Spread of NDM-producing *Klebsiella pneumoniae* in a tertiary Greek hospital

KONSTANTINA KONTOPOULOU¹, GEORGIOS MELETIS²*, STYLIANI PAPPA², SOFIA ZOTOU¹, KATERINA TSIOKA², PANAGIOTA DIMITRIADOU¹, ELENI ANTONIADOU² and ANNA PAPA²

¹ Laboratory of Biopathology, General Hospital of Thessaloniki “G. Gennimatas”, Thessaloniki, Greece
² Department of Microbiology, Medical School, Aristotle University of Thessaloniki, Thessaloniki, Greece
³ Intensive Care Unit, General Hospital of Thessaloniki “G. Gennimatas”, Thessaloniki, Greece

Received: January 17, 2021 • Accepted: February 04, 2021
Published online: March 3, 2021

ABSTRACT

Bacterial carbapenem resistance, especially when mediated by transferable carbapenemases, is of important public health concern. An increased number of metallo-β-lactamase (MBL)-producing *Klebsiella pneumoniae* strains isolated in a tertiary hospital in Thessaloniki, Greece, called for further genetic investigation.

The study included 29 non-repetitive carbapenem resistant *K. pneumoniae* isolates phenotypically characterized as MBL-producers collected in a tertiary hospital in Greece. The isolates were screened for the detection of carbapenemase genes (*K. pneumoniae* carbapenemase (*bla*KPC), Verona-integron-encoded MBL-1 (*bla*VIM-1), imipenemase (*bla*IMP), oxacillinase-48 (*bla*OXA-48) and New Delhi MBL (*bla*NDM)). The genetic relationship of the isolates was determined by Random Amplified Polymorphic DNA (RAPD) analysis. The whole genome sequences (WGS) from two NDM-positive *K. pneumoniae* isolates were further characterized.

The presence of New Delhi MBL (*bla*NDM) gene was confirmed in all *K. pneumoniae* isolates, while *bla*KPC and *bla*VIM-1 genes were co-detected in one and two isolates, respectively. The RAPD analysis showed that the isolates were clustered into two groups. The whole genome sequence analysis of two *K. pneumoniae* isolates revealed that they belonged to the sequence type 11, they carried the *bla*NDM-1 gene, and exhibited differences in the number and type of the plasmids and the resistant genes.

All MBL-producing *K. pneumoniae* isolates of the study harbored a *bla*NDM gene, while WGS analysis revealed genetic diversity in resistance genes. Continuous surveillance is needed to detect the emergence of new clones in a hospital setting, while application of antimicrobial stewardship is the only way to reduce the spread of multi-resistant bacteria.

KEYWORDS

*Klebsiella pneumoniae*, NDM, carbapenemases, Greece

INTRODUCTION

*Klebsiella pneumoniae* is a notorious opportunistic nosocomial pathogen involved in serious and often life-threatening hospital-acquired infections; therefore, the emergence of multi-drug-resistant, and especially carbapenem-resistant strains, is considered as a major public health problem [1, 2]. Carbapenem resistance is mainly mediated by enzymes able to hydrolyze carbapenems and almost all other β-lactams [3]. They are encoded by carbapenemase-encoding genes, mainly *bla*KPC, *bla*VIM, *bla*IMP, *bla*OXA-48, and lately, *bla*NDM, that
emerged in the Indian subcontinent [4] and subsequently has been reported in Europe and other continents [5–8]. According to the most recent annual surveillance report from the European Centre for Disease Prevention and Control (ECDC), Greece presents high percentage of carbapenem-resistant isolates among invasive K. pneumoniae strains [9] and is considered endemic for KPC and VIM-producing K. pneumoniae [10, 11]. Since 2011, when the first detection of NDM-producing strains was reported in Greece [12], several sporadic cases and outbreaks have been reported causing great concern [13–17]. Up to now the NDM K. pneumoniae strains in Greece belong to the sequence type (ST) 11. The present study presents the molecular epidemiology and resistance mechanisms of NDM-encoding K. pneumoniae isolated in a tertiary hospital in Greece.

