.7. RAPD Analysis

Initial screening of genomic DNA samples was carried out with 10 different decameric oligonucleotide primers—RBaC 1 - 10 (Bangalore Genei, Pvt. Ltd.) to check for the reproducibility of the fingerprints. Of the few primers which produced consistently reproducible pattern of discrete bands, the primer, RBaC5 with the sequence AGGGGCGGCA (Accession. no. AM911680) was selected for generating fingerprints of the isolates. As described earlier, 25 µl reactions were subjected to thermal cycling according to manufacturer's instructions. The photographs of RAPD gels were used to construct the dendrogram employing NTSYS pc2.0 (Numerical Taxonomy and Multivariate Analysis System) software by unweighed pair-group method arithmetic mean (UPG-MA).

2.8. Statistical Analysis

Statistical analysis was carried out using SPSS (Statistical Package for Social Sciences) software version 20. Significance of difference between two independent proportions was analyzed by Z-test. A two-tailed $P < 0.01$ was considered significant.

3. Result

3.1. Bacterial Isolates

A total of 44 *E. coli* isolates were collected all of which were found to produce an amplicon having same molecular weight as that of *E. coli* MTCC 41. This particular band was sequenced for confirmation of the reliability of identification. The sequence showed identity with *E. coli* 16S rRNA sequences.

3.2. Antibiotic Sensitivity

All the isolates were found to be resistant to AMP, *β*-lactam/*β*-lactam inhibitor combination—PIT, 1st and 2nd generation quinolone—NA and CIP, 3rd generation cephalosporins—CTX and CAZ. Significantly high resistance was observed against AZM [(77%) ($P < 0.01$)]. However, comparatively decreased resistance was observed in isolates against GEN (57%), MRP (59%) and C (25%).

3.3. Plasmid Isolation and ESBL Production

Plasmids could be isolated from 25 (57%) of the isolates which were designated as E1 to E25. All of the plasmid bearing isolates were found to be ESBL producers as they gave positive results during screening (zone of inhibition was ≤22, ≤27 and ≤27 around CAZ, AT, and CTX respectively) which were further confirmed by employing CAZ and CTX antibiotics alone and in combination with clavulanic acid [ceftazidime-clavulanic acid (CAC—30/10 mcg) and cefotaxime-clavulanic acid (CEC—30/10 mcg)]. The zone of inhibition around CAC disc was found to increase in diameter (>5 mm) than that around CAZ in all the isolates. CEC was found to be ineffective.

3.4. Phylogenetic Analysis of *bla*NDM-1 Sequences

*bla*NDM-1 specific amplicons were observed in only four (16%) isolates (E7, E8, E10 and E23) which were found to vary in size from ~580 to 850 bp **Figure 1**. The DNA sequence identity was confirmed using BLASTN analysis. These four DNA sequences together with six similar sequences, retrieved from NCBI GenBank database, based on the criteria mentioned above, were used to construct the phylogenetic tree in order to understand the nearest neighbour of the study sequences. The genetic divergence and homogeneity of the sequences are apparent in the phylogenetic tree **Figure 2**. Among the DNA sequences obtained from our study, those from isolates E23 and E8 were found to form distinct lineages, whilst those from E7 and E10 shared similarity. Interestingly, these sequences were closely placed in the phylogenetic tree and their genetic similarity with sequences from other countries like Japan (Accession no: KP347609.1), Korea (Accession no: CP012754.1) and china (Accession no: KP987216.1) as well as with two sequences reported from India (Accession no: KR872634.1 and KR872624.1) was also clearly discernible in the tree.

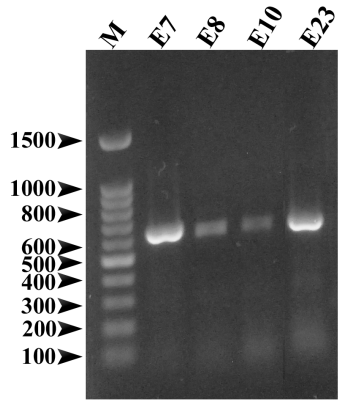

Figure 1. Agarose gel (1.0%) showing *bla*NDM-1 amplicons from plasmid DNA of four *E. coli* isolates: Lane M denotes 100 bp DNA ladder. The other four lanes show *bla*NDM-1 amplicons from isolates—E7, E8, E10 and E23.

