Clonal diversity of New Delhi metallobetalactamase-1 producing Enterobacteriaceae in a tertiary care centre


Abstract

Purpose: New Delhi metallobetalactamase-1 (NDM-1) production is a major mechanism of resistance to carbapenems among the Enterobacteriaceae and is a cause for concern in the field of microbial drug resistance. This study was performed to detect NDM-1 in Enterobacteriaceae and to determine the clonal relatedness of NDM-1 producing Escherichia coli and Klebsiella pneumoniae isolated from patients admitted in a tertiary care centre.

Materials and Methods: A total of 111 clinically significant Enterobacteriaceae isolates, resistant to cephalosporin subclass III were screened for carbapenemase production by the modified Hodge test. Minimum inhibitory concentration to imipenem and meropenem was determined and interpreted according to Clinical Laboratory Standards Institute 2011 criteria. Presence of blaNDM-1 was detected by polymerase chain reaction. To ascertain clonal relatedness, random amplification of polymorphic deoxyribonucleic acid (RAPD) was carried out for representative NDM-1 producers.

Results: blaNDM-1 was detected in 64 study isolates, of which 27 were susceptible to carbapenems. RAPD revealed a high degree of clonal diversity among NDM-1 producers except for a small clustering of isolates in the neonatal intensive care unit. Conclusion: There is extensive clonal diversity among the NDM-1 producing E. coli and K. pneumoniae. Hence, antibiotic selection pressure rather than horizontal transfer is probably an important operating factor for the emergence of NDM-1. This calls for increased vigilance, continuous surveillance and strict enforcement of antibiotic policy with restricted use of inducer drugs.

Key words: Clonal diversity, Enterobacteriaceae, New Delhi metallobetalactamase-1

Introduction

Acquired carbapenemases confer extensive antibiotic resistance in Enterobacteriaceae and represent a public health threat.[1] The most wide spread of them have so far been VIM, IMP, KPC and OXA-48. More recently, New Delhi metallobetalactamase-1 (NDM-1) has been reported from many countries and its origin has been traced to Asia. This metallo β (beta) lactamase (MBL) is plasmid borne and has the propensity to spread between species through horizontal transfer. NDM-1 producing Enterobacteriaceae are implicated in a wide range of clinical conditions such as bacteremia, ventilator associated pneumonia, soft-tissue and urinary tract infections.[2] Timely and rapid detection of NDM-1 producers is therefore crucial to institute infection control measures.

NDM-1 frequently coexists with other carbapenemases particularly VIM types and OXA-48, plasmid mediated cephalosporinases (extended spectrum β-lactamases, Amp C), aminoglycoside and macrolide resistance genes (16S ribosomal ribonucleic acid methylase and esterase), rifampicin modifying enzymes and sulfamethoxazole resistance genes thus conferring multidrug drug resistant profile. Thus far, most NDM-1 producers remain susceptible to tigecycline and polymyxins.[3,4]

In order to control these multidrug resistant organisms better, an understanding of the mediating mechanisms and their molecular epidemiology is essential. Molecular typing is useful to ascertain their clonal relatedness and aids in identifying the source of infection in addition to early recognition and containment of possible outbreaks. There are sparse reports of single centre molecular epidemiological studies carried out on NDM-1 producers particularly from the Indian subcontinent.[5-9]

This study was therefore undertaken to determine the clonal relatedness of NDM-1 producing Escherichia coli and Klebsiella pneumoniae isolated from patients admitted
in a tertiary care centre in an effort to understand their molecular epidemiology.

Materials and Methods

Setting

The study was conducted in a 1600 bedded University Teaching Hospital between April and October 2010. The study protocol was approved by the Institutional Ethics Committee.

Clinical isolates

A total of 111 clinically significant, non-repetitive Enterobacteriaceae resistant to one of the cephalosporins subclass III, isolated from 96 patients admitted to intensive care units (ICU) and 15 patients in non-ICU settings of the health-care facility were collected. It included K. pneumoniae (52), E. coli (25), Citrobacter freudii (16), Enterobacter cloacae (16) and Providencia retgeri (2). Species identification was carried out by Microscan Walkaway 96 – using gram-negative panels (Siemens Health-care Diagnostics Inc. – Sacramento CA, USA). The source of the isolates was blood (23), respiratory secretions (24), exudative specimens (19) and urine (45).

