

Original Research Article

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Molecular Detection and Subtyping of New Delhi Metallobetalactamases (NDM-1) in Clinical Isolates of *Escherichia coli* and *Klebsiella pneumoniae* in a Tertiary Care Hospital, South India

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ABSTRACT

Keywords

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Enterobacteriaceae are inhabitants of human, animal gastrointestinal tract and various other environmental sites. *Escherichia coli* and *Klebsiella pneumoniae* initially responded to betalactam antibiotics but there was an increase in resistance to betalactam antibiotics due to beta lactamase produced by them. Resistance to carbapenems has now become more prevalent due to production of carbapenemases. NDM-1 is a novel metallo betalactamase that is capable of hydrolysing all beta lactam antibiotics except Aztreonam. It was aimed for Molecular detection and subtyping of NDM-1 in *Escherichia coli* and *Klebsiella pneumoniae*. Hundred carbapenem resistant isolates (42 isolates of *Escherichia coli* and 58 isolates of *Klebsiella pneumoniae*) were selected randomly for Molecular detection and sequencing of subtypes. A total of 82% strains were found to be NDM-1 positive yielding 475bp amplicon by molecular methods. Nine samples were sequenced for subtypes, of which five were NDM-1, two were NDM-4, and remaining two were NDM-3, and NDM-5. Sequencing and finding out of the subtypes may help to select new drug targets and design novel antibiotics.

Introduction

Enterobacteriaceae are inhabitants of wide variety of niches, including the human, animal gastrointestinal tract and various other environmental sites. The family Enterobacteriaceae includes a large number and diversity of genera.

During the past decade, there has been an emergence of carbapenems-resistant *Enterobacteriaceae* that produce carbapenemases, enzymes that efficiently hydrolyze carbapenems, as well as most β -lactam drugs (Queenan *et al.*, 2007). New Delhi Metallo-beta-lactamases-1 (NDM-1) are one such carbapenemase that makes

bacteria resistant to a broad range of beta-lactam antibiotics. These include the antibiotics of the carbapenems family, which are a mainstay for the treatment of antibiotic-resistant bacterial infections. Bacteria that produce carbapenemases are often referred to in the news media as "superbugs" because infections caused by them are difficult to treat.

Such bacteria are usually susceptible only to polymyxins and tigecycline. NDM-1 was first detected in a *Klebsiella pneumoniae* isolate from a Swedish patient of Indian origin in 2008. It was later detected

in bacteria in India, Pakistan, the United Kingdom, the United States, Canada, and Japan (Kumarasamy *et al.*, 2010).

Materials and Methods

Study population

After Ethical clearance from the institution, the study was conducted between April 2014 and August 2015 on various clinical samples. Two thousand and forty two isolates of *Escherichia coli* and thousand hundred and ninety two isolates of *Klebsiella pneumoniae*, were identified by IMViC tests. Of which Five hundred and sixty isolates of *Klebsiella pneumoniae* (47%) and hundred and forty eight isolates of *Escherichia coli* (7%) were resistant to carbapenems. Among them hundred isolates were selected randomly for the study (42 isolates of *Escherichia coli* and 58 isolates of *Klebsiella pneumoniae*)

Isolation of carbapenem resistant *Escherichia coli* and *Klebsiella pneumoniae*

The isolates were screened for the production of Carbapenam resistance as per CLSI 2014, using Ertapenam 10ug and Merapenam 10ug disc. The zone diameter of inhibition less than 19-21mm for Ertapenam and less than 16-21mm for Merapenam indicates Carbapenamase production. As per CLSI 2014 Ertapenam non susceptibility was the most sensitive indicator of Carbapenam resistance. Isolates were also tested by automated identification system (VITEK 2) for carbapenam resistance, based on Minimal Inhibitory Concentration. Among them a total of 100 isolates (42 isolates of *Escherichia coli* and 58 isolates of *Klebsiella pneumoniae*) were selected randomly for molecular study.

DNA extraction

The isolates were incubated overnight at 35°C in Luria broth. About 2-3 ml of inoculum was centrifuged at 5000rpm for 10 minutes. After

centrifugation the supernatant was discarded and the pellet was taken in a sterile Eppendorf tube. The pellets were resuspended in distilled water and kept in a water bath at 95°C for 20 minutes. It was then cooled and centrifuged at 5000 rpm for 10 minutes. The supernatant was the extracted DNA and is stored at -20°C.

