The Balkan region: NDM-1-producing Klebsiella pneumoniae ST11 clonal strain causing outbreaks in Greece

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Objectives: Despite the fact that the NDM-1 carbapenemase has successfully disseminated worldwide, outbreaks remain uncommon in the European region. We describe the characteristics of the first outbreaks caused by NDM-1-producing Klebsiella pneumoniae clonal isolates in Greece.

Methods: Between January 2010 and June 2013, 132 non-repetitive carbapenem-resistant Enterobacteriaceae isolates, which gave a positive modified Hodge test and were phenotypically suspected of metallo-β-lactamase production, were recovered from patients hospitalized at Ioannina University Hospital. Resistance genes were identified by PCR and sequencing. Plasmid profiling, conjugation experiments, enterobacterial repetitive intergenic consensus PCR, PFGE and multilocus sequence typing (MLST) were performed. Patient records were retrieved to access patterns of acquisition.

Results: Molecular testing verified the presence in 78 K. pneumoniae isolates, collected from 71 patients, of the \( \text{bla}_{\text{NDM-1}} \) gene. The \( \text{bla}_{\text{CTX-M-15}}, \text{bla}_{\text{OXA-1}} \) and \( \text{bla}_{\text{TEM-1}} \) genes were also present in most isolates. The \( \text{bla}_{\text{NDM-1}} \) gene was located on a narrow host range IncFII-type plasmid, of \(~95\,\text{kb}\) flanked upstream by a non-truncated \( \text{IS}_{\text{Aba125}} \) element and downstream by the \( \text{ble}_{\text{MBL}} \) gene. Genotyping clustered all K. pneumoniae isolates into a single clonal type with one subtype and MLST assigned them to sequence type 11. Two outbreaks were noted, the first between November and December 2011 involving four patients and the second initiated in May 2012 and ongoing, involving the remaining patients. All but two cases were characterized as hospital acquired. No links to immigration or travel history to endemic areas were established.

Conclusions: This survey highlights the successful undetected dissemination of yet another carbapenemase in Greece and strengthens the hypothesis of a latent NDM-1 cluster in the Balkan region.

Keywords: K. pneumoniae, carbapenemases, sequence types

Introduction

Carbapenems are commonly considered as a last-resort antibiotic option in dealing with infections caused by multidrug-resistant Enterobacteriaceae. Over the past 10 years their effectiveness has been challenged by the emergence of isolates able to hydrolyse carbapenems along with most β-lactam antibiotics. These carbapenemases have traditionally been assigned to Ambler classes A, B and D, with KPC, VIM, IMP type and OXA-48 enzymes exhibiting worldwide dissemination and prevalence. Recently, a novel class B metallo-β-lactamase (MBL), the New Delhi metallo-β-lactamase-1 (NDM-1), has altered this setting and provides yet another source of serious concern not only to the medical community but this time also to the general public.

To date, nine minor variants of NDM-1 (NDM-2 to -10) have been identified (http://www.lahey.org/studies/). This gene has found a variety of hosts among Enterobacteriaceae species but also in other Gram-negatives, such as Vibrio cholerae, Pseudomonas spp. and Acinetobacter baumannii. Despite the fact that a chromosomal location has been identified in certain isolates, the NDM carbapenemase has been primarily linked to multiple separate acquisition events mediated by plasmids of various sizes belonging to an unprecedented palette of incompatibility groups with both broad and narrow host ranges, such as IncF, IncA/C, IncL/M, IncH, IncN and IncX3. Following the emergence of NDM producers from the Indian subcontinent, such isolates have disseminated worldwide, giving rise to reports of their presence in the UK and other European countries.
countries, Canada, Australia, North and South America, New Zealand, Asia and the Arabian Peninsula. In the European region outbreaks remain restricted and uncommon. The Balkans have been highlighted as yet another likely secondary reservoir accountable for the spread of NDM producers. Greece, which is addressing an endemic situation regarding Klebsiella pneumoniae clonal strains harbouring class A (KPC-type) and/or class B (VIM-type) carbapenemases, has had awareness raised across laboratories to the novel enzymes may be imported. Re-evaluated according to the instructions issued by the Hellenic National Public Health Authorities (http://www2.keelpno.gr/blog/?p=1140&lang=en), nosocomial active surveillance protocols indicate that carbapenem-resistant Gram-negative isolates are routinely checked by phenotypic tests for MBL and KPC production and selected isolates are referred for further investigation. Thus, we were able to identify the first nosocomial outbreaks in Greece attributable to carbapenem-resistant K. pneumoniae isolates harbouring the NDM-1 carbapenemase.

