Epidemic diffusion of KPC carbapenemase-producing *Klebsiella pneumoniae* in Italy: results of the first countrywide survey, 15 May to 30 June 2011

T Giani[1], B Pini[2], F Arena[1], V Conte[1], S Bracco[2], R Migliavacca[3], the AMCLI-CRE Survey Participants[4], A Pantosti[5], L Pagani[3], F Luzzaro[2], G M Rossolini (gianmaria.rossolini@unisi.it)[1,6,7]

1. Department of Medical Biotechnologies, University of Siena, Siena, Italy
2. Microbiology and Virology Unit, A. Manzoni Hospital, Lecco, Italy
3. Department of Clinical Surgical Diagnostic and Pediatric Sciences, Section of Microbiology, University of Pavia, Pavia, Italy
4. The AMCLI-CRE Survey Participants are listed at the end of this article
5. Department of Infectious, Parasitic and Immune-Mediated Diseases, Italian National Health Institute, Rome, Italy
6. Department of Experimental and Clinical Medicine, University of Florence, Italy
7. Clinical Microbiology and Virology Unit, Department of Laboratory Medicine, Careggi University Hospital, Florence, Italy

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Carbapenem-resistant *Enterobacteriaceae* (CRE) are emerging as a public health problem in various settings. In Italy, a rapid and remarkable increase of carbapenem-non-susceptible *Klebsiella pneumoniae* has been reported since 2010. Here we report on the results of a countrywide cross-sectional survey, carried out from 15 May to 30June 2011 to investigate the diffusion of CRE in Italy and to characterise the most prevalent resistance mechanisms and their dissemination patterns. CRE were reported from most (23 of 25) participating laboratories, with an overall proportion of 3.5% and 0.3% among consecutive non-duplicate clinical isolates of *Enterobacteriaceae* from inpatients (n=7,154) and outpatients (n=6,595), respectively. *K. pneumoniae* was the most frequent species (proportion of carbapenem-non-susceptible isolates: 11.9%), while a minority of CRE of other species were detected. Carbapenemase production was detected in the majority (85%) of CRE. KPC-type enzymes were by far the most common (89.5% of carbapenemase producers), followed by VIM-1 (9.2%) and OXA-48 (1.3%). KPC-producing *K. pneumoniae* (KPC-KP) were detected in most centres and contributed majorly to the epidemic dissemination of CRE recently observed in our country. Dissemination of KPC-KP was mostly sustained by strains of clonal complex 258 (ST-258 producing KPC-2 or KPC-3, and ST-512 producing KPC-3), while a minority belonged to ST-101.

Introduction

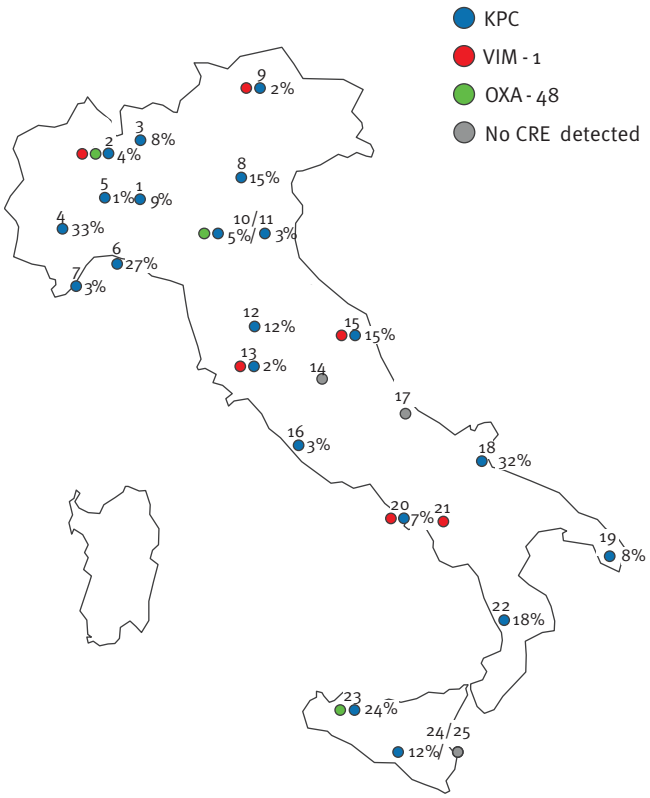
The increasing resistance to carbapenems among *Enterobacteriaceae* has become a public health problem of major concern [1,2]. Carbapenem-resistant *Enterobacteriaceae* (CRE) usually exhibit complex multidrug resistance phenotypes that leave very few therapeutic options [3,4], and infections caused by CRE are associated with increased morbidity and mortality in comparison with those caused by carbapenem-susceptible strains (72% versus 22%) [5].

