First occurrence of KPC-2-possessing *Klebsiella pneumoniae* in a Greek hospital and recommendation for detection with boronic acid disc tests

Athanassios Tsakris1*, Ioulia Kristo2, Aggeliki Poulou3, Fani Markou3, Alexandros Ikonomidis2 and Spyros Pournaras2

1Department of Microbiology, Medical School, University of Athens, Athens, Greece; 2Department of Microbiology, Medical School, University of Thessaly, Larissa, Greece; 3Department of Microbiology, Serres General Hospital, Serres, Greece

Received 12 June 2008; returned 20 June 2008; revised 6 August 2008; accepted 7 August 2008

**Objectives:** To investigate the first KPC carbapenemase-producing *Klebsiella pneumoniae* isolate from a Greek hospital, including phenotypic methods to aid recognition of this resistance type.

**Methods:** A carbapenem-resistant clinical isolate of *K. pneumoniae* was recovered from a hospitalized Greek patient. Detailed susceptibility testing was carried out by the agar dilution method. The isolate was screened by phenotypic and genotypic assays for the presence of various β-lactamases. Boronic acid disc tests were performed to show the ability of these tests to detect production of the KPC enzymes. The potential for conjugal transfer of carbapenem resistance was examined by biparental matings, plasmid analysis and PCR studies.

**Results:** The isolate possessed on the same self-transferable plasmid the KPC-2 carbapenemase and the SHV-12 extended-spectrum β-lactamase. Although the isolate did not produce an AmpC-type enzyme, the production of KPC-2 was associated with positive boronic acid disc tests using cephamycins and cefotaxime as well as cefepime and carbapenems as substrates.

**Discussion:** KPC-2-possessing *K. pneumoniae* clinical isolates seem to have been introduced in our region. Boronic acid disc tests using boronic acid in combination with carbapenems or cefepime may help the phenotypic detection of KPC enzymes and their distinction from plasmid-mediated AmpC enzymes.

**Keywords:** carbapenemases, extended-spectrum β-lactamases, combined disc test

**Introduction**

The plasmid-encoded KPC-type carbapenem-hydrolysing enzymes have emerged among Gram-negative species, particularly *Klebsiella pneumoniae*, in the north-eastern regions of the USA.1 KPC-producing isolates of multiple Gram-negative species have also been detected in other regions of the USA, as well as in Colombia and China, and in an endemic situation in Israel.1 Additionally, KPC-possessing *K. pneumoniae* clinical isolates have been recovered from patients hospitalized in French and Swedish hospitals,2-4 and in two of these cases, the patients were previously hospitalized in Greek intensive care units.3,4 In this report, we describe the characteristics of a carbapenem-resistant KPC-producing *K. pneumoniae* isolate, which was recovered from a patient hospitalized in a northern Greek hospital. The use of boronic acid as KPC inhibitor in phenotypic detection tests was also tested.

**Materials and methods**

After 8 days of intravenous ampicillin/sulbactam treatment (1.5 g every 8 h), *K. pneumoniae* M410 was repeatedly recovered from urine samples of a young woman hospitalized in Serres General Hospital in 2008. The patient suffered from eclampsia complicated with urinary tract infection. She had delivered by a caesarean section 12 days prior to the bacterial isolation. The isolate was confirmed to be *K. pneumoniae* by using API20E (bioMérieux, Marcy l’Etoile, France) and displayed resistance to all β-lactams
including carbapenems. The isolate remained susceptible to aminoglycosides, tigecycline and colistin, but was resistant to co-trimoxazole and fluoroquinolones. Based on the susceptibility profile, the patient was successfully treated with an aminoglycoside regimen and discharged in a good clinical condition.

Detailed susceptibility analysis was carried out by the agar dilution method following the CLSI guidelines and interpretative criteria. For tigecycline, the US Food and Drug Administration recommendation was used (susceptible, \(\leq 2\) mg/L; and resistant, \(\geq 8\) mg/L), and for colistin, the CLSI recommendation for Acinetobacter spp. was used (susceptible, \(\leq 2\) mg/L; and resistant, \(\geq 4\) mg/L). Screening for the presence of a carbapenemase was performed with the modified Hodge test using Escherichia coli ATCC 25922 as the indicator strain and 10 \(\mu\)g imipenem discs. The MBL Etest (AB Biodisk, Solna, Sweden), where MBL stands for metallo-\(\beta\)-lactamases, and the combined disc test with imipenem and EDTA, performed in Mueller–Hinton agar plates, were used to screen for the production of class B carbapenemases. The presence of extended-spectrum \(\beta\)-lactamases (ESBLs) was tested with the CLSI confirmatory test and a modified test, which uses clavulanate in combination with boronic acid. The presence of AmpC-\(\beta\)-lactamase was phenotypically tested by determining cefoxitin and imipenem MICs in agar with and without 200 mg/L cloxacinil and by using the AmpC detection Etest strips (AB Biodisk), which contain cefotetan with or without cloxacinil.