Antimicrobial susceptibility testing

Disc diffusion method was used to test for susceptibility to aztreonam (30 μg), cefepime (30 μg), piperacillin-tazobactam (100/10 μg), ciprofloxacin (5 μg), amikacin (30 μg), imipenem (10 μg) and meropenem (10 μg) (Hi-Media Laboratories, India). The results were interpreted as per Clinical Laboratory Standards Institute (CLSI) 2011 guidelines.10 Susceptibility to tigecycline was performed using 15 μg disc (BBL™ BD, USA) and interpretation of zone of inhibition was done using the United States, Food and Drug Administration, tigecycline susceptibility breakpoints criteria.11

Minimum inhibitory concentration

Minimum inhibitory concentration (MIC) to imipenem and meropenem was determined by broth microdilution method and results interpreted according to CLSI document M100-S 21.10 MIC to colistin was determined by the E-test (Biomerieux SA, France).

Phenotypic tests

Carbenemase production was screened by the modified Hodge test (MHT)10 and MBL production by inhibitor potentiated disk diffusion test using ethylene diamine tetra acetic acid (EDTA).12

Polymerase chain reaction

All study isolates were subjected to Polymerase chain reaction (PCR) using primers targeting bla_NDM-1 irrespective of their susceptibility profile to carbapenems. Coexistence of other carbapenemases was also looked for by PCR targeting bla_KPC, bla_OXA-48, bla_VIM and bla_PEM.13 For optimization of PCR, strains previously confirmed by PCR and gene sequencing were used as positive controls and E. coli ATCC 25922 was used as negative control.

Deoxyribonucleic acid sequencing

PCR products of representative isolates were purified using PCR Deoxyribonucleic acid (DNA) purification kit (QIA quick Gel Extraction Kit, Qiagen, Valencia, CA, USA) and subjected to automated DNA sequencing (ABI 3100, Genetic Analysyer, Applied Biosystems, Foster city, CA, USA). The aligned sequences were analysed with the Bioedit sequence program and similarities searches for the nucleotide sequences was performed with the BLAST program (http://www.ncbi.nlm.nih.gov). The sequences were submitted to genbank under the accession numbers (JX 215367, JX 215368, JF702917, JF702918 and JF702919).

Random amplification of polymorphic deoxyribonucleic acid

Random amplification of polymorphic deoxyribonucleic acid (RAPD) was done for 23 NDM-1 producers, which included K. pneumoniae (16) and E. coli (7). The isolates were selected based on the location of the source patients, time of isolation, species, isolation from invasive infections and susceptibility profile. They were representative of pathogens in the multidisciplinary adult, paediatric, neonatal and neurosurgery ICUs of the health care facility.

The following primers were used in the study: M13, 5‘-GAGGGTTGGCCTTCT-3‘; DAF4, 5’-CGGCACGGCC-3‘.16 This fingerprinting technique was optimised according to the laboratory conditions, with varied concentrations of primers, DNA template and MgCl₂. A primer concentration of 10 pg/μl, MgCl₂ of 4 mM and DNA template of 15 ng/μl yielded clear banding patterns.17

DNA preparation and PCR reaction conditions

DNA was extracted from a single colony of an overnight culture on nutrient agar. It was suspended in 25 μl of distilled sterile water and boiled for 10 min. After a short centrifugation of 1000 rpm for 5 min, 10 μl of the template DNA from supernatant was added to 30 μl PCR reaction mixture consisting of 5 10X PCR buffer with 15 mM MgCl₂, 8 μl of 200 μM each deoxyribonucleoside triphosphates, 10 ng/μl of both forward and reverse primers, 5 μl of deionized water and 1.0 U of Taq polymerase (Merek, Darmstadt, Germany). PCR amplification was carried out using thermal cycler Perkin Elmer Model 2700 (Applied Biosystems, Massachusetts, USA). The PCR thermal profile described by Grundmann et al., was followed for M13 primers and for DAF-4 primer, a slight modification in time duration for extension from 20 s to 30 s was carried out.16 Entire RAPD products were loaded into 1% agarose gel containing ×1 tris-acetate-EDTA buffer. Molecular size markers (100 bp
and 500 bp DNA ladder; Merck, Darmstadt, Germany) were run in parallel on all gels. Resolved DNA products were stained with ethidium bromide, 50 ng/ml (Hi-Media Laboratories, Mumbai) and viewed under ultraviolet light using gel documentation system (Vilber Lourmat – France).\(^{[11]}\)

**Analysis of RAPD profile**

Relationships among isolates was examined by analysing the agarose gel electrophoretogram of RAPD fingerprints of each isolate with Advanced BIO 1-D Software (Vilber Lourmat, Bio1D++ Software, USA). Each of the RAPD bands was identified in comparison with the band size of molecular weight markers (100 bp ladder). The dendrogram picture was obtained with the band size inputs using Nei and Li (Dice) similarity coefficients.\(^{[8]}\) Cluster analysis was performed by the Unweighted Paired Group Method With Arithmetical Averages (UPGMA). Each isolate was examined twice, with DNA extracts prepared on different days. Each batch of RAPD experiment samples were loaded along with negative controls. Clustering of molecular size markers (100-bp ladder) was used as a measure of the level of inherent variability following merging and clustering of data from different gels. All typing experiments and clustering of fingerprint data were initially performed ‘blind’ and were correlated with the epidemiological, clinical and susceptibility data only at the conclusion of the study.\(^{[19]}\)