Materials and methods

Bacterial isolates and patients

In total, 132 isolates (127 K. pneumoniae, 4 Enterobacter cloacae and 1 Proteus mirabilis) collected over a period of 3.5 years (January 2010 to June 2013) were investigated. Isolates were assessed following two separate referrals. Initially 24 selected, non-repetitive carbapenem-resistant isolates (23 K. pneumoniae and 1 E. cloaca) collected from November 2012 to February 2013, suspected of MBL or MBL and KPC production following phenotypic screening for class A and B carbapenemases, with the combined phenylboronic acid (PBA) and EDTA zone inhibition enhancement double disc test using meropenem as a substrate, were referred for further characterization. While the majority of isolates were either VIM or VIM/KPC producers, molecular testing also identified 11 NDM-harbouring K. pneumoniae. This prompted the re-evaluation of both current and archive Enterobacteriaceae that exhibited resistance to at least one carbapenem (MIC ≥ 4 mg/L for imipenem and meropenem and MIC ≥ 2 mg/L for ertapenem) according to the updated CLSI criteria and had been found positive for Ambler class B carbapenemase production by phenotypic testing.

Following identification, records were assessed and patients' demographic characteristics, including medical history, travel history, underlying comorbidities, surgical interventions, prior hospitalizations or exposure to healthcare facilities and previous antibiotic consumption, were recorded. Nosocomial infections were defined according to standard CDC definitions. The sites of infection/colonization were determined by the presumed portal of entry.

Bacterial identification, susceptibility testing and phenotypic assays

Species identification and MICs were determined using the VITEK 2 automated identification system (bioMérieux, Marcy l’ Étoile, France). MICs of carbapenems, tigecycline and colistin were re-evaluated using Etest strips (bioMérieux) on Mueller–Hinton agar plates. Tigecycline MICs were interpreted following US FDA recommendations (susceptible, ≤ 2 mg/L; resistant, ≥ 8 mg/L). Carbapenemase activity was assessed with the modified Hodge test using imipenem and meropenem discs according to CLSI guidelines. Screening for class A and B carbapenemases was carried out by a combined-disc test with meropenem as a substrate without and with PBA, EDTA or both. The likelihood of extended-spectrum β-lactamase (ESBL) coproduction was evaluated using a modified CLSI ESBL combined-disc test.

PCR amplification and sequencing

Single PCR reactions for each gene were used to search isolates for Ambler class A, B and D carbapenem-hydrolysing β-lactamase genes and genes encoding OXA-1, OXA-9, ESBLs including the SHV, TEM, CTX-M and IBC/GES enzymes and plasmid-mediated AmpC. The blaNDM gene was sought using primers NDM-F (5’-GGG CAG TCG TCT CCA ACG GT-3’) and NDM-R (5’-GTA GTG CTC ATG GTC GGC AT-3’), which amplify an internal fragment of 475 bp (kindly provided by Professor Neil Woodford, Microbiology Services Colindale, Health Protection Agency, London, UK). Isolates were screened for the presence of the Is1ab125 element and the novel blaOXA-3 gene previously reported in NDM-producing Enterobacteriaceae. Mapping of the blaNDM surrounding environment was performed using a panel of primers as previously described. PCR products were subjected to direct sequencing.

