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Evaluation of carbapenem-resistant *Enterobacteriaceae* in an Italian setting: Report from the trench

D. Barbarini [a], G. Russello [b], F. Brovarone [b], C. Capatti [b], R. Colla [c], M. Perilli [d], M.L. Moro [e], E. Carretto [b,*]

[a] Clinical Virology and Microbiology Laboratory – Fondazione IRCCS Policlinico San Matteo, Pavia, Italy
[b] Clinical Microbiology Laboratory – IRCCS Arcispedale Santa Maria Nuova, Reggio Emilia, Italy
[c] Laboratory of Analysis Area Nord, AUSL Reggio Emilia, Italy
[d] Department of Science and Biomedical Technologies, University of L'Aquila, Italy
[e] Area di Programma Rischio Infettivo – Agenzia Sanitaria e Sociale Regione Emilia Romagna, Italy

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ABSTRACT

The spread of carbapenem resistant Enterobacteriaceae (CRE) has recently become a matter of concern in public health, mainly due to the wide distribution of carbapenemase genes. Italy is a country considered endemic for the spread of bla_{KPC} *Klebsiella pneumoniae* (KP). The aim of this study was to depict the epidemiological trend of CRE in one Italian hospital over a long period (3 years surveillance, from May 2011 to April 2014). Based on defined MIC cut-off for specific carbapenems, 164 strains isolated from 146 different patients were analyzed both phenotypically and genotypically to establish the resistance genes. Molecular typing was performed using the RAPD technique. 77 strains were demonstrated to harbor the bla_{KPC} gene (73 KP, 4 *Escherichia coli* – EC), 51 strains the bla_{VIM} gene (44 KP, 3 EC, 2 *Enterobacter cloacae* and 2 *Klebsiella oxytoca*), 8 the bla_{NDM} gene (3 KP, 4 EC and one *Providencia stuartii*), 3 the bla_{OXA-48} gene (2 KP, 1 EC), whereas 25 out of the 164 isolates (of different genera and species) had a negative multiplex-PCR amplification for all the targets tested. 39 out of the 164 strains analyzed (23.8%) revealed discrepancies between the MICs obtained with automated instrument and gradient MICs of more than two logs of difference; the broth microdilution provided a better agreement with the results obtained with the gradient MIC. The use of RAPD allowed to distinguish different clusters, closely related, both for bla_{KPC} and for bla_{VIM} KP.

1. Introduction

The spread of carbapenem resistant *Enterobacteriaceae* (CRE) has recently become a matter of concern in public health. The pattern of resistance is mainly due to the dissemination of carbapenemase genes. The spread of these multi-drug resistant organisms appears even more striking considering that the first identification of CRE took place almost 20 years ago (USA, 1996) and became public knowledge only in 2001 (Yigit et al., 2001). To date, three main classes of carbapenemases have been identified (Queenan and Bush, 2007). Ambler class A beta-lactamases are enzymes that can be either chromosomally encoded (bla_{NMC}, bla_{SME}, bla_{IMI-1}, bla_{SFC-1}) or plasmid encoded (bla_{KPC} and, less common, bla_{IMI-2}, bla_{GES}). Verona integron-encoded metallo-β-lactamase (bla_{VIM}), bla_{IMP}, and the New Delhi metallo-β-lactamase (bla_{NDM}) belong to class B metallo-β-lactamases (MBLs). The characterization of bla_{NDM} was firstly published in December 2009 (Yong et al., 2009) and its presence was then detected in almost the entire world (Cornaglia et al., 2011). Bla_{OXA-48} carbapenemases belong to Ambler class D. Finally, of somehow minor impact and perhaps underestimated, resistance to carbapenems can also be caused by other mechanisms, such as hyperexpression of *AmpC* gene or to decreased permeability of the outer membrane because of porin loss in combination with the expression of AmpC enzymes or ESBLs.

The origin of carbapenemase genes was well-defined from the geographical point of view: KPC β-lactamases were first observed in USA (Yigit et al., 2001) but are nowadays widely diffused worldwide, particularly in Greece (Tsakris et al., 2008) and Italy (Canton et al., 2012), VIM gene was firstly described in Italy (Lauretti et al., 1999), NDM enzyme was firstly isolated in Sweden in a patient with a prolonged stay in India, then in the UK and worldwide (Kumarasamy et al., 2010; Poirel et al., 2010; Yong et al., 2009),

* Corresponding author at: Clinical Microbiology Laboratory – IRCCS Arcispedale Santa Maria Nuova – Viale Risorgimento, 80 – 42123 Reggio Emilia, Italy. Tel.: +39 0522296363; fax: +39 0522296750.
E-mail address: edoardo.carretto@asmn.re.it (E. Carretto).

