Research Article

Carbapenem Resistance among Enterobacter Species in a Tertiary Care Hospital in Central India

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Objective. To detect genes encoding carbapenem resistance among Enterobacter species in a tertiary care hospital in central India.

Methods. Bacterial identification of Enterobacter spp. isolates from various clinical specimens in patients admitted to intensive care units was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques. Antibiotic sensitivity test was performed by standard Kirby Bauer disc diffusion technique. PCR amplification and automated sequencing was carried out. Transfer of resistance genes was determined by conjugation.

Results. A total of 70/130 (53.84%) isolates of Enterobacter spp. were found to exhibit reduced susceptibility to imipenem (diameter of zones of inhibition ≤ 13 mm) by disc diffusion method. Among 70 isolates tested, 48 (68.57%) isolates showed MIC values for imipenem and meropenem ranging from 32 to 64 mg/L as per CLSI breakpoints. All of these 70 isolates were found susceptible to colistin in vitro as per MIC breakpoints (< 0.5 mg/L). PCR carried out on these 48 MBL (IP/IPI) E-test positive isolates (12 Enterobacter aerogenes, 31 Enterobacter cloacae, and 05 Enterobacter cloacae complex) was validated by sequencing for beta-lactam resistance genes and result was interpreted accordingly.

Conclusion. The study showed MBL production as an important mechanism in carbapenem resistance in Enterobacter spp. and interspecies transfer of these genes through plasmids suggesting early detection by molecular methods.

1. Introduction

Beta-lactams are one of the most frequently used classes of antimicrobials in hospital settings, crucial for the treatment of infections caused by Gram-negative bacteria. Enterobacter spp. are common pathogens of Enterobacteriaceae family responsible for nosocomial infections, especially bloodstream infections in intensive care units. Enterobacter may produce severe diseases including those of abdomen, lower respiratory tract, urinary tract, meningeal, eye, bone, and surgical site infections [1]. As per National Nosocomial Infection Surveillance System, more than one-third of the Enterobacter spp. are resistant to extended-spectrum cephalosporins in intensive care units [2]. However, of late due to the presence of extended-spectrum beta-lactamase (ESBL) and AmpC enzymes in Enterobacter spp., Carbapenems have become the drug of choice to treat such infections [3]. There has been an increase in incidence of multidrug resistance in these organisms due to dissemination of resistance determinant genes mediated by transposons, plasmids, and gene cassettes in integrons. To understand the widespread occurrence of the beta-lactamasases in Enterobacter spp., we conducted a study to detect beta-lactam resistance genes along with plasmid replicon typing of carbapenem resistant Enterobacter spp. isolates recovered from clinical specimens in a tertiary care hospital in central India.

2. Materials and Methods

2.1. The Bacterial Isolates. A prospective study was conducted in a 1000 bedded tertiary care centre in Pune, India, from October 2011 to May 2013. A total of 130 Enterobacter spp. isolates (45 Enterobacter aerogenes, 62 Enterobacter cloacae, and 23 Enterobacter cloacae complex) were recovered from clinical specimens from different patients (one isolate per patient) admitted to the medical and surgical intensive care units. Collection of sample was done using strict aseptic precautions and was immediately processed without any delay. The isolates were obtained from various clinical specimens such as cerebrospinal fluid, bone marrow, blood, pus,
urine, lower respiratory secretions (endotracheal secretions, bronchoalveolar lavage, and bronchial wash), sputum, tissues, and other sterile body fluids. Bacterial identification was performed by routine conventional microbial culture and biochemical tests using standard recommended techniques [4]. The organisms were identified up to the species level using VITEK-GNI cards (bioMérieux, Marcy l’Etoile, France).

2.2. Antimicrobial Susceptibility Testing. The antimicrobial susceptibility was performed by the Kirby Bauer’s disc diffusion technique on Mueller-Hinton agar, as per Clinical Laboratory Standard Institute (CLSI) guidelines [5]. The antibiotics tested were as follows (potency in μg/disc): Amoxicillin (10), Cefuroxime (30), Cefotaxime (30), Piperacillin (100), Ticarcillin (75), Piperacillin-Tazobactam (100/10), Ticarcillin-Clavulanic acid (75/10), Ceftazidime (30), Cefepime (30), Aztreonam (30), Imipenem (10), Meropenem (10), Ertapenem (10), Colistin (10), Gentamicin (10), Tobramycin (10), Amikacin (30), Netilmicin (30), Ciprofloxacin (5), Levofloxacin (5), Moxifloxacin (10), and Aztreonam (30) (HiMedia Laboratories Pvt. Ltd., Mumbai, India). K. pneumoniae (HiMedia Laboratories Pvt. Ltd., Mumbai, India).

The ESBL as well as carbapenemase producing clinical isolates were determined by the modified Hodge test by the BLAST facility (http://blast.ncbi.nlm.nih.gov/). DNA sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (http://blast.ncbi.nlm.nih.gov/).

