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ORIGINAL ARTICLE

Emergence of two novel sequence types (3366 and 3367) NDM-1- and OXA-48-co-producing *K. pneumoniae* in Italy

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Abstract

The aim of this study was to analyze the alarming spread of NDM-1- and OXA-48-co-producing *Klebsiella pneumoniae* clinical isolates, collected between October 2016 and January 2018 in a neonatal intensive care unit of the University Hospital, Catania, Italy, through whole genome sequencing. All confirmed carbapenem-resistant *K. pneumoniae* (CRKp) isolates were characterized pheno- and geno-typically, as well as by whole genome sequencing (WGS). A total of 13 CRKp isolates were identified from 13 patients. Pulsed-field gel electrophoresis (PFGE) was performed, and the multilocus sequence typing (MLST) scheme used was based on the gene sequence as published on the MLST Pasteur website. Core genome MLST (cgMLST) was also performed. All isolates co-carried $bla_{\text{oxa-48}}$ and $bla_{\text{NDM-1}}$ genes located on different plasmids belonging to the IncM/L and IncA/C2 groups, respectively. The 13 strains had identical PFGE profiles. MLST and cgMLST showed that *K. pneumoniae* was dominated by CRKp ST101 and two novel STs (ST3666 and ST3367), identified after submission to the MLST database for ST assignment. All isolates shared the same virulence factors such as type 3 fimbriae, genes for yersiniabactin biosynthesis, yersiniabactin receptor, and iron ABC transporter. They carried the wzi137 variant associated with the K17 serotype. To the best of our knowledge, this is the first report of two novel STs, 3366 and 3367, NDM-OXA-48-co-producing *K. pneumoniae* clinical isolates, in Italy.

Keywords ST3366 · ST3367 · ST101 · NDM · OXA-48 · Carbapenemase · *K. pneumoniae*

Introduction

Since the first Italian report on the diffusion of carbapenem-resistant *Klebsiella pneumoniae* (CRKP), the detection of these multidrug-resistant (MDR) microorganisms has increased steadily [1]. In recent years, colistin resistance has appeared in these isolates [2, 3], challenging our possibilities to treat infections sustained by these microorganisms [4], in which various combinations, including colistin, have been used [5]. The spread of MDR CRKp clones has been an area of intensive investigations over the past decades: these strains were typically characterized by their sequence type (ST), with ST258 predominantly isolated worldwide, including Italy, within the past 15 years [6].

New clones originated and evolved from ST258 as variants carrying either the KPC gene and/or other carbapenem resistance determinants (i.e., OXA and NDM families), variably assembled together. This phenomenon contributed to increase their diversity worldwide, as recently described in recent European studies [7–9].

OXA-48 Kp was originally found in Turkey in 2003 and NDM-1 Kp in 2008 from a traveler from India [10]; major clones such as ST11, ST54, ST101, and ST437 carrying OXA-48 or NDM-1 are now changing the original ST258 scenario, describing a complex polyclonal and changeable CRE Kp [11, 12].

Recently, sporadic reports describing different clones carrying new gene combinations (carrying OXA and NDM) have been found in our country [13, 14].

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Here, we describe the isolation of carbapenemase-co-producing *K. pneumoniae* detected in a neonatal ward in the University of Catania, Italy.

Materials and methods

Between 2016 and 2018, 13 carbapenem-resistant *K. pneumoniae* were isolated from blood (n.4), urine (n.6), gastric aspirate (n.2), and rectal swab (n.1) from 13 different hospitalized patients of the neonatal intensive care unit of the University Hospital, Catania, Italy.

The identification and antimicrobial susceptibility testing of all isolates were preliminarily performed by the VITEK 2 system (bioMerieux, Marcy l'Etoile, France). In addition, minimum inhibitory concentrations (MICs) were performed by microdilution method interpreted according to EUCAST guidelines v.8.0. (http://www.eucast.org). The following antibiotics were tested: meropenem (MEM), piperacillin/tazobactam (TZP), amoxicillin-clavulanic acid (AMC), aztreonam (ATM), cefotaxime (CTX), cefepime (FEP), ceftazidime (CAZ), ceftazidime/avibactam (CAZ/AVI), amikacin (AMK), gentamicin (GEN), ciprofloxacin (CIP), cotrimoxazole (SXT), tigecycline (TGC), colistin (CST), and fosfomycin (FOF). Carbapenemase phenotyping was carried out using the KPC + MBL Confirm ID kit (Rosco Diagnostica A/S, Taastrup, Denmark) as recommended by the manufacturer.