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OXA-48 have been identified mostly in the Mediterranean and southern European countries with a rapid spread in France and Turkey (Nordmann, 2014; Poirel et al., 2012).

Although a number of bacterial species can harbor carbapenemase genes, they are most commonly described in *Klebsiella pneumoniae* (KP) isolates, which can express different resistance mechanisms such as bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48} and bla_{IMP}, due to plasmid distribution.

The spread of CRE has dramatically increased in Italy since 2009. At that time, the percentage of invasive isolates of KP resistant to carbapenems was 1.3%, rising to more than 10% in 2010 and reaching the rate of 34.3% in 2013 (European Centre for Disease Prevention and Control, 2010, 2014).

Recently, Monaco et al. pointed out that colistin resistance has emerged in Italy as an overlapping topic in carbapenem-resistant KP, making the problem of the carbapenemase-producing Enterobacteriaceae (CPE) even more alarming (Monaco et al., 2014).

After a number of different KPC+ KP outbreaks in different hospitals in our region (Emilia-Romagna, Italy), guidelines to control and prevent the further spread of CPE, were developed by the Infectious Risk Unit of the Agenzia Sanitaria e Sociale Regionale: these were based on the most recent scientific evidence and were applied from August 2011 (Gagliotti et al., 2011). This centrally-coordinated intervention lead to a partial containment of the spread of CPE in the Region (Gagliotti et al., 2014). The epidemiological trend of CRE, within one of the main Emilia-Romagna hospitals which took part in this region-wide intervention, is described below.

2. Materials and methods

2.1. Setting and study period

The study was performed in the Clinical Microbiology Laboratory of the IRCCS Arcispedale Santa Maria Nuova of Reggio Emilia, Italy. The Laboratory is located in Reggio Emilia, the fourth largest city in the region Emilia-Romagna. It works as a reference laboratory for the detection and confirmation of CPE for the main Hospital (923 acute care beds) and for a number of smaller district general hospitals in Reggio Emilia province. The province has an area of 2292.89 km^2 (885.05 mi^2), and a total population of 536.618 inhabitants. Geographical details are provided as KML file in the Supplemental material. The study was carried out in a 3-year period, from May 2011 to April 2014, for an overall duration of 36 months.

2.2. Study population and bacterial samples

During the study period all strains of *Enterobacteriaceae* showing reduced susceptibility to carbapenems, defined as strains with ertapenem MICs $\geqslant$ 1 mcg/ml, and/or imipenem MICs $\geqslant$ 2 mcg/ml and/or meropenem MICs $\geqslant$ 2 mcg/ml, were further characterized using phenotypic tests. The isolates were collected from both clinical samples and from surveillance screening (rectal swabs). Basic clinical information of the patients were collected. The molecular analysis was performed only on the first strain isolated from any patient per year (regardless of the site of isolation), or in case of isolation of different CPE genera or species in the same patient. Isolates from the same patient after more than 1 year were analyzed with molecular techniques as well. For molecular analysis, five reference strains known to harbor carbapenemase genes were included as positive internal quality control for molecular testing (bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48} and bla_{IMP}). These strains were collected from the external quality assessment services (UK NEQAS) or from previously published studies (Gaibani et al., 2011).

