Outbreak of OXA-48 carbapenemase-producing Klebsiella pneumoniae in Greece involving an ST11 clone

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Objectives: First detected in Enterobacteriaceae isolates in Turkey, the OXA-48 carbapenemase has gradually disseminated in the wider Mediterranean area and Europe. Despite reports from other European regions, until now no such isolates have been detected in Greece. We describe the characteristics of the first outbreak caused by OXA-48-producing Klebsiella pneumoniae in Greece.

Methods: From December 2011 to March 2012, 13 ertapenem-resistant K. pneumoniae isolates, which were positive by the modified Hodge test while remaining negative by phenotypic screening for metallo-β-lactamase (MBL) and KPC production, were recovered from nine patients. Patient records were retrieved to access patterns of acquisition. Resistance genes were identified by PCR and sequencing. ompK35, ompK36 and the genetic environment of the blaOXA-48 gene were investigated. Plasmid profiling, conjugation experiments, PFGE and multilocus sequence typing (MLST) were performed.

Results: All isolates harboured the blaOXA-48 gene along with the blaCTX-M-15 and blaOXA-1 genes. The blaOXA-48 gene was located on a self-transferable IncL/M-type plasmid of ~62 kb, which harboured no other resistance genes. IS1999 was located upstream of the blaOXA-48 gene. Genetic disruptions of the ompK35 and ompK36 genes were not detected. The isolates belonged to a unique PFGE clone and MLST assigned them to sequence type ST11. All cases were characterized as hospital acquired and none of them was linked to immigration or history of travel in endemic areas.

Conclusions: Carbapenem resistance due to MBL and KPC carbapenemases is currently on an endemic scale in Greece and this report highlights the wider undetected dissemination of yet another carbapenemase in this region.

Keywords: K. pneumoniae, clonal strains, sequence types

Introduction

Klebsiella pneumoniae isolates are increasingly expressing their potential to become multidrug resistant by acquiring a diversity of carbapenem-hydrolysing enzymes.1,2 As such, over the past decade and following its initial identification in Turkey,3 the OXA-48 enzyme has been detected not only in countries of the Mediterranean Basin,4 but also in various Western European countries with no close geographical proximity to Turkey,1,5–9 and has been linked to hospital outbreaks in the UK, France, Spain, Ireland and the Netherlands.1,7

This oxacillinase hydrolyses carbapenems weakly, while sparing expanded-spectrum cephalosporinases;3,9 however, when it is associated with extended-spectrum β-lactamase (ESBL) production and permeability defects, the level of resistance conferred to cephalosporins and carbapenems may be significantly higher.7–9 OXA-48 is usually located on Tn1999, a composite transposon with two copies of the insertion sequence IS1999,10 in conjugative plasmids of ~62 kb.6–9

In Greek hospitals there is an ongoing endemic of K. pneumoniae clonal strains harbouring class A (KPC-type) and/or class B (VIM-type) carbapenemases.1,11,12 The recognition in the clinical laboratory of Tzaneion General Hospital, of carbapenem-resistant K. pneumoniae isolates that were positive by the modified Hodge test (MHT), but negative by the phenotypic screening tests for metallo-β-lactamase (MBL) and KPC carbapenemase production, prompted their further investigation. Thus, we were able to identify the first nosocomial outbreak in Greece of a K. pneumoniae strain expressing the OXA-48 carbapenemase.
Materials and methods

Bacterial isolates and patients

Over a 4 month study period (December 2011 to March 2012), initiated with the identification of the first isolate under investigation, all ertapenem-resistant Enterobacteriaceae (MIC >1 mg/L) with screening tests indicative of carbapenemase production and negative phenotypic testing for class A or B carbapenemases were referred for further investigation. Following identification, patients’ demographic characteristics were recorded.

Bacterial identification, susceptibility testing and phenotypic assays

Species identification was performed with the Vitek 2 automated identification system (bioMérieux, Marcy l’Etoile, France) and confirmed with the API 20E system (bioMérieux). MICs were determined by the Vitek 2 system. In addition, MICs of β-lactams and selected antibiotics were evaluated by Etest (AB Biodisk, Solna, Sweden) and were interpreted according to the updated CLSI criteria. Preliminary screening for the presence of a carbapenemase was performed with the MHT according to CLSI guidelines. The MBL Etest (AB Biodisk, Solna, Sweden) and the combined-disc tests using meropenem without and with phenylboronic acid, EDTA or both were used to screen for class A and class B carbapenemases. Coproduction of an ESBL was tested using a modified CLSI ESBL combined-disc test.