Genotyping of study isolates

Whole DNA of the strains under investigation and selected isolates from the same time period, harbouring KPC, both KPC and VIM or only VIM-type carbapenemase, were analysed by enterobacterial repetitive intergenic consensus PCR (ERIC-PCR) using primers ERIC-1 and ERIC-2. Fingerprints were compared visually and patterns differing by at least one amplification band were classified as different. Clonal relationship was further investigated by PFGE of XbaI-digested genomic DNA performed with a CHEF-DR III system (Bio-Rad, Hemel Hempstead, UK), with running time of 23 h and pulse times ranging from 3 to 20 s. PFGE patterns were compared visually following previously described criteria. Multilocus sequence typing (MLST) was performed on representative isolates. Internal fragments of the seven housekeeping genes were amplified using the primers given at the Institute Pasteur MLST Databases web site (www.pasteur.fr/recherche/genopole/PGPb/mlst). The resulting PCR products were purified and sequenced. Sequence types (STs) were assigned using online database tools.

Plasmid analysis and conjugation experiments

Experiments focused on the potential for conjugal transfer of carbapenem resistance. Biparental mating out assays, were carried out, with Escherichia coli strain 26R793 (lac+, RifR) as the recipient strain. Isolates KP36, KP37, KP39 and KP48, which harboured, in regard to β-lactam resistance genes, only the blaNDM gene, and isolates KP38 and KP41, which harboured the blaOXA-1 gene, were selected and used. Donor and recipient cells from Luria-Bertani broth cultures were mixed in a ratio of 1:5 and transconjugant clones were screened on MacConkey agar plates containing rifampicin (100 mg/L) and amoxicillin (100 mg/L) or ertapenem (0.25 mg/L). Conjugal events were studied at both 30 and 37°C. All β-lactamase genes were sought by PCR amplification. Plasmid DNA was isolated from 50 mL overnight cultures of both donor and transconjugant strains using a Plasmid Midi Kit (Macherey-Nagel, Düren, Germany) according to the manufacturer’s protocol. E. coli 39R861 was used as a plasmid size standard for comparison. Plasmid incompatibility groups were determined by a PCR-based replicon-typing scheme.

Results

Carbapenem-resistant clinical isolates and patients

In total, after screening 132 Enterobacteriaceae we identified 78 NDM-1-producing K. pneumoniae, which were harvested from 71
patients and were implicated in two distinct outbreaks. The first extended from November 2011 to December 2011 and the second from May 2012 to June 2013 (Figure 1). Patient characteristics for both outbreaks are summarized in Table S1 (available as Supplementary data at JAC Online). The first outbreak involved four \textit{K. pneumoniae} isolates retrieved from four patients in the haematology department. All patients were of Greek descent and none had a recent history of travelling abroad. The second outbreak was significantly more long-lasting and is ongoing, currently spanning 14 months. A total of 67 patients were involved, from whom 74 NDM-producing \textit{K. pneumoniae} were harvested. With the exception of two patients of Albanian origin (one was identified in the midst of the outbreak, in September 2012, and the second in May 2013), the rest were locals with no prior travel history to endemic areas. Patients were hospitalized in nearly all hospital wards, including the general and cardiology intensive care units (ICUs). Isolates were retrieved from a variety of clinical samples, mainly blood and urine cultures followed by wound swabs, intravenous catheters, ascitic fluid samples, aspirates, sputum samples and faecal swabs. The deaths of six patients were directly attributable to the bloodstream infections caused by the NDM-1-producing isolates.

\textbf{Initial outbreak description}

Index \textit{K. pneumoniae} was retrieved on day 12 of hospitalization from a random urine culture of a female patient (P1) in the haematology department. Subsequently, similar isolates were found in three more patients, limited to the haematology ward (Table S1).