2.3. Phenotypic characterization and susceptibility to carbapenems

In order to initially define the presence and type of the carbapenemases involved, we used disk diffusion synergy tests for detection of MBL and KPC (meropenem disks supplemented with aminophenylboronic acid, dipicolinic acid and cloxacillin (Rosco, Denmark) (Doyle et al., 2012). A suspension of 0.5 McFarland of each strain was used to inoculate a plate of Mueller-Hinton agar and left to dry; disks of meropenem alone and with combinations as above were placed into the agar afterwards. In order to improve the detection of bla_{OXA-48} strains, we also implemented the test using a disk of 30 μg disk of temocillin placed in the center of the plate. The results were read using the manufacturer's indication (based on the enlargement of the zone of inhibition towards the meropenem disks with supplements). Lack of inhibition around temocillin with a negative synergy test for meropenem – meropenem/dipicolinic acid was considered suggestive of an OXA-48 positive strain. ESBL resistance was confirmed using the procedure suggested by CLSI (CLSI, 2011). AmpC presence was established by synergy test using ertapenem versus combinations ertapenem + boronic acid and ertapenem + cloxacillin (test provided by Liofilchem, Italy – unpublished data). For all strains, antimicrobial susceptibility to carbapenems was evaluated using an automated instrument, Vitek2 (BioMérieux, France); gradient MIC was assessed by using MIC test strips (Liofilchem, Italy). The broth microdilution method, performed according to the recommendations of CLSI (CLSI, 2011), was used to correctly define strains showing a difference of more than two logs with the two above tests.

2.4. Molecular analysis of the carbapenemase genes

Genomic DNA was extracted using the UltraClean™ microbial DNA isolation kit (MoBio Laboratories, USA) according to the manufacturer's instructions. A multiplex PCR (based on a slight modification of the protocol of Poirel et al.) was used on all isolates to detect five carbapenemase genes (bla_{KPC}, bla_{VIM}, bla_{NDM}, bla_{OXA-48} and bla_{IMP}) (Poirel et al., 2011). The following modifications were applied to the original procedure: reactions were performed as single multiplex PCR reaction in a final volume of 25 μl, the Taq used was AmpliTaq (Applied Biosystems, USA) and primers used for amplification of bla_{KPC}, which provided a 893 bp band, were: forward (5′–3′): ATGTCACTGTATCGCCGTCT and reverse (5′–3′): TTTTCAGACCCTTACTGCCC. All different experiments were performed using internal controls for the five different genes.

2.5. Molecular typing

Selected KP strains were analyzed for typing purposes using both the Enterobacterial Repetitive Intergenic Consensus (ERIC-PCR) and the random amplification of polymorphic DNA (RAPD). The most discriminating methodology was then applied to type the different KP isolates. The protocol described by Cartelle et al. was used for ERIC-PCR, without modifications (Cartelle et al., 2004). Five different primers were used for RAPD, four of them originally used to type staphylococci (Damiani et al., 1996). The primers used are listed in Table 1.

Amplification reactions were performed in a 25 μl volume containing reaction buffer, 5 mM $MgCl_2$, 10 mM dNTPs, 1 μM of each single primer, 40 ng of DNA and 5 U/μl of Taq Polymerase (Amplitaq™, Applied Biosystems, Thermo Scientific USA). After 5 minutes of denaturation at 95 °C, RAPD amplifications were cycled 35 times through the following temperature profile: 95 °C – 30 s; 45 °C – 1 min and 72 °C – 2 min, followed by an elongation phase at 72 °C for 10 min. For the primer ED1, the annealing temperature was set at 38 °C. For gel electrophoresis, 5 μl of each amplification product were loaded onto a 2% (w/v) agarose gel.

Table 1
Primers used in this study.

NP2: 5′-CGGGGGACTGTTGGGCGCCATCT-3′
NP3: 5′-GAAGCAGCCCGGTAGTAGGTTGAG-3′
NP4: 5′-CTAATGCAGGAGTCGCATAAGGGAGA-3′
NP5: 5′-AGCGCTGTGAGAAAGATGA-3′
ED1: 5′-CCGTCGGAGCC-3′

Two profiles were considered different if they showed a single band of diverse size, regardless of the intensity of the bands, according to the ESGEM guidelines (van Belkum et al., 2007). PCR products were then stored at −20 °C.

3. Results

3.1. Study population and bacterial isolates

During the surveillance period 218 CRE strains from 146 different patients were isolated, both from clinical samples and from surveillance screening. 183 of the 218 isolates were identified as KP, 19 were *Escherichia coli* (EC), 10 *Enterobacter cloacae*, 2 *Klebsiella oxytoca*, 2 *Enterobacter aerogenes*, 1 *Citrobacter freundii* and 1 *Providencia stuartii*. 122 strains were isolated from rectal swabs, 65 from urinary samples, 14 from blood cultures, 8 from wounds, 7 from respiratory samples and 2 from indwelling catheters.

164 out of the 218 isolates were further analyzed with molecular techniques according to the eligibility criteria; they are summarized in Table 2.