MLST of *K. pneumoniae* isolates was performed as previously described [2], and STs were assigned using the MLST Pasteur website (http://www.pasteur.fr/mlst). All *K. pneumoniae* isolates were genotyped, after *Xba*I digestion, by pulsed-field gel electrophoresis (PFGE) and profiles analyzed by Tenover criteria [2, 15].

All strains were sequenced at the San Raffaele Hospital, Milan, Italy, by the Illumina NextSeq500 platform, (Illumina Inc., San Diego, CA, USA), with a paired-end run of 2 by 150 bp, after Nextera XT paired-end library preparation. Sequencing reads were assembled using SPAdes genome (version 3.6.1) with accurate de novo settings as previously described [16].

The resistome and virulence genes of all the 13 *K. pneumoniae* isolates were determined by uploading the assembled contigs into the online tool BIGSdb-Kp (http://bigsdb.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public).

Plasmid incompatibility types were identified using PlasmidFinder [17], an identity of 95% was utilized as a cut-off. Plasmid identification was performed by an in-house pipeline: the contigs of > 90% identity and a minimum length of 60% overall coverage were mapped against known NDM and OXA-48 plasmids present in the NCBI database (https://www.ncbi.nlm.nih.gov/genome/plasmids). Core genome MLST

Table 1 Molecular characteristics and susceptibilities of NDM-1-/OXA-48-co-producing *K. pneumoniae*

Strains	Isolation date	Specimens	Sequence type	cgMLST	PFGE pattern	Antibiotics MICs (mg/l)													
						MEM	TZP	AMC	ATM	CTX	FEP	CAZ	CAZ/AVI	AMK	GEN	CIP	SXT	TGC	CST	FOF*
KP 730	24/10/2016	Urine	101	A	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	2048	> 2048	64	0.5	1	32	64
KP 729	26/10/2016	Urine	101	A	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	2048	> 2048	64	0.5	1	16	128
KP 728	30/10/2016	Urine	101	A	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	2048	> 2048	32	0.125	0.25	32	16
KP 727	02/11/2016	Urine	101	A	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	32	0.5	1	16	128
KP 735	07/11/2016	Urine	3367	B	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	32	2	1	8	32
KP 733	25/01/2017	Urine	3367	B	A	512	> 256	> 256	> 256	> 256	> 256	> 256	> 256	512	> 2048	32	4	0.5	4	64
KP 724	03/07/2017	Blood	101	C	A	128	> 256	> 256	> 256	> 256	> 256	> 256	> 256	256	> 2048	32	0.5	2	8	64
KP 731	28/09/2017	Gastric aspirate	101	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	64	0.5	1	32	128
KP 726	30/09/2017	Blood	101	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	1024	> 2048	64	0.5	1	32	32
KP 723	14/10/2017	Gastric aspirate	101	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	512	0.25	1	16	> 1024
KP 722	23/10/2017	Blood	3366	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	64	1	2	8	64
KP 725	23/10/2017	Blood	3366	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	> 2048	> 2048	64	0.5	1	16	64
KP 734	10/01/2018	Rectal swab	3366	C	A	256	> 256	> 256	> 256	> 256	> 256	> 256	> 256	1024	> 2048	64	0.5	0.5	16	> 1024

MEM, meropenem; *TZP*, piperacillin/tazobactam; *AMC*, amoxicillin-clavulanic acid; *ATM*, aztreonam; *CTX*, cefotaxime; *FEP*, cefepime; *CAZ*, ceftazidime; *CAZ/AVI*, ceftazidime/avibactam; *AMK*, amikacin; *GEN*, gentamicin; *CIP*, ciprofloxacin; *SXT*, cotrimoxazole; *TGC*, tigecycline; *CST*, colistin; *FOF*, fosfomycin. *MIC was performed by agar dilution method

(cgMLST) analysis was performed using the SeqSphere+ software (Ridom, Germany) exploiting a genome-wide allelic numbering schema named core genome multilocus sequence typing (cgMLST) [18]. All the strains were mapped on the reference genome NTUH-K2044 (NC_012731.1) by BWA software (v 0.6.2), and 2358 targets were extracted (about 40% of reference genome). All the targets were covered by at least 5 reads with a Phred value higher than 30. A threshold of ≤ 4 allelic differences was used to define clusters.