At least two mechanisms can be responsible for acquired carbapenem resistance in *Enterobacteriaceae*: (i) reduced outer membrane permeability by porin loss in combination with the production of an extended-spectrum beta-lactamase (ESBL) or of AmpC-type beta-lactamase; and (ii) production of beta-lactamases capable of hydrolysing carbapenems (carbapenemases) [2]. While the former mechanism is a result of mutation and has a low overall propensity to disseminate, acquired carbapenemases are encoded by transferable genes that can disseminate among different strains and different species, and carbapenemase production is the leading carbapenem resistance mechanism in *Enterobacteriaceae* [2,6,7]. Several types of acquired carbapenemases have been detected in CRE, with KPC-, VIM-, NDM- and OXA-48-type enzymes being the most prevalent, although with a notable geographical variability [2,6-9].

In Europe, individual cases or outbreaks of CRE have been reported in several countries [6] but data from the EARS-NET database show that, until 2009, the proportion of CRE has remained overall low in most countries except Greece and Cyprus, where high-level endemicity of carbapenem-nonsuscetible *Klebsiella pneumoniae* has been reported since the mid-2000s [10]. In Italy, sporadic cases or outbreaks caused by CRE of various species and with different resistance mechanisms have been reported since the early 2000s [11-24], but only since 2010 an abrupt and notable increase in the proportion of carbapenem-non-susceptible *K. pneumoniae* has ben reported by the EARS-NET surveillance system

Figure 1

Location of the laboratories participating in the survey on carbapenem-resistant *Enterobacteriaceae*, Italy, 15 May–30 June 2011 (n=25)



1: Milan; 2: Varese; 3: Lecco; 4: Turin; 5: Novara; 6: Genoa; 7: Sanremo; 8: Verona; 9: Bolzano; 10-11: Modena; 12: Florence; 13: Siena; 14: Perugia; 15: Ancona; 16: Rome; 17: Pescara; 18: San Giovanni Rotondo; 19: Lecce; 20: Naples; 21: Avellino; 22: Cosenza; 23: Palermo; 24-25: Catania. The types of carbapenemases detected in different laboratories, and the proportion of KPC-producing *Klebsiella pneumoniae* versus the total number of *K. pneumoniae* isolates are indicated.

[10]. This trend has recently been confirmed by data from the Micronet sentinel surveillance network [25]. However, the resistance mechanisms responsible for this increase have not been investigated.

In this work we report the results of a countrywide cross-sectional survey promoted by the Italian Society of Clinical Microbiologists (AMCLI) and carried out in mid-2011, to investigate the diffusion of CRE in Italy and to characterise the most prevalent resistance mechanisms and their dissemination patterns. Results confirmed that CRE have reached epidemic dissemination in Italy, and revealed that this condition was mostly related with the clonal diffusion of *K. pneumoniae* producing KPC-type carbapenemases (KPC-KP) of clonal complex (CC) 258.