The temporal distribution per month (i.e. the time of the first isolation) of the KPC+ and VIM+ isolates is shown in the Fig. 1. At least three small outbreaks can be identified: in August 2011, sustained mainly by VIM+ KP, in February 2012, sustained mainly by KPC+ KP, as well as it happened in February 2014.

Among the 146 patients, 121 were colonized/infected by CPE and 25 by strains with reduced susceptibility to carbapenems due to other mechanisms.

Seventeen different episodes of infections were documented: 14 bacteraemias (among them, in 4 cases the strains were also isolated from urinary samples, in 1 case from BAL and in 1 case from vascular prosthetic material), 2 wound infections (WI) and 1 lower respiratory tract infection (LRTI). The 14 episodes of bacteraemias were sustained in 7 cases by VIM+ KP, in 4 cases by KPC+ KP, in 3 cases by *E. cloacae* with AmpC hyper-expression; the two WI by a NDM+ KP and a KPC+ KP, whereas the LRTI by a KPC+ KP. We therefore documented 14 infections due to carbapenemase producers strains in 121 patients (11.6%). All these patients have been previously colonized by the strain which caused the infection.

3.2. Phenotypic characterization and susceptibility to carbapenems

The disk diffusion synergy tests detected 77 possible KPC strains (synergy only with the association meropenem – boronic acid). 55 cases showed synergy only with the association meropenem-dipicolinic acid (defining MBL-producers strains). Temocillin showed an inhibition zone of more than 12 mm in 74 out of the 77 potential KPC isolates and only in one MBL positive isolate. Among the 29 strains that did not show any synergistic effect with the disk diffusion test, 7 were resistant to temocillin: 3 of those strains proved to be bla_{OXA-48} positive, while 4 were not amplified by the multiplex PCR used. The ESBL phenotype was detected in 14 cases, whereas in 11 cases a synergy for both ertapenem/boronic acid and ertapenem/cloxacillin versus ertapenem alone confirmed the strain as being AmpC positive. These strains were usually only

Table 2
Study synopsis. Legend: KPC = strains harboring the bla_{KPC} gene; VIM = strains harboring the bla_{VIM} gene; NDM = strains harboring the bla_{NDM} gene; OXA-48 = strains harboring the bla_{OXA-48} gene; IMP = strains harboring the bla_{IMP} gene; AmpC + PL = strains with expression (or hyper-expression) of the AmpC determinant, possibly combined with porin loss or efflux pumps; ESBL + PL = strains expressing the extended-spectrum beta-lactamase resistance profile, possibly combined with porin loss or efflux pumps.

Microorganisms	Resistance determinants							Total
	KPC	VIM	NDM	OXA-48	IMP	AmpC + PL	ESBL + PL	
Klebsiella pneumoniae	73	44	3	2	0	0	7	129
Escherichia coli	4	3	4	1	0	0	7	19
Enterobacter cloacae	0	2	0	0	0	8	0	10
Klebsiella oxytoca	0	2	0	0	0	0	0	2
Enterobacter aerogenes	0	0	0	0	0	2	0	2
Citrobacter freundii	0	0	0	0	0	1	0	1
Providencia stuartii	0	0	1	0	0	0	0	1
Total	77	51	8	3	0	11	14	164

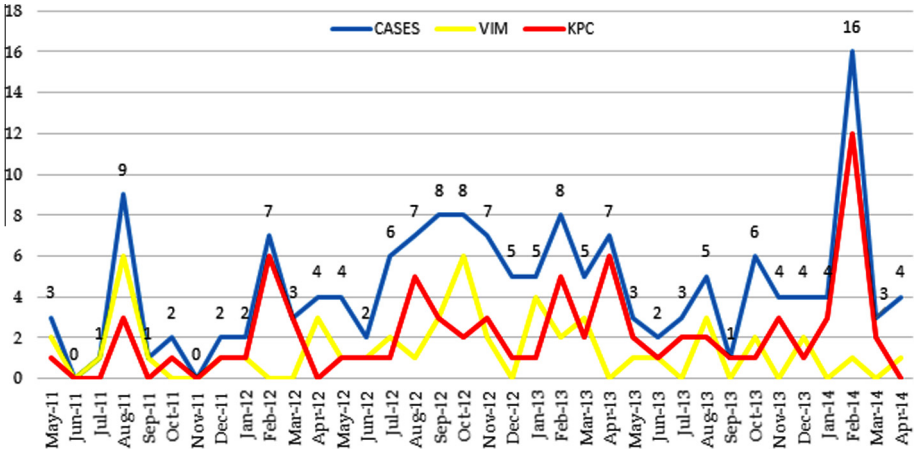

Fig. 1. Temporal distribution of the KPC+ and VIM+ isolates analyzed in the study.

