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Research note

Genomics of *Klebsiella pneumoniae* ST16 producing NDM-1, CTX-M-15, and OXA-232

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ABSTRACT

Objectives: Genomic characterization of the internationally spread sequence type (ST) 16 carbapenem-resistant *Klebsiella pneumoniae*.
Methods: The complete genomes of three carbapenem producing ST16 *K. pneumoniae* from Italian patients were analysed by single-nucleotide polymorphism-based phylogeny, core genome multilocus sequence typing, resistance, plasmid, and virulence content and compared with ten genomes of ST16 strains isolated in other countries. Plasmids carrying $bla_{\text{NDM-1}}$ or $bla_{\text{OXA-232}}$ carbapenemase genes were assembled and sequences were analysed.
Results: The internationally spread ST16 *K. pneumoniae* clone showed variability in terms of distribution of NDM-1 and OXA-232 type carbapenemases. In some ST16 strains, up to six plasmids can be simultaneously present in the same cell, including ColE-like plasmids carrying $bla_{\text{OXA-232}}$ and IncF plasmids carrying $bla_{\text{NDM-1}}$. The differences observed in plasmid, resistance, and virulence content and core genome suggested that there is not a unique, highly conserved ST16 clone, but instead different variants of this lineage circulate worldwide.
Conclusions: The ST16 *K. pneumoniae* clone has spread worldwide and may become a high-risk clone.
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Introduction

Klebsiella pneumoniae represents one of the most common opportunistic hospital-associated pathogens and is a major source for multi-antibiotic resistance, including carbapenem resistance, because of the spread of high-risk clones [1]. In *K. pneumoniae* most of the carbapenemase genes have been identified on large, self-conjugative plasmids [1], except $bla_{\text{OXA-232}}$, which is frequently encoded by small ColE-like plasmids [2]. Moreover, carriage of different carbapenemase genes in the same strain has been observed, such as the association of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-232}}$ in sequence type (ST) 14 [3].

In this study, we report the characterization of ST16 *K. pneumoniae* strains, producing NDM-1, OXA-232 or both, causing an outbreak in the Treviso Hospital area, northern Italy. ST16 has been reported worldwide, showing different antimicrobial resistance profiles. In 2011, *K. pneumoniae* ST16 harbouring CTX-M-15 was the cause of nosocomial infections in Denmark and Sweden [4], NDM-5-carrying ST16 *K. pneumoniae* was described in Denmark [5], and later the co-presence of NDM-1 and OXA-232 in ST16 *K. pneumoniae* was observed in Italy [6].

Materials and methods

Ethics declaration

This was an observational study on strains selected from anonymized databases. The study did not require the approval of an ethics committee.

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Table 1
Plasmid and resistance content of the fully sequenced ST16 *K. pneumoniae* strains analysed in this study