During the period in question, central venous catheters used for chemotherapy were inserted in the general ICU, where patients remained for 1–2 days. Such an intervention had taken place for all four patients. The time between isolate detection and exposure to the ICU environment ranged from 2 to 32 days. Retrospective analysis of carbapenem-resistant \textit{K. pneumoniae} collected during 2011 from the ICU department, failed to identify other NDM producers. Two different possibilities are therefore likely: either P1 introduced this pathogen to the haematology environment as part of an unidentified community pool or the patient was colonized during hospitalization in the ICU from a source we were unable to detect, given that environmental samples and patient/staff carrier status were not investigated at the time.

\textbf{Ongoing outbreak description}

In the subsequent outbreak (May 2012 to June 2013), 67 patients were identified (Figure 1 and Table S1). It would appear, however, that we may have experienced two separate models of acquisition and transmission, one of widespread intra-hospital dissemination and the other of community acquisition.

The index case, P5, was a female patient admitted to the ICU who developed a bloodstream infection on day 8 of hospitalization. Initial rectal screening was not performed and therefore carrier status upon admission cannot be determined. Recent exposure to healthcare facilities was established, so it was unclear whether this infection should be characterized as community or hospital acquired.

With the exception of two patients, P29 and P54, whose infections were community acquired, having had no previous exposure to the healthcare infrastructure and yielding isolates retrieved from urine and blood samples requested upon admission, the origin of the remaining isolates remains debatable. The large number of patients identified, the retrieval of these pathogens at least 48 h following hospital admission and the overlapping periods of hospitalization noted reinforce the perspective of an intra-hospital dissemination scenario.

Further investigation of patient trafficking following admission revealed that, regardless of the initial ward to which patients were admitted, the general ICU, the cardiology ICU, the surgical ward and the physical medical rehabilitation centre seem to have acted as clusters from which the NDM-producing strains disseminated via patient transfer to the other wards.

\textbf{Susceptibility testing}

The 78 NDM-1-producing isolates were highly resistant to most \(\beta\)-lactam antibiotics, including penicillins and their combinations with inhibitors, expanded-spectrum cephalosporins and carbapenems (imipenem MICs >32 mg/L, meropenem MICs 16 to

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{fig1.png}
\caption{Histogram showing the identification of patients with NDM-1-producing \textit{K. pneumoniae} isolates.}
\end{figure}
>32 mg/L and ertapenem MICs 24 to >32 mg/L). Only seven isolates remained susceptible to aztreonam (MICs ≤ 1 mg/L), which is not hydrolysed by metallo-carbapenemases, thus suggesting the presence of additional β-lactamases in the remaining isolates. All K. pneumoniae were resistant to amikacin and ciprofloxacin while nine remained susceptible to gentamicin and four to co-trimoxazole. MICs of colistin were consistently ≤ 0.5 mg/L with the exception of 11 isolates (MICs 11–32 mg/L). Finally, 20 isolates exhibited intermediate susceptibility to tigecycline (MICs 3–4 mg/L) and 11 were tigecycline resistant (MICs 8–16 mg/L).

**Phenotyping and molecular testing**

Phenotypic testing of the NDM-1-producing K. pneumoniae using the modified Hodge test was positive for the production of a carbapenemase while the subsequent combined-disc tests gave positive results for the production of a class B carbapenemase. With the exception of seven isolates, the isolates tested positive with the modified CLSI ESBL combined-disc test and were considered to express an ESBL. PCR amplification and sequencing verified the presence in all K. pneumoniae of the blaNDM-1 gene. The majority of isolates co-harbour the blaOXA-1, blaCTX-M-15 and blaTEM-1 genes (Table S1). Molecular analysis verified that the seven isolates that gave a negative screening test for ESBL production did not possess the blaCTX-M-15 gene; four of these isolates possessed only the blaNDM-1 gene. The blaCTX-M-15 gene was also not identified in three more isolates. Finally, one harboured the blaOXA-1, blaCTX-M-15 and blaNDM-1 genes but not the blaTEM-1 gene. Analysis of the genetic environment of the blaNDM-1 gene revealed that it was flanked upstream by a non-truncated ISAba125 element and downstream by the bleMBL gene.