Table 3
MICs obtained for the different strains analyzed.

	Imipenem MICs (Vitek2)							Imipenem MICs (E-test strips)							
	⩾16	8	4	2	1	0.5	⩽0.25	⩾32	16	8	4	2	1	0.5	⩽0.25
KPC	58	13	2	3	1	0	0	58	8	4	3	4	0	0	0
VIM	28	13	10	0	0	0	0	22	5	9	9	5	1	0	0
NDM	7	0	0	0	0	0	0	5	0	1	1	0	0	0	0
OXA-48	3	0	0	0	0	0	0	0	0	1	1	0	1	0	0
OXA-48 + NDM	1	0	0	0	0	0	0	0	0	0	1	0	0	0	0
AmpC + PL	1	0	0	1	2	3	4	0	0	0	2	1	3	3	2
ESBL + PL	0	0	1	1	3	5	4	0	1	0	1	1	6	2	3
	Meropenem MICs (Vitek2)							Meropenem MICs (E-test strips)							
	⩾16	8	4	2	1	0.5	⩽0.25	⩾32	16	8	4	2	1	0.5	⩽0.25
KPC	68	0	0	5	4	0	0	58	5	8	1	4	1	0	0
VIM	38	0	2	3	8	0	0	13	6	5	9	9	6	1	2
NDM	6	1	0	0	0	0	0	4	0	2	1	0	0	0	0
OXA-48	1	0	0	0	2	0	0	0	0	0	0	1	2	0	0
OXA-48 + NDM	1	0	0	0	0	0	0	0	0	0	0	0	1	0	0
AmpC + PL	1	0	0	1	0	0	9	0	0	0	1	0	0	3	7
ESBL + PL	0	1	1	5	6	1	0	0	0	1	0	8	2	2	1
	Ertapenem MICs (Vitek2)							Ertapenem MICs (E-test strips)							
	=	⩾8	4	2	1	⩽0.5	=	⩾32	16	8	4	2	1	0.5	⩽0.25
KPC	=	69	6	2	0	0	=	57	9	5	4	2	0	0	0
VIM	=	23	21	2	0	5	=	18	4	6	12	7	3	0	1
NDM	=	7	0	0	0	0	=	4	3	0	0	0	0	0	0
OXA-48	=	2	1	0	0	0	=	1	0	2	0	0	0	0	0
OXA-48 + NDM	=	1	0	0	0	0	=	0	0	1	0	0	0	0	0
AmpC + PL	=	2	3	4	1	1	=	1	0	0	1	6	3	0	0
ESBL + PL	=	11	3	0	0	0	=	6	1	4	3	0	0	0	0

Table 4
Comparison of MICs obtained with different methods. IC = internal laboratory code; BM = broth microdilution technique.