Polymerase chain reaction

The extracted DNA was mixed with forward, reverse primers for NDM-1, master mix and PCR water and amplified in step one AB applied Bio system Real time PCR machine. PCR cycle consists of the following steps (Jeremiah *et al.*, 2014). Initial denaturation at 94°C for 3 min, Denaturation at 94°C for 30 s Annealing at 60°C for 25 Extension at 72°C for 30 s, Final extension at 72°C for 3 min for 30 cycles. The amplified products are stored at -20°C. Gel Electrophoresis was run. The images of the gel were captured by Gel doc and viewed. The presence of the NDM-1 gene was confirmed by the formation of bands at 475bp.

Gene sequencing and blasting

The amplified products which were confirmed for the presence of NDM-1 were sent for gene sequencing to Eurofins Genomics Bangalore. The sequenced genes were blasted using BIO EDIT software and results were interpreted in the NCBI (National centre for Biotechnology Information) website.

Results and Discussion

Two thousand and forty two isolates of *Escherichia coli* and thousand hundred and ninety two isolates of *Klebsiella pneumoniae*, were identified in various clinical samples during the study period, of which Five hundred and sixty isolates of *Klebsiella pneumoniae* (47%) and hundred and forty

eight isolates of *Escherichia coli* (7%) were resistant to carbapenems. Among them Forty two isolates of *Escherichia coli* and Fifty eight isolates of *Klebsiella pneumoniae* were selected for molecular study.

Polymerase chain reaction

Among the hundred isolates, eighty two (82%) were found to be NDM-1 positive (475bp) by PCR. Gel pattern of NDM-1 is depicted (Fig 1). Thirty seven (88%) of *Escherichia coli* and Forty five *Klebsiella pneumoniae* (77%) were NDM-1 producers.

Subtypes of NDM-1

Nine samples of amplified DNA (25ul) were sent randomly for sequencing at Euro fin genomics India pvt Ltd in Bangalore. Among them four were *Escherichia coli* and five were *Klebsiella pneumoniae*. The results send by the Eurofins Genomics were subjected to gene blasting using the BIO EDIT software and the sequence results were interpreted in NCBI (National centre for Biotechnology and Information) web site as shown in figures 2 and 3. On interpretation it was found that Amplicon-1, Amplicon -2, Amplicon -4, Amplicon -6 and 8 had amino acid sequences of NDM-1 and Amplicon -3 was NDM-5 and Amplicon -5 was NDM-3 and Amplicon -7 and Amplicon -9 were NDM-4. NDM-5 showed a point mutation variant differing from NDM-1 having a Met to Leu (M-L) substitution at 154 and Val to Leu (V-L) substitution at 88. NDM-3 showed a point mutation variant differing from NDM-1 having substitution of Asp to Asn (D-N) at 95. NDM-4 differs from NDM-1 by a single amino acid substitution (M-L) at 154 (Met to Leu) which is shown in table 1.

Gram negative bacteria belonging to the family of Enterobacteriaceae are the most frequently isolated organisms from clinical specimens. They are incriminated in virtually

any type of infectious diseases and recovered from any specimen received in the laboratory. *Escherichia coli* and *Klebsiella pneumoniae* cause sepsis, endotoxic shock, urinary tract infections, meningitis in neonates and pneumonia in immune compromised.

They are normally treated based on the antibiotic sensitivity, site of infection and co-morbidity conditions (Longo *et al.*, 2011). *Escherichia coli* and *Klebsiella* infections initially responded to betalactam antibiotics, but there was a dramatic increase in resistance to beta lactam antibiotics due to betalactamases produced by them. This resistance was superseded by use of second and third generation cephalosporins and combination of betalactam antibiotics with betalactamases inhibitors (Nosocomial burn wound infections, 2004).

Further with the emergence of ESBLs and Amp C resistance, carbapenems became the drug of choice. There has been a rapid increase in carbapenems resistance in Enterobacteriaceae in the recent years. The carbapenems resistance may be due to carbapenamases production, Efflux pump and Amp C enzyme production with membrane impermeability. However, carbapenamases (betalactamases) were found to be the predominant mechanism of this resistance. Among the newly emerged carbapenamases, New Delhi Metallo betalactamases (NDM-1) represents a war between the bugs and drugs. NDM-1 producing organisms are called super bugs (BorA *et al.*, 2012).