2.5. DNA Extraction and Molecular Detection. DNA was extracted from the bacterial isolates using the spin column method (QIAGEN; GmbH, Hilden, Germany) as per manufacturer’s instructions. PCR-based detection of ESBL genes (blaCTXM, blaSHV, blaTEM, and blaOXA), Ambler class B MBLs (blaIMP, blaVIM, blaSPM, blaGIM, blaSIM, and blaNDM-1), Ambler class D (blaOXA-23, blaOXA-24, and blaOXA-48), and serine class A carbapenemases (blaKPC, blaGES, and blaNMC) were carried out on the isolates by using Gene Amp 9700 PCR System (Applied Biosystems, Singapore) [9–12]. PCR products were run on 1.5% agarose gel, stained with ethidium bromide visualized under UV light and photographed. The amplicons were purified using QIAquick PCR purification kit (QIAGEN; GmbH, Hilden, Germany).

2.6. DNA Sequencing and Sequence Analysis. Automated sequencing was performed on an ABI 3730XL DNA analyzer using the Big Dye system (Applied Biosystems Foster City, CA, USA). Sequences were compared with known sequences using the BLAST facility (http://blast.ncbi.nlm.nih.gov/).

2.7. Conjugation Experiments. Transfer of resistance genes by conjugation was assayed by mating experiments in Luria-Bertani broth using Enterobacter isolates (Parental strains) as donors and an azide-resistant E. coli J53 as the recipient strain using 1:10 ratio. The transconjugants were selected on Luria-Bertani agar with selection based on growth on agar in the presence of ceftazidime (30 μg/mL) and sodium azide (100 μg/mL). Plasmids were separated and compared by coelectrophoresis with plasmid of known sizes from E. coli (V517 and 39R861) on a horizontal 0.5% agarose gel at 50 volts for 3 hrs. Bands were visualized with UV transilluminator after staining with 0.05% ethidium bromide.

2.8. Strain Molecular Typing. Repetitive element based PCR (REP-PCR), Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR), and Randomly Amplified Polymorphic DNA (RAPD) assays were performed to characterize Enterobacter strains recovered from patients [13, 14].

2.9. Plasmid Analysis. Plasmid from the parental strains and their transconjugants was extracted by using Qiagen plasmid mini kit (GmbH, Hilden, Germany) as per manufacturer’s instructions. Extracted plasmid DNA was subjected to plasmid based replicon incompatibility (Inc) typing by using eighteen pairs of primers to perform five multiplex and three single PCRs which recognized F, FIA, FIB, FIC, B/O, X, Y, N, P, W, T, A/C, H11, H12, II-Ic, L/M, K, and FII replicons as described previously [15]. Plasmid replicons were determined for the ESBL as well as carbapenemase producing clinical isolates.
3. Result and Discussion

A total of 70/130 (53.84%) isolates of Enterobacter spp. were found to exhibit reduced susceptibility to imipenem (diameter of zones of inhibition ≤13 mm) by disc diffusion method. Among 70 isolates tested, 48 (68.57%) isolates showed MIC values for imipenem and meropenem ranging from 32 to 64 mg/L as per CLSI breakpoints. Twenty-two, out of 70 isolates tested, showed MIC values below 8 mg/L. All of these 70 isolates were found susceptible to Colistin in vitro as per MIC breakpoints (<0.5 mg/L). Phenotypic characterization of Enterobacter spp. isolates (N = 130) from clinical samples is shown in Table 1. PCR carried out on these 48 MBL isolates revealed that blaNDM-1 was carried on plasmids ranging in sizes from 35 to 170 kb and blaVIM was carried on 70 to 200 kb size plasmids.

### Table 1: Phenotypic characterization and distribution of Enterobacter spp. isolates (N = 130) from clinical samples.

<table>
<thead>
<tr>
<th>Samples</th>
<th>Number of isolates</th>
<th>Carbapenem resistance by disc diffusion</th>
<th>MHT</th>
<th>CDST</th>
<th>DDST</th>
<th>MBL E-test</th>
</tr>
</thead>
<tbody>
<tr>
<td>Urine</td>
<td>40</td>
<td>22</td>
<td>9</td>
<td>10</td>
<td>10</td>
<td>11</td>
</tr>
<tr>
<td>Blood</td>
<td>32</td>
<td>21</td>
<td>13</td>
<td>13</td>
<td>15</td>
<td>18</td>
</tr>
<tr>
<td>Pus</td>
<td>22</td>
<td>18</td>
<td>10</td>
<td>11</td>
<td>11</td>
<td>13</td>
</tr>
<tr>
<td>Sputum</td>
<td>8</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>Body fluids (synovial, pleural, and ascitic fluid)</td>
<td>10</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Endotracheal</td>
<td>8</td>
<td>4</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>BAL</td>
<td>6</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Tissue</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Total</td>
<td>130</td>
<td>70</td>
<td>38</td>
<td>39</td>
<td>42</td>
<td>48</td>
</tr>
</tbody>
</table>