Strain IC	Isolate	Resistance	Meropenem MICs (mcg/ml)		
			Vitek-2	MIC test strip	BM CLSI
4	*Klebsiella pneumoniae*	VIM	1	4	4
10	*Klebsiella pneumoniae*	KPC	1	8	4
14	*Klebsiella pneumoniae*	KPC	2	16	4
22	*Klebsiella pneumoniae*	KPC	2	8	8
23	*Escherichia coli*	NDM	8	>32	8
30	*Klebsiella pneumoniae*	KPC	2	16	8
35	*Klebsiella pneumoniae*	KPC	2	32	4
49	*Escherichia coli*	VIM	⩾16	0.25	2
57	*Klebsiella pneumoniae*	VIM	⩾16	4	4
63	*Klebsiella pneumoniae*	VIM	⩾16	8	2
78	*Klebsiella oxytoca*	VIM	⩾16	4	0.5
83	*Klebsiella oxytoca*	VIM	⩾16	2	0.5
95	*Klebsiella pneumoniae*	VIM	⩾16	1	4
96	*Klebsiella pneumoniae*	VIM	⩾16	2	4
104	*Klebsiella pneumoniae*	NDM	⩾16	8	32
111	*Klebsiella pneumoniae*	VIM	⩾16	2	4
117	*Enterobacter cloacae*	VIM	⩾16	4	4
120	*Klebsiella pneumoniae*	VIM	⩾16	8	4
123	*Klebsiella pneumoniae*	KPC	⩾16	8	4
129	*Klebsiella pneumoniae*	VIM	⩾16	4	4
134	*Klebsiella pneumoniae*	VIM	⩾16	8	8
137	*Klebsiella pneumoniae*	KPC	⩾16	8	8
139	*Klebsiella pneumoniae*	KPC	⩾16	8	8
140	*Klebsiella pneumoniae*	KPC	⩾16	8	8
141	*Klebsiella pneumoniae*	KPC	⩾16	8	4
143	*Klebsiella pneumoniae*	VIM	⩾16	2	8
158	*Klebsiella pneumoniae*	NDM	⩾16	4	8
159	*Klebsiella pneumoniae*	VIM	1	0.25	8
163	*Klebsiella pneumoniae*	VIM	⩾16	1	4
174	*Klebsiella pneumoniae*	VIM	⩾16	4	4
184	*Klebsiella pneumoniae*	neg	8	2	8
187	*Klebsiella pneumoniae*	KPC	⩾16	2	2
188	*Klebsiella pneumoniae*	VIM	⩾16	8	32
190	*Enterobacter cloacae*	VIM	⩾16	2	4
193	*Klebsiella pneumoniae*	KPC	⩾16	4	4
197	*Klebsiella pneumoniae*	KPC	⩾16	8	4
212	*Klebsiella pneumoniae*	VIM	⩾16	2	2
218	*Escherichia coli*	OXA-48 + NDM	⩾16	1	4
219	*Enterobacter aerogenes*	neg	⩾16	4	8

resistant to ertapenem (only one of them also exhibited resistance to both imipenem and meropenem) and for them hyperexpression of the AmpC gene was supposed, eventually combined with a porin loss.

The MICs of the analyzed strains are shown in Table 3. As expected, all the bla_{KPC} isolates showed MICs for carbapenems higher compared with those obtained for strains harboring MBL genes.

39 out of the 164 strains showed a difference of more than 2 logs between Vitek2 and gradient MIC for meropenem and/or the other carbapenems. To evaluate the meropenem susceptibility for these strains, they were further characterized using the broth microdilution technique, which appeared to have a better agreement with the data provided by the gradient MIC technique (Table 4).

3.3. Molecular analysis of the carbapenemase genes

An example of the products of amplification obtained is shown in Fig. 2. The molecular analysis detected 77 strains harboring the bla_{KPC} gene (73 KP, 4 EC), 51 strains harboring the bla_{VIM} gene (44 KP, 3 EC, 2 *E. cloacae* and 2 *K. oxytoca*), 8 strains with the bla_{NDM} gene (3 KP, 4 EC and one *P. stuartii*), 3 strains with the bla_{OXA-48} gene (2 KP, 1 EC). Interestingly, the 8 strains expressing bla_{NDM} were only isolated from 4 different patients: two had a contemporary isolation of KP + EC, one had a single KP strain and in one patient we isolated KP + EC + *P. stuartii* (one EC harboring both bla_{NDM} and bla_{OXA-48}).

25 out of the 164 isolates (8 *E. cloacae*, 7 KP, 7 EC, 2 *E. aerogenes* and 1 *C. freundii*) had a negative multiplex-PCR amplification for all the five targets tested.

3.4. Molecular typing

In a preliminary phase, 10 different clinical KP strains and an internal control (i.e. a reference strain from UK NEQAS, which we reasonably assumed to be unrelated to the strains we analyzed) were studied using both ERIC-PCR and RAPD-PCR with five different primers. The latter technique was able to provide a better discrimination compared with the ERIC-PCR; for this reason, we used this method to analyze the KP strains.

Considering the 73 KP-KPC+ strains, among the five different primers used NP5 appeared to be the most discriminant. This primer allowed the generation of 8 different fingerprints (Fig. 3). The largest cluster of strains accounted for 54 isolates, and four profiles were characteristic for a single strain. Strains belonging to the main cluster were isolated during all the study period. The different fingerprints were quite similar, with few polymorphic bands.