**Results**

**Antimicrobial susceptibility testing**

All study isolates were resistant to cefotaxime, ceftriaxone, aztreonam, ceftazidime, piperacillin-tazobactam and ciprofloxacin. Resistance to amikacin and/or meropenem was detected in 49 isolates out of 100. Resistance to imipenem and/or meropenem was detected in 95 isolates out of 100. Resistance to imipenem and/or meropenem was detected in 95 isolates out of 100. MIC\(_{90}\) values for imipenem were 4 mg/L and for meropenem 2 mg/L. There was universal susceptibility to colistin and tigecycline.

**Phenotypic tests**

While MHT was positive in 95 isolates, the MBL screen test was positive in 54.

**PCR**

Bla\(_{NDM-1}\) was detected in 64 (57.65%) isolates of which six coproduced bla\(_{VIM}\). They were obtained from blood (17), respiratory secretions (9), exudative specimens (13) and urine (25). The MHT was positive in 57 and MBL screening test in 43, thereby producing a sensitivity of 89.1% and 79.6% respectively. A total of 59 of NDM-1 producers were from patients in the ICU and five were from the non-ICU patients. The latter group of patients were either hospitalised for a prolonged period or had a history of previous hospitalisation within the preceding 6 months. The distribution of carbapenemase encoding genes in the study isolates is shown in Table 1. A total of 27 NDM-1 producers were susceptible to carbapenems with their MIC ranging from 0.03 mg/L to 1 mg/L. Bla\(_{IMP}\), bla\(_{OXA-48}\) and bla\(_{KPC}\) were not detected in any of the study isolates.

Of the 49 isolates which exhibited resistance to carbapenems, 31 carried bla\(_{NDM-1}\) alone, six harboured both bla\(_{NDM-1}\) and bla\(_{VIM}\) and in one isolate bla\(_{VIM}\) alone was detected. In the remaining 11, none of the genes looked for were detected.

**RAPD**

To study the genomic relatedness among the NDM-1 producing E. coli (7) RAPD was performed. The dendrogram constructed according to the banding pattern using UPGMA revealed, five isolates as belonging to different clones and two isolates with clonal relatedness. Results of dendrogram constructed by DAF-4 primer showed 100% distance coefficient for two isolates that possessed similar clonality, whereas M13 primers had only a 70% distance coefficient for them [Figure 1].

In case of NDM-1 producing, K. pneumoniae selected for RAPD profiling, the dendrogram showed a high degree of genotypic diversity with 12 clonal clusters exhibited by 16 isolates. Of them, 10 isolates revealed 10 different clones; four other isolates showed similar clonality and two other isolates exhibited significant clonal relatedness among them. Results of dendrogram constructed by DAF-4 primer and M13 primers showed 100% distance coefficient for the four isolates that possessed similar clonality [Figures 2-4].

Clinical characteristics and the outcome of infections caused by the NDM-1 producing E. coli and K. pneumoniae isolates subjected to RAPD is depicted in Table 2.

**Discussion**

MBLs are important mediators of resistance to carbapenems in Enterobacteriaceae. Of them, NDM-1 has emerged as a major causative mechanism. This enzyme first identified in K. pneumoniae and E. coli was recovered

| Table 1: Carbanapenemase encoding genes in Enterobacteriaceae |
|-----------------|----------------|----------------|
| Organism        | NDM-1 (n=58)  | NDM-1 and VIM (n=6) | VIM (n=1) |
| Klebsiella pneumoniae (n=31) | 27 | 3 | 1 |
| Escherichia coli (n=13) | 10 | 3 | - |
| Citrobacter freundii (n=10) | 10 | - | - |
| Enterobacter cloacae (n=10) | 10 | - | - |
| Providencia rettgeri (n=1) | 1 | - | - |

NDM: New Delhi metallobetalactamase-1, VIM: Verona imipenemase
in Sweden from a traveller returning from India. Since then there are a spate of reports from many countries, indicating their rapid and alarming dissemination. Most reports suggest that the likely source of NDM-1 acquisition is the Indian subcontinent and is related to ‘medical tourism’.4-6,10