MATERIAL AND METHODS

Strains

From August 15, 2019 to February 15, 2020, during the routine phenotypic screening for carbapenemase production among carbapenem-resistant Gram-negative pathogens isolated at a 270-bed tertiary hospital in Greece, an unusual increase of metallo-β-lactamase (MBL)-producing K. pneumoniae was observed prompting for further investigation. Twenty-nine non-repetitive K. pneumoniae isolates which phenotypically gave an evidence for MBL production were included in the study. Seventeen isolates were recovered from blood, urine and pus samples and twelve were recovered during surveillance (5 from pharyngeal and 7 from rectal swab cultures). Most patients (17/29, 63%) were hospitalized in the intensive care unit (ICU).

Bacterial identification and antimicrobial susceptibility testing were performed by the Vitek2 automated system (bioMérieux, Marcy l’Etoile, France), while the MICs of imipenem and meropenem were further determined by E-test (bioMérieux, Marcy l’Etoile, France); results of antimicrobial testing were interpreted in accordance to Clinical and Laboratory Standards Institute (CLSI) 2019 guidelines.

Detection of β-lactamase genes

Phenotypic testing for the presence of MBL and/or KPC carbapenemases was performed with the combined disk test (CDT) using meropenem disks without and with phenyl boronic acid and/or EDTA [18]. The presence of blaKPC, blaIMP, blaOXA-48-like and blaNDM genes was investigated by a multiplex PCR [19], while an additional PCR was applied for the detection of blaVIM-1 gene [20]. The genetic relationship of the isolates was determined by random amplification of polymorphic DNA test (RAPD) [21]. The clustering analysis on RAPD patterns and the dendrogram construction were performed based on the pairwise similarities among the RAPD profiles applying the UPGMA method in the BioNumerics software package (version 5.1, Applied Maths available from: http://www.applied-maths.com/bionumerics).

Whole Genome Sequence analysis

The whole genome sequence (WGS) of two randomly selected NDM-producing K. pneumoniae isolates (213A and 248D) was obtained and analyzed by next generation sequencing. Specifically, following DNA extraction, the concentration of double strand (ds) DNA was measured with Qubit using Qubit ds DNA HS assay kit (Q32851, Life Technologies) and NGS was performed using the Ion Torrent S5 PGM Platform (Life Technologies Corporation, Grand Island, NY, USA); shearing, purification, ligation, barcoding, size selection, library amplification and quantitation, emulsion PCR and enrichment were performed according to manufacturer’s instructions and the products were loaded on a 316 chip. The Ion PGM-Hi Q (200) chemistry (Ion PGM Hi-Q Sequencing kit, A25592) was applied (Thermo Fisher Scientific).

Identification of multi-locus sequence type (MLST), plasmids and antimicrobial resistance genes was performed applying the tools using MLST 2.0, Resfinder 4.1, KmerResistance 2.2, Comprehensive Antibiotic Resistance Database (CARD), and PlasmidFinder 2.1 [22–26].

RESULTS

All isolates were resistant to imipenem, meropenem, ceftazidime, piperacillin/tazobactam, while 37.94 and 44.83% were non-susceptible to amikacin and gentamicin, respectively; aztreonam and colistin resistance was observed in 79.31 and 10.34% of the isolates, respectively. The blaNDM gene was detected in all K. pneumoniae isolates, while blaKPC and blaVIM genes were co-detected in one (33AP) and two (3051D and 322D) isolates, respectively (Table 1).

RAPD DNA profiles of the 29 K. pneumoniae isolates showed bands ranging from 200- to 3,000 base pairs. Based on RAPD patterns the isolates were divided into two major groups (A and B) which differed by 28.4%. A clear chronological grouping of the isolates was observed, with group A containing isolates collected during August-November 2019 (with 4 subgroups), and group B containing isolates collected during December 2019–February 2020 (with 7 subgroups) (Fig. 1). The in-group difference was <10%.