Strain	KL8	KL11	KL29	FDAARGOS_440	QS17-0029	CNR48	AR_0087	DA33141	UCLAOXA232KP	UCLAOXA232KP
Country	ITALY	ITALY	ITALY	CANADA	THAILAND	FRANCE	NA	SWEDEN	USA	USA
Date	7/3/17	22/3/17	21/9/16	01/2015	04/2017	2012	NA	NA	12/10/14	11/3/15
NCBI GenBank accession numbers	REGZ00000000.1	REGY00000000.1	REHA00000000.1	CP023919 (Chr) CP023922 CP023923 CP023924	CP024038 (Chr) CP024039 CP024040 CP024041 CP024042 CP024043 CP024044	LS399318 (Chr) LT994835 LT994840	CP029738 (Chr) CP029739 CP029740	CP029587 (Chr) CP029588 CP029589	CP012561 (Chr) CP012562 CP012563 CP012564 CP012565 CP012566 CP012567	CP012568 (Chr) CP012569 CP012570 CP012571 CP012572
Acquired resistance genes[a]										
bla$_{TEM-1A}$		pos	pos	pos	pos					
bla$_{TEM-1B}$				pos		pos		pos		
bla$_{CTX-M-15}$	pos	pos	pos	pos	pos	pos		pos	pos	
bla$_{OXA-9}$		pos		pos	pos					
bla$_{OXA-1}$						pos		pos	pos	
bla$_{NDM-1}$	pos	pos		pos	pos					
bla$_{OXA-232}$	pos		pos	pos	pos				pos	
bla$_{DHA-1}$						pos				
bla$_{SHV-12}$						pos	pos			
aadA1		pos		pos	pos					
aadA2	pos	pos		pos	pos	pos		pos		
aac(6')-Ib		pos		pos	pos					
aac(6')-I-cr					pos	pos		pos	pos	
aac(6')-IIc						pos				
aph(3'')-Ib						pos				
aph(6)-Id						pos				
aph(3')-Ia						pos				
aac(6')-Ib3									pos	pos
sul1	pos	pos		pos	pos	pos		pos	pos	pos
dfrA1									pos	pos
dfrA12	pos	pos		pos	pos	pos		pos		
dfrA19						pos				
catB4						pos		pos	pos	pos
mph(A)		pos		pos		pos				
tet(A)						pos	pos	pos		
tet(B)	pos	pos		pos	pos					
qnrB1			pos	pos			pos			
qnrB4						pos				
erm(B)				pos						
rmtB				pos						
rmtF									pos	pos
mcr-1					pos					
ere(A)						pos				
ARR-2									pos	pos
ARR-3									pos	pos
Plasmids	**Replicons**[b]									
IncF	FII_36	FII_36	FII_36	FII_36	FII_36				FII(pKPX1)	FII(pKPX1)
	FII_22	FII_22	FII_22	FII_22	FII_22					
	FIA_1	FIA_1	FIA_1	FIA_1	FIA_1					
	FIB_20	FIB_20	FIB_20	FIB_20	FIB_20				FIB(pKPHS1)	FIB(pKPHS1)
		FIIK_2	FIIK_2	FIIK_2	FIIK_2	FIIK_5	FIIK_2	FIIK_5	FIIK_5	FIIK_5
		FIB_K (pQil)	FIB_K (pQil)	FIB_K (pQil)	FIB_K (pQil)	FIB_K (pKN3)		FIB_K (pKN3)	FIB_K (pKN3) FIIK_7 (IncR)	FIB_K (pKN3) FIIK_7 (IncR)

ColE	ColKP3 ColE440_2 ColE440_1	ColE440_2 ColE440_1	ColKP3 ColE440_2 ColE440_1	ColKP3	ColKP3 ColE440_2 ColE440_1		ColE440_2	ColKP3
IncHI2						HI2 HI2A		
IncX					X4			X3

NA, not available; Chr, accession number of the bacterial chromosome, the other accession numbers listed below the chromosome refer to plasmid sequences identified in the same strain and separately submitted in Genbank.
[a] Acquired resistance genes determined by ResFinder3.0 (https://cge.cbs.dtu.dk/services/ResFinder/).
[b] Replicons detected by PlasmidFinder 2.0 (https://cge.cbs.dtu.dk/services/PlasmidFinder/).

Whole genome sequencing

Whole genome sequencing (WGS) was performed using the Illumina MiSeq instrument (Illumina Inc., San Diego, CA, USA). *De novo* assembly was performed by SPADES (Galaxy Version 3.11.1). Plasmid and resistance genes were detected using PlasmidFinder [7] and ResFinder [8], respectively. Replicon alleles were assigned at the plasmid multilocus sequence typing (pMLST) site (https://pubmlst.org/plasmid/).

The core genome MLST on 629 alleles (cgMLST629_S) was identified using the *Klebsiella* Sequence Typing tool (http://bigsdb.pasteur.fr/klebsiella/klebsiella.html) [9,10].

The whole-genome shotgun projects of the KL8, KL11, and KL29 strains have been deposited at DDBJ/EMBL/GenBank under the accession nos. REGZ00000000.1, REGY00000000.1 and REHA00000000.1, respectively.

Plasmid analysis

Purified plasmid DNA was used to transform Library Efficiency DH5α-competent cells (Invitrogen, Carlsbad, CA, USA), selecting the transformants on Luria–Bertani agar plates, containing ampicillin 50 mg/L. Transformants were analysed by plasmid restriction analysis and screened by PCR for the presence of bla_{NDM-1}, $bla_{CTX-M-15}$, and $bla_{OXA-232}$ resistance genes.

