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 μg/mL as per CLSI breakpoints (<0.5 μg/mL). 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 blood stream 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-lactamases 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,
Laboratory Standard Institute (CLSI) guidelines [5]. The
2.2. Antimicrobial Susceptibility Testing. The antimicrobial
susceptibility was performed by the Kirby Bauer’s disc dif-
fusion technique on Mueller-Hinton agar, as per Clinical
Laboratory Standard Institute (CLSI) guidelines [5]. The
antibiotics tested were as follows (potency in μg/disc):
Ampicillin (10), Cefuroxime (30), Cefotaxime (30), Pipera-
cillin (100), Ticarcillin (75), Piparacillin-Tazobactam (100/
10), Ticarcillin-Clavulanic acid (75/10), Ceftazidime (30),
Cefepime (30), Aztreonam (30), Imipenem (10), Meropenem
(10), Ertaopenem (10), Colistin (10), Gentamicin (10), Tobra-
mycin (10), Amikacin (30), Netilmicin (30), Ciprofloxacin
(5), Levofloxacin (5), Lomefloxacin (10), and Ofloxacin (5)
(ForMedia Laboratories Pvt. Ltd., Mumbai, India). P. aerugi-
osa ATCC 27853, E. coli ATCC 25922, E. coli ATCC 35218,
and K. pneumoniae ATCC 700603 were used as quality
control strains.

2.3. MIC Determination. Minimum inhibitory concentra-
tions (MICs) of antibiotics were determined by VITEK-2
AST-GN25 and AST-GN280 susceptibility cards in accord-
ance with CLSI recommendations and manufacturers’
instructions, except tigecycline and colistin, for which the
2012 European Committee on Antimicrobial Susceptibility
Testing break points were used [5, 6]. MICs were fur-
ther determined by the E-test (bioMérieux, Marcy l’Etoile,
France). According to Centers for Disease Control and
Prevention (CDC), CRE are defined as Enterobacteri-
aeae that are nonsusceptible to penicillins, third-generation
cephalosporins (ceftriaxone, cefotaxime, and ceftazidime),
and one of the Carbapenems (doripenem, meropenem, and
imipenem).

2.4. Phenotypic Screening for the Carbapenemase Produc-
tion. Isolates with reduced susceptibility to meropenem and
imipenem (diameter of zones of inhibition ≤13 mm) by disc
diffusion method were screened for the production of car-
bpapenemase. The phenotypic detection of the carbapenemase
production was performed by the modified Hodge test by
using ertapenem and meropenem discs (10 μg) as per CLSI
guidelines [5]. For MHT K. pneumoniae ATCC BAA-1705
and BAA-1706 were used as positive and negative controls,
respectively. Metallo-beta-lactamase production detected by
double-disc synergy tests (DDST) with both imipenem and
meropenem discs (10 μg) plus disc containing (750 μg) of
EDTA as described earlier by Lee et al. [7] and combined-
disc synergy test (CDST) as described previously by Franklin
et al. [8] by using imipenem/meropenem (10 μg) discs and
one disc with 292 μg EDTA. K. pneumoniae ATCC BAA-2146
and P. aeruginosa ATCC 27853 were used as positive and
2.5. DNA Extraction and Molecular Detection. DNA was
etracted from the bacterial isolates using the spin col-
umn 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, blaOIM, blaGIM, blaSIM, and blaNDM1),
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 plas-
mid 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, HI1, HI2, II-1c, L/M, K, and FII replicons as
described previously [15]. Plasmid replicons were determined
for the ESBL as well as carbapenemase producing clinical isolates.
Table 1: Phenotypic characterization and distribution of *Enterobacter* spp. isolates \((N = 130)\) from clinical samples.

| Samples                      | Number of isolates | Carbapenem resistance by disc diffusion | MHT | CDST | DDST | MBL E-test |
|------------------------------|--------------------|----------------------------------------|-----|------|------|-------------|
| Urine                        | 40                 | 22                                     | 9   | 10   | 10   | 11          |
| Blood                        | 32                 | 21                                     | 13  | 13   | 15   | 18          |
| Pus                          | 22                 | 18                                     | 10  | 10   | 11   | 13          |
| Sputum                       | 8                  | 1                                      | 1   | 1    | 1    | 1           |
| Body fluids (synovial, pleural, and ascitic fluid) | 10     | 0                                      | 0   | 0    | 0    | 0           |
| Endotracheal                 | 8                  | 4                                      | 3   | 3    | 3    | 3           |
| BAL                          | 6                  | 4                                      | 2   | 2    | 2    | 2           |
| Tissue                       | 4                  | 0                                      | 0   | 0    | 0    | 0           |
| Total                        | 130                | 70                                     | 38  | 39   | 42   | 48          |

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 (IP/IP) E-test positive isolates (12 *Enterobacter aerogenes*, 31 *Enterobacter cloacae*, and 05 *Enterobacter cloacae* complex) was validated by sequencing for beta-lactam resistant genes and results were interpreted accordingly. Distribution of carbapenem resistant genes among *Enterobacter* spp. depicted in [Table 2].

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

| Organism                     | VIM-2 | VIM-6 | NDM-1 |
|------------------------------|-------|-------|-------|
| *E. aerogenes* \((N = 12)\)  | 4     | 2     | 6     |
| *E. cloacae* \((N = 31)\)    | 10    | 8     | 13    |
| *E. cloacae* complex \((N = 5)\) | 4   | 1     | 0     |

3.1. Strain Molecular Typing. REP-PCR, ERIC-PCR, and RAPD assays as per banding pattern confirmed presence of eight, four, and three clones among *E. cloacae* (A–D), *Enterobacter aerogenes* (A–D), and *Enterobacter cloacae* complex (A–C), respectively. *E. cloacae* strain typing showed 8 clones, among them three blood, two in urine, and three in pus, respectively, while in case of *Enterobacter aerogenes*, two clones were detected in medical and two were in surgical wards. *Enterobacter cloacae* complex showed two different clonality in Medical ICU whereas in surgical ICU isolates were from single clone.

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 *bla*\_NDM-1. Majority of *bla*\_SHV showed association with multiple replicons (either IncFII, IncFIB or IncFIC). The *bla*\_NDM-1 gene in *Enterobacter* spp. was located on IncA/C, IncFII, and IncN plasmid. The *bla*\_VIM was carried on plasmids belonging to IncP, IncW, IncFII, and IncFIB replicons. *bla*\_CTX-M-15 was associated with multiple replicons of plasmid (IncFIA, IncFIB). The *bla*\_OXA identified on plasmids was associated with IncP, IncH12, IncFIC, and IncW replicons. *Enterobacter*
infections can be acquired from exogenous as well as endogenous sources being ubiquitous in nature as a saprophyte in soil and sewage and as a commensal in human gastrointestinal 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 infections 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 colonization 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, immunosurveillance 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 resistance in Enterobacter strains varies with geographic locations. Whereas resistance to beta-lactam antibiotics, aminoglycosides, 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) metallobeta-lactamase production seen in blood stream infections, followed by 18.57% (13/70) surgical site infections, 15.71% (11/70) 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: blaIMP from Turkey [42], blaIMP,8 from Taiwan [43], blaVIM-4 from Italy [44–46], blaVIM-2 from Korea [47], and blaVIM-12 from Greece [48]. In our study, we detected blaVIM-2 and blaVIM-6 among Enterobacter spp. Presence of BlaOXA-44 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 Ageeets 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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