Objective:
The serine carbapenemase KPC (Klebsiella pneumoniae carbapenemase) has emerged as a beta-lactamase capable of inactivating carbapenem antibiotics. The emergence of carbapenem-resistant enterobacteria is therefore worrisome, since consequently the antimicrobial treatment options are very restricted. In the present study we have reported the presence of KPC β-lactamase producing enterobacterial isolates from a tertiary referral hospital in north India.

Methods:
The isolates were subjected to phenotypic confirmatory test by boronic acid and clavulanic acid inhibition and presence of carbapenemase activity. They were further tested for PCR detection of \( \text{bla}^{\text{KPC}} \) and the associated genetic component.

Results:
Three enterobacterial isolates were found to be phenotypically similar to that of KPC enzyme and genotypically showing positive results with primers specific for \( \text{bla}^{\text{KPC}} \) gene were found to be located on integron.

Conclusion:
The findings indicate the need for continuous surveillance of this resistant determinant in this part of the world.

Key Words: Serine Carbapenemases, KPC, Integron
INTRODUCTION

Carbapenem remains the most appropriate treatment option for extended spectrum beta lactamase (ESBL) and AmpC beta-lactamase producing organisms. The emergence of carbapenem-resistant enterobacteria is therefore worrisome, since consequently the antimicrobial treatment options are very restricted. *Klebsiella pneumoniae* carbapenemases (KPCs) are one of the most frequently reported Ambler’s class A carbapenemases and are generally plasmid-encoded enzymes. KPC enzymes have the ability to hydrolyze β-lactams of all classes including the carbapenems. Hydrolysis capacity is high for penicillins and first generation cephalosporins while it is low for carbapenems, oxyiminocephalosporins, monobactam and cephemycins. The first member of KPC family, KPC-1 was discovered in 1996 from North Carolina. Soon after its expansion in United States, particularly in New York, hospital outbreaks of infection caused by KPC-producing isolates in Israel were reported. KPC producers are also an emerging public health concern in Europe, China and Central and South America. Although predominant in *Klebsiella pneumoniae*, they have also been discovered in other species from Enterobacteriaceae family and there is recent report of plasmid mediated KPC from *Pseudomonas aeruginosa* and *Acinetobacter baumannii*. Detection of these KPC producers remains difficult, and cannot rely on results of antibiotic susceptibility testing. Most worrisome is the treatment of infections caused by these organisms due to heterogeneous expression of these multidrug resistant organisms that result in long hospital stay and high mortality rates. Here, in the present study we have reported the presence of KPC β-lactamase producing enterobacterial isolates from a tertiary referral hospital in north India.

MATERIALS AND METHODS

Three enterobacterial isolates Kp 58, Ec 445 and Ec 449, were obtained from patients admitted in a tertiary referral hospital of north India. The first isolate was *Klebsiella pneumoniae* (Kp 58), isolated from a blood culture of an 18 years old male from intensive care unit (ICU) in July 2008. The second isolate was *Escherichia coli* (Ec 445), isolated from a urine specimen of a 60 years old male treated for pyelonephritis, the nephrology ward in March 2009. The third one was *E. coli* (Ec 449), isolated two days later from a urine sample of a 3 year old male admitted to pediatric ward in March 2009. Written informed consent was obtained from the patient(s) for publication of this manuscript and accompanying images. This work has been ethically approved by the chairperson of the ethical committee of Institute of Medical Sciences.

All the three isolates showed Minimum inhibitory concentration ≥ 2 µg/ml against ertapenem and were tested for boronic acid and clavulanic acid inhibition. They were further tested for carbapenemase activity by modified Hodge test. A previously confirmed carbapenemase producing *E. coli* was taken as positive control for Hodge test. Isolates were also subjected to detection of AmpC β-lactamase activity by modified three dimensional testing, ESBL detection by combined disc diffusion method according to CLSI recommendation and metallo β-lactamase production by imipenem–EDTA disc test. Isolates were subjected to polymerase chain reaction (PCR) detection of *blaKPC* using primers described previously. The reaction mixture (total volume 25µl) contained 5 µl of template, 15 pmol (each) primers, 10mM dNTPs, 10X reaction buffer and 1 U Taq polymerase (Genei, Bangalore, India). The reaction condition was initial denaturation of 95°C for 5min followed by 35 cycles of
denaturation at 95°C for 1 min, annealing at 56°C for 30 s and extension at 72°C for 1 min 30 s. The amplification was ended by a final extension cycle at 72°C for 1 min. The reactions were amplified in MJ Mini Thermal cycler (Bio-Rad, USA). The amplicons were sequenced and compared by performing BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi). Negative controls used in the PCR reaction were E. coli ATCC 25922, three previously confirmed isolates of E. coli producing bla_{CTX-M}, bla_{CIT} and bla_{NDM-1}. For PCR detection of ESBL and AmpC β-lactamase, presence of integron was detected by integrase gene PCR. To find their genetic association with bla_{KPC}, 5’-CS GGC ATC CAA GCA GCA AG primers and reverse primers of bla_{KPC} was used in the PCR reaction.