NDM-1 was first reported in 2008 in *Klebsiella pneumoniae* and *Escherichia coli*. Both of them were recovered from a Swedish patient of Indian origin who was previously admitted in a hospital in New Delhi, India. Thereafter, NDM-1 has increasingly been reported from India and from several other parts of the world.

Besides this, most of these NDM-1 positive bacteria are also resistant to a wide variety of Antimicrobials like aminoglycosides, flouroquinolones, macrolides and sulphonamides and carry several additional resistance mechanisms, leaving only few or no therapeutic options (Krishna, 2010). In our study period carbapenem resistance was observed in 47% of *Klebsiella pneumoniae* and 7% of *Escherichia coli* in our hospital.

As per CLSI 2014 Ertapenem is a good marker for detection of Carbapenems resistance as others do not predict this resistance well. We randomly selected 42 *Escherichia coli* followed by 58 *Klebsiella pneumoniae* which were carbapenems resistant for molecular analysis. Molecular tests are the most accurate and usually taken

as Gold standard. In our study we used the following primers (475bp) to detect NDM-1 by conventional PCR. NDM Forward: 5'-GGGCAGTCGCTTCC AACGGT and

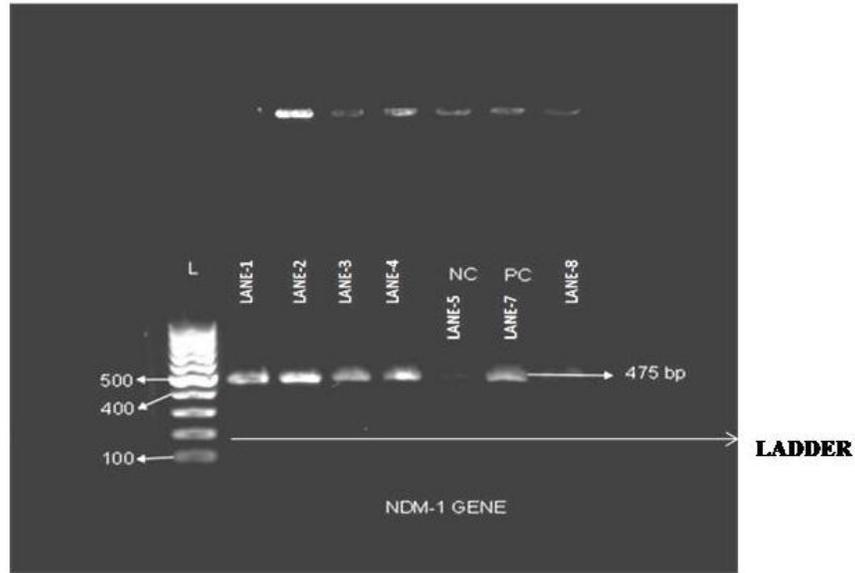
NDM Reverse: 5'- GTAGTGCTCAGTGTC GGCAT

Amplification, sequencing and subsequent blasting were performed to identify the subtypes. Eighty-two (82%) strains were found to be NDM-1 positive (475bp) of which Thirty seven (45%) were *Escherichia coli* and Forty five were *Klebsiella pneumoniae* (54%). The presence of NDM-1 has also been reported in Bangladesh, Australia, Netherlands, USA, Canada, Japan, and China indicating that blaNDM-1is widely spread all over the world (Berrazeg *et al.*, 2014).

Table.1 DNA sequencing results of NDM-1 carbapenamase genes studied

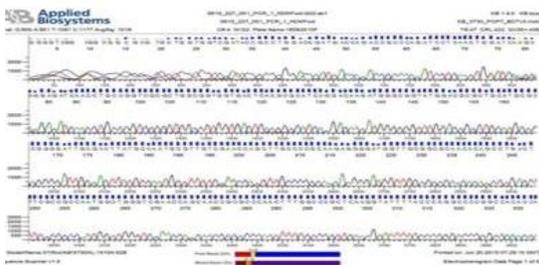
Amplicon	Aminoacid Substitution	Interpretation
Amplicon-1	Aminoacid sequences of NDM-1	NDM-1
Amplicon-2	Aminoacid sequences of NDM-1	NDM-1
Amplicon-3	Met to Leu (M-L) substitution at 154 and Val to Leu (V-L)substitution at 88.	NDM-5
Amplicon-4	Aminoacid sequences of NDM-1	NDM-1
Amplicon-5	Substitution of Asp to Asn (D-N) at 95.	NDM-3
Amplicon-6	Aminoacid sequences of NDM-1	NDM-1
Amplicon-7	Single amino acid substitution (M-L) at 154 (Met to Leu).	NDM-4
Amplicon 8	Aminoacid sequences of NDM-1	NDM-1
Amplicon 9	Single amino acid substitution (M-L) at 154 (Met to Leu).	NDM-4