Methods

Study design

Twenty-five large clinical microbiology laboratories from 23 Italian cities, distributed across the national territory and covering most Italian regions, participated in the study (Figure 1). During the period from 15 May to 30 June 2011, each laboratory collected consecutive non-replicate clinical isolates of *Enterobacteriaceae*, from any site of infection, that exhibited minimum inhibitory concentrations (MICs) for imipenem and/or meropenem and/or ertapenem higher than 1 mg/L (for isolates of *Proteeae*, i. e. *Morganella morganii*, *Proteus* spp. and *Providencia* spp., only meropenem and ertapenem MICs were considered). The collected isolates were transferred to reference laboratories for confirmation of species identification and carbapenem MICs, and for characterisation of the carbapenem resistance mechanisms and analysis of clonal relatedness. For each isolate, information on the clinical specimen and type of ward (in case of isolates from inpatients) were provided. Moreover, each participating laboratory provided information on the total number of consecutive non- duplicate clinical isolates of *Enterobacteriaceae* observed during the collection period.

Characterisation of bacterial isolates and of resistance determinants

Bacterial identification and antimicrobial susceptibility testing were carried out by the collecting laboratories using either the Phoenix Automated Microbiology System (Becton Dickinson Diagnostic Systems, Sparks, United States) or the Vitek-2 System (bioMérieux, Marcy l'Etoile, France). Confirmatory identification was carried out by Matrix-assisted laser desorption ionisation – time of the flight (MALDI-TOF) mass spectrometry (Vitek-MS, bioMérieux). Confirmatory MIC testing for imipenem, meropenem and ertapenem was carried out by Etest (bioMérieux). All collected isolates confirmed to be non-susceptible to imipenem (not considered for *Proteeae*) and/or meropenem and/or ertapenem according to the EUCAST breakpoints [26] were considered as CRE for the purposes of this study. For KPC-KP, MICs of colistin, tigecycline and gentamicin were determined by the reference broth microdilution method [27], and results were interpreted according to the EUCAST breakpoints [26].

CRE were evaluated for carbapenemase production by meropenem plus EDTA and meropenem plus phenylboronic acid using the disk diffusion method [28,29], and for the presence of the most common carbapenemase genes (bla_{KPC}-, bla_{VIM}-, bla_{NDM}-, bla_{OXA-48}-type) by dot-blot hybridisation [30]. PCR amplification [13,31-33] and sequencing of PCR amplicons were used to identify the carbapenemase genes detected by hybridisation. All CRE isolates testing negative in disk diffusion test and/or in hybridisation assays were further investigated for production of carbapenemase activity by modified Hodge test [34] and by spectrophotometric assay with crude extracts [35].

TABLE 1

Proportions of carbapenem-non-susceptible *Enterobacteriaceae* detected in the first countrywide survey, Italy, 15 May–30 June 2011 (n=13,749)

Species	Isolates from inpatients		Isolates from outpatients		All isolates	
	Total	CRE (%)	Total	CRE (%)	Total	CRE (%)
Escherichia coli	3,844	4 (0.10)	4,765	1 (0.02)	8,609	5 (0.06)
Klebsiella pneumoniae	1,346	219 (16.3)	618	15 (2.4)	1,964	234 (11.9)
Klebsiella oxytoca	203	0	116	1 (0.86)	319	1 (0.31)
Enterobacter cloacae	361	15 (4.2)	144	0	505	15 (3.0)
Enterobacter aerogenes	147	4 (2.7)	68	2 (2.9)	215	6 (2.8)
Serratia marcescens	117	4 (3.4)	39	1 (2.6)	156	5 (3.2)
Proteus mirabilis	624	1 (0.16)	491	0	1,115	1 (0.09)
Citrobacter freundii	79	0	56	1 (1.8)	135	1 (0.74)
Hafnia alvei	24	2 (8.3)	8	0	32	2 (6.2)
Other species	409	0	290	0	699	0
Total	**7,154**	**249 (3.5)**	**6,595**	**21 (0.32)**	**13,749**	**270 (2.0)**

CRE: carbapenem-resistant *Enterobacteriaceae*.

All collected isolates confirmed to be non-susceptible to imipenem (not considered for *Proteeae*) and/or meropenem and/or ertapenem according to the EUCAST breakpoints [26] were considered as CRE for the purposes of this study.

Analysis of clonal relatedness

Genotyping of *K. pneumoniae* isolates by pulsed-field gel electrophoresis (PFGE) profiling of genomic DNA was carried out after digestion with XbaI with a CHEF-DRIII apparatus (Bio-Rad, Hemel Hempstead, United Kingdom) [36], and results interpreted as recommended by Van Belkum et al. [37]. Multi-locus sequence typing (MLST) of *K. pneumoniae* isolates was performed as previously described [38], and sequence types (STs) were assigned using the MLST web site [39].

Results

Proportions of carbapenem-resistant *Enterobacteriaceae* from inpatients and outpatients in Italy

During the study period (15 May–30 June 2011), a total of 13,749 consecutive non-replicate clinical isolates of *Enterobacteriaceae* were isolated at the 25 Italian laboratories participating in the survey. Overall, 270 isolates (2.0%) were confirmed as CRE. The proportion of CRE was approximately 10-fold higher among isolates from inpatients (3.5%) than among those from outpatients (0.3%) (Table 1).

Proportions of CRE in different species are shown in Table 1. *K. pneumoniae* was the most affected species (proportion: 11.9%) and contributed to the majority of CRE (234 of 270, 86.7%). Lower CRE proportions, but still higher than 2%, were observed among *Enterobacter* spp., *Serratia marcescens*, and *Hafnia alvei*. In *Escherichia coli* the proportion of CRE was very low (0.06%).

CRE were reported from 23 of the 25 participating laboratories (Figure 1). Carbapenem-nonsusceptible isolates of *K. pneumoniae* were detected at any of these 23 laboratories, with proportions ranging from 1.2 to 32.7% (mean: 11.5%) (Figure 1).

Carbapenem resistance mechanisms in carbapenem-resistant *Enterobacteriaceae*

Carbapenemase production was detected in the majority of CRE (85%) (Table 2). KPC-type enzymes were by far the most common (89.5% of carbapenemase producers). Other types of carbapenemases included VIM-1 and OXA-48 (9.2% and 1.3% of carbapenemase producers, respectively). KPC-type enzymes were only detected in *K. pneumoniae* and in one *E. coli*. VIM-1, although much less prevalent, was detected in a wider variety of bacteria: *K. pneumoniae*, *Klebsiella oxytoca*, *E. coli* and *Enterobacter cloacae*. OXA-48 was only detected in three *K. pneumoniae* isolates (Table 2). KPC-producers were detected in 21 of 25 centres, showing a countrywide distribution. VIM-1-producers were detected in six centres, while the three OXA-48-producers were from three different centres (Figure 1). Other types of carbapenemases, including NDM, were not detected.

TABLE 2

Mechanisms of resistance in carbapenem-nonsusceptible isolates of *Enterobacteriaceae*, Italy, 15 May–30 June 2011 (n=270)

Species	Isolates	Carbapenemase				Non-carbapenemase
		Total (%)	KPC	VIM-1	OXA-48	Total (%)
Escherichia coli	5	2 (40.0)	1	1	0	3 (60.0)
Klebsiella pneumoniae	234	223 (95.3)	204	16	3	11 (4.7)
Klebsiella oxytoca	1	1 (100.0)	0	1	0	0
Enterobacter cloacae	15	3 (20.0)	0	3	0	12 (80.0)
Others[a]	15	0	0	0	0	15 (100.0)
Total	**270**	**229 (84.8)**	**205**	**21**	**3**	**41 (15.2)**

[a] Including *Enterobacter aerogenes* (n=6), *Serratia marcescens* (n=5), *Proteus mirabilis* (n=1), *Citrobacter freundii* (n=1), and *Hafnia alvei* (n=2).

Carbapenem-non-susceptible *Klebsiella pneumoniae*: proportion in clinical specimens, distribution in hospital wards, and carbapenem MICs

The overall proportion of carbapenem non-susceptibility was approximately seven-fold higher in *K. pneumoniae* isolates from inpatients than in those from outpatients (16.3% versus 2.4%, Table 1). Considering isolates from inpatients, the proportion of carbapenem non-susceptibility was higher among bloodstream isolates than among isolates from other specimens (Table 3), revealing that carbapenem-non-susceptible *K. pneumoniae* strains circulating in Italy retained a remarkable potential for causing invasive infections. In the case of outpatients, carbapenem-non-susceptible isolates of *K. pneumoniae* were obtained only from urine, which was by far the most common specimen, and most of them were KPC-producers (Table 3).

Concerning the in-hospital distribution, 42.5% of the 219 carbapenem-nonsusceptible *K. pneumoniae* from inpatients were from intensive care units (ICUs), while 32.4% were from medical wards, 21.5% from surgical wards, and 3.6% from other areas.

Carbapenem MICs of the 234 carbapenem-nonsusceptible *K. pneumoniae* are reported in Table 4. Virtually all isolates were resistant to ertapenem, while some were intermediate or susceptible to the other carbapenems. A susceptible or intermediate phenotype to imipenem and/or meropenem was observed with most of the VIM producers, with the OXA-48 producers and with the non-carbapenemase producers, while most of the KPC producers were resistant also to these drugs.

Molecular epidemiology and susceptibility to non-beta-lactam agents of KPC-producing *Klebsiella pneumoniae*

To gather information on the molecular epidemiology of KPC-KP circulating in Italy, the 204 KPC-KP isolates were characterised by MLST and by PFGE genotyping, and their KPC allelic variants were determined by sequencing.

TABLE 3

Proportions of carbapenem-non-susceptible *Klebsiella pneumoniae* from different clinical sources, Italy, 15 May–30 June 2011 (n=1,346)

Source	Isolates from inpatients[a]		Isolates from outpatients[b]	
	Total	CNS-KP (%)	Total	CNS-KP (%)
Blood	179	40 (22.3)	7	0
Lower respiratory tract	219	40 (18.3)	38	0
Urine	647	93 (14.4)	503	15 (3.0)
Other	301	46 (15.3)	70	0
Total	**1,346**	**219 (16.3)**	**618**	**15 (2.4)**

CNS-KP: carbapenem-non-susceptible *Klebsiella pneumoniae*.

[a] Mechanisms of resistance: KPC (n=190; 86%);VIM (n=15; 7%); OXA-48 (n=3; 2%); non-carbapenemase producer (n=11; 5%).
[b] Mechanisms of resistance: KPC (n=14; 93%); VIM (n=1; 7%).

TABLE 4

Susceptibility of carbapenem-non-susceptible *Klebsiella pneumoniae* to various carbapenems, Italy, 15 May–30 June 2011 (n=234)

Resistance mechanism	Meropenem					Imipenem					Ertapenem				
	Range	MIC50	MIC90	%S	%R	Range	MIC50	MIC90	%S	%R	Range	MIC50	MIC90	%S	%R
KPC (n=204)	4 to >32	>32	>32	0	94	4 to >32	32	>32	0	96	16 to >32	>32	>32	0	100
VIM (n=16)	2 to >32	2	32	62	19	1 to >32	2	8	50	6	4 to >32	8	>32	0	100
OXA-48 (n=3)	1 to 8	1	1	66	0	0.5 to 8	1	1	66	0	>32	>32	>32	0	100
Non-carbapenemase (n=11)	0.25 to 4	0.5	1	90	0	0.25 to 1	0.5	1	100	0	1 to >32	8	32	0	90
Total CPE (n=223)	1 to >32	>32	>32	6	87	0.5 to >32	>32	>32	5	87	4 to >32	>32	>32	0	100
TOTAL (n=234)	0.25 to >32	>32	>32	10	82	0.25 to >32	32	>32	10	83	1 to >32	>32	>32	0	99

CPE: carbapenemase-producing *Enterobacteriaceae*; MIC: minimum inhibitory concentration; R: resistant; S: susceptible.

MICs in mg/L, interpreted according to EUCAST breakpoints [26]. Percentage of isolates with intermediate susceptibility not shown in the table.

MLST revealed that most isolates belonged in CC 258, either ST-258 or ST-512 (a single locus variant of ST-258), while a minority were ST-101. ST-258 and ST-512 isolates were detected in many centres, with an overall countrywide distribution, while ST-101 isolates were detected in only two centres, where ST-512 was also present (Figure 2). Isolates of ST-258 carried either bla_{KPC-2} or bla_{KPC-3} alleles, while those of ST-512 and of ST-101 carried bla_{KPC-3} and bla_{KPC-2}, respectively (Figure 2).

PFGE genotyping revealed an oligoclonal population of KPC-KP, with a prevalent PFGE profile, named A, consisting of several variants (A0 to A6) and including the isolates of CC-258. In particular, the A0, A4 and A5 variants included isolates of ST-512 producing KPC-3, while the A1 and A2 variants included isolates of ST-258 producing KPC-3, and the A3 and A6 variants included isolates of ST-258 producing KPC-2 (Figure 2). Each variant was detected in multiple centres (Figure 2), confirming the epidemic propensity of this lineage. An additional PFGE profile, named B and consisting of only a few variants (B0 to B2), included the isolates of ST-101 producing KPC-2 and was detected in two centres (Figure 2).

Susceptibility testing of the 204 KPC-KP against non-beta-lactam agents which often retain activity against these strains, revealed that 22.4% were resistant to colistin, 20.9% were non-susceptible to tigecycline, and 15.8% were non-susceptible to gentamicin. Concerning co-resistances, 1.5% of the KPC-KP were non-susceptible to all three drugs, while 6.4% were non-susceptible to tigecycline and colistin, 1% to gentamicin and colistin, and 2.7% to tigecycline and gentamicin. No association was detected between ST and non-susceptibility to colistin or tigecycline, while non-susceptibility to gentamicin was more frequent in isolates of ST-101 (85.8% versus 12.6% in ST-258 or 10% in ST-512, $p<0.05$).

Discussion

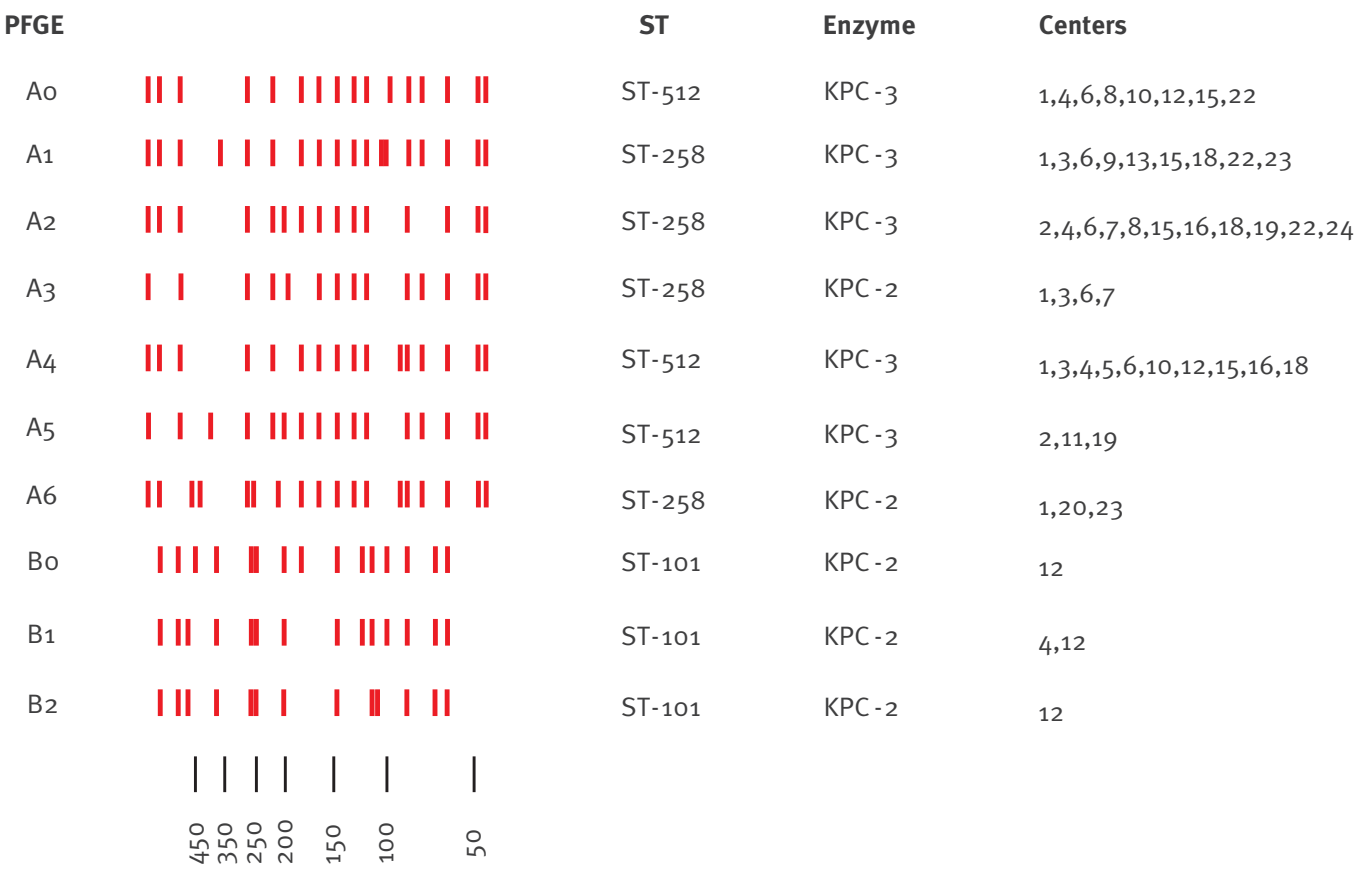
A rapid dissemination of carbapenem-non-susceptible *K. pneumoniae* has been reported in Italy since 2010 [10,24]. Results of this cross-sectional survey, carried out in mid-2011, confirmed that CRE have undergone an epidemic diffusion in Italy, with a widespread distribution across the national territory (although with variable proportions in different areas), and that the phenomenon was mostly related with rapid dissemination of carbapenem-non-susceptible *K. pneumoniae*, while the contribution by CRE of other enterobacterial species was much more limited.

Production of KPC-type carbapenemases was the most prevalent carbapenem resistance mechanism. Production of other carbapenemases and non-carbapenemase-mediated mechanisms were also detected, but remained in the background. However, continuous surveillance should be enforced, considering a recent outbreak caused by NDM-1-producing *K. pneumoniae* [18].

Expansion of KPC-KP strains belonging to variants (ST-258 and ST-512) of the hyperepidemic CC-258, detected for the first time in Italy in late 2008 [13], was responsible for a major part of the CRE epidemic in Italy. A similar

Figure 2

XbaI PFGE profiles of KPC-KP in combination with MLST results and KPC-type alleles, Italy, 15 May–30 June 2011 (n=204)



PFGE: pulsed-field gel electrophoresis; KPC-KP: KPC-type carbapenemase-producing *Klebsiella pneumoniae*; MLST: multi-locus sequence typing.

DNA size standards for PFGE profiles are indicated at the bottom. Distribution by centres of different PFGE-types is also indicated: 1: Milan; 2: Varese; 3: Lecco; 4: Turin; 5: Novara; 6: Genoa; 7: Sanremo; 8: Verona; 9: Bolzano; 10-11: Modena; 12: Florence; 13: Siena; 14: Perugia; 15: Ancona; 16: Rome; 17: Pescara; 18: San Giovanni Rotondo; 19: Lecce; 20: Naples; 21: Avellino; 22: Cosenza; 23: Palermo; 24-25: Catania.

phenomenon was also observed in other countries and further underscores the propensity for dissemination of multi-resistant *K. pneumoniae* strains belonging to ST-258 and ST-512 [40-47]. Emergence of KPC-KP belonging to ST-101 was also observed, although only in two of the participating centres. This finding, along with recent reports of ST-101 isolates of KPC-KP from Italy, Brazil and the United States [21,42,48,49], emphasises the emerging role in dissemination of KPC of this clonal lineage which is also involved in the dissemination of other carbapenemases, such as OXA-48 and OXA-181 [50,51] as well as extended-spectrum beta-lactamases [52,53].

Since aggressive infection control was shown to be effective in controlling the dissemination of KPC-KP [54-56], present results mandate for strong and prompt intervention in Italy. Implementation of infection control measures on a countrywide scale appears now to be necessary, in addition to the actions that have already been taken at local and regional level (the Italian public healthcare system has a typically regional organisation) with positive results. In a hospital in Catania, Sicily, it was possible to control the spread of a KPC-3-producing *K. pneumoniae* clone without closing the ICU, by applying a multimodal infection control programme [16]. The Emilia-Romagna region issued in July 2011 guidelines and protocols to monitor and control the spread of carbapemenase-producing *Enterobacteriaceae* in all the healthcare structures of the region. The increasing trend of KPC-producing *K. pneumoniae* slowed down in the second half of 2011 and early 2012, and in hospitals that had been able to implement all control activities the number of cases had a remained stable or showed a sustained decrease [57]. Dissemination of carbapenem-non-susceptible *K. pneumoniae* was not restricted to ICU settings, but affected all major hospital sectors. Although at least some of these patients could have been transferred from ICU settings, this propensity to dissemination on

multiple wards should be considered when planning infection control strategies.

Although cases and outbreaks of CRE have been reported in many European countries [6], only Greece, Cyprus and Italy have experienced such an extensive CRE epidemic to date. However, the phenomenon observed in these countries deserves considerable attention by public health authorities in Europe, considering the high mobility of people (tourists, workers, patients) within Europe, and the difficulties in containing the epidemic diffusion of CRE.

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Participants of the AMCLI-CRE Survey (the first Italian survey on carbapenem-resistant *Enterobacteriaceae*, promoted by the Italian Society of Clinical Microbiologists)

Milan, Niguarda Ca' Granda Hospital - C. Vismara; Varese, Circolo and Fondazione Macchi University Hospital - A. Colombo; Turin, S. Giovanni Battista "Molinette" Hospital - R. Serra; Lecco, A. Manzoni Hospital – F. Luzzaro; Novara, Maggiore Hospital - V. Kroumova; Genoa, Galliera Hospital - M. Mori; Sanremo, Azienda Sanitaria Imperiese Hospital - P. A. Dusi; Verona, Borgo Roma University Hospital - A. Mazzariol; Bolzano, Azienda Sanitaria dell'Alto Adige Hospital – E. Pagani; Modena, S. Agostino Estense Hospital - R. Gargiulo; Modena, Modena University Hospital - C. Venturelli; Firenze, Careggi University Hospital - P. Pecile; Siena, Santa Maria alle Scotte University Hospital – G.M. Rossolini; Perugia, Santa Maria della Misericordia University Hospital - A. Mencacci; Ancona, ''Torrette'' University Hospital - E. Manso; Rome, San Camillo Forlanini Hospital - M. Tronci; Pescara, Santo Spirito Hospital - P. Fazii; San Giovanni Rotondo, IRCCS Casa Sollievo della Sofferenza Hospital - M. Labonia; Lecce, V. Fazzi Hospital - M. Pizzolante; Neaples, A. Cardarelli Hospital - G. Amato; Avellino, San Giuseppe Moscati Hospital - G. Buonopane; Cosenza, Annunziata Hospital - P. Cavalcanti; Palermo, Mediterranean Institute of Transplantation (ISMETT) - P.G. Conaldi; Catania, Policlinico and Vittorio Emanuele University Hospitals - S. Stefani; Catania, Policlinico Hospitals - F. Di Vincenzo.

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