Characterization of novel plasmid-mediated $\beta$-lactamases (SHV-167 and ACT-16) associated with New Delhi metallo-$\beta$-lactamase-1 harbouring isolates from neonates in India

Neonatal sepsis due to carbapenem-resistant bacteria is difficult to treat due to limited therapeutic options. The detection of the new carbapenemase New Delhi metallo-$\beta$-lactamase-1 (NDM-1) from neonates has further complicated the situation (Roy et al., 2011a). The potent metallo-$\beta$-lactamase NDM-1 efficiently hydrolyses all classes of $\beta$-lactam antibiotics (penicillins, cephalosporins and carbapenems) and is also associated with multiple determinants that enable the bacteria to become resistant to other antibiotic classes (Nordmann et al., 2011). In the presence of NDM-1 other $\beta$-lactamases may go unobserved because of the spectrum of activity of NDM-1 against all $\beta$-lactam antibiotics. Thus, under the canopy of the NDM-1 these $\beta$-lactamases also get the opportunity to spread. This communication reports association of two novel $\beta$-lactamases, SHV-type $\beta$-lactamase (SHV-167) and AmpC-type $\beta$-lactamase (ACT-16), in two NDM-1-carrying Enterobacteriaceae isolated from the blood of two septicemic neonates admitted to a neonatal intensive care unit.

The first neonate presented with late onset sepsis in April 2010, and blood culture yielded Klebsiella pneumoniae (Isolate-1). The neonate was treated with ofloxacin and was discharged. The second neonate presented with features of intestinal obstruction and was referred to the unit for ventilation and surgical care in October 2010. Later the baby developed symptoms of septicemia, and Enterobacter cloacae (Isolate-2) was isolated from the blood. The patient was treated with ofloxacin and colistin and subsequently left the unit against medical advice. There was no overlap of hospital stay between the neonates.

The identity of the two isolates was confirmed by ID 32 E kit (bioMérieux). Disc diffusion tests using antibiotics (BD Diagnostics) were performed according to CLSI guidelines (CLSI, 2008). Both isolates were resistant to a range of antibiotics, including $\beta$-lactams, quinolones and aminoglycosides. Isolate-1 was susceptible only to colistin, ofloxacin and tigecycline but Isolate-2 was susceptible to colistin, ofloxacin, minocycline, tigecycline and chloramphenicol. MICS were determined using Etest (AB Biodisk) (Table 1). Tests with cephalosporin/clavulanic acid combination discs for extended-spectrum $\beta$-lactamase (ESBL) production, meropenem/ABP (3-amino phenyl boronic acid; Sigma-Aldrich) for KPC (K. pneumoniae carbapenemase), and imipenem/EDTA for metallo-$\beta$-lactamase (MBL) production were carried out. Both isolates produced MBL but both showed inconclusive phenotypic results for ESBLs. Investigation of expression of AmpC in the presence of MBL was also performed using cefoxitin, cefoxitin/ABP and cefoxitin/ABP/EDTA combination discs. Isolate-2 was found to co-produce AmpC.

To detect carbapenem-resistant genes, PCRs for blaKPC, blaVIM,IMP,SPM-1,GIM-1,SIM-1, blaNDM,AME,IMI,REL,GES and blaOXA-23,OXA-24,OXA-48,OXA-58,OXA-31 were undertaken. PCRs for ESBLs (blaSHV,TEI,OXA-1,CTX-M) and AmpC genes (blaCTX,ACC,MOX,DIR,EBF,FOX) were also carried out (Roy et al., 2011a).

Sequence analysis of PCR products was performed using the Lasergene DNASTAR sequence analysis software (DNASTAR). The deduced protein sequences were analysed with the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST). Both isolates possessed NDM-1; no other carbapenemases were detected. Isolate-1 also possessed CTX-M-15 and a novel SHV-type, SHV-167 (GenBank accession no. AB733453). Analysis of the predicted amino acid sequence of SHV-167 revealed three substitutions (G87D, H112Y and A146V) compared with SHV-1. However, SHV-167 differed from SHV-1 by only one amino acid substitution (G87D). Going by the sequence similarity of SHV-167 to SHV-71, we predict it is a common broad-spectrum $\beta$-lactamase like SHV-71 (Mendonça et al., 2009). Isolate-2 carried CTX-M-15 along with OXA-1 and a novel AmpC gene, ACT-16 (GenBank accession no. AB737978). ACT-16 has substantial amino acid substitution compared with ACT-1 and is closest to ACT-7 with six substitutions (R3K, I14L, T21A, P58S, N262K and T362K) (Table 1).

16S rRNA methylase genes (Roy et al., 2011b) were also detected: armA and rmtC in Isolate-1 and Isolate-2 respectively. Plasmid-mediated quinolone-resistant genes (Kim et al., 2009) detected were: qnrB and aac(6’)-Ib-cr in both isolates. In both cases, integron class 1 was present. Immunodetection of the porins (Roy et al., 2011a) showed loss of OmpF in both the isolates but normal levels of OmpA and OmpC (Table 1).