Fig.1 Gel electrophoretic analysis of amplified NDM-1 gene products



LANE-1&2- Escherichia coli (+ve for NDM-1);LANE 3&4 –Klebsiellapnenmoniac(+ve for NDM-1)

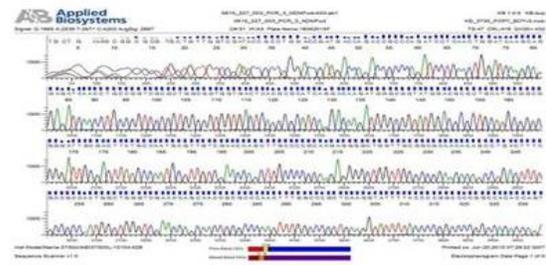
Fig.2 DNA sequencing results of amplified NDM-1



>0615_227_14_AMPlicon_1
AACGATTGGCCTTGCTGCTGATCAGGCAGCCACCAAAAGCGATGT
CGGTGCGTCGATCCCAACGGTGATATTGTCACTGGTGTGGCCGGGG
CGGGTAAAAATACCTTGAGCCGGCCAAAGTTGGCCGGGTTGCTGGTT
CGACCCAGCCATTGGCGGCGAAAGTCAGGCTGTGTTGCCCGCAACCA
TCCTCTTGGCGGGCAAGCTGGTTCGACAAACGCAATTGGCATAAGTCG
CAATCCCGCCGATGCAAGCGGTCATAACCGCCATCTGTCTGAT
GCGGTGAGTCAACACCGCCAGCGGACCGGACGGTGTGATCTCTGCT
TGATCCAGTTGAGGATCTGGGCGGTC

AMINOACID ELAST
IVRDGGRVLYVDYTAWIDDQTAQILNWKQEINLPVALAVVTHAHQDKM
GGMDALHAAGIATYANALSNQLAPQEGMVAQAQHSIFAANGWVEPATA
PNFGPLKVFYPPGPGHTSDNITVGDGTDIARGGCLIKDS

NDM-1

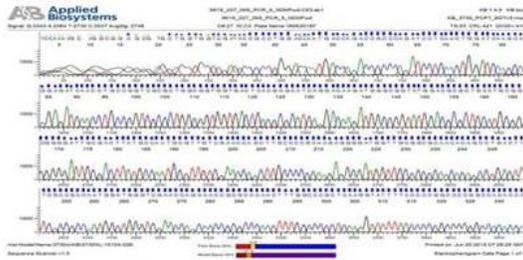


>0615_227_14_AMPlicon_3
ITTTGCTGTTGGTCGATACCGCCTGGACCGATGACCAGACCGCCAGA
TCCTCAACTGGATCAAGCAGGAGATCAACCTGCCGGTGGCGTGGCGG
TGGTGACTCACCGCATCAGGACAAGATGGCGGATATGACCGGCTG
CATGCGGCGGGGATTCGACTTATGCCAAATGCGTGTGCAACACGCTT
GCCCGCAAGAGGGGCTGGTTCGCGGCGCAACAACGCTGACTTTCGCC
GCCAATGGCTGGTTCGAAACAGCAACCGGCGCAACTTTGCCCGGTC
AAGGTATTTACCCCGGCCCGGCCACACAGTGAACAATACACCGTT
GGGATCGACGGCACCGACATCGCTTTGGTGCTGCCTGATCAAGGAC
AGCAAGGC

AMINOACID ELAST
3LLVDTAWIDDQTAQILNWKQEINLPVALAVVTHAHQDKMGGMDALH
AAGIATYANALSNQLAPQEGLYAAQHSIFAANGWVEPATAPNFGPLKV
FYPGPGHTSDNITVGDGTDIARGGCLIKDSKA