The two isolates from which the WGS was obtained, presented high minimum inhibitory concentrations to imipenem, meropenem, ceftazidime, piperacillin/tazobactam, and aztreonam; 213A was sensitive to amikacin and gentamicin and colistin resistant, while 248D was resistant to amikacin and gentamicin and colistin sensitive. The main characteristics of their whole genome analysis are seen in Table 2. Both isolates belonged to MLST ST11 and carried the blaNDM-1 gene. In silico analysis revealed that only IncFIA (H11) plasmid was present in both isolates, with the rest belonging to various incompatibility groups (Table 2).
Regarding genes conferring resistance to antibiotics, most were detected in \textit{K. pneumoniae} 248D (Table 2). Co-occurrence of beta-lactamas was observed. Besides the \textit{bla}	extsubscript{NDM-1} gene, several additional chromosomal and plasmid-mediated beta-lactamase genes were detected, including \textit{bla}	extsubscript{CTX-M-15}, \textit{bla}	extsubscript{OXA-1}, \textit{bla}	extsubscript{SHV-11}, \textit{bla}	extsubscript{TEM-1B}, and \textit{bla}	extsubscript{VEB-1} genes. Both strains harbored the plasmid-mediated quinolone resistance genes \textit{qoxA} and \textit{qoxB}, while genes conferring resistance to doxycycline, penicillins and rifampicin were detected only in 248D. Both strains harbored the aminoglycoside-modifying enzymes genes \textit{aac(6)-Ib-cr}, and \textit{aph (3)-Ib}, while 248D co-harbored the \textit{aac(6)-IIa}, \textit{ant(2’)-Ia}, \textit{ant(3’)-Ie} genes and the 16S rRNA methylase gene \textit{rmtB}. A variety of genes associated with efflux pump and reduced permeability were also detected.

**DISCUSSION**

Since the early 2000s, Greece is affronting a critical situation regarding the presence of carbapenemase-encoding genes in hospitals, as VIM-1 carbapenemases emerged in 2002, KPC-2 in 2007, NDM-1 in 2011, OXA-48 in 2012 [27] and, since 2010, \textit{K. pneumoniae} strains co-producing two carbapenemases have been also detected [28]. As reported recently, the Greek situation differs greatly from other European countries where OXA-48, is the most frequently encountered carbapenemase [16]. The increased number of metallo-beta-lactamase (MBL)-producing \textit{K. pneumoniae} isolates observed in the summer of 2019 in a tertiary hospital in Thessaloniki, Greece, called for further genetic investigation since MBL–producing \textit{K. pneumoniae} are associated with high rates of morbidity and mortality in nosocomial settings [29]. It was shown that all isolates of the study harbored the \textit{bla}	extsubscript{NDM-1} gene, while three NDM-producing isolates carried additional carbapenemase genes (two \textit{bla}	extsubscript{VIM} and one \textit{bla}	extsubscript{KPC}). A previous 4-year (January 2013–December 2016) survey in 8 Greek hospitals showed that 71% of 481 phenotypically MBL positive carbapenem non-susceptible \textit{K. pneumoniae} isolates carried the \textit{bla}	extsubscript{NDM-1} gene [30]. Since 2016, there was a clear predominance of KPC over metallo-beta-lactamases in the country [31, 32] which recently seems to change. This could be at least partially attributed to the implementation of ceftazidime/avibactam [33] that shows optimal results against KPC-producers [34].

Epidemiological data showed that the detection of NDM-producing \textit{K. pneumoniae} was not limited in certain units.
although most (63%) were isolated from patients hospitalized in ICU. Twelve isolates were taken during colonization control, which shows the value of surveillance in order to detect silent dissemination, since carbapenem-resistant Enterobacterales colonization may rapidly result in infection, especially in critically ill patients [35].

A low level of similarity (<28%) was observed among the RAPD patterns of the two time-dependent groups, suggesting the circulation of two different clones. Even within groups, the isolates differed each other (although less than 10%), reflecting the great heterogeneity of the isolates.

The analysis of the whole genome sequences of the two K. pneumoniae isolates revealed that they belonged to ST11, which is a common NDM-bearing lineage in several countries worldwide, and it seems that currently is established in Greece. A noteworthy variability in the genetic characteristics was observed between the two isolates, regarding the number and content of plasmids and the number and types of antibiotic resistance genes (Table 2). The IncFIA (H1) plasmid was present in both isolates, and it is known that plasmids of the IncF group represent one of the most common plasmid types contributing to the spread of antibiotic resistance genes in Enterobacteriaceae [36]. In previous studies in Greece the identified plasmids in NDM-producing ST11 K. pneumoniae isolates belonged to the IncFII group [12, 30]. In the present study, an IncFII plasmid was detected in one isolate, however, a variety of other plasmids were identified, including IncC and IncR. The content of resistance genes differed between the two isolates, which reflects the difference in antibiotic resistance. A discrepancy was seen in amikacin resistance of 213A isolate since it was found sensitive (MIC 5 16 mg/L); however, aac (6)-Ib-cr gene which confers resistance to certain aminoglycosides (amikacin, isepamicin, and tobramycin) and fluoroquinolones was detected in the WGS. This can be explained by the fact that AAC (6'-)Ib-cr enzymes are less effective against aminoglycosides compared to other members of the same subclass [37]. As expected, 213A