Boronic acid disc tests were performed to show the ability of these tests to detect production of the KPC enzyme. Discs containing boronic acid were prepared in-house, as recommended previously. The tests were performed by inoculating Mueller–Hinton agar by the standard diffusion method and plating discs containing different \(\beta\)-lactams (imipenem, meropenem, ertapenem, cefepime, cefotaxin, cefotetan, ceftazidime, aztreonam and aztreonam) with or without 400 \(\mu\)g boronic acid onto the agar. The test was considered positive when the zone diameter around the disc containing the antibiotic substrate and boronic acid was \(\geq 5\) mm than the zone diameter around the disc containing the antibiotic substrate alone.

Isoelectric focusing was performed on a polyacrylamide gel [ampholytes (pH 3–10)]. \(\beta\)-Lactamase genes were amplified using a panel of primers to detect all types of MBLs, KPCs, plasmid-mediated AmpCs in single PCR reactions for each gene, OXA carbapenemases, ESBLs and TEM enzymes. The primers for KPC are located from nucleotide positions –39 to +68 of the total, 882 bp, \(bla_{KPC}\) gene (GenBank EU176014) and produce a 989 bp amplicon. In all reactions, previously characterized isolates from our collection carrying all types of \(\beta\)-lactamases were used as positive controls. PCR products were purified using the ExoSAP-IT reagent (USB Corporation, Cleveland, OH, USA) and used as templates for sequencing on both strands with an ABI Prism 377 DNA sequencer (Applied Biosystems, Foster City, CA, USA).

The potential for conjugal transfer of carbapenem resistance was examined in biparental matings using E. coli 26R793 (Lac\(^{-}\) Rif\(^{+}\)) as the recipient strain. Donor and recipient cells were mixed in a ratio of 1:5, and transconjugants were selected on MacConkey agar plates containing 100 mg/L rifampicin and ertapenem at concentrations of 0.5–2 mg/L. Plasmid extraction on the clinical isolate and transconjugant was performed by using an alkaline lysis protocol and the QIAGEN Plasmid Midi Kit (Qiagen, Hilden, Germany), and the E. coli strain 39R861 that harbours plasmids of \(\sim 147\), 63, 35.8 and 6.9 kb as a control.

**Results**

The MICs of a variety of \(\beta\)-lactam agents tested against K. pneumoniae M410 are shown in Table 1. The isolate was resistant to all carbapenems (imipenem, meropenem and ertapenem MICs of 32, 32 and 64 mg/L, respectively) as well as to various penicillins, \(\beta\)-lactam/inhibitor combinations, extended-spectrum cephalosporins and aztreonam. It also showed resistance to ciprofloxacin (MIC, 64 mg/L) and trimethoprim (MIC, >256 mg/L), but not to colistin (MIC, 1 mg/L), tigecycline (MIC, 2 mg/L), gentamicin (MIC, 2 mg/L) and amikacin (MIC, 16 mg/L). The application of the modified Hodge test showed the production of a carbapenemase, but the MBL Etest and the combined disc test were negative for the production of class B carbapenemases. Phenotypic testing for ESBL production by the CLSI confirmatory test gave a positive result only for ceftazidime. However, the application of the CLSI confirmatory test in the presence of clavulanate and boronic acid was positive for both compounds (ceftazidime and ceftaxime), clearly indicating the production of an ESBL. Inhibition with cloxacinil did not change imipenem and cefotixin MICs, and the AmpC detection Etest assay was negative, suggesting the absence of a plasmid-mediated AmpC.

<table>
<thead>
<tr>
<th>Antibiotic*</th>
<th>K. pneumoniae M410</th>
<th>Tc E. coli 26R793 (pM410)</th>
<th>E. coli 26R793</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>16</td>
<td>0.5</td>
</tr>
<tr>
<td>Meropenem</td>
<td>32</td>
<td>16</td>
<td>0.03</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>64</td>
<td>32</td>
<td>0.06</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;256</td>
<td>256</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>128</td>
<td>32</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefepime</td>
<td>64</td>
<td>32</td>
<td>0.12</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>1</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>64</td>
<td>16</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin + CLA</td>
<td>128</td>
<td>64</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>2</td>
</tr>
<tr>
<td>Piperacillin + TZB</td>
<td>&gt;256</td>
<td>128</td>
<td>1</td>
</tr>
</tbody>
</table>

*CLA, clavulanic acid at a fixed concentration of 2 mg/L; TZB, tazobactam at a fixed concentration of 4 mg/L.
However, the inhibitor did not affect the activity of ceftazidime or aztreonam, most likely due to the presence of the SHV-type ESBL, which is not affected by boronic acid.

Plasmid analysis and conjugation experiments showed that the M410 clinical isolate contained two plasmids of ~70 and 20 kb and that both $\text{bla}_{\text{KPC-2}}$ and $\text{bla}_{\text{SHV-12}}$ genes were co-transferred along with resistance to trimethoprim on the largest (70 kb) plasmid. $E. \ coli$ transconjugant MICs of $\beta$-lactam agents were similar to those for M410, except for those of cefoxitin, cefotaxime and piperacillin/tazobactam, which were lower than those for the parental strain (Table 1). Isoelectric focusing of the transconjugant 26R793 (pM410) revealed $\beta$-lactamases with pI values of 6.8 and 8.2, which was consistent with the acquisition of KPC-2 and SHV-12 enzymes, respectively. The application of boronic acid disc tests on the $E. \ coli$ transconjugant also showed positive results for cefotetan, cefoxitin and cefotaxime as well as for all carbapenems and cefepime (Figure 1c and d). When the 70 kb plasmid band of 26R793 (pM410) was extracted from the gel and used as a template for the amplification of $\text{bla}_{\text{KPC}}$ and $\text{bla}_{\text{SHV}}$ genes, the specific 989 bp and 1014 bp products were detected, respectively, suggesting that both resistance determinants were carried on this plasmid.

**Discussion**

Carbapenem resistance is one of the major threats for the antimicrobial treatment of Gram-negative infections. In our regions, carbapenem resistance has emerged among $K. \ pneumoniae$ clinical isolates and is mostly attributed to the production of class B MBLs. This is the first report describing carbapenem resistance due to a KPC-type carbapenemase detected in our hospital settings. The present isolate, similarly to cases described in the USA, also possessed the SHV-12 and TEM-1 enzymes, which contributed to the $\beta$-lactam-resistant phenotype. It is of note that phenotypic testing for ESBL production using clavulanate in combination with boronic acid enhanced the detection of ESBL in the KPC-producing isolate, similarly to previous observations for AmpC producers.

KPC-type-producing Enterobacteriaceae are becoming established in several regions, and accurate detection methods for
these enzymes as well as for other coexisting β-lactamases are urgently required for both therapeutic and epidemiological purposes.16,13 The early identification of KPC phenotypes often relies on indirect indicators such as reduced susceptibility to carbapenems.11 Also, accurate ertapenem susceptibility testing along with the modified Hodge test have been suggested for screening KPC-producing isolates.6 However, in our regions where carbapenem resistance might also be caused by other resistance mechanisms, such as production of MBLs, these tests cannot be used as markers of KPC enzymes. A previous study gave an indication that class A carbapenemase-producing isolates may be wrongly inferred to have AmpC enzymes using the chromogenic Cica-β-Test, which examines the hydrolysis of a chromogenic oxyimino-cephalosporin substrate in the presence of boronic acid.14 In accordance with this observation, our case illustrates that screening for AmpC production with boronic acid assays of KPC-producing isolates, although they did not produce a plasmid-mediated AmpC enzyme. It is also suggested that boronic acid assays using cefepime or carbapenem discs may be used in clinical laboratories to differentiate KPC producers. Further studies are required to evaluate the potential use of boronic acid for the phenotypic detection of KPC-producing enterobacterial isolates as well as the interpretative guidelines for the accurate detection of plasmid-mediated AmpCs among clinical isolates with KPC enzymes.

Funding
No specific funding was received for this study.

Transparency declarations
None to declare.

References