**ERIC-PCR, REP-PCR, PFGE and MLST typing**

ERIC-PCR and PFGE clustered all K. pneumoniae isolates into a single clonal type A, with one subtype A1, which differed from the formerly known KPC-, VIM- and KPC- and VIM-producing clones from our hospital (Figure 2). MLST of representative isolates from both outbreaks, including those with a community onset, assigned the K. pneumoniae to a single sequence type (ST11).

**Conjugation experiments and plasmid analysis**

Conjugation experiments failed to transfer carbapenem resistance, regardless of the incubation temperature used, in all donor cells with the exception of isolate KP36, in which carbapenem resistance was transferred at 37°C at a very low conjugation rate of ≈1.3 × 10⁻⁷. Analysis of the selected transconjugants showed that they acquired the blaNDM-1 gene. Susceptibility patterns are shown in Table 1. Resistance to amikacin was also transferred. Plasmid analysis conducted on the initial isolates and the resulting transconjugants indicated that the blaNDM-1 gene was harboured on a plasmid of ≈95 kbp belonging to the narrow host range IncFII incompatibility group.

**Discussion**

The emergence of NDM-producing Enterobacteriaceae is the foremost trend in carbapenemase-producing Enterobacteriaceae worldwide. First detected in 2008 in a clinical urine sample from a Swedish patient following hospitalization in India, this carbapenemase has its own success story. Despite the initial controversy, which fascinated the media following the announcement of the ‘NDM-1 superbug’, reports have effectively linked the emergence of this enzyme to the Indian–Pakistani sub-continent. Challenging sanitation infrastructures, lack of uncontaminated potable water and abuse of over-the-counter antibiotic administration offered the ideal setting for the development of a latent endemic situation in the area.

International travel between countries and continents, regardless of whether or not it is for medical purposes, has played a significant role in the dissemination of NDM producers, given that most reports have been linked to prior hospitalization or travel and community acquisition originating in the Indian or

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**Figure 2.** PFGE clonal types and subtypes of XbaI-digested genomic DNA of the K. pneumoniae isolates in the study. Lanes 2 – 5, 9, 10, 14, 16 and 18 – 20, outbreak NDM-1-harbouring K. pneumoniae; lanes 6 and 11 – 13, representative VIM-1-producing K. pneumoniae; lanes 17 and 21, representative KPC-2-producing K. pneumoniae; lane 15, KPC-2 and VIM-1-producing K. pneumoniae. Lanes marked with M show multimers of phage lambda DNA (48.5 kb) molecular mass marker.
Table 1. Antimicrobial susceptibilities of clinical isolate KP36 and its transconjugant carrying pNDM-1

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>K. pneumoniae clinical isolate KP36</th>
<th>Transconjugant E. coli 26R793 (pNDM-1)</th>
<th>E. coli 26R793 (lac&lt;sup&gt;-&lt;/sup&gt;, Rif&lt;sup&gt;+&lt;/sup&gt;)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Imipenem</td>
<td>&gt;32</td>
<td>12</td>
<td>0.25</td>
</tr>
<tr>
<td>Meropenem</td>
<td>&gt;32</td>
<td>3</td>
<td>0.03</td>
</tr>
<tr>
<td>Ertopenem</td>
<td>&gt;32</td>
<td>4</td>
<td>0.06</td>
</tr>
<tr>
<td>Astrezenom</td>
<td>0.5</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>0.12</td>
</tr>
<tr>
<td>Cefepime</td>
<td>&gt;32</td>
<td>2</td>
<td>0.12</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>1</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>&gt;64</td>
<td>&gt;64</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
</tr>
<tr>
<td>Amoxicillin/clavulanate</td>
<td>&gt;32</td>
<td>&gt;32</td>
<td>4</td>
</tr>
<tr>
<td>Piperacillin/tazobactam</td>
<td>&gt;128</td>
<td>&gt;128</td>
<td>1</td>
</tr>
<tr>
<td>Tigecycline</td>
<td>0.25</td>
<td>0.06</td>
<td>0.12</td>
</tr>
<tr>
<td>Colistin</td>
<td>0.25</td>
<td>0.12</td>
<td>0.12</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>&gt;32</td>
<td>0.06</td>
<td>0.06</td>
</tr>
<tr>
<td>Amikacin</td>
<td>&gt;32</td>
<td>16</td>
<td>2</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>&gt;32</td>
<td>0.5</td>
<td>0.25</td>
</tr>
</tbody>
</table>