Fig. 1. RAPD profiles of the 29 K. pneumoniae isolates of the present study
isolate was sensitive to gentamicin, in contrast to the 248D isolate which contained a plethora of aminoglycoside-modifying enzymes genes and the 16S rRNA methylase gene rmtB (Table 2).

Limitation of the study was the low number of whole genome sequenced isolates, and especially the lack of WGS of group A isolates. However, even the analysis of two isolates of the same group showed that although the NDM-producing K. pneumoniae strains belonged to the same sequence type and were isolated in the same unit of the hospital within one-week interval, they differed greatly in their genetic characteristics. As indicated previously, the “one size fits all” approaches to identifying effective antimicrobial regimens against carbapenem-resistant K. pneumoniae strains are not effective [38].

In conclusion, all MBL-producing K. pneumoniae isolates recovered from a Greek hospital during a 6-month period carried a blanNDM gene, while WGS analysis showed that isolates, even within similar genetic group, exhibit high genetic diversity. Continuous surveillance is necessary to detect the emergence of new bacterial clones in hospital settings, while application of antimicrobial stewardship is the only way to reduce the spread of multi-drug resistant bacteria.

Nucleotide sequence accession numbers

The whole genome sequences of Thessaloniki-248D-2020 and Thessaloniki-213A-2020 were submitted to European Nucleotide Archive (ENA) under the study PRJEB41773 and received the Accession numbers ERS5489252 and ERS5489253, respectively.

**Table 2. Genetic characteristics of K. pneumoniae isolates 213A and 248D**

<table>
<thead>
<tr>
<th>Isolate</th>
<th>213A</th>
<th>248D</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sequence size</td>
<td>5377931</td>
<td>5787820</td>
</tr>
<tr>
<td>No of contigs</td>
<td>363</td>
<td>505</td>
</tr>
<tr>
<td>GC content (%)</td>
<td>57.4</td>
<td>57.0</td>
</tr>
<tr>
<td>Sequence type</td>
<td>11</td>
<td>11</td>
</tr>
<tr>
<td>Plasmid replicons</td>
<td>IncR, IncFIA (H1)</td>
<td>ColRNAI, IncC, IncFIA (H11), IncFIB(K), IncFII(K)</td>
</tr>
<tr>
<td>Resistance to:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>β-lactams</td>
<td>NDM-1, OXA-1, SHV-11</td>
<td>NDM-1, OXA-1, OXA-10, SHV-11, CTX-M-15, TEM-1B, VEB-1</td>
</tr>
</tbody>
</table>
| Aminoglycosides | aac(6’)-Ib-cr, aph(3’)-Ib | aac(3’)-Ie, aph(3’)-Ib, aph(3’)-la, aac(6’)-Ib-cr, 
                             aph(6)-Id, aadA1, ant(2’)-Ia, rmtB |
| Quinolones | aac(6’)-Ib-cr, oqxA, oqxB | aac(6’)-Ib-cr, oqxA, oqxB |
| Sulphonamides | sul2 | sul2 |
| Trimethoprim | dfrA14, oqxA, oqxB | dfrA14, oqxA, oqxB |
| Fosfomycin | fosA | fosA |
| Phenicol | oqxA, oqxB | cmlA1, oqxA, oqxB |
| Tetracycline (doxycycline) |  | tet(A), tet(D), tet(G) |
| Penicillins |  | TEM-1 |
| Rifampicin |  | ARR-2 |

FUNDING INFORMATION

The current work was supported by the European Union’s Horizon 2020 grant VEO (grant number 874735).

COMPETING INTERESTS

The authors declare that they have no competing interests.

REFERENCES

6. Johnson AP, Woodford N. Global spread of antibiotic resistance: the example of New Delhi metallo-β-lactamase (NDM)-mediated


Acta Microbiologica et Immunologica Hungarica 68 (2021) 3, 162–168

167