Antimicrobial Susceptibility testing was performed by disc diffusion method against cefuroxime (30 µg), cefoxitin (30 µg), co-trimoxazole (25 µg), ciprofloxacin (5 µg), piperacillin/tazobactam (100/10 µg), netilmicin (30 µg), gentamicin (10 µg), kanamycin (10 µg), tigecycline (15 µg) and polymixin B (300 µg) (Hi-Media, Mumbai, India). The results were interpreted according to the CLSI guidelines. MIC was determined by agar dilution method against cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam (Aristo Pharmaceuticals Ltd., Mumbai, India), imipenem (United Biotech, Solan, India), meropenem (Astra Zeneca Pharmaceuticals Ltd., Bangalore, India) and ertapenem (Invanz, Bhiwandi, India). Tests were performed and interpreted according to CLSI guidelines. Strains were typed by random amplification of polymorphic DNA (RAPD) using primer 7. The reaction conditions were as described previously.

**RESULTS**

The three isolates showed inhibition by both clavulanic acid and boronic acid. The carbapenemase activity was confirmed by modified hodge test. All of them were negative by imipenem-EDTA disc test for the presence of metallo β-lactamases. MIC study showed the isolates were resistant to cephalosporins while false susceptibility was observed against imipenem and meropenem (Table 1).

**Table 1: MIC (µg/ml) of KPC positive isolates against different beta-lactam drugs**

<table>
<thead>
<tr>
<th>Antibiotics</th>
<th>Kp 58</th>
<th>Ec 445</th>
<th>Ec 449</th>
<th>E. coli 25922</th>
<th>NDM -ve control</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefotaxime</td>
<td>≥1024</td>
<td>≥1024</td>
<td>64</td>
<td>≤ 2</td>
<td>≥1024</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>≥1024</td>
<td>≥1024</td>
<td>64</td>
<td>≤ 2</td>
<td>≥1024</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>≥1024</td>
<td>≥1024</td>
<td>128</td>
<td>≤ 2</td>
<td>≥1024</td>
</tr>
<tr>
<td>Cefepime</td>
<td>≥1024</td>
<td>≥1024</td>
<td>32</td>
<td>≤ 2</td>
<td>≥1024</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>≥1024</td>
<td>≥1024</td>
<td>32</td>
<td>≤ 2</td>
<td>≥1024</td>
</tr>
<tr>
<td>Imipenem</td>
<td>2</td>
<td>2</td>
<td>0.25</td>
<td>≤ 0.125</td>
<td>≥16</td>
</tr>
</tbody>
</table>

On disc diffusion testing they were susceptible only against tigecyclin and polymixin. On performing PCR amplification with bla_{KPC} primers was found in all of them. The reaction was repeated four times which yielded the same result in each occasion. None of the control strains showed any amplification in the PCR reaction. Multiplex PCR for bla_{AmpC} and bla_{ESBL} did not show any amplification for the KPC positive strains. Sequencing of bla_{KPC} PCR product showed 99 to 100% similarities among three of them by BLAST search. All the three isolates were harbouring class I integron. Association of integron and bla_{KPC} was established as two of the E. coli isolates showed an amplicon of 950 bp in size when a forward primer from 5’ conserved sequence of integron I and bla_{KPC} reverse primer was used. On typing of the strains, RAPD showed the isolates were of different clonality.
DISCUSSION
So far, in this continent KPC producing organisms has been detected in Israel and China. In India, carbapenemase mediated resistance in enterobacteriaceae is generally due to presence of metallo β-lactamase. However, two previous studies reported the presence of KPC β-lactamase which included one from north and the other from southern part of this country. But no genotypic characterization was done for the earlier reported isolates. While observing the genetic environment of blaKPC gene, their location in class I integron is well established. The strains in this study were isolated from patients housed in different locations within the hospital and were non-clonal as evident from RAPD experiment, underlines the horizontal transmission of the drug resistant determinants in hospital environment which was also reflected in the heterogenous nature of the strains examined. In this study, none of the isolates were found to be in-vitro resistant to imipenem and meropenem by routine disc diffusion method. It shows the potential risk of interpreting the isolates as ESBLs in routine clinical microbiology laboratory.

CONCLUSION
In light of our current struggle with New Delhi Metallo β-lactamase (NDM-1) producing Enterobacteriaceae, the presence of another resistance mechanism would further augment its grave situation. Thus a coordinated approach by clinicians and microbiologists in hospital settings is required for their laboratory detection, continuous monitoring of their genetic environment and formulation of proper antimicrobial policy to prevent or at least slow down the horizontal transmission of this drug resistant determinant.

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