The presence of plasmids was determined using the Qiagen Plasmid Midi kit and compared with plasmids of known sizes. Large plasmids, approximately 155 kb, along with smaller ones of 7 kb and 2.8 kb in Isolate-1 and approximately 119 kb, 8 kb and 1.5 kb in Isolate-2, were detected. Conjugal transfer of one large plasmid (approx. 155 kb and 119 kb respectively) from each of the isolates to the sodium azide-resistant recipient Escherichia coli strain J53 by a solid mating assay using plates containing ampicillin (20 $\mu$g $^{-1}$) and sodium azide (100 $\mu$g $^{-1}$) was successful. The transferable plasmid in both cases possessed the novel $\beta$-lactamases along with NDM-1, other resistance determinants and integron class
The diversity of the genetic features associated with the NDM-1 gene has been established (Poirel et al., 2011). The ability of NDM-1 to move across different strains, species and genera of bacteria with dexterity is probably the reason for its association with diverse genes. An earlier study by the current authors had shown the presence of a novel AmpC, CMY-59, along with NDM-1 in an Escherichia coli isolate (Roy et al., 2011b). Other novel β-lactamases, like CTX-M-62, have also been shown to be associated with NDM-1 (Partridge et al., 2012). The presence of novel or veteran β-lactamases in promiscuous plasmids which might go undetected because of their co-existence with NDM-1 is indeed worrisome. β-lactamases can hydrolyse penicillins or cephalosporins but cannot compete with the spectrum of activity of MBLs like NDM-1 which can hydrolyse even carbapenems. This leads to the difficulty of detecting such β-lactamases by phenotypic methods using inhibitors like clavulanic acid in the presence of MBLs, as was also observed in this study. The presence of the β-lactamases CTXM-15 and SHV-167 in both of the isolates went unobserved by the cephalosporin/clavulanic acid combination disc test. With molecular methods of detection unavailable in many laboratories, these β-lactamases may simply give us the slip. Consequently, failure to detect such enzymes in clinical isolates and also the inability to identify patients who are asymptomatically colonized by bacteria possessing such enzymes may lead to the spread of such β-lactamases in the hospital. This study thus emphasizes the need to re-evaluate the detection systems for clinical laboratories so that these β-lactamases can be detected in an appropriate fashion.

### Table 1. Susceptibility and molecular characterization of two Enterobacteriaceae isolates and their transconjugants (TC) harbouring novel β-lactamases

<table>
<thead>
<tr>
<th>Isolate no.</th>
<th>Organism</th>
<th>MIC values (mg l⁻¹)*</th>
<th>Genetic determinants</th>
<th>Integron</th>
<th>Porin</th>
<th>Plasmid size (kb)</th>
<th>Plasmid type</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>CT</td>
<td>ETP</td>
<td>MP</td>
<td>AK</td>
<td>GM</td>
<td>CI</td>
</tr>
<tr>
<td>Isolate-1</td>
<td>Klebsiella pneumoniae</td>
<td>≥256</td>
<td>≥32</td>
<td>24</td>
<td>≥256</td>
<td>≥1024</td>
<td>3</td>
</tr>
<tr>
<td>TC-1</td>
<td>Escherichia coli J53</td>
<td>≥256</td>
<td>≥32</td>
<td>16</td>
<td>≥256</td>
<td>≥1024</td>
<td>1</td>
</tr>
<tr>
<td>Isolate-2</td>
<td>Enterobacter cloacae</td>
<td>≥256</td>
<td>≥32</td>
<td>≥32</td>
<td>≥256</td>
<td>≥1024</td>
<td>4</td>
</tr>
<tr>
<td>TC-2</td>
<td>Escherichia coli J53</td>
<td>≥256</td>
<td>≥32</td>
<td>8</td>
<td>≥256</td>
<td>≥1024</td>
<td>1</td>
</tr>
</tbody>
</table>

ND, Not determined.

*MIC values for Escherichia coli J53: CT, 0.047 mg l⁻¹; ETP, 0.064 mg l⁻¹; MP, 0.047 mg l⁻¹; AK, 1.5 mg l⁻¹; GM, 0.19 mg l⁻¹; CI, 0.047 mg l⁻¹. CT, cefotaxime; ETP, ertapenem; MP, meropenem; AK, amikacin; GM, gentamicin; CI, ciprofloxacin; TGC, tigecycline; CL, colistin.

**Acknowledgements**

We extend our thanks to Dr Arun K. Singh for his support during the study. We wish to thank the anonymous reviewers for their constructive comments which helped in the improvement of the paper.

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http://jmm.sgmjournals.org
George A. Jacoby, Anne Marie Queenan, Olivier Moquet and Kyungwon Lee for providing control strains for the PCRs and Heinz Schwarz and Helen I. Zgurskaya for the antibodies. The study was partially supported by a fund from Department of Science and Technology (DST), West Bengal, and S.D. was supported by a fellowship from the same funding source. S.M. was supported by a fellowship from CSIR, India. The authors declare that they have no conflict of interest.

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Abbreviation: MBL, metallo-β-lactamase.

The GenBank/EMBL/DDBJ accession numbers for SHV-167 and ACT-16 are AB733453 and AB737978 respectively.