PCR amplifications and sequence analysis of β-lactamase and specific porin genes

Isolates were screened by PCR amplification using a panel of primers for the detection of MBLs, KPCs, OXA-48, ESBLs, including the SHV, TEM, CTX-M and IBC/GES enzymes, and plasmid-mediated AmpCs. PCR was also used to screen for IS1999 elements as well as the blaOXA-1 and blaOXA-9 β-lactamase genes. The structural genes ompK35 and ompK36 were amplified in order to define the DNA sequences of the outer membrane porin genes.

PFGE and multilocus sequence typing (MLST)

PFGE of XbaI-digested genomic DNA of the isolates under investigation and representative K. pneumoniae of the same time period, harbouring either the KPC or both the KPC- and VIM-type carbapenemases, was performed with a CHEF-DR III system (Bio-Rad, Hermal Hempstead, UK), with a running time of 23 h and pulse times ranging from 3 to 20 s. MLST was used to assess the relatedness of the K. pneumoniae isolates (www.pasteur.fr/recherche/genopole/PPB/mlst) with sequence types (STs) assigned using online database tools.

Plasmid analysis and conjugation experiments

The potential for conjugal transfer of carbapenem resistance was examined using representative isolates K1, K3, K4, K7 and K12 and Escherichia coli strain 26R793 (lac, RifR) as the recipient strain. Transconjugant clones were screened on MacConkey agar plates containing rifampicin (100 mg/L) and amoxicillin (100 mg/L) or ertapenem (0.5 mg/L). β-Lactamase genes were sought by PCR amplification. Plasmid extraction was performed by using an alkaline lysis protocol. Plasmid incompatibility groups were determined by a PCR-based replicon-typing scheme.

Results

Patients of the study and carbapenem-resistant clinical isolates

The characteristics of the nine patients involved in the outbreak are summarized in Table 1. The patients were hospitalized in five different hospital wards, their ages ranged from 35 to 89 years (mean age of 66.3 years) and none was linked to immigration or had a recent history of travelling in countries known for the dissemination of OXA-48-producing pathogens. All patients prior to the isolation of carbapenem-resistant pathogens had received antimicrobial regimes, which included ciprofloxacin, third- or fourth-generation cephalosporins, carbapenems, clindamycin and aztreonam.

Outbreak description

Index isolate K1 was retrieved from a female patient admitted to the cardiology intensive care unit (ICU) on day 18 of hospitalization. We presume that this pathogen was subsequently introduced to the general ICU environment by this patient following her transfer due to the rapid deterioration of her status. Patients infected thereafter from other wards were at some point of their hospitalization transferred either to the cardiology ICU or the general ICU or were hospitalized during overlapping periods of time in the cardiology ICU with the index patient. Infection control measures were strengthened to include the reinforcement of diligent hand hygiene prior to and after patient handling, the use of disposable gloves and the disinfection of inanimate surfaces related to the patients in question. Patient 9 was identified approximately 1 month after the outbreak had been limited, was directly admitted from a long-term care facility to the medical ward and could not be directly associated with the outbreak.

All clinical isolates were recovered 3–28 days following admission and were therefore characterized as hospital acquired. Apart from Patient 9, three more, including index Patient 1, also had a history of previous hospitalization in our facilities or in other hospitals or long-term care facilities over the preceding year.

Susceptibility testing

The susceptibility patterns are shown in Table 2. All K. pneumoniae were resistant to penicillins, expanded-spectrum cephalosporins, aztreonam, ciprofloxacin, gentamicin and co-trimoxazole and exhibited heterogeneous carbapenem resistance patterns. MICs of ertapenem ranged from 4 to 12 mg/L. It should be noted that while isolates K1–K6 and K8–K11 were either susceptible or intermediate to imipenem and susceptible to meropenem, K12 and K13 were resistant to both imipenem and meropenem, as was K7, which exhibited the slightly higher ertapenem MIC (12 mg/L).