Results

All strains were MDR, uniformly resistant to carbapenems, fluoroquinolones, aminoglycosides, colistin, and ceftazidime/avibactam. Twelve out of the 13 strains remained susceptible to cotrimoxazole and 11 out of 13 to tigecycline (Table 1). Furthermore, all strains were MBL producers and resistant to temocillin.

The 13 Kp strains were identical for their PFGE profiles but not for their STs, this latter resolving 8 strains belonging to ST101 and 5 with new ST profiles that were submitted to the MLST database for ST number assignment. MLST analysis revealed that three isolates shared a novel allelic profile (2-6-1-5-4-186-6), assigned as ST3366 and two isolates with a novel MLST profile (2-6-1-5-4-67-6), assigned as ST3367. Our new MLST Pasteur number ST3366 differs from ST101 because of a single nucleotide in the *rpoB* gene (position 59 G → T) creating the novel allele 186. Our new MLST Pasteur number ST3367 differs from ST101 because of a single nucleotide in the *rpo*B gene (position 65 T → C) creating a new MLST profile.

The cgMLST method accurately characterized transmission events of the 13 *K. pneumoniae* isolates in three clusters: A containing only ST101, B containing only ST3367, and finally C containing both ST3366 and ST101 due to the close relationship between ST101 and ST3366, while ST101 was not so close to ST3367 (Fig. 1). Four isolates were included in cluster A, two isolates in cluster B, and seven isolates in cluster C. It is of interest that the allelic distance between ST3366 and ST101, as well as ST3367 and ST101, corresponds to a single mutation (RS11130) in the ribosomal RNA large subunit methyltransferase *rpo*B.

All 13 strains analyzed possessed genes encoding proteins associated with virulence, such as fimbriae type 3 (MrkA and MrkD), genes for yersiniabactin biosynthesis (irp1, irp2, ybtS), yersiniabactin receptor (fyuA), and iron ABC transporter (kFuABC). They carried the wzi137 variant associated with the K17 serotype.

The assembled genomes and informatics analysis related to the resistome showed the presence of twelve antimicrobial resistance genes identified in accordance with the phenotype of resistance pattern (Table 1), including $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$, $bla_{\text{SHV-28}}$, $bla_{\text{TEM-1}}$, $bla_{\text{oxa-48}}$, $bla_{\text{oxa-9}}$, *aac3_IIa, aac6_Ib, aad*A1, and *arm*A. Mutations in the gyrB1, parC25, and parE1 genes, implied in FQs resistance, were also detected.

Plasmid analyses showed that the sequence types ST101, ST3366, and ST3367 of *K. pneumoniae* isolates had all identical plasmid compositions. The $bla_{\text{oxa-48}}$ and $bla_{\text{NDM-1}}$ genes were located on different plasmids with high identity to the previously described 74 kb pKPN1482-3 [Genbank CP020844] and 134 kb pTR2 [Genbank KJ187752], belonging to the IncM/L and IncA/C2 group, respectively.