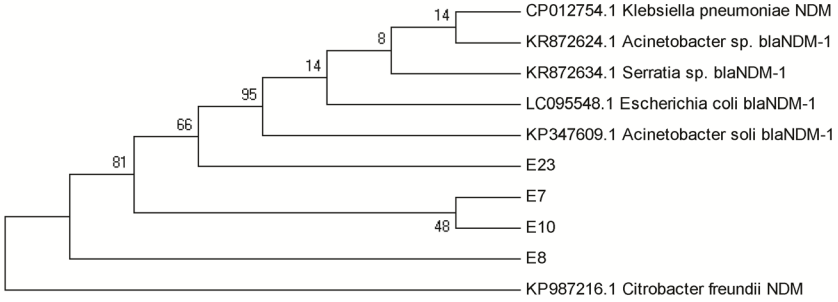

Figure 2. Phylogenetic analysis based on *bla*NDM-1 gene sequences obtained from the four *E. coli* isolates in this study and six sequences retrieved from GenBank database (NCBI). Numbers on nodes represent bootstrap support values.

3.5. Plasmid Profile and RAPD Analysis

All the isolates were found to exhibit some genotypic similarities, as evident from their plasmid profile **Figure 3(a)** and RAPD dendrogram. Three major plasmid profile types (a-c) could be noticed, with molecular weights ranging from 1.3 to >21 kb **Table 1**, on agarose gel electrophoresis of the isolated plasmids. The isolates which were found to share the plasmid profiles were grouped under type a, and those with similar profiles were included under subtypes of a (a1, a2, a3 and a4) **Figure 3(b)**. The 2nd type of profile (b) included isolates showing unique profiles and the 3rd type (c) included those which exhibited a single band of >21 kb on agarose gel. Isolates which displayed the same plasmid profile also showed an apparent congruence in their resistance phenotypes as well. Minor similarities were also observed in the subtypes of profile a. Out of 25 isolates, 13 (52%) belonged to type a, 10 (40%) to type b and the remaining to type c profile. It is interesting to note that, all the *bla*NDM-1 positive isolates were found to be included under type a, except E23, with E7 and E10 showing identical profile (a4) and E8 with a1profile **Table 1**. The isolate E23, which exhibited a unique plasmid profile, was collected from Calicut area.

The RAPD profiles generated employing random decameric primer-RBaC 5 were analysed to construct a dendrogram using NTSYS pc2.0 software which also displayed a similar picture. The dendrogram showed two major clusters-A and B **Figure 4**. The major clusters could be further subdivided into sub-clusters-A1, A2 and B1, B2. All the isolates collected from Calicut area were found to be included in the same sub-cluster A1. Interestingly, the two isolates with similarity index 1.000, E21 and E22 (sub-cluster A1) were also from the same place of collection. Likewise, isolates E15, E18 and E19 (sub-cluster A2) with similarity index 1.000 also showed a similar antibiotic resistance phenotype. Notably, all isolates within this sub-cluster except E15 exhibited identical plasmid profile-a1 **Table 1**. Among the *bla*NDM-1 positive isolates, E10, however, failed to be amplified with the random primer, while the other three (E7, E8 and E23) were found to be included in a same distant subcluster of A1 with E7 and E8 showing greater similarity. This observation is line with the results of our plasmid profile analysis.

4. Discussion

Increasing emergence of ESBL producing *E. coli*, co-producing other *β*-lactamases and exhibiting co-resistance to many classes of antibiotics, poses a significant threat worldwide [26] [27]. This is also the case in the Indian subcontinent, where the emergence of a new metallo-*β*-lactamase (MBL) gene—the *bla*NDM-1, was reported recently [28] [29]. The subsequent worldwide reports on the emergence of *bla*NDM-1 producing bacteria clearly showed the role of plasmid vectors in their dissemination as they were found to be residents on large plasmids. Occurrence of *bla*NDM-1 producing *E. coli* from patients with a history of previous hospitalization in India was first reported in 2011 from Germany [30]. Nielsen *et al.*, 2012, reported that an NDM-1 producing *E. coli* obtained in Denmark has a genetic profile similar to an NDM-1 producing *E. coli* isolate from UK, suggesting dissemination of a common plasmid into various sequence types of *E. coli* [31]. In contrast, a new NDM-1-producing *E. coli* has been first reported from China from patients without any travel history [32]. However, such studies and reports from Kerala are sparse with few available in published literature. In this study, prevalence of ESBL producers was observed among clinical isolates of *E. coli* collected from two tertiary care centers in Kerala. Co-existance of *bla*NDM-1 has been observed in four isolates. The genetic similarity between isolates