Phenotyping and molecular testing

The MHT was positive for the production of a carbapenemase, while the combined-disc tests gave negative results for the production of class A and class B carbapenemases. The modified CLSI ESBL combined-disc test was indicative of ESBL production. PCR amplification and sequencing verified the presence in all K
Table 1. Characteristics of patients infected with blaOXA-48-producing K. pneumoniae isolates

<table>
<thead>
<tr>
<th>Patient no.</th>
<th>Age (years)</th>
<th>Sex</th>
<th>Site of isolation</th>
<th>Date of isolation (day/month/year)</th>
<th>Reason for hospitalization</th>
<th>Underlying disease/complication</th>
<th>Underlying disease/complication</th>
<th>Antibiotic regimen administered</th>
<th>Status</th>
<th>Outcome</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>89</td>
<td>F</td>
<td>blood</td>
<td>29/12/2011</td>
<td>card-ICU arrhythmia</td>
<td>aortic valve stenosis, myocardial infarction</td>
<td>none</td>
<td>imipenem/colistin/tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>2</td>
<td>87</td>
<td>F</td>
<td>CVC</td>
<td>16/01/2012</td>
<td>cardiology</td>
<td>arrhythmia deep vein thrombosis gastrointestinal tract infection</td>
<td>none</td>
<td>tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>3</td>
<td>81</td>
<td>M</td>
<td>wound swab</td>
<td>24/01/2012</td>
<td>surgical</td>
<td>gastrointestinal cancer</td>
<td>none</td>
<td>tigecycline</td>
<td>improved</td>
<td>improved</td>
</tr>
<tr>
<td>4</td>
<td>47</td>
<td>F</td>
<td>CVC</td>
<td>19/02/2012</td>
<td>cardiology</td>
<td>endocarditis soft-tissue infection</td>
<td>none</td>
<td>tigecycline</td>
<td>improved</td>
<td>improved</td>
</tr>
<tr>
<td>5</td>
<td>85</td>
<td>F</td>
<td>blood</td>
<td>19/02/2012</td>
<td>genetic ICU</td>
<td>head trauma</td>
<td>none</td>
<td>tigecycline</td>
<td>improved</td>
<td>improved</td>
</tr>
<tr>
<td>6</td>
<td>24</td>
<td>M</td>
<td>blood</td>
<td>22/02/2012</td>
<td>gen-ICU</td>
<td>cardiac arrest</td>
<td>none</td>
<td>tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>7</td>
<td>78</td>
<td>F</td>
<td>blood</td>
<td>22/02/2012</td>
<td>gen-ICU</td>
<td>cardiac arrest</td>
<td>none</td>
<td>tigecycline/tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>8</td>
<td>35</td>
<td>M</td>
<td>blood</td>
<td>22/02/2012</td>
<td>gen-ICU</td>
<td>trauma</td>
<td>none</td>
<td>tigecycline</td>
<td>improved</td>
<td>improved</td>
</tr>
<tr>
<td>9</td>
<td>71</td>
<td>M</td>
<td>CVC</td>
<td>22/03/2012</td>
<td>surgical</td>
<td>infection</td>
<td>none</td>
<td>tigecycline/tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>10</td>
<td>102</td>
<td>M</td>
<td>CVC</td>
<td>23/03/2012</td>
<td>surgical</td>
<td>aspiration</td>
<td>none</td>
<td>tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
<tr>
<td>11</td>
<td>103</td>
<td>M</td>
<td>ulcer</td>
<td>24/03/2012</td>
<td>medical ICU</td>
<td>aspiration</td>
<td>none</td>
<td>tigecycline/tigecycline</td>
<td>deceased</td>
<td>improved</td>
</tr>
</tbody>
</table>

F, female; M, male; CVC, central venous catheter; card-ICU, cardiology ICU; gen-ICU, general ICU; MDS, myelodysplastic syndrome.

**PFGE, MLST and conjugation experiments**

PFGE clustered the OXA-48-producing K. pneumoniae isolates into a single PFGE clonal type with two distinct subtypes (Ia and Ib), which differed from both the formerly known KPC- and/or VIM-producing clones of our hospital (data not shown). MLST assigned the OXA-48-producing isolates to ST11. Plasmid profiling revealed two large plasmids of ~140 and ~62 kb, respectively, and either two or three smaller plasmids ranging from ~2 to 7 kb. Conjugation experiments were successful in transferring carbapenem resistance at a high rate, as previously noted.Regardless of the donor isolate, the recipient cells exhibited similar increases in β-lactam MICs (Table 2). Transconjugants acquired solely the blaOXA-48 gene and the ~62 kb plasmid, which was assigned to the IncL/M incompatibility group.

