Short Communication

Prevalence of New Delhi Metallo-β-Lactamase-1 (blaNDM-1) Gene in Children from Tertiary Care Hospital of Pakistan

Farheen Aslam*, Hira Lodhi, Rasheeda Bashir, Faiza Saleem and Shagufta Naz
Department of Biotechnology, Lahore College for Women University, Lahore, Pakistan

ABSTRACT

Carbapenems are hydrolyzed by carbapenamase, present in the bacteria, which is a growing clinical threat. bla Ndm gene encodes for new Delhi metallo-beta lactamase, which can hydrolyze all types of beta-lactams. The objective of the study was to screen multiple drug resistant strains of bacteria for New Delhi-metallo-beta-lactamase (bla-Ndm1) gene. Blood samples (5ml) of children suffering from different infections, under treatment in a tertiary care hospital, were screened for blaNDM-1 gene. Blood samples of 116 patients having tested for multiple drug resistance were analyzed for blaNDM-1 gene by PCR. Sixteen samples were found to be positive for blaNDM-1 gene. The bacterial species harboring blaNDM-1 gene were 25% Enterobacter cloacae, 18.75% Klebsiella sp., 12.5% Pseudomonas sp., 12.5% Citrobacter freundii, 12.5% Acinetobacter Baumannii, 12.5% E. coli and 6.25% shigella sp. Nucleotide sequencing of PCR product of Klebsiella sp, Enterobacter cloacae and Citrobacter freundii showed 100% sequence homology. It is concluded that there is high prevalence of blaNDM-1 among carbapenem resistant enterobacteriaceae isolated from patient suffering from different diseases at local tertiary care hospital of Lahore.

Gram negative multidrug resistant pathogen (especially Enterobacteriaceae) are of main concern in bacterial infections (Diene and Rolain, 2013). Transposons, plasmids and integrons are vehicles for gene transfer (Bennett, 2008). Among Gram negative bacterial species resistance is spreading by mobile genetic element through horizontal gene transfer (Majewski et al., 2012). Immune compromised and neonates are more prone to multi drug resistant (MDR) pathogens (Mittal et al., 2015).

Beta-lactamases are divided into four major classes A, B, C and D. Metallo-beta-lactamase belongs to B class and again divided into three more subclasses i.e. B1, B2 and B3 (Queenan and Bush, 2007; Hall et al., 2004).

Enterobacteriaceae having blaNDM-1 gene is high zinc dependent, metallo-beta-lactamase (MBL) was named as New Delhi metallo-β-lactamase-1 (NDM-1) (Medić et al., 2012). Zinc dependent MBL bacteria resist broad range of beta-lactam (King and Strynadka, 2013).

New delhi metallo-beta lactamase blaNDM-1 was first reported in 2009 from a patient of Sweden who was of Indian origin, he acquired urinary track infection of Klebsiella pneumonia (Rolain et al., 2010). The K. pneumonia was found to be resistant to all antibiotics except colistin (Yong et al., 2009). blaNDM-1 gene was found to be present on 180KDa plasmid of K. pneumonia, the gene was found to be transferrable to 140 KDa plasmid of Escherichia coli (Yong et al., 2009). The plasmids acquired all the genes of antibiotic resistance and their rapid spread in clinical isolates posed a threat to clinical therapy (Rolain et al., 2010). It has been identified that blaNDM-1 gene was formed by the fusion of pre-existing MBL gene with aminoglycoside resistance gene aphA6 (Toleman et al., 2012).

The blaNDM-1 gene primarily identified in K. pneumonia (Dortet et al., 2014) and Escherichia coli isolates has now been reported in Citrobacter freundii, Morganella morgani, Providencia sp. and Enterobacter cloacae (Johnson and Woodford, 2013). blaNDM-1 bearing Enterobacteriaceae were present to be geographically extensive in the Indian subcontinent, being retrieved from 10 areas of India, 8 areas of Pakistan, 1 area of Bangladesh but also in the USA, Canada, China, Japan, the Netherlands, the Sultanate of Oman and United Kingdom (Johnson and Woodford, 2013).