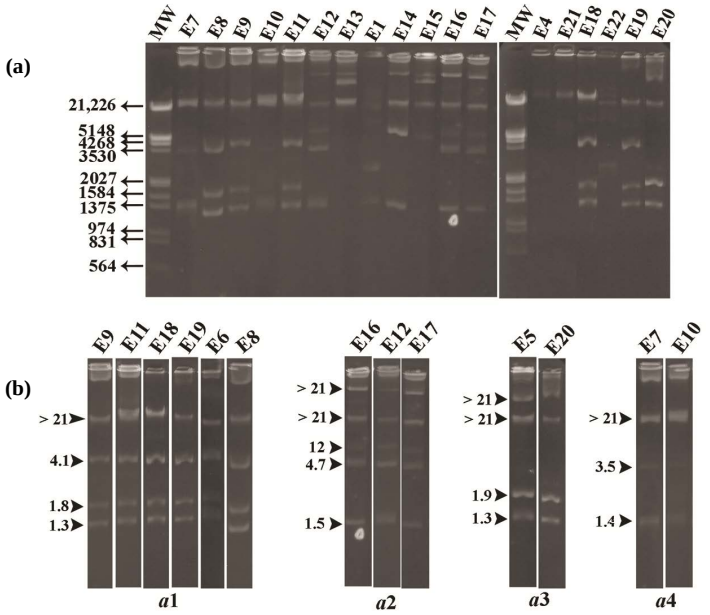

Figure 3. Plasmid profiles. (a) From 18 representative *E. coli* isolates; lanes denoted MW represent Lambda DNA EcoRI/Hind III double digest molecular weight marker; strain designations have been indicated against the lanes; (b) A regrouping of lanes showing identical patterns—a1, a2, a3 and a4.

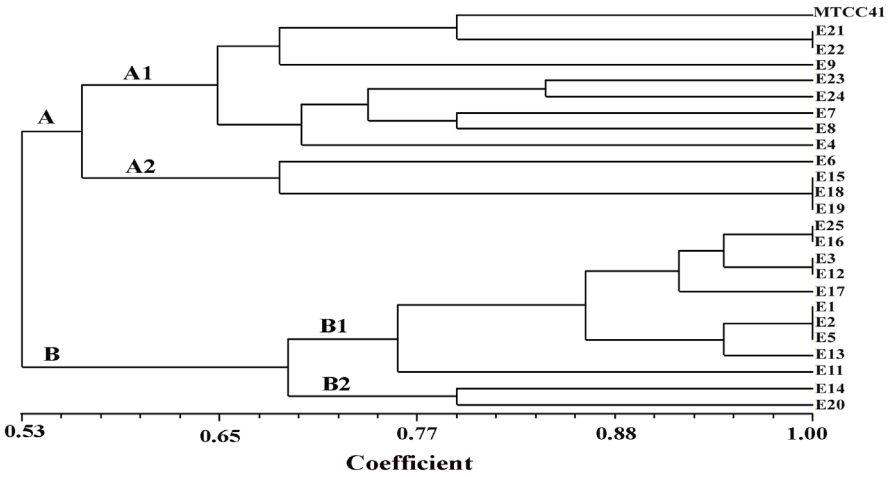

Figure 4. RAPD Dendrogram showing clonal relatedness among *E. coli* isolates. Clustering was done by UPGMA.

Table 1. Grouping of plasmid-bearing isolates based on plasmid profile characteristics.

Plasmid profile characteristics	Plasmid profile types	Molecular weight of plasmids (kb)	Designation of isolates
Multiple bands similar in two or more isolates	a1	>21, 4.1, 1.8, 1.3	E6, E8, E9, E11, E18, E19
	a2	2 bands of >21 kb, 12, 4.7, 1.5	E12, E16, E17
	a3	2 bands of >21 kb, 1.9, 1.3	E5, E20
	a4	>21, 3.5, 1.4	E7, E10
Multiple bands unique to each isolate	b	>21 to 1.1 kb	E1, E2, E3, E13, E14, E15, E22, E23, E24, E25
Single band	c	>21 kb	E4, E21

was apparent in the antibiogram, plasmid profile and RAPD dendrogram. The similarities observed in the plasmid profiles of *bla*NDM-1 positive isolates as well as their *bla*NDM-1 gene sequences, suggest a common plasmid vehicle related dissemination of the *β*-lactamase gene. Diversity among isolates from different geographical areas, however, was also apparent in our study.

5. Conclusion

In conclusion, a predominance of ESBL producing *E. coli* isolates was observed in this study. The *bla*NDM-1 specific amplicons in these plasmid-bearing isolates were found to be genetically similar to each other. Further, these sequences also showed similarities with those previously reported from Japan, Korea and China. Thus, this study warns urgent implementation of strict control measures to prevent spread of resistance genes and warrants the need for constant surveillance. In the face of sparse information available from Kerala State on the emerging drug resistance in clinical bacteria, it is imperative to further expand the study in terms of larger samplings of pathogens from across the state and country followed by critical evaluation.

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