### Table 2: Distribution of carbapenem resistance genes among Enterobacter spp. (N = 48).

<table>
<thead>
<tr>
<th>Organism</th>
<th>VIM-2</th>
<th>VIM-6</th>
<th>NDM-1</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. aerogenes (N = 12)</td>
<td>4</td>
<td>2</td>
<td>6</td>
</tr>
<tr>
<td>E. cloacae (N = 31)</td>
<td>10</td>
<td>8</td>
<td>13</td>
</tr>
<tr>
<td>E. cloacae complex (N = 5)</td>
<td>4</td>
<td>1</td>
<td>0</td>
</tr>
</tbody>
</table>

3.2. Plasmid Replicon Typing. Plasmids purified from the clinical isolates were typed by PCR based replicon typing. IncFIA, IncFIB, IncFIC replicons were associated with blaTEM-1. Majority of blaTEM-1 showed association with multiple replicons (either IncFII, IncFIB or IncFIA, IncFIB); five isolates showed single replicon association (IncFIC). The blaNDM-1 gene in Enterobacter spp. was located on IncA/C, IncFII, and IncN plasmid. The blaVIM was carried on plasmids belonging to IncP, IncW, IncFII, and IncFIB replicons. BlaCTX-M-15 was associated with multiple replicons of plasmid (IncFIA, IncFIB). The blaOXA identified on plasmids was associated with IncP, IncH2, IncFIC, and IncW replicons. Enterobacter
infections can be acquired from exogenous as well as endoge-
nous sources being ubiquitous in nature as a saprophyte in soil and sewage and as a commensal in human gastroin-
testinal tract. It is present in the feces of humans, animal excreta, dairy products, plants, plant materials, insects, and water [16–18]. Outbreaks of *Enterobacter* infection associated with contaminated intravenous solutions, blood products, distilled water, endoscopes, stethoscopes and other health care devices have been reported [19–22]. *Enterobacter* infec-
tions in a health care settings, seems to arise endogenously from a previously colonized site in an infective individual, mainly the colonization of the gastrointestinal tract with *Enterobacter* spp. in the debilitated patients. Sometimes colo-
nization of more than one strain is seen among those patients who already have been hospitalized and were on antibiotic therapy. Colonization leads to infection by this organism. Prolonged hospital stay, debilitating underlying illnesses, immuno surveillance and indwelling devices/implants have been risk factors for *Enterobacter* spp. infection in hospital settings [23]. *E. cloacae* and *E. aerogenes* are the two most common *Enterobacter* species causing nosocomial infections, most frequently associated with disease. Antimicrobial resist-
ance in *Enterobacter* strains varies with geographic locations. Whereas resistance to betalactam antibiotics, aminoglyco-
sides, trimethoprim-sulfamethoxazole, and quinolones is more prevalent in southern Europe, Belgium, and Israel, in Greece, resistance to cefotaxime, ceftazidime, ceftriaxone, and aminoglycosides is prevalent in 60 to 70% of strains. 2–10% resistance to fluoroquinolones have been documented in various reports [24–30]. The emergence of AmpC, ESBL, and carbapenemase producers along with multiple resistant isolates poses a serious problem in the hospital settings. In our study, among *Enterobacter* spp. 25.71% (18/70) metallo-
beta-lactamase production seen in blood stream infections, followed by 18.57% (13/70) surgical site infections, 15.71% (17/70) urinary tract infections, 8.57% (6/70) respiratory secretions. In 2010, CDC first reported carriage of NDM-1 in *E. cloacae* from patients who received medical care in India [31], following which various reports for the same were published by various authors. Khan and Nordmann reported presence of blaNDM-1 from cases of diabetic foot ulcer [32]. Lascols et al. and Castanheira et al. also reported carriage of blaNDM-1 among *E. cloacae* [33, 34]. Emergence of blaNDM-1 producing *E. cloacae* clinical isolates was reported from Singapore [35], China [36], Australia [37], United States [38], Kuwait [39], Turkey [40], and Canada [41]. MBLs other than NDM-1 also have been reported by various authors in *E. cloacae*: blaMPM-3 from Turkey [42], blaMPM-3 from Taiwan [43], blaVIM-4 from Italy [44–46], blaVIM-2 from Korea [47], and blaVIM-13 from Greece [48]. In our study, we detected blaVIM-2 and blaVIM-4 among *Enterobacter* spp. Presence of BlaVIM-48 in *E. cloacae* have been reported in literature [49, 50]. However, our isolates were negative for OXA-48 like gene. Three studies from abroad by Brink et al. [51], Dai et al. [36], and Ageeves et al. [52] reported presence of blaKPC-2 in *E. cloacae*. Our study showed negative result for blaKPC. Carbapenems are one of the important antibiotics used to treat serious infections caused by Enterobacteriaceae. Multidrug resistance in Enterobacteriaceae is associated with significant morbidity and mortality. Therefore, it is important to check constantly the prevalence of resistance to carbapenem in Gram-negative organisms. Multidrug resistance due to the presence of MBL carrying genes is a point of concern as few drugs can be used for the treatment. The transfer of these genes through plasmids increases the spread of drug resistance from one species to another. Hence, early detection of these drug resistance genes by molecular methods is essential in limiting the spread of infection due to these organisms.

**Conflict of Interests**

The authors declare that there is no conflict of interests regarding the publication of this paper.

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