Because of association of blaNDM-1 with india and Pakistan, a number of studies have been done on prevalence of this enzyme in these regions. The prevalence of blaNDM-1 gene was 18.5% from stool samples collected from patients from local hospital of Pakistan (Perry et al., 2011).

Present study was conducted to determine the
with denaturation at 94°C for thirty seconds, annealing DNA polymerase and 0.5 μg of genomic DNA. The reaction began with an initial denaturation at 61°C for 30 seconds and elongation at 72°C for thirty minutes.

**Materials and methods**

Blood samples of 240 children (0-15 years) having different infections in different wards of a tertiary care hospital were collected and inoculated in blood culture bottles at 37°C for 7 days and was observed twice a day, for signs of microbial growth. About 2.5 ml blood was taken from the patient through syringe and transferred into blood culture bottles immediately. Blood culture bottles containing 25ml brain-heart infusion broth (BHI) were used for blood inoculation. When there was indication of growth, the samples were sub cultured on Blood agar and MacConkey agar. All other samples were sub cultured after 48 h of incubation. Plates were incubated aerobically for up to 48 h. Bottles with no growth were incubated for 7 days. The samples were collected from March 2014 to August 2014. Culturing of bacteria and their identification were done at the Department of Microbiology of Children’s Hospital and Institute of Child health using API 10S (Biomerieux, France).

Antibiotic sensitivity was performed by Kirby Bauer disc diffusion method. Different antibiotics were tested which includes amikacin (AK-30 μg), cefuroxime (CMX-30 μg), cefixime (CFM-30 μg), cefotaxime (CTX-30 μg), ceftriaxone (CRO-10 μg), imipenem (IPM-10 μg), tazobactam-piperacillin (TZP), ciprofloxacin (CIP), levofloxacin (LEV), meropenem (MEM), imipenem (IPM-10 μg) and tazobactam-piperacillin (TZP-10 μg). The plates were incubated at 37°C for 24 h. After incubation, the zones of inhibition of the antibiotics were calculated in millimeters sensitive, intermediate sensitive or resistant according to the CLSI guidelines.

Multiple drug resistant bacteria were processed for DNA extraction and amplification of blaNDM-1 gene. DNA was isolated according to Sambrook and Russels method (Sambrook and Russell, 2001).

Specific region of blaNDM-1 gene were amplified by using forward primer 5’-GTC GCG AAG CTG AGC ACC GTA TTA G-3’ and reverse primer 5’-ATG CCG GCC GTA GTG ATT GCG3’. The PCR reaction mixture comprised 1X PCR buffer (75 mM Tris–Cl, pH 8.8, 20 mM (NH4)2SO4 and 0.01 % Tween 20), 1mM MgCl2, 0.1 mM dNTPs, 10 pmole of each forward and reverse primer, 5 units of Taq DNA polymerase and 0.5 μg of genomic DNA. The reaction began with an initial denaturation of 94°C for three minutes, which was followed by 30 cycles with denaturation at 94°C for thirty seconds, annealing at 61°C for 30 seconds and elongation at 72°C for thirty seconds. At the end, final elongation at 72°C for 5 min. PCR product was analyzed on agarose gel electrophoresis. The positive clones were sent to DNA Core Facility, Macrogen, Korea.

### Results and discussion

Antimicrobial susceptibility was done according to standard CLSI guidelines. Out of 240, 116 (48.3%) samples were considered to be multiple-drug resistant. Bulk of the strains were resistant to amikacin (AK), sulbactam-cefoperazone (SCF), tazobactam-piperacillin (TZP), ciprofloxacin (CIP), levofloxacin (LEV), meropenem (MEM) imipenem (IPM) ceftriaxone (CRO), cefazidime (CAZ) cefotaxime (CTX) cefuroxime (CMX) cefixime (CFM).