*Bla* _NDM-1_ is located on plasmids of different sizes and location. They also have high transmissibility and disseminate among other species leading to their identification in _E. coli, K. pneumoniae, Enterobacter_ species, _C. freundii, Morganella_ species and _Providencia_ species.2 Several studies have indicated extensive genotypic heterogeneity in the NDM-1 producing _E. coli_ and _K. pneumoniae_. However, clonal spread leading to outbreak has been documented in only one centre from India.6,8,9

This study provides some insights into the complex molecular epidemiology of NDM-1 in this tertiary care centre. Genotyping of seven _E. coli_ has revealed substantial genetic diversity and heterogeneity. There were two _E. coli_ isolates with similar RAPD pattern, but were from patients who were hospitalised at different points of time. Similarly, _K. pneumoniae_ exhibits 12 different lineages of the 16 isolates analysed. Among them, 4 belonged to a single cluster. These were from neonates in the ICU and cultured during the same period of time. This is perhaps indicative of a horizontal transfer. However, such transfer is not the main mechanism of dissemination of NDM-1 producers. Surveillance of water supplies and selective environmental sampling during the study period did not reveal a NDM-1 source. In addition, strict implementation of infection prevention and isolation protocols could have curtailed a possible outbreak.

The extensive diversity of the NDM-1 producers is consistent with the findings of previous studies.6,8,9 Our centre being a tertiary care teaching hospital, patients are transferred from a wide range of health-care facilities in the geographic region. Therefore, colonisation or infection with resistant strains is likely to occur before transfer, implying the import of such strains on admission. The wide diversity encountered in this study suggests multiple source or origin.

The genomic variability suggests strong selection
Figure 2: Dendrogram illustrating the DAF-4 (up) and M13 (down) random amplification of polymorphic deoxyribonucleic acid clustering relationships observed among 4 isolates of *Klebsiella pneumoniae* (1^st^ set)

pressure on bacterial population, underlining the necessity for proper management of antibiotic therapy, within health-care units. In essence, the epidemiology of NDM-1 producers in our hospital is substantially representative of a divergent acquisition of multiple clones with limited dissemination between patients. Though an antibiotic policy with restriction of inducer drugs is implemented within this hospital, such measures are seldom implemented in other centres leading to an immense selection pressure.

The gene encoding NDM-1 was the most prevalent, being detected in 57.65% (64). In Indian studies, the prevalence of NDM-1 producers among carbapenem resistant *Enterobacteriaceae* ranged between 31.2% and 91.6%. In one study, 235 ertapenem non-susceptible isolates of *Enterobacteriaceae* causing intra-abdominal infections were screened for β-lactamase encoding genes. It revealed the presence of *bla*<sub>NDM1</sub> in 33 isolates, of which 32 coproduced one or more β-lactamases such as Extended-Spectrum Beta-Lactamase, Amp C or OXA-48. Only one isolate harboured *bla*<sub>NDM1</sub> alone and *bla*<sub>KPC</sub> was not detected in any isolate.

It is notable that we detected a significant number of NDM-1 producers (27/64) with MIC to carbapenems within the susceptible range. Since their MIC ranged from 0.03 mg/L to 1 mg/L, it becomes mandatory that all *Enterobacteriaceae*, which exhibit resistance to one of the cephalosporins subclass III, are screened for the presence of NDM-1.

Identification of *E. coli* producing NDM-1 is a cause for concern as it a major community acquired pathogen with possible high rates of colonisation. Similarly, presence of NDM-1 in *K. pneumoniae* is a threat in health-care settings as it is a common nosocomial pathogen and has a propensity to spread rapidly.

Screening tests for NDM-1 lead to erroneous interpretation due to their poor sensitivity and specificity.
These findings are notable, because MHT has been the recommended test by the CLSI for the phenotypic detection of carbapenemase producing bacteria in the clinical microbiology laboratory. False positive results with MHT may be obtained due to production of CTX-M with reduced outer membrane permeability. The prevalence of CTX-M being very high in India, the value of this test may be undermined. Therefore, PCR remains the gold standard for the detection of NDM-1.

In 11 carbapenem resistant isolates, none of the genes looked for were detected. Of them, MHT and MBL screen tests were positive in 10 and 6 isolates, respectively. The possible mechanisms operative in these could be other MBLs, CTX-M production with porin defects or hyper production of Amp C β-lactamases.

Therapeutic options against serious infections due to NDM-1 producers are limited to tigecycline and polymyxins. However, tigecycline may not reach desired serum levels to treat systemic infection leaving polymyxins as the last resort.
To conclude, NDM-1 producers in our centre were distinct strains, suggesting that minimal cross-transmission occurred between patients. Antibiotic selection pressure is probably the most important factor for their emergence. This calls for increased vigilance, continuous surveillance and strict enforcement of antibiotic policy with restricted use of inducer drugs.

**References**


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