Plasmid assembly was obtained mapping ST16 contigs on reference plasmid sequences, checking overlapping paired ends and confirming the assembly by the PCR-based gap closure method. Plasmids were annotated at the RAST server (http://rast.nmpdr.org/).

Phylogenetic analysis

Single-nucleotide polymorphism (SNP) analysis was performed by the Kn3SNP (Galaxy version 3.1) software at the ARIES public Galaxy server (https://w3.iss.it/site/aries/) on *K. pneumoniae* genomes from three Italian ST16, ten international ST16, and 22 belonging to other STs (differing from ST16 for one, two, or three alleles, respectively).

Results

Characteristics of the outbreak strains

An outbreak involving 23 infected/colonized patients occurred from 24 February to 29 March 2017 in three different wards of the Treviso Hospital and two long-term care facilities (LTCFs) in the Treviso area. The outbreak clone was identified as ST16 by MLST and pulsed field gel electrophoresis. Retrospectively, nine ST16 carbapenem-resistant strains were isolated in these hospitals before the outbreak from September 2016 to January 2017 and included in the study (Table S1).

The 32 Italian ST16 showed different distribution of two major carbapenemase genes: 14 strains were positive for bla_{NDM-1}, two for $bla_{OXA-232}$, and 16 for both bla_{NDM-1} and $bla_{OXA-232}$ (Table S1). Six carbapenem-resistant strains were also CTX-M-15 extended-spectrum β-lactamase (ESBL) producers.

KL8 (LTCF, March 2017), producing NDM-1, CTX-M-15, and OXA-232, and KL11 (Treviso hospital, March 2017), producing NDM-1 and CTX-M-15, were selected for WGS as prototypic strains of the outbreak clone. KL29 (LTCF, September 2016), producing CTX-M-15 and OXA-232 was also sequenced as prototypes of the strains collected before the outbreak (Table 1).

Core genome and SNP-based phylogenetic analysis

The three Italian ST16 strains were compared with ten internationally isolated ST16 strains (USA, Canada, Sweden, France, Thailand, Table 1). The cgMLST analysis [9,10] showed that there were between 13 and 85 out of 629 core genome locus differences among ST16 genomes. KL11 showed 13, 15, 24, and 85 locus differences compared with QS17-0029 (CP024038.1) from Thailand, positive to $bla_{\text{OXA-232}}$, $bla_{\text{NDM-1}}$, $bla_{\text{CTX-M-15}}$, and *mcr-1* [11], FDAARGOS_440 (CP023919.1) isolated in Canada positive to $bla_{\text{NDM-1}}$, $bla_{\text{OXA-232}}$, and $bla_{\text{CTX-M-15}}$, UCLAOXA232KP (CP012568) from the USA, positive to $bla_{\text{OXA-232}}$ and $bla_{\text{CTX-M-15}}$, and AR_0087 (CP029738) identified in the USA, negative for carbapenemase and ESBL genes, respectively. KL29, the ST16 isolated in Italy in 2016, differed for 32 and 43 loci from outbreak strains KL11 and KL8, respectively.

The neighbour-joining phylogeny tree showed that all ST16 clustered in the same branch, but separated into different clades (Fig. 1). The three Italian ST16 strains clustered in Clade I, together with QS17-0029 and FDAARGOS_440 from Thailand and Canada, respectively. Four ST16 *K. pneumoniae* from the USA clustered in Clade II. The two ST16 genomes from Sweden producing CTX-M-15 but negative for carbapenemase were closely related to those of Clade I but clustered in a separate clade. As was previously found analysing the cgMLST, the carbapenemase ESBL-negative AR_0087 strain was confirmed to be the most divergent ST16 genome among those under investigation.

The NDM-1-IncF plasmids

Major differences among ST16 strains were found in resistance and plasmid content (Table 1). Up to six plasmids were simultaneously detected in ST16 genomes. Strains from Italy, Canada, Thailand, and the USA carried one or two carbapenamase genes, among $bla_{\text{OXA-232}}$ and $bla_{\text{NDM-1}}$ and most of the strains also carried the $bla_{\text{CTX-M-15}}$ ESBL gene. Additional resistance genes, including the colistin resistance *mcr-1* determinant in the isolate from Thailand, or the 16S rRNA methylase *rmtB* in the isolate from Canada, contributed to diversification of ST16 antimicrobial resistance gene content.

Complete assembly of the IncF plasmid carrying the $bla_{\text{NDM-1}}$ gene was obtained for strain KL8 (pKL8-NDM plasmid; MH523448). The same plasmid was also identified in strain KL11.

pKL8-NDM was highly conserved with NDM-positive IncF plasmids in ST16 strains from different continents. Using BlastN, the pKL8-NDM plasmid showed 99% nucleotide identity and 96% and 88% coverage with pMR0617ndm (CP024039.1) of strain QS17-0029 and *plasmid unnamed1* (CP023923.1) of FDAARGOS_440, respectively. All included the F_{22}:F_{36}:A_1:B_{20} replicons [12] and the IS*26*-ΔIS*Ecp1*-ΔIS*Aba125*-$bla_{\text{NDM-1}}$-ble_{MBL}-*trpF-dsbD* module

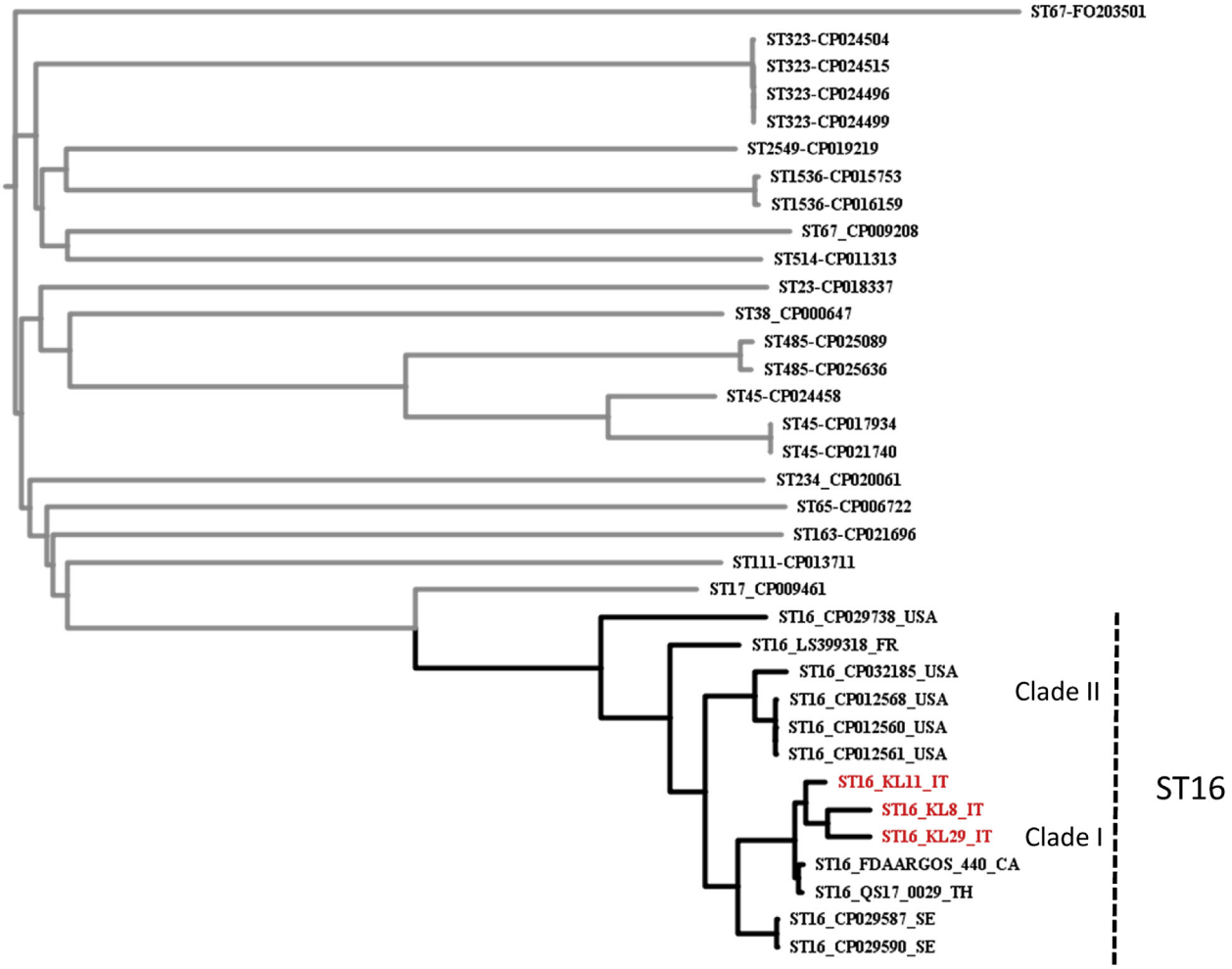

Fig. 1. Phylogenetic tree of different sequence types (STs) of *K. pneumoniae*. Black branches of the parsimony tree indicate the branch generated by ST16 WGSs. The three Italian ST16 genomes are highlighted by red characters. The whole group of the ST16 in the tree is underlined by a dashed vertical bar. The phylogenetic tree was visualized using the Fig Tree program v. 1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/).

located in a complex class 1 integron, carrying the *aadA2* and *dfrA12* resistance gene cassettes (Fig. S1). The arginine deaminase (ADI) cluster (*arcA*, *arcB*, *arcC*, and *arcD* genes) was trapped between the two F_{22}:F_{36} replicons [13].

Interestingly, the F_{22}:F_{36}:A_1:B_{20} scaffold was detected in strain KL29, but not the variable region including the bla_{NDM-1} and linked resistance genes (Table 1).

The OXA-232 ColE plasmids

The $bla_{OXA-232}$ gene was located on a 6141-bp plasmid carrying the ColKP3 replicon. This plasmid was obtained by transformation from strains KL8 and KL29 (pKL29-OXA-232; MH523449). It showed the best match (99% nucleotide identity and 100% coverage) with OXA-232 plasmids from QS17-0029 (CP024042), FDAARGOS_440 (CP023924) and the plasmid of the ST14 strain PittNDM1 from USA (CP006802) [3]. All these ColE-like plasmids carried the mobilization system (MobA-D), a replication gene (*repA*), truncated parts of erythromycin esterase (Δ*ereA*) and the transcriptional regulator (Δ*lysR*). Furthermore, all plasmids showed 206 bp upstream of the $bla_{OXA-232}$ corresponding to a vestigial, deleted ΔISE*cp1* insertion sequence.

Location of $bla_{CTX-M-15}$ in ST16 K. pneumoniae

The $bla_{CTX-M-15}$ gene was localized in the chromosome of the three KL8, KL11, and KL29 strains, flanked by the insertion sequence IS*Ecp1* and the truncated transposon ΔTn*2*, integrated in the metal-dependent hydrolase tRNA synthetase gene (MH523447). The same structure and integration site was also found in the chromosome of QS17-0029 [11] (between positions 2449972 and 2452365 in CP024038), while it was absent in the chromosome of FDAARGOS_440. However, QS17-0029 (CP024040) and FDAARGOS_440 (CP023922) and strains from Sweden (CP029588) and France (LT994840) carried a plasmid copy of the $bla_{CTX-M-15}$ gene located on IncFIIk.

Discussion

The small number of isolates investigated in this study limited the possibility of describing the emergence and evolution of the outbreak strains within the hospital. However, by comparison with internationally isolated *K. pneumoniae* strains, we demonstrated that ST16 showed considerably divergent genomes, suggesting the existence of different lineages. The most clinically relevant difference lay in the acquisition of multiple plasmids conferring multidrug resistance, including carbapenem resistance. The bla_{NDM-1} and $bla_{OXA-232}$ genes, located on different plasmids, were detected within some strains of the ST16 clone, with up to six plasmids simultaneously resident within the same cell. The versatility to acquire and exchange resistance plasmids suggests that ST16 may become a high-risk clone resulting in important healthcare-associated infections.

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Transparency declaration

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.cmi.2018.11.004.

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