**Discussion**

In the present study, we describe the first outbreak in Greece that was caused by a single clone of K. pneumoniae carrying the OXA-48, CTX-M-15 and OXA-1 enzymes. Taking into account that in Greece, KPC- and/or VIM-producing K. pneumoniae strains have become endemic during the last decade, the initial identification of these OXA-48-producing clonal strains posed an extra challenge and was made possible through the incorporation of routine phenotypic testing for carbapenemase production into everyday practice. Given that the presence of OMP genes was verified, no disruptions that could result in porin loss were revealed and no significant differences in the acquired transconjugant resistance profiles were seen, increased levels of resistance to carbapenems seen in certain isolates were very likely caused by decreased expression of outer membrane porins and/or increased efflux as described elsewhere.

Isolates were assigned to ST11 of the K. pneumoniae lineage, which represents a single locus variant of ST258 and is currently widely disseminated among KPC-producing K. pneumoniae strains from China and other Asian regions. Although the predominant K. pneumoniae carbapenemase-producing clone in Greece has been assigned to ST258, the isolates in our outbreak belonged to the less frequent ST11. In Europe, outbreaks or solitary case reports involving OXA-48-producing isolates have implicated diverse sequence types, such as STs 496, 395, 392, 152, 147, 101, 17 and 14. It is of note that a recent study has indicated the likelihood of the extensive spread of a single ST395 OXA-48-producing K. pneumoniae clone in the Mediterranean area and Europe. However, to date and with regard to oxacillinase-type carbapenemases, ST11 in Europe has been linked solely to a single clone harbouring the OXA-48 variant, OXA-181.

The introduction of this clonal strain in our hospital environment has been attributed to index Patient 1, probably due to colonization from prior hospitalizations. This observation, which is

pneumoniae of the blaOXA-48 gene as well as the blaOXA-1 and blaCTX-M-15 genes. Analysis of the genetic environment located upstream of the blaOXA-48 gene revealed the presence of IS1999, thus indicating that the gene was located on Tn1999. Sequence analysis of the outer membrane protein genes ompK35 and ompK36 revealed no genetic disruptions, either in the form of point mutations or insertion sequences.
highly indicative of the possible pre-existing dissemination of such isolates in Greece and their ineffective detection and isolation, is further strengthened by the retrieval of the OXA-48-bearing K. pneumoniae isolate from Patient 9. It should be clarified that despite the absence of initial carrier screening in the case of Patient 9, upon identification, in order to exclude an undetected colonization of the medical ward, rectal surveillance swabs were collected from all patients in the ward and none yielded an OXA-48 producer. Thus, in the absence of a plausible cross-infection history and in light of the patient’s direct admission from a long-term care facility, it can be presumed that this could be an isolated incident not associated with our outbreak. Following the initial detection and characterization of this clone, infection control measures were reinforced and no novel OXA-48-possessing isolates have been reported to date.

Our report highlights the dissemination of the OXA-48 β-lactamase in Greece. Taking into account the high prevalence of other carbapenemase-bearing K. pneumoniae clones in Greece\textsuperscript{11,12} and the documented potential for the horizontal and interspecies dissemination of the bla\textsubscript{OXA-48} carrier plasmid, early and accurate detection in conjunction with effective infection control measures are of utmost importance.

### Funding

This study was supported by internal funding.

### Transparency declarations

None to declare.

### Table 2. Antimicrobial susceptibility patterns of the study clinical isolates, their transconjugants and the recipient strain E. coli 26R793

<table>
<thead>
<tr>
<th></th>
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</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>0.75–2</td>
<td>6</td>
<td>4</td>
<td>0.75–1</td>
<td>0.25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>1</td>
<td>8</td>
<td>4–8</td>
<td>0.19–1</td>
<td>0.06</td>
</tr>
<tr>
<td>Ertapenem</td>
<td>4–6</td>
<td>12</td>
<td>6</td>
<td>1.5–2</td>
<td>0.06</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.064</td>
<td>0.06</td>
</tr>
<tr>
<td>Cefepime</td>
<td>32 to &gt;128</td>
<td>&gt;128</td>
<td>32–64</td>
<td>0.25–0.38</td>
<td>0.25</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>0.125–0.38</td>
<td>0.125</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>8–16</td>
<td>16</td>
<td>8</td>
<td>1–2</td>
<td>1</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>64 to &gt;256</td>
<td>2</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.5–2</td>
<td>1</td>
<td>1</td>
<td>0.12</td>
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<tr>
<td>Colistin</td>
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<td>1</td>
<td>0.5</td>
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<td>&gt;4</td>
<td>&gt;4</td>
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<tr>
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<td>2</td>
<td>2</td>
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<tr>
<td>Gentamicin</td>
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<td>&gt;32</td>
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<td>1</td>
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<tr>
<td>Co-trimoxazole</td>
<td>&gt;32</td>
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### References


