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Molecular epidemiology of KPC-producing *Klebsiella pneumoniae* from invasive infections in Italy: increasing diversity with predominance of the ST512 clade II sublineage

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Objectives: The spread of carbapenem-resistant Enterobacteriaceae (CRE) represents one of the most worrisome problems for clinical medicine worldwide. In Italy, the Antibiotic-Resistance-Istituto Superiore di Sanità surveillance network, in collaboration with the Committee for Antimicrobial Agents of the Italian Society of Clinical Microbiologists, promoted a study to investigate the carbapenem-resistance mechanisms, clonal relatedness and capsular typing of a recent collection of carbapenem-resistant *Klebsiella pneumoniae* (CR-KP).

Methods: A total of 17 laboratories distributed across Italy collected all consecutive non-replicate CR-KP isolated from invasive infections during two different study periods (2011–12 and 2013). Carbapenemase genes were searched for by filter hybridization and confirmed by PCR and sequencing. KPC-producing *K. pneumoniae* (KPC-KP) were typed by PFGE and MLST. Capsular types were identified by *wzi* gene typing.

Results: Of the collected *K. pneumoniae* isolates ($n=461$), the overall proportion of CR-KP was 36.2% ($n=167$). The majority (97%) of the CR-KP were positive for the bla_{KPC} gene. Among the KPC-KP population, nine different STs were detected with the majority of isolates (94%) belonging to the clonal group (CG) 258. A subpopulation that belonged to ST512 and showed an identical PFGE profile represented the majority (57%) of KPC-KP strains, with a countrywide distribution. Capsular characterization showed the predominance of the *wzi154*, *cps-2* capsular type (88.8% of all CG258 strains). ST258 strains were associated with both *cps-1* and *cps-2* capsular types, while ST512 was associated with *cps-2* only.

Conclusions: Although a trend to a polyclonal evolution of the Italian KPC-KP was noted, this study showed that the KPC-KP population remained largely oligoclonal with the wide diffusion of an ST512 lineage carrying *cps-2* capsular type and producing the KPC-3 enzyme.

Introduction

Carbapenem-resistant Enterobacteriaceae (CRE) are spreading globally and have become endemic in several countries, including Italy.[1–3] CRE infections are difficult to treat due to their complex multidrug-resistance phenotypes[4,5] and are associated with increased morbidity and mortality in comparison with infections caused by carbapenem-susceptible strains.[6] The most important mechanism of carbapenem resistance in Enterobacteriaceae is the production of carbapenemases, mostly of the KPC, VIM, NDM and OXA-48 types, which exhibit notable geographical variability.[1,2,7,8] KPC-type carbapenemase-producing *Klebsiella pneumoniae* (KPC-KP) emerged in 1996 in the USA[9] and since then has disseminated globally, becoming endemic in some countries including Italy.[7,10] The rapid and efficient dissemination of KPC-KP has mostly been caused by the clonal expansion of strains of clonal group (CG) 258 including ST258 and related variants (e.g. ST512).[7,11,12]

Recent comparative genomic studies revealed that ST258 comprises at least two different lineages, namely clade I and clade II, which differ by a chromosomal region of 215 kb carrying the capsular polysaccharide (CPS) gene cluster.[12–15] Characterization of the *cps* locus and prediction of the serotypes (K type) is based on sequencing of the conserved *wzi* and/or *wzc* genes, which are essential for CPS assembly and conserved in all capsular types.[16,17] Several multiplex-PCR approaches have been developed for rapid characterization of the *cps* locus.[18–20] Some K types are significantly associated with serious human infections (e.g. K1) and CPS may be involved in the global success of ST258.[12,14]

ST258 clade I carries the *cps-1* gene cluster (characterized by the *wzi29* allele variant and associated with the K41 serotype), while clade II carries the *cps-2* gene cluster (characterized by *wzi154*, untypeable with conventional methods).[14] Furthermore, a strong association between bla_{KPC-2} and clade I, and between bla_{KPC-3} and clade II, has been noted.[11,12,17,21]

In Italy, the results from a cross-sectional nationwide survey performed in 2011 showed that the KPC-KP represented the majority (87%) of CRE and that the dissemination of KPC-KP was mostly caused by strains of CG258, with a minority of strains belonging to ST101.[10] Further characterization of representative strains from the same Italian nationwide survey showed that the *cps-2* variant of the CPS gene cluster was the most prevalent among isolates of CG258 and was carried by both ST258 and ST512 strains. In contrast, the *cps-1* type was less widespread and associated with isolates of ST258 only.[17]

In this work, we report the results of a countrywide survey that aimed to investigate carbapenem-resistance determinants, clonal relatedness and capsular types of carbapenem non-susceptible *K. pneumoniae* isolated from invasive infections.

Methods

Study design

A multicentre cross-sectional collection of carbapenem-resistant *K. pneumoniae* (CR-KP) was promoted by the Italian Antibiotic-Resistance-Istituto Superiore di Sanità (AR-ISS) surveillance network, in collaboration with the Committee for Antimicrobial Agents of the Italian Society of Clinical Microbiologists. A total of 17 laboratories, from 14 cities, of the AR-ISS network (Figure 1) collected all consecutive non-replicate clinical isolates of *K. pneumoniae* from blood or CSF with MICs >1 mg/L for meropenem and/or imipenem during two different periods, 1 October 2011–31 March 2012 and 15 February 2013–15 June 2013. All isolates that matched these criteria were considered as CR-KP for the scope of this work. All laboratories provided information on the total number of consecutive non-duplicate clinical isolates of *K. pneumoniae* observed during the collection period. Collected isolates were sent to reference laboratories for phenotypic and genotypic characterization.

Bacterial identification and phenotypic characterization of resistance mechanisms

Bacterial identification and antimicrobial susceptibility testing were carried out by the network laboratories using either a Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, MD, USA) or a Vitek-2 System (bioMérieux, Marcy-l'Étoile, France). Reference laboratories confirmed species identification by MALDI-TOF MS (Vitek-MS, bioMérieux) and screened the collected isolates for carbapenemase production by combination disc test using meropenem plus EDTA or phenylboronic acid.[22,23]

Molecular characterization

The presence of the most prevalent carbapenemase genes (bla_{KPC}, bla_{VIM}, bla_{IMP}, bla_{NDM} and bla_{OXA-48}-like) was investigated by dot-blot DNA hybridization using the method described by Srinivasan *et al.*[24] All hybridization-positive results were confirmed by PCR using primers and conditions previously described.[10] Amplification products were sequenced by an external facility (Macrogen Inc., Seoul, Korea) and analysed using the BLAST program at NCBI (http://www.ncbi.nlm.nih.gov/BLAST).

Isolates testing negative by the combination disc test and/or in a hybridization experiment were further investigated for production of carbapenemase by spectrophotometric assay on crude extracts.[25]

Clonal relatedness

Clonality was analysed for all KPC-KP by PFGE after digestion of genomic DNA with XbaI as previously described[10] and results were interpreted according to the criteria reported by van Belkum *et al.*[26] The same capital letter (e.g. A) was assigned to identical PFGE profiles. PFGE profiles that differed by <4 bands were considered as profile variants (e.g. A1). PFGE patterns differing by ≥4 bands were considered to belong to a different profile (e.g. B). Representative isolates for each different PFGE profile or profile variants were subjected to MLST analysis.[27] STs were assigned using the *K. pneumoniae* MLST web site (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public).

Capsular type characterization

Capsular type characterization was performed for KPC-producing isolates only. All isolates were tested using a previously developed multiplex PCR able to recognize the two major capsular types associated with CG258 strains as well as those frequently associated with invasive disease or pathogenicity.[17] The *wzi* gene of the isolates that tested negative with the multiplex PCR was amplified, sequenced and analysed according to the scheme available at the Bacterial Isolate Genome Sequence Database (BIGSdb) (http://bigsdb.web.pasteur.fr/perl/bigsdb/bigsdb.pl?db=pubmlst_klebsiella_seqdef_public).[14]

Results

Proportion of CR-KP from reporting laboratories

The 17 laboratories of the AR-ISS network recorded, in the two study periods, a total of 461 invasive infections caused by *K. pneumoniae* (246 and 215 in the first and second study periods, respectively). Details are reported in Table S1 (available as Supplementary data at *JAC* Online). Overall, 167 isolates (36.2%) were CR-KP, of which 164 (98%) were confirmed to be carbapenemase-producing *K. pneumoniae* (CP-KP). The CP-KP proportion observed in the second study period was slightly higher than that observed in the first one (39.5% versus 33.3%, respectively), but the difference was not statistically significant ($P=0.16$). Remarkable differences, however, were observed among proportions in various laboratories and in local trends; only three laboratories did not report CR-KP isolates in both study periods. However, two of these laboratories collected only very few *K. pneumoniae* isolates during the study periods (Table S1).

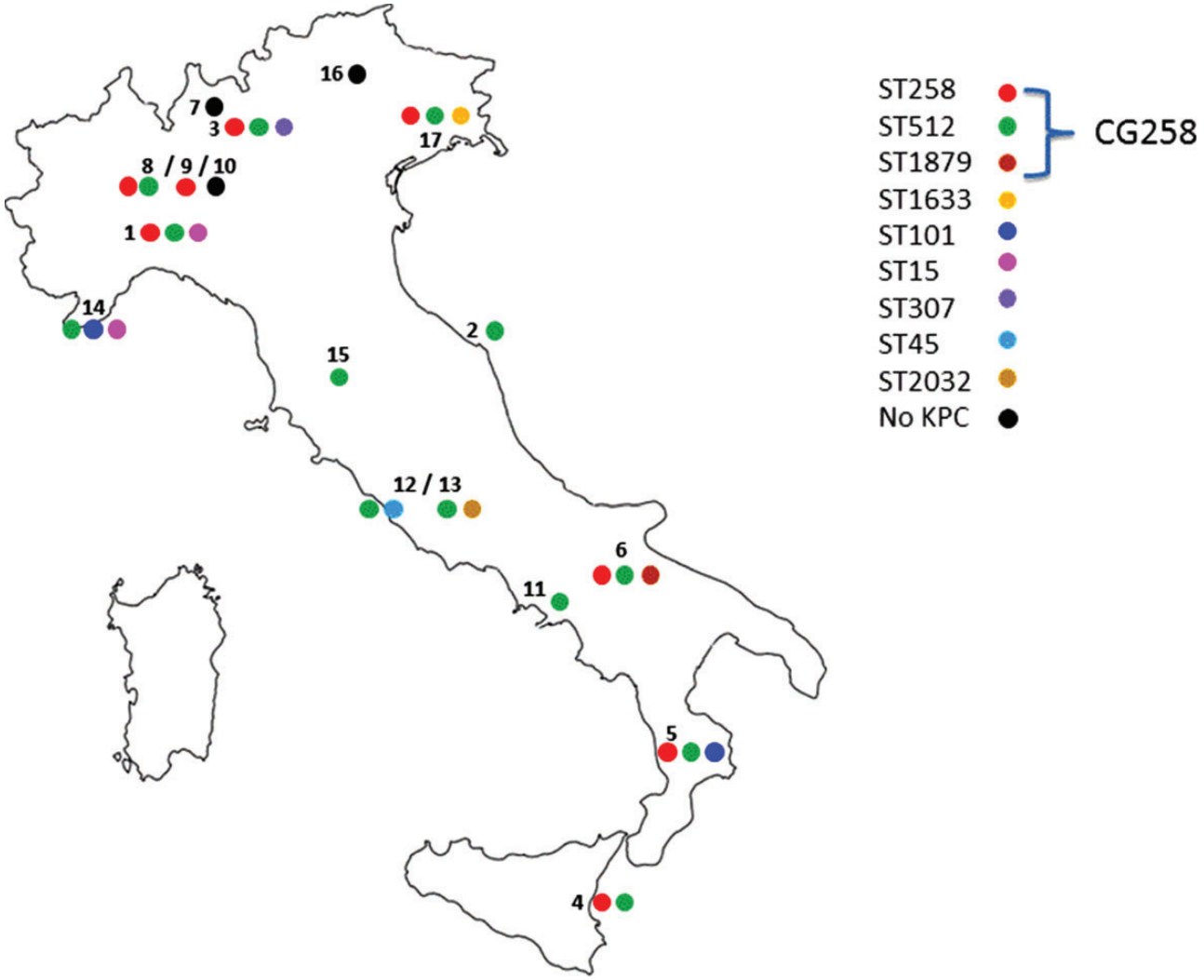

Figure 1. Geographical location of the 17 laboratories participating in the survey. The KPC-KP STs detected in different laboratories are indicated by different colours. Cities were as follows: 1, Alessandria; 2, Ancona; 3, Bergamo; 4, Catania; 5, Cosenza; 6, Foggia; 7, Lecco; 8–10, Milan; 11, Naples; 12 and 13, Rome; 14, San Remo; 15, Sienna; 16, Trento; and 17, Venice. This figure appears in colour in the online version of *JAC* and in black and white in the print version of *JAC*.

Characterization of carbapenem-resistance mechanisms

Overall, 162 out of the 167 CR-KP (97%) were positive for the bla_{KPC} gene and 2 (1.2%) for the bla_{VIM-1} metallo-β-lactamase gene. The remaining three isolates (1.8%) tested negative for phenotypic (including spectrophotometric assay) and genotypic tests, suggesting the presence of a non-carbapenemase-mediated mechanism of resistance. Other types of carbapenemases, including OXA-48 and NDM variants, were not detected. Regarding the bla_{KPC} types, the majority of the 162 KPC-KP (87%) carried the bla_{KPC-3} variant, while the remaining (13%) carried the bla_{KPC-2} variant.

KPC-KP population analysis

Nine different STs were detected among KPC-KP (ST512, ST258, ST1879, ST101, ST1633, ST15, ST307, ST45 and ST2032) with the majority of isolates (94%) belonging to CG258 (ST258 and its single-locus variants ST512 and ST1879) (Table 1). Isolates of CG258 were found in all centres reporting KPC-KP isolates with a countrywide distribution (Table 1 and Figure 1), confirming the spreading ability of these lineages. Although not statistically significant, an increment in the proportion of ST512 versus ST258 was observed in the most recent study period (42% in 2013 versus 28% in 2011–12). Overall, other STs accounted for 6% of the KPC-KP population and were sporadically detected with a more restricted geographical distribution (Table 1). Two novel STs, ST2032 and ST1879, were described, while ST1633 (a single-locus variant of ST101) was reported for the first time in Italy.

ST512, ST258 and ST15 showed 7, 12 and 3 PFGE profile variants, respectively (Table 1 and Figure S1). The two major PFGE profiles, A and B, included isolates belonging to both ST258 and ST512 (Table 1 and Figure S1). However, the majority (57%) of KPC-KP isolates belonged to the same PFGE A2 variant of ST512 that was detected in 10 out of 14 centres reporting KPC-KP isolates (Table 1 and Figure 1).

Table 1. Correspondence among ST, PFGE and KPC enzyme variants produced by the 162 KPC-KP; the total numbers of isolates that belonged to specific ST and PFGE profiles are also reported

ST	PFGE type	Total number of isolates	Centres	*wzi* variant (*cps* type)	KPC variant
512	A0	1	1	154 (*cps-2*)	KPC-3
	A1	9	5;6;8	154 (*cps-2*)	KPC-3
	A2	93	1;2;6;8;11;12;13;14;15;17	154 (*cps-2*)	KPC-3
	A3	5	4;12;13	154 (*cps-2*)	KPC-3
	A4	1	6	154 (*cps-2*)	KPC-3
	A5	1	14	154 (*cps-2*)	KPC-3
	B0	2	3;14	154 (*cps-2*)	KPC-3
258	A6	5	1	29 (*cps-1*)	KPC-2
	A7	14	4;6;8;17	154 (*cps-2*)	KPC-3
	A8	3	1;5	154 (*cps-2*)	KPC-3
	A9	1	4	154 (*cps-2*)	KPC-3
	A10	3	8;9	29 (*cps-1*)	KPC-2
	B1	2	8	29 (*cps-1*)	KPC-2
	B2	3	4	154 (*cps-2*)	KPC-3
	B3	2	1	29 (*cps-1*)	KPC-2
	B4	2	3	29 (*cps-1*)	KPC-2
	B5	1	3	29 (*cps-1*)	KPC-2
	B6	2	3;8	29 (*cps-1*)	KPC-2
	B7	1	4	154 (*cps-2*)	KPC-3
1879	A11	1	6	154 (*cps-2*)	KPC-3
101	C0	2	5;14	137	KPC-2
15	D0	1	1	24	KPC-3
	D1	1	1	24	KPC-3
	E0	2	14	24	KPC-2
307	F0	1	3	173	KPC-3
2032	F1	1	13	174	KPC-3
1633	G0	1	17	137	KPC-3
45	H0	1	12	101	KPC-3

Characterization of the cps gene clusters in KPC-KP isolates

All the 152 strains belonging to CG258 were typeable using the multiplex PCR method.[17] Of these, 88.8% carried *cps-2* and 11.2% carried *cps-1*. The *cps-2* capsular type was detected in all but one centre reporting KPC-KP isolates and was associated with isolates belonging to ST258, ST512 and ST1879, while *cps-1* was associated with ST258 strains only, with a distribution restricted to centres located in northern Italy (Table 1). ST512 was invariably associated with the *cps-2* capsular type.

During the study periods a significant increment in the percentage of isolates with *cps-2* capsular type among CG258 strains was documented (83.5% in 2011–12 versus 93.7% in 2013; $P<0.05$). According to what has been previously reported,[11,12,17,21] *cps-1* was always associated with the presence of bla_{KPC-2}, and *cps-2* with the presence of bla_{KPC-3}. The *cps-1* and *cps-2* capsular gene clusters were restricted to CG258 strains.

The remaining strains that were untypeable ($n=10$) by the multiplex PCR method belonged to STs not included in CG258. ST101 and its single-locus variant ST1633 carried the *wzi137* variant associated with the K17 serotype. ST307 and ST2032 strains carried *wzi173* and *wzi174* (two variants not previously associated with specific serotypes), respectively. The remaining five strains belonging to ST15 and ST45 carried *wzi24* or *wzi101* (the predicted K24 serotype) (Table 1).

Discussion

The results of this survey confirmed the highly endemic nature of CR-KP across the entire Italian territory. The observed proportions were overall similar to those reported by the EARS-NET surveillance report for the same years (29.1% for 2012 and 34.7% for 2013).[28] As reported in the 2011 Italian survey,[10] KPC production continued to represent the most frequent mechanism of resistance to carbapenems in *K. pneumoniae*, while other mechanisms remained in the background or were not detected.

Although the sporadic detection of several non-CG258 STs (ST101, ST15, ST307, ST45, ST1633 and ST2032) was reported,

the KPC-KP population structure remained largely oligoclonal, with 94% of isolates represented by CG258 strains and the majority of them belonging to a single PFGE subtype.

Nevertheless, compared with the previous Italian survey, where only CG258 and strains of ST101 were reported (the latter sporadically), a trend to a polyclonal evolution of the KPC-KP population was observed, as also previously reported at a local level.[29,30] This could suggest an evolution of the KPC-KP epidemic similar to what has been observed in Greece, where the later phases of the KPC-KP epidemic were associated with the diffusion of several STs different from the original epidemic ST258 clone.[31] Considering this ongoing epidemiological evolution, characterization of a more recent collection of KPC-KP from the same centres will be of interest. It would also be of interest to analyse the types and diversity of KPC-encoding plasmids associated with the predominant KPC-KP clones and compare them with those circulating in the early stages of the Italian KPC-KP epidemic.[32]

Our study also confirmed, on a broader scale and over a longer time frame, the presence of two distinct *cps* types among KPC-KP strains of CG258.[17,21] ST258 was associated with both *cps-1* and *cps-2*, while ST512 was always associated with *cps-2*. To further support this finding, we determined *in silico* the *wzi* allelic variant of the 455 CG258 genomes available in the nr and wgs NCBI databases (accessed on 15 January 2016) and confirmed the predominance of the *wzi154* variant (*cps-2*) (76% of the CG258 sequenced genomes). This analysis also confirms the invariable association of ST512 with *cps-2* and suggests that ST258 *wzi154* could be the ancestor of ST512.

The reasons for the predominance of strains with the *cps-2* capsular type remain to be clarified. However, a different virulence potential could contribute to the success of a variant within a clone. In fact, the lower virulence potential of *cps-2* ST258 strains compared with *cps-1* ST258 strains, as indicated by results of previous works carried out in the *Galleria mellonella* model, could play a role in this phenomenon.[21,33] In this work we analysed isolates from invasive infections only. Further investigation on isolates from colonization and other types of infection would be of interest to study a possible correlation between the different virulence potential of the two capsular types and their ability to cause colonization and infections.

Results from capsular typing also underlined the presence in Italy of ST15 strains carrying *wzi24* associated with K24 and belonging to CG14. Strains belonging to CG14 of the K2 and K24 serotypes usually carry a relevant virulence factor content and have been associated with serious human infections.[34] This finding underscores the potential threat represented by the emergence and diffusion of carbapenem-resistance clones with increased virulence potential.[35,36]

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Transparency declarations

None to declare.

Supplementary data

Table S1 and Figure S1 are available as Supplementary data at *JAC* Online (http://jac.oxfordjournals.org/).

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