A total of 116 carbapenem resistant strains were collected from children belonging to different regions of a tertiary care hospital. Region wise distribution of 116 carbapenem resistant strains showed that 41 (35.3%) carbabenen resistant strain were identified from Lahore, 15 (12.9%) from Sheikhupura, 11 (9.5%) from Gujranwala, 9 (7.8%) from Kasur, 7 (6.0%) from Hafizabad, 6 (5.2%) from Okara, 5 (4.3%) from Sialkot, 4 (3.4%) from Bahawalnagar, 4 (3.4%) from Gujraat, 3 (2.6%) from Nankana, 2 (1.7%) from Jhang, 2 (1.7%) from Rawalpindi, 2 (1.7%) from Sargodha, 1 (0.9%) from Mandi bahaudin, 1 (0.9%) from Pakpatan, 1 (0.9%) from Sahiwal and 1 (0.9%) from Vehari.

#### Table I. Sequences, GC content and melting temperature of primers of blaNDM-1 gene.

<table>
<thead>
<tr>
<th>Name of Primer</th>
<th>Sequence (5’-3’</th>
<th>GC content</th>
<th>Melting Temperature (°C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>HIR-F</td>
<td>GTC GCG AAG CTG AGC ACC GTA TTA G</td>
<td>60 %</td>
<td>62.6</td>
</tr>
<tr>
<td>HIR-R</td>
<td>TGA GTG ATT GCG</td>
<td>58 %</td>
<td>60.8</td>
</tr>
</tbody>
</table>

#### Table II. Presence of blaNDM-1 gene reported in following species of bacteria.

<table>
<thead>
<tr>
<th>Sample #</th>
<th>Strain</th>
<th>Gene</th>
<th>Amplified product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Klebsiella spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>2</td>
<td>E. cloacae</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>9</td>
<td>Klebsiella spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>14</td>
<td>Citrobacter freundii</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>18</td>
<td>E.coli</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>19</td>
<td>Pseudomonas spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>21</td>
<td>Pseudomonas spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>23</td>
<td>E. cloaece</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>26</td>
<td>Citrobacter freundii</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>33</td>
<td>E. cloacae</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>35</td>
<td>A. baumanii</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>36</td>
<td>Shigella spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>37</td>
<td>Klebsiella spp.</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>50</td>
<td>A. baumanii</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>56</td>
<td>E. coli</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
<tr>
<td>58</td>
<td>E. cloacae</td>
<td>blaNDM-1</td>
<td>767 bp</td>
</tr>
</tbody>
</table>
The most prevalent specie with blaNDM-1 gene was Enterobacter cloacae, 4(25%), Klebsiella spp. 3(18.75%), Pseudomonas spp. 2(12.5 %), Citrobacter freundii, 2(12.5%), Acinetobacter Baumannii, 2(12.5%), E. coli 2(12.5%) and shigella spp.1(6.25%).

The blaNDM-1 gene was identified from the neonatal emergency/neonatal unit 6(37.5%), from medical ward is 5(31.2%), from surgical ward 2(12.5%), from hematology/oncology ward 2(12.5%) and surgical neonatal intensive care unit 1 (6.25%).

BlaNDM1-gene was cloned in pCR2.1 vector restricted with Hind III.

There is 100 % sequence similarity (Fig. 1) of blaNDM1 gene between Klebsella spp. Citrobacter freundii and Enterobacter cloacae, it means the same gene of blaNDM1 was transmitted to all the strains of bacteria through a vector.

Fig. 1. Multiple sequence alignment of blaNDM1 gene from klebsella spp. Citrobacter freundii and Enterobacter cloacae.

Many bacteria from Enterobacteriaceae group are multiple drug resistant because of carbapenemase production especially metallo-beta-lactamase, which is encoded by blaNDM1-gene. Double Disk Synergy Test (DDST) and Combined Disk Test (CDT) were done for phenotypic identification of metallo-beta-lactamase. In this study 116 (100%) strains are MBL producers. Combined Disk Test shows 100% strains are MBL producer while Double Disk Synergy Test shows 94.8% strains are MBL producer. In Rawalpindi, Pakistan, 39(78%) out of 50 strains were found to be metallo-beta-lactamase producer (Kaleem et al., 2010). A total of 24 out of 74 (32.4%) carbapenem resistant isolates were found to be MBL producer in Mumbai and India (Deshpande et al., 2010). A study in Greece, showed 24 out of 74 (32.4%) strains were metallo-beta-lactamase producers (Falagas et al., 2010). There is high prevalence of carbapenem resistant metallo-beta-lactamase producers in developing countries due to insufficient socioeconomic conditions, practicing self-medication, scarcity in educational awareness, non-assent to antibiotic protocols, poor good health care facilities and lack of infection control precautions in hospital.

All the carbapenem resistant isolates were extracted from blood unlike, to the study preceded in Karachi where highest number of carbapenem resistant Enterobacteriaceae isolates was mainly from urology ward, causing urinary tract infection (Sufian et al., 2013).

In this study it was found that 16 out of 116 MBL strains carrying blaNDM-1 gene with maximum cases in Lahore. A study in India showed, 4 out 20 (20%) metallo-beta-lactamase producing strains have blaNDM-1 gene (Khajuria et al., 2013). Similarly, in Dhaka, Bangladesh 8 out of 31 (22.8%) MBL isolates have blaNDM-1 gene. In a study from two tertiary care hospitals out of 356 isolates, 131 showed metallo-beta-lactamase production with 31 (23.6%) isolate show blaNDM-1 gene (Nahid et al., 2013).

The occurrence of blaNDM-1 gene is maximum in Enterobacter cloacae 4(25%) then in Klebsiella spp. 3 (18.75%). Pseudomonas spp. 2(12.5 %), Citrobacter freundii, 2(12.5%), Acinetobacter Baumannii, 2 (12.5%), E. coli 2 (12.5%) and shigella spp.1 (6.25%).

There is presence of blaNDM-1 gene in neonatal emergency/neonatal unit 6 (37.5%), 5 (31.2%) were identified from Medical ward, 2 (12.25%) from surgical ward, 2(12.25%) and Hematology/oncology ward. 1 (6.25%) was identified from Surgical neonatal Intensive care unit. There is major occurrence of carbapenem resistant, gram negative, Enterobacteriaeae in paediatric patients. Pediatricians have very narrow treatment options and if the problem is not controlled appropriately, it may lead to treatment failure. This delinquent can only be recovered with devotion to actual infection control, to take general public knowledge to adopt cleanliness, proper use of antibiotics and avoid self-medication. A devoted hospital management team plays the vitally important role in abolition of such resistant mechanisms.

Out of 116 multiple drug resistant, MBL producers only 16 have blaNDM-1 gene because many resistant genes coexists with other resistant genes. Multiple drug resistant blaNDM-1 gene positive isolates also co-harbored many resistant genes like blaCTX-M, blaTEM-1, bla-OXA-1,
blaOXA-10. 16S RNA methyl transfer gene (RMT) confers aminoglycoside resistance are of different types (ARMA, RMTA, RMT-B, RMT-C), Quinolone resistance genes (QNR), Reduced susceptibility to ciprofloxacin AAC(6)-IB-CR gene and QEP-A efflux pump encoding gene. Bla NDM1 producer can gather other genes of resistance in a single bacteria. This high level of resistance did not take place in a single genetic event (Poirel et al., 2011).

There is high prevalence of blaNDM-1 among carbapenem resistant enterobacteriaceae isolated from patient suffering from different diseases at local tertiary care hospital of Lahore. Spread of multiple drug resistant isolates limits the treatment options. Efforts are needed to limit the spread of these MDR in hospitals. World health organization emphasize to control infections in hospitals and halt the spread of these MDR strains and make national policies to restrict the use of antibiotics.

Conclusions
Carbapenemase producing gram negative Enterobacteriaceae have emerged as serious life threatening infectious agents especially for hospitalized paediatric patients which may ultimately result in treatment failure. In the present study, the prevalence rate of carbapenemase producing Klebsiella 42.2%, Enterobacter cloacae 17.2%, Acinetobacter baumannii 12.9%, Escherichia coli 9.5%, Pseudomonas spp. 9.5%, Citrobacter freundii 5.2%, Salmonella 1.7% and Proteus spp. is 0.9% which were 100% MBL producers. Results of this study show that the intake of carbapenems should be restricted to avoid the spread of this resistance.

Statement of conflict of interest
The authors have declared no conflict of interest.

References