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Short Communication

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

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FA conceived and designed the experiments; supervised and analyzed the data and wrote the paper. H L design and perform the experiments. RB, FS and SN reviewed the manuscript.

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New Delhi metallo-beta-lactamase, Modified hodge test, Carbapenems, Double disk synergy test, Metallo-beta-lactamase

ABSTRACT

Carbapenems are hydrolyzed by carbapanamase, present in the bacteria, which is a growing clinical threats. *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 teriary 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 Baumanii,* 12.5% *E. coli* and 6.25% *shigella* sp. Nucleotide sequencing of PCR product of *Klebsella* sp, *Enterobacter cloacea* and *Citrobacter freudii* showed 100% sequence homology. It is concluded that there is high prevalence of *blaNDM*-1 among carbapenem resistant entero-bacteriaceae 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) pathogen (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 *bla*NDM-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 *Klebsella 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 *bla*NDM-1 gene primarily identified in *K. pneumonia* (Dortet *et al*., 2014) and *Escherichia coli* isolates has now been reported in *Citrobacter freundii*, *Morganella morganii*, *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

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prevalence of metallo-beta-lactamases in clinical isolates.

Materials and methods

Blood samples of 240 children (0-15years) having different infections in different wards of a tertiary care hospital was 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 (CXM-30 µg), cefixime (CFM-30 µg), cefotaxime (CTX-30 µg), ceftazidime (CAZ-30 µg), ceftriaxone (CRO-30 µg), sulbactam-cefoperazone (SCF-10 µg), ciprofloxacin (CIP-10 µg), levofloxacin (LEV-10 µg), meropenem (MEM-10 µg), 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 GCA TTA G-3' and reverse primer 5'-ATG CGG GCC GTA TGA GTG ATT GCG3'. The PCR reaction mixture comprised 1X PCR buffer (75 mM Tris–Cl, pH 8.8, 20 mM $(NH_4)_2SO_4$ and 0.01 % Tween 20), 1mM $MgCl_2$, 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), ceftazidime (CAZ) cefotaxime (CTX) cefuroxime (CXM) 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, 4 (3.4%) 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 %) fromVehari.

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

Name of Primer	Sequence (5`-3`)	GC content	Melting Temperature (°C)
HIR-F	GTC GCG AAG CTG AGC ACC GCA TTA G	60 %	62.6
HIR-R	ATG CGG GCC GTA TGA GTG ATT GCG	58 %	60.8

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

Sample #	Strain	Gene	Amplified product size
1	*Klebsiella* spp.	*bla*NDM-1	767 bp
2	*E. cloacae*	*bla*NDM-1	767 bp
9	*Klebsiella* spp.	*bla*NDM-1	767 bp
14	*Citrobacter freundii*	*bla*NDM-1	767 bp
18	*E.coli*	*bla*NDM-1	767 bp
19	*Pseudomonas* spp.	*bla*NDM-1	767 bp
21	*Pseudomonas* spp.	*bla*NDM-1	767 bp
23	*E. cloacae*	*bla*NDM-1	767 bp
26	*Citrobacter freundii*	*bla*NDM-1	767 bp
33	*E. cloacae*	*bla*NDM-1	767 bp
35	*A. baumanii*	*bla*NDM-1	767 bp
36	*Shigella* spp.	*bla*NDM-1	767 bp
37	*Klebsiella* spp.	*bla*NDM-1	767 bp
50	*A. baumanii*	*bla*NDM-1	767 bp
56	*E. coli*	*bla*NDM-1	767 bp
58	*E. cloacae*	*bla*NDM-1	767 bp

The most prevalent specie with *bla*NDM-1 gene was *Enterobacter cloacae,* 4(25%), *Klebsiella* spp. 3(18.75%), *Pseudomonas* spp. 2(12.5 %), *Citrobacter freundii*, 2(12.5%), *Acinetobacter Baumanii,* 2(12.5%), *E. coli* 2(12.5%) and *shigella* spp.1(6.25%).

The *bla*NDM-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%).

*Bla*NDM1-gene was cloned in pCR2.1 vector restricted with *Hind* III.

There is 100 % sequence similarity (Fig. 1) of *bla*NDM1 gene between *Klebsella* spp. *Citrobacter freudii* and *Enterobacter cloacae,* it means the same gene of *bla*NDM1 was transmitted to all the strains of bacteria through a vector.

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Klesella          GATACCGCCTGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATC
Citrobacter       GATACCGCCTGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATC
Enterobacter      GATACCGCCTGGACCGATGACCAGACCGCCCAGATCCTCAACTGGATCAAGCAGGAGATC
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Klesella          AACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATG
Citrobacter       AACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATG
Enterobacter      AACCTGCCGGTCGCGCTGGCGGTGGTGACTCACGCGCATCAGGACAAGATGGGCGGTATG
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Klesella          GACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCC
Citrobacter       GACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCC
Enterobacter      GACGCGCTGCATGCGGCGGGGATTGCGACTTATGCCAATGCGTTGTCGAACCAGCTTGCC
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Klesella          CCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTC
Citrobacter       CCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTC
Enterobacter      CCGCAAGAGGGGATGGTTGCGGCGCAACACAGCCTGACTTTCGCCGCCAATGGCTGGGTC
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Klesella          GAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCAC
Citrobacter       GAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCAC
Enterobacter      GAACCAGCAACCGCGCCCAACTTTGGCCCGCTCAAGGTATTTTACCCCGGCCCCGGCCAC
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Klesella          ACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTG
Citrobacter       ACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTG
Enterobacter      ACCAGTGACAATATCACCGTTGGGATCGACGGCACCGACATCGCTTTTGGTGGCTGCCTG
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Klesella          ATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCACTAC
Citrobacter       ATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCACTAC
Enterobacter      ATCAAGGACAGCAAGGCCAAGTCGCTCGGCAATCTCGGTGATGCCGACACTGAGCACTAC
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Klesella          GCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGC
Citrobacter       GCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGC
Enterobacter      GCCGCGTCAGCGCGCGCGTTTGGTGCGGCGTTCCCCAAGGCCAGCATGATCGTGATGAGC
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Klesella          CATTCCGCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGA
Citrobacter       CATTCCGCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGA
Enterobacter      CATTCCGCCCCGATAGCCGCGCCGCAATCACTCATACGGCCCGCATGGCCGA

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Fig. 1. Multiple sequence alignment of *bla*NDM1 gene from *klebsella* spp. *Citrobacter freindii* 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-β-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-β-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-β-lactamase producers (Falagas *et al*., 2010). There is high prevalence of carbapenem resistant metallo-β-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 *bla*NDM-1 gene with maximum cases in Lahore. A study in India showed, 4 out 20 (20%) metallo-β-lactamase producing strains have *bla*NDM-1 gene (Khajuria *et al*., 2013). Similarly, in Dhaka, Bangladesh 8 out of 31 (22.8%) *MBL* isolates have *bla*NDM-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 *bla*NDM-1 gene (Nahid *et al*., 2013).

The occurrence of *bla*NDM-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 Baumanii,* 2 (12.5%), *E. coli* 2 (12.5%) and *shigella* spp.1 (6.25%).

There is presence of *bla*NDM-1 gene in neonatal emergency/neonatal unit 6 (37.5%), 5 (31.25%) 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, *Enterobacteriaceae* 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 *bla*NDM-1 gene because many resistant genes coexists with other resistant genes. Multiple drug resistant *bla*NDM-1 gene positive isolates also co-harbored many resistant genes like *bla*CTX-M, *bla*TEM-1, *bla*-OXA-1, *bla*OXA-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 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 baumanii* 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.

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