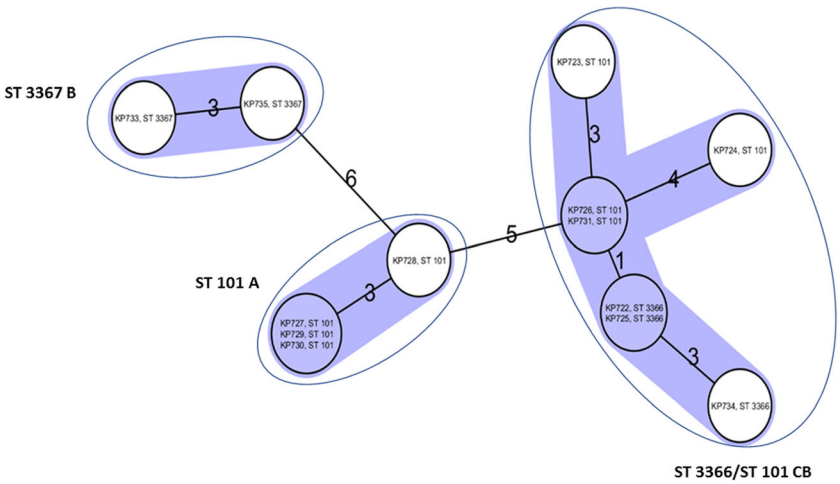

Fig. 1 Clonal relationship of all 13 *K. pneumoniae* isolates, indistinguishable by PFGE, in a minimum-spanning tree based on core genome multilocus. Each circle represents a single genotype, i.e., an allelic profile based on up to 2358 target genes present in the isolates with the "pairwise ignoring missing values" option turned on in the SeqSphere+ software during comparison. The number on connecting lines represents the number of alleles that differ between the connected genotypes. The allelic distance between ST3366 and ST101 corresponds to RS11130 mutation

Discussion

The aim of this study was to use WGS to analyze the emerging co-producing NDM-1 and OXA-48 *K. pneumoniae* infections that occurred between October 2016 and January 2018 in a neonatal intensive care unit of the University Hospital, Catania, Italy.

All clinical isolates included a common MLST, previously associated with MDR infections in hospitals (ST101), and 2 novel STs (ST3366 and ST3367) which differ from ST101 by a single nucleotide of *rpo*B gene, which can be considered a susceptible target being under rifampin pressure. This is concordant with the cgMLST results.

Most of the isolates, belonging to a single lineage (ST101), were indistinguishable by PFGE, while cgMLST analysis allowed an accurate characterization of the transmission events, identifying 3 clonal clusters in 13 patients between 2016/2018. Comparing the cgMLST results with epidemiological data, we observed that cluster A strains were isolated between October and November 2016, cluster B strains were isolated 2 months later (November 2016–January 2017), and cluster C strains were detected between September 2017 and January 2018. In particular, the isolates KP722 and KP725 (ST3366) clustered more tightly to ST101 (by 1 allele) than ST3367 (by 6 alleles).

The analysis of virulence factors revealed that all strains exhibited the cps locus serotype K17, recently found in ST101 KPC2 bla_{oxa-9} strain in India [19]. *K. pneumoniae* K17 has been found in neonatal and rheumatology patients [20, 21]. The presence in these strains of the mannose-insensitive type-3 fimbriae, encoded by the *mrk*ABCD cluster, seems to be linked to the GI tract colonization or to virulence in the lung, even if there are no clear results [22]. One of the most clinically significant roles of these traits resides in biofilm formation: in fact, type 3 fimbriae are expressed during biofilm formation on catheters [23, 24]. Controversial results were obtained with type-1 fimbriae expression. Furthermore, type-3 fimbriae can have a role in entry and persistence of Kp in VAP [22].

Yersiniabactin siderophore, encoded by *irp* and *ybt* genes, was also been found. Interestingly, yersiniabactin has been observed in only approximately 18% of classical *K. pneumoniae* however in at least 90% of hypervirulent strains [25]. It seems that this siderophore is overexpressed in isolates from the respiratory tract during lung infection [26]. Our strains also possessed the ferric uptake operon *kfu*ABC but none of them demonstrated the presence of *rmp*A *wzy*-K1 (*mag*A) genes, which are often associated with a hyper-mucoviscosity of the bacteria [27].

Co-expression of carbapenemase genes, together with other virulence genes, reduces treatment options; we therefore have to focus on the rapid identification of CRE colonized patients and implementation of effective infection control measures [28–30].

In conclusion, our study shows that analyzing the genetic features of these novel clusters in relation to their resistance and pathogenicity may be helpful for patient management and outbreak surveillance in hospital settings. Furthermore, the dissemination of co-producing NDM and OXA-48 Enterobacteriaceae is worrisome, narrowing the already limited therapeutic options against these microorganisms.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval Not applicable.

Informed consent Not applicable.

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