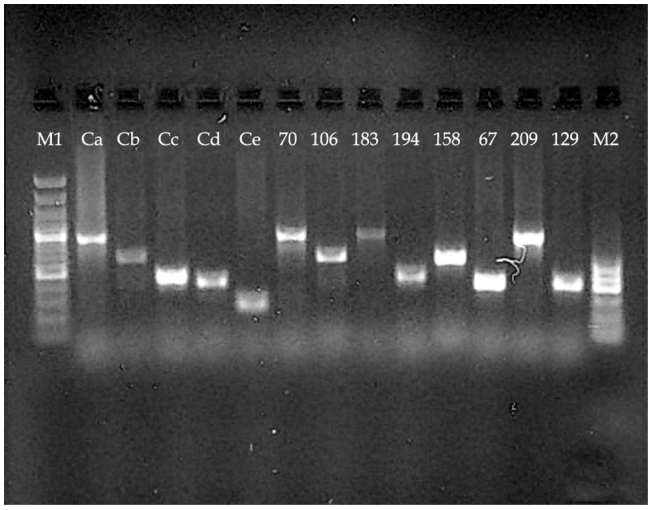

Fig. 2. The multiplex PCR performed in the study. Legend: M1 = molecular markers, 100–3000 bp; Ca = KPC+ control; Cb = NDM+ control; Cc = OXA-48+ control; Cd = VIM+ control; Ce = IMP+ control. Other lines: #70, #183, #209 KPC+ isolates; #106, #158 NDM+ isolates; #194 OXA-48+ isolate; #67 e #129 VIM+ isolates; M2 = molecular markers, 100–600 bp.

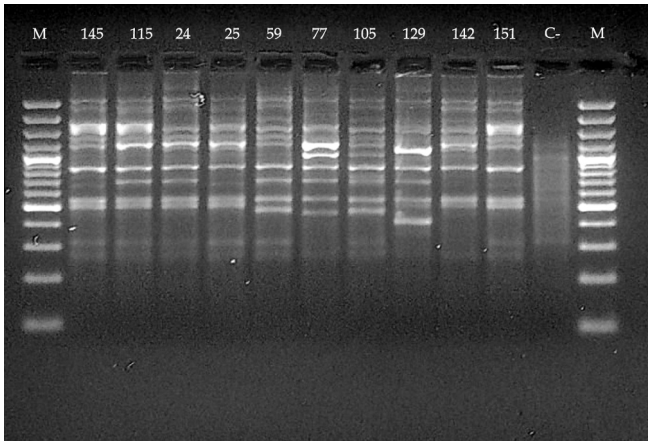

Fig. 3. RAPD profiles of KPC positive *Klebsiella pneumoniae* using primers NP5. Lanes 1 and 13, M = molecular markers, 100–3000 bp; lanes 2–11, the different profiles obtained (number = strains' label); lane 12, negative control. The profiles #24 and #129 belong to two internal controls (i.e., strains previously supposed to be unrelated with those analyzed).

The most discriminant primers for the 44 KP-VIM+ strains were NP3 and NP5, which allowed to cluster the isolates in 6 different group (with a total agreement in clustering for the two primers). The largest group of KP-VIM+ isolates comprised 26 strains, with a temporal distribution from July 2011 to June 2013.

4. Conclusions

The study assessed the epidemiology of CRE over a 3-year period in a relatively small geographical Italian province. Italy is well known as a country where KP-KPC have spread widely and nowadays is experiencing a situation of endemicity. Recently, a paper of Gaiarsa et al. evaluated, from the genetic point of view, the epidemiology of *K. pneumoniae* in Italy; some of the KP strains harboring the bla_{KPC} gene examined in that paper were also analyzed in the present study and were clustered with other strains isolated from different Italian settings (Gaiarsa et al., 2014).

On the other hand, carbapenemase genes different from bla_{KPC} have so far been considered less of a problem, whose magnitude has perhaps not been fully assessed, with only sporadic reports being published (Gaibani et al., 2013; Kocsis et al., 2013). As an example, although VIM carbapenemase was firstly recognized in Italy (Lauretti et al., 1999), the exact magnitude of its distribution in our country is still difficult to establish.

The circulation of different carbapenemase genes was hereby well documented: in our study population 4 different genes were found (bla_{KPC} was detected in 46.9% of the isolates, bla_{VIM} in 31.9%, bla_{NDM} in 4.3%, bla_{OXA-48} in 1.8%). No IMP genes were detected in our study population. In 25 out of the 164 isolates (15.2%) the decreased susceptibility to carbapenems (mostly to ertapenem) was due to resistance mechanisms different from carbapenemase production.