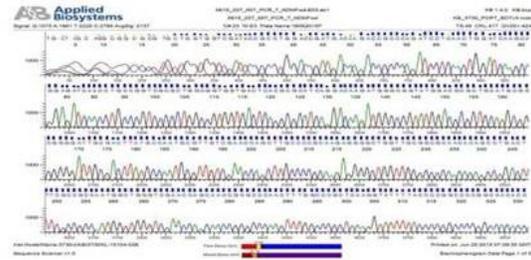
NDM-5

Fig.3 DNA DNA sequencing results of amplified NDM-1



>0615_227_14_AMPLICON-5
 CTGGTGGTCGATACCGCCTGGACCAATGACCGAGCCGCCAGATC
 CTCAACTGGATCAAGCAGGAGATCAACTGCGCGTGGCGGTGGGGTGGTACTACCGCGGATCAGGACAAGATGGGGGATGGACCG
 GCTGCATGCGCGGGGATGCGACTTATGCCAATGCGTGTGCGAA
 CCAGCTTCCCCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCT
 GACTTTCGCGCAATGGCTGGTGAACAGCAACCGCGCCCAA
 CTTGGCCCGCTCAAGGTAITTTACCCCGCCCGCCACACCAATG
 GACAATATCACCGTTGGGATCGACGGCACCAGACATCGCTTTGGT
 GGCTGCTGATCAAGGACAGC

AMINOACID ELAST
 LYVDIAWINDQTAQLNWKQENLPVALAVVTHAHQDKMGMD
 ALHAAGIATYANALSNQLAPQEGMVAHQSLTFAANGWVEPATP
 NFGPLKVFYFPGPGHSDNITVGDGDIAGGGLIKDS
NDM-3



>0615_227_14_AMPLICON-7
 GCGTGTGGTGGTCGATACCGCCTGGACCGATGACCAGACC
 GCCCAGATCCTCAACTGGATCAAGCAGGAGATCAACTGCC
 GGTGCGCTGGCGGTGGTACTCACGGCATCAGGACAAGA
 TGGCGGATGGACCGCGTGCATGCGCGGGGATGCGACT
 TATGCCAATGCGTTGCGAACACAGCTTGCCTCCGCAAGAGGG
 GCTGGTTGCGGCGCAACACAGCGTACTTTCGCGCCAAATG
 GCTGGTGAACCGCAACCGCGCCAACTTTGGCCCGCTC
 AAGGTAITTTACCCCGCCCGCCACACCAAGTGAACAAT
 GACCCGTGGGATCGACGGCACCAGACATCGCTTTGGTGGCT
 GCCTGATCAAGGACAGCAAGGCCA

AMINOACID ELAST
 VLVDIAWIDDQTAQLNWKQENLPVALAVVTHAHQDKM
 GGM DALHAAGIATYANALSNQLAPQEGLVAAQSLTFAANG
 WVEPATPNFGPLKVFYFPGPGHSDNITVGDGDIAGGGLIK
 DSKA
NDM-4

In a similar study from south India, NDM-1 was detected in 75% of *K. pneumoniae* and 66% of *E. coli* by PCR and the remaining isolates were assumed to possess other mechanisms of resistance like efflux pumps, other carbapenemases, Amp C production and membrane impermeability (Nagaraj *et al.*, 2012). DNA and protein sequencing were started in the 1970s, when the virus Lambda (50,000 nucleotides) was sequenced by Sanger *et al.*, DNA sequencing was initially carried out for small genomes such as viruses and organelles, and complete sequencing of a bacterial genome were started later on with the improvements in sequencing technologies. DNA mutations has resulted in changes in the amino acid sequences and produced an ever increasing range of subtypes or variants in each of carbapenemase.

Fifteen variants of NDM-1 have been identified. These variants were identified after sequencing and blasting. They differ by

various aminoacid substitutions. We found NDM-1 in 5 strains (56%), NDM-4 in 2 strains (22%) NDM-3 and NDM-5 in one strains each (11%). Further characterization of the 18 strains (5 were *Escherichia coli* and 13 *Klebsiella pneumoniae* which were negative for NDM-1 gene) for the mechanism of resistance was not done in our study. These isolates probably have other carbapenemases like VIM or it may have efflux changes or Amp C beta lactamase production with outer membrane impermeability.

Presently NDM-1 producing bacterial infections can be managed with Colistin and Tigecycline combination. Colistin should not be given alone as it provokes resistance in vivo due to hetero resistant strains. Fosfomicin may also serve as an option for combination therapy. Detection of NDM-1 like carbapenemases in members of Enterobacteriaceae will help in the management and prevention of spread of such

bacteria in hospital and community which in turn will save the patients and valuable drugs. Besides epidemiological surveillance sequencing would help to identify new drug targets, which can be used in the design of novel antibiotics.

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