Pakistan region. The Balkan region is a second area that remains controversial for two reasons: there is debate about whether the introduction of the gene here was also linked to medical tourism; and there is the notion of an undetected latent pool in the midst of the European region.

Given the number of patients implicated in the current survey, it is essential to emphasize that these two outbreaks, especially the second, represent the most long-lasting and multidisciplinary outbreaks reported in Europe. Our study, along with the recent identification of isolated NDM producers in Greece, with one isolate linked to the Ioannina University Hospital, further reinforces what previous reports have suggested in terms that these are from a latent Balkan reservoir. In contrast to other reports from Europe, in which these pathogens have been introduced via patient transfer, our cases had no history of travel to endemic areas. The community-acquired incidents emphasize another unnerving factor: the likely presence of a community pool contributing to autochthonous acquisition.

To date, the K. pneumoniae that have been linked directly to the Balkan region have been assigned to ST15, which is a single-locus variant (SLV) of ST14 and ST25. In the rest of Europe, reports involving NDM-producing isolates have implicated a more diverse palette of STs, such as ST11, ST14, ST15, ST16, ST37, ST147, ST231 and ST340. Despite the fact that no globally predominant clone has been highlighted in the case of the NDM carbapenemase, ST11, ST14 and ST147, which are known to be successful epidemic clones, appear to be frequent carriers of the enzyme. The isolates in our study were clustered into one distinct clone with one subtype and were assigned to the ST11 K. pneumoniae lineage. ST11 represents an SLV of K. pneumoniae lineage. ST11 has successfully established its presence as a circulating clone, the ST11 NDM producers reported in Europe had an epidemiological link to the Indian subcontinent. This successful acquisition of yet another carbapenemase enzyme by ST11 is notable because it raises concerns regarding its potential for future spread.

In our report, the likelihood of novel carbapenemase genes disseminating in endemic areas and yet remaining undetected due to insufficient molecular epidemiological screening is clearly highlighted. Routine bacteriological surveillance was able to verify the presence of an MBL in these isolates; however, given the prior presence of VIM-producing isolates and the lack of clinical data to raise awareness, the significance of these isolates went unnoticed. Efficient identification was achieved with the implementation of stricter epidemiological surveillance that included collaboration between peripheral hospitals and reference centres.

Infection control measures were strengthened following the identification of this outbreak. They included raising staff awareness through educational meetings, reinforcing diligent hand hygiene before and after patient handling, using disposable gloves and disinfecting environmental surfaces/equipment related to the patients in question. However, screening of fecal carriage status remains an issue as it has not yet been efficiently implemented, mainly due to staff and resource shortages. Given that NDM-producing bacteria exhibit the potential to persist at sites of colonization for prolonged periods of time, asymptomatic community carriers acting as reservoirs are also extremely difficult to investigate.

In summary, our report presents the dissemination of yet another β-lactamase in Greece and highlights how phenotypic and molecular detection of carbapenemases is essential for the accurate depiction of the underlying situation. Failure to effectively implement such methods in endemic regions undoubtedly contributes to delayed identification of novel carbapenem resistance traits. The notion of a latent Balkan reservoir can be advocated and the presence of a community pool that is difficult to assess presents a novel challenge.

Acknowledgements

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Transparency declarations

None to declare.

Supplementary data

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

Outbreaks of NDM-1-producing K. pneumoniae in Greece