The presence of carbapenemase was prevalent in KP, as expected; and in this microorganism, the bla_{KPC} gene was predominant. However other genera and species of *Enterobacteriaceae* were represented, harboring different resistance genes (Table 2).

The high reliability of the phenotypic assays (disk synergy testing) was remarkable, since no discrepancies with the molecular tests were found. In particular, the synergy with boronic acid was so convincing that in our opinion the test appears to be strong enough to define the strain as bla_{KPC} producer without having to resort to molecular testing.

By contrast, synergy testing with dipicolinic acid, although very effective in our experience, cannot be considered valid enough for surveillance purposes, as it is not able to discriminate among bla_{VIM} and bla_{NDM} strains. In an endemic setting it is therefore necessary to implement molecular analysis, perhaps in a second level center, at least on a weekly basis.

Temocillin proved to be a useful surrogate marker of bla_{OXA-48}, but should always be used taking into account all other synergistic effects. In particular, resistance to temocillin could be considered a marker for bla_{OXA-48} only for strains with no other evident synergies and the test should always be confirmed further (by growth on selective media and by molecular biology as gold standard).

The molecular approach is also necessary to demonstrate the contemporary presence of more than one resistance genes. A patient included in the study had multiple injuries after a car crash and in a three-month period we isolated in sequence a KP with a bla_{OXA-48}, an EC harboring both bla_{OXA-48} and bla_{NDM}, a *P. stuartii* with bla_{NDM} and finally another EC with only bla_{NDM}. He was a native of North Africa with a recent transfer to Italy and this would explain the presence of these carbapenemase, which are unusual in our country.

In our setting, resistance to carbapenems is mediated by production of carbapenemases as primary mechanism; however, decreased susceptibility (mostly to ertapenem) can be detected and is probably due to overexpression of AmpC, or sometimes to a combination of AmpC and ESBL trait along with outer membrane porin loss or hyper-expression of efflux pumps. Further phenotypic assays are required to better define this situation. Molecular analysis should be always performed on these isolates: in the present study two *Enterobacter cloacae*, well known to exhibit AmpC resistance, also harbored the bla_{VIM} gene.

14 out of the 121 patients with CPE developed infections, which always happened in patients known to be colonized, as showed by routine screening with rectal swabs. This low percentage of infection, compared with other data of the literature, is most likely due to active surveillance, which detects a wide number of colonized patients and not only infected patients which are the tip of the iceberg. Molecular typing using the RAPD technique on the strains isolated from surveillance and from diagnostic cultures in infected patients showed always the same fingerprint, indirectly demonstrating that infections always followed a previous colonization. Interestingly, half of the bacteraemias were sustained by VIM+ KP.

A number of papers have recently stressed the clinical importance of carbapenems in treating infections due to CPE (Petrosillo et al., 2013; Tzouvelekis et al., 2012). MIC values are important predictors of carbapenem efficacy; nevertheless, it is well known that automated instruments could fail in correctly evaluating the correct MICs of these microorganisms (Tenover et al., 2006; Woodford et al., 2010). In this paper we demonstrated that in 39 out of the 164 strains analyzed (23.8%) there were major discrepancies between the MICs obtained with automated instrument and gradient MICs (i.e. more than two logs of difference), particularly for MBL genes. The broth microdilution technique was in a better agreement with the results obtained with the gradient MIC, providing the same value in 13 cases (33.3%) or within one log of difference in 11 cases (28.2%). This should be taken into consideration while pondering over treatment options. Confirmatory techniques are therefore required before reporting carbapenem MICs values in CPE infections. It is interesting to note that all the KP strains analyzed in the present study were susceptible to colistin, and that the problem emphasized by Monaco et al. does not seem to have an impact in our setting (at least for the moment) (Monaco et al., 2014).

Regarding the molecular typing, we are aware of some limitations in our study. MLST have not been performed on the strains and RAPD are difficult to be evaluated and compared. The decision to perform this kind of analysis was based on the fact that the geographical setting was not particularly big and we postulated the circulation of ST258 and ST512, as demonstrated in our country (Giani et al., 2009, 2013); moreover, Deleo et al. recently demonstrated that carbapenem-resistant ST258 KP is a single genetic clone that has disseminated worldwide (Deleo et al., 2014). The paper of Gaiarsa et al. also showed a very high homogeneity of strains isolated in different Italian institutