Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece

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Received 7 January 2009; returned 24 March 2009; revised 21 May 2009; accepted 22 May 2009

Objectives: KPC-possessing *Klebsiella pneumoniae* have been found to be widespread in several regions but are still rarely detected in Europe. We describe the characteristics of an outbreak caused by KPC producers in a tertiary care Greek hospital.

Methods: During a 12 month period (October 2007–September 2008), 47 patients in Hippokration University Hospital yielded *K. pneumoniae* isolates that exhibited reduced susceptibility to carbapenems and were phenotypically positive for carbapenemase production but negative for metallo-β-lactamase (MBL) production. Single patient isolates were tested by Vitek 2, Etest, agar dilution MICs, phenotypic assays and PFGE. Carbapenemase and other β-lactamase genes were identified by PCR and sequencing. Patient records were retrospectively reviewed to access co-morbidities, antibiotic exposure prior to infection and outcome.

Results: The 47 *K. pneumoniae* isolates exhibited various susceptibilities to imipenem and meropenem; all were non-susceptible to ertapenem and several other antibiotics but most were susceptible to gentamicin, colistin and tigecycline. PFGE classified the isolates into two clonal types, with the predominant type, which was closely related to that of hyperepidemic strains from the USA and Israel, comprising three subtypes. All isolates carried the *bla*KPC-2 gene; 45 also carried *bla*SHV-12 and 29 *bla*TEM-1. Patients were hospitalized in nine different units. The median length of hospital stay prior to KPC isolation was 21 days; 38 patients (80.9%) had evidence of clinical infection due to a KPC producer and 16 (34%) had bacteraemia. The crude mortality rate was 27.7%. A β-lactam/β-lactamase inhibitor combination was the most frequently administered antimicrobial prior to KPC isolation (20 patients; 42.5%), whereas only nine patients (19.1%) had prior carbapenem use.

Conclusions: This study presents for the first time a wide intrahospital spread of KPC-producing *K. pneumoniae* clones in a European hospital. The KPC producers were rapidly disseminated in several units, indicating the difficulty in restraining such multidrug-resistant clones when they have been established in a hospital environment.

Keywords: SHV-12, extended-spectrum β-lactamases, antibiotic exposure, length of hospital stay, crude mortality

Introduction

*Klebsiella pneumoniae* often cause severe hospital infections against which carbapenems are frequently used when isolates produce extended-spectrum β-lactamases (ESBLs). Resistance to carbapenems, although relatively common among Gram-negative non-fermenting bacteria, still remains uncommon in Enterobacteriaceae.¹ However, identification of carbapenem-resistant *K. pneumoniae* is increasing in various regions worldwide such as the north-eastern USA, where carbapenem

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Outbreak of KPC-2-possessing *Klebsiella pneumoniae* in Greece

Resistance is mainly due to KPC enzymes, while in southern Europe metallo-β-lactamases (MBLs) prevail.

KPC enzymes belong to molecular class A and are capable of hydrolysing carbapenems, penicillins, cephalosporins and aztreonam. The initial report of a KPC β-lactamase was from a carbapenem-resistant *K. pneumoniae* strain isolated in North Carolina, and subsequently KPC producers have also been detected in other regions of the USA, as well as in Latin America, China and endemically in Israel. There are currently seven recognized variants, with KPC-2 and KPC-3 being more commonly isolated. Recently, sporadic *K. pneumoniae* isolates producing KPC-2 enzyme or the close variant KPC-3 have been detected in European countries such as Greece, the UK and France. This evolution raises the disturbing possibility that the dissemination of KPC enzymes has already begun in Europe, posing another threat for carbapenem activity, considering the increasing rates of MBL-producing *K. pneumoniae*. In addition, the spread of KPC producers may be underestimated, as the detection of KPC- and also MBL-producing *K. pneumoniae* may be unsuccessful because these enzymes do not always confer obvious carbapenem resistance.

In Hippokration University Hospital, *K. pneumoniae* producing MBLs of the VIM type are commonly recovered from severe clinical infections. However, during October 2007 we noticed that several of our *K. pneumoniae* isolates with reduced susceptibility to carbapenems gave negative results to the phenotypic tests for MBL production. This prompted a study of the mechanisms underlying this phenotype and a systematic review of patients infected or colonized with such isolates. In the present report, we describe for the first time the extensive spread of KPC-2-producing *K. pneumoniae* clinical isolates in a European hospital. This outbreak began in 2007 and is still ongoing in our healthcare facilities.

Materials and methods

Hospital setting and bacterial collection

The Hippokration University Hospital is the largest tertiary care hospital in Northern Greece, with >900 beds and >60000 admissions each year; it has an intensive care unit (ICU), a transplantation unit, haematological units and two neonatal ICUs. During the 1 year study period (October 2007–September 2008) all single patient *K. pneumoniae* isolates that were identified by the Vitek 2 system as having imipenem or meropenem MICs >1 mg/L and by the EXPERT software as ‘possible carbapenemase producers’ were qualified for further analysis. When more than one isolate was recovered per patient, we included in the study the isolate recovered from the most clinically significant site.

Phenotypic testing

The identification and the initial susceptibility testing of *K. pneumoniae* isolates were performed with the Vitek 2 automated system (bioMérieux, Marcy l’Etoile, France). MICs of imipenem, meropenem, ertapenem, colistin and tigecycline were determined by Etest (AB Biodisk, Solna, Sweden) and agar dilution following the CLSI guidelines and interpretative criteria. For tigecycline the US Food and Drug Administration recommendation was used (susceptible, MIC ≤ 2 mg/L; resistant, MIC ≥ 8 mg/L), and for colistin the CLSI recommendation for *Acinetobacter* spp. was used (susceptible, MIC ≤ 2 mg/L; resistant, MIC ≥ 4 mg/L).

Phenotypic screening for the presence of carbapenemases was performed with the cloverleaf test, and the production of MBLs was tested for by the MBL Etest (AB Biodisk, Solna, Sweden) and the combined disc test with imipenem and EDTA in Mueller–Hinton agar plates. The boronic acid disc test was performed to screen for KPC producers using 400 μg of boronic acid as the enzyme inhibitor and different β-lactams as antibiotic substrates.

*PCR assays and DNA sequencing*

The detection of β-lactamase genes was performed by PCR, using a panel of primers for detection of KPCs (989 bp amplicon encompassing –39 to +68 of the *bla*KPC coding region with position +1 being that of A in the ATG start codon), acquired AmpCs in single PCRs for each gene, OXA-type carbapenemases, MBLs and ESBLs. Previously characterized isolates from our collection carrying every type of the β-lactamases tested were used as positive controls. Nucleotide sequencing of both strands of the PCR products derived with the *bla*KPC-specific and *bla*TEM-specific primers was performed with an ABI Prism 377 DNA sequencer (Perkin-Elmer, Applied Biosystems Division, Foster City, CA, USA).

*Macrogenetic analysis*

PFGE of SpeI-digested genomic DNA of the KPC-producing *K. pneumoniae* strains was performed with a CHEF-DRII system (Bio-Rad, Hemel Hempstead, UK), with a running time of 23 h and pulse times ranging from 3 to 20 s. PFGE was also performed for comparison on three contemporary *K. pneumoniae* isolates from Hippokration University Hospital that were carbapenemase-negative. The PFGE patterns were compared visually.

Retrospective review of patients’ data

We retrospectively examined the medical records of patients that harboured KPC producers, to ascertain factors that may have influenced the acquisition of the organism and the persistence of the outbreak. The hospital location of patients, anonymized demographic data, clinical characteristics, underlying illnesses, prior exposures to antibiotics for ≥3 days and in-hospital mortality were extracted. Clinical infections versus colonization and the outcome of infected patients were defined. The infections were considered hospital-acquired if they occurred >48 h after admission. Nosocomial infections were defined by standard CDC definitions.

Results

During the study period, 47 patients at Hippokration University Hospital yielded 62 *K. pneumoniae* clinical isolates that had reduced susceptibility or resistance to carbapenems and were phenotypically positive for carbapenemase production but negative for MBL production; one isolate per patient was selected for study. The 47 single patient isolates exhibited resistance to many antimicrobial classes, most of them being resistant to β-lactams (including β-lactam/β-lactamase inhibitor combinations and carbapenems), fluoroquinolones, co-trimoxazole and aminoglycosides (amikacin, netilmicin and tobramycin); all isolates were susceptible to colistin and tigecycline and as many as 43 to gentamicin. It should be noted that 15 of the 47...
representative isolates were initially identified as susceptible to imipenem and/or meropenem by the Vitek 2 system but were classified as resistant by the Vitek EXPERT software. By Etest, all isolates were intermediate or resistant to meropenem and resistant to imipenem and ertapenem, exhibiting growth of heterogeneous colonies inside the elliptic zone of inhibition around meropenem and imipenem. By agar dilution, 15 isolates were found to be susceptible to meropenem, seven to imipenem but none to ertapenem. The isolates were phenotypically identified as possible KPC producers by the negative MBL tests and positive boronic acid disc synergy tests using as substrates carbapenems or cefepime. The presence of \( \text{bla}_{KPC} \) was confirmed in all isolates by PCR. Both strands of the KPC-specific PCR product from all isolates were sequenced and it was found that they contained an open reading frame of 882 bp with no nucleotide changes compared with \( \text{bla}_{KPC-2} \) (GenBank accession number AF297554). In 45 isolates the additional presence of an SHV-type ESBL gene was revealed by PCR analysis, and in all cases sequencing identified the \( \text{bla}_{SHV-12} \) gene. Co-amplification of \( \text{bla}_{SHV-12} \) with the intrinsic \( \text{bla}_{SHV-1}-\text{like} \) gene was not detected in any isolate. The only heterozygotes observed in sequencing were those of \( \text{bla}_{SHV-12} \) and a \( \text{bla}_{SHV-12}\)-like gene that differed by two nucleotides from the original \( \text{bla}_{SHV-12} \). This co-amplification was identified in three isolates. TEM-1 \( \beta \)-lactamase was also detected in 29 of the isolates. Other ESBLs or plasmid-mediated AmpCs were not detected in any of the isolates.

PFGE analysis of the 47 \( \text{K. pneumoniae} \) isolates identified two different clonal types. The predominant one contained all 45 SHV-12-carrying isolates and included three subtypes differing by one or two bands from each other [Figure S1, available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/)]. Contemporary carbapenem-negative \( \text{K. pneumoniae} \) isolates from Hippokration University Hospital that were run for comparison exhibited various unrelated PFGE patterns (data not shown). It is of importance that the PFGE pattern of the predominant clonal subtype (Ia), produced under the same conditions, seemed to be identical to the pattern of hyperendemic KPC-producing strains from New York\(^\text{12}\) (strains 8 and 9 in Figure 1B of Navon-Venezia et al.\(^\text{12}\)) and related to that of other strains from the USA and Israel.\(^\text{12}\)

Various characteristics of the 47 patients infected or colonized with KPC-possessing \( \text{K. pneumoniae} \) isolates are presented

### Table 1. Clinical characteristics of the 47 patients from whom KPC-producing \( \text{K. pneumoniae} \) were isolated

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>No. (%) of patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Clinical status</td>
<td></td>
</tr>
<tr>
<td>infection</td>
<td>38 (80.9)</td>
</tr>
<tr>
<td>colonization</td>
<td>9 (19.1)</td>
</tr>
<tr>
<td>Underlying disease</td>
<td></td>
</tr>
<tr>
<td>chronic renal failure</td>
<td>16 (34.0)</td>
</tr>
<tr>
<td>malignancy</td>
<td>13 (27.7)</td>
</tr>
<tr>
<td>liver cirrhosis</td>
<td>11 (23.4)</td>
</tr>
<tr>
<td>diabetes mellitus</td>
<td>5 (10.6)</td>
</tr>
<tr>
<td>severe neurological disorder</td>
<td>3 (6.4)</td>
</tr>
<tr>
<td>cardiovascular disease</td>
<td>2 (4.3)</td>
</tr>
<tr>
<td>morbid obesity</td>
<td>1 (2.1)</td>
</tr>
<tr>
<td>Outcome</td>
<td></td>
</tr>
<tr>
<td>recovery</td>
<td>34 (72.3)</td>
</tr>
<tr>
<td>death</td>
<td>13 (27.7)</td>
</tr>
<tr>
<td>Previous antimicrobial use</td>
<td></td>
</tr>
<tr>
<td>( \beta )-lactam/( \beta )-lactamase inhibitor combination(^\text{a})</td>
<td>20 (42.6)</td>
</tr>
<tr>
<td>expanded-spectrum cephalosporin(^\text{a})</td>
<td>15 (31.9)</td>
</tr>
<tr>
<td>aminoglycoside</td>
<td>13 (27.7)</td>
</tr>
<tr>
<td>fluoroquinolone</td>
<td>11 (23.4)</td>
</tr>
<tr>
<td>carbapenem(^\text{a})</td>
<td>9 (19.1)</td>
</tr>
<tr>
<td>colistin</td>
<td>8 (17.0)</td>
</tr>
<tr>
<td>co-trimoxazole</td>
<td>5 (10.6)</td>
</tr>
<tr>
<td>tigecycline</td>
<td>2 (4.3)</td>
</tr>
</tbody>
</table>

\(^{\text{a}}\)Several patients had received more than one compound of each category.

in Table 1. The patients from whom KPC-positive isolates were recovered were hospitalized in nine different units or clinics of the hospital, with the most common of those being the general ICU (14 patients), the transplantation unit (13 patients) and a surgical clinic (10 patients). One to three cases were detected in six additional services (three cases each in renal and neurosurgical and one case each in medical, neurology, gynaecology and haematology departments). The index isolate was recovered from the surgical drainage of a patient hospitalized in the
transplantation unit and belonged to the most prevalent clonal subtype Ia (which included 34 isolates) that was subsequently spread to eight services of the hospital. Subtype Ib (nine isolates) was detected in four different services, while subtype Ic (two isolates) and clone II (two isolates) were each detected in different services. The monthly prevalence of KPC-positive isolates is shown in Figure 1. All isolates were acquired intrahospitally, since the patients were hospitalized for at least 10 days prior to the recovery of the organism. The median length of hospitalization prior to the isolation was 21 days (range, 10–87 days). Patients had a male to female ratio of 1.5 and a mean age of 59.5 years (range, 31–85 years). Patients harbouring KPC producers were usually admitted for solid organ transplantation (13 patients, 27.7%), abdominal surgery (10 patients, 21.3%), respiratory infection (8 patients, 17.0%) or abdominal infection (4 patients, 8.5%). Thirty-eight patients (80.9%) had evidence of clinical infection due to the KPC producers. The organisms were most often implicated in bacteraemias (16 patients, 34.0%), urinary tract infections (8 patients, 17.0%) and wound infections (7 patients, 14.9%). Most patients had one or more underlying disease (41 patients, 87.2%), with chronic renal failure, malignancy and liver cirrhosis being predominant (Table 1). The crude mortality of patients with KPC-producing isolates was 27.7%. Multiple antimicrobials had usually been administrated prior to the isolation of the KPC producer; antibiotics that were given for more than 3 days were noted. Among those antibiotics, a β-lactam/β-lactamase inhibitor combination was the most frequently administered antimicrobial regimen prior to KPC isolation (20 patients, 42.6%), whereas only 9 patients (19.1%) had prior carbapenem use (Table 1). Infections due to KPC producers were usually treated with colistin and/or tigecycline, while in several cases gentamicin or co-trimoxazole was also co-administered.

Discussion

KPC-producing *K. pneumoniae* were first isolated in the northeastern USA and since then they have been increasingly detected.1,2,13 According to a recent report from the SENTRY Antimicrobial Surveillance Program,14 KPC β-lactamases are now the most prevalent carbapenemases among *K. pneumoniae* in North and Latin America. Nevertheless, KPC-producing *K. pneumoniae* are only sporadically isolated in Europe,2 and the increasing carbapenem resistance of Enterobacteriaceae is still mainly attributed to the production of MBL enzymes.3,14 The present study describes a large hospital outbreak of KPC-producing *K. pneumoniae* in a European hospital. All outbreak isolates were produced to the KPC-2 enzyme and all but two harbour SHV-12 ESBL. The two SHV-12-negative isolates exhibited lower MICs of ceftazidime (16 mg/L), compared with the SHV-12-positive isolates that were within the resistance range (data not shown). This observation suggests the contribution of the SHV-12 ESBL to the resistance to ceftazidime, which is weakly hydrolysed by the KPC enzymes.2 PFGE of the 47 isolates identified two strain patterns, with the predominant pattern comprising three subtypes that were related to clones identified previously in the USA and Israel and carried the close KPC-2 variant, KPC-3.12 It is noteworthy that isolates belonging to the same PFGE type have been found previously to carry either KPC-2 or KPC-3.13

The potential of KPC producers to disseminate, together with the difficulties in their detection due to the low carbapenem MICs that they may confer, raise the need for accurate detection methods for these enzymes as well as for other co-existing β-lactamases. In the outbreak isolates, the specific tests for MBLs were negative, while the cloverleaf test, which has been applied for the detection but not discrimination of carbapenemases,3 was positive. It is also of note that the recently proposed disc synergy testing for KPC enzymes6 using carbapenems in combination with boronic acid was consistently positive.

The majority of our KPC-positive isolates belonged to a major clonal type and may have been disseminated up to now mainly via cross-infections, as genetically unrelated strains of the PFGE type II have been limited. The outbreak is continuing in Hippokration University Hospital and efforts to control it include infection control measures and rigorous local surveillance that up to now have not restricted the dissemination of KPC producers. The control of the outbreak may be affected by the spread of the isolates in unrelated units and also by the severe underlying illnesses of the patients, such as liver cirrhosis and renal failure that required transplantation. It is also of interest that a large proportion of patients had received combinations of β-lactam/β-lactamase inhibitors prior to the isolation of the KPC producer, while considerably fewer patients had received carbapenems. Our study showed that outbreaks due to KPC-producing *K. pneumoniae* are difficult to constrain, as was also indicated from the USA experience.15 It is believed that if a nosocomial pathogen is not controlled soon after its introduction into a hospital, the monoclonal outbreak may evolve to polyclonal endemicity, which can be especially difficult to control. In that respect, more intensive efforts should be applied to restrict the predominant KPC-producing clone and the further spread of *bla*<sub>KPC</sub> to unrelated clones and species.

Funding

No specific funding was received for this study.

Transparency declarations

None to declare.

Supplementary data

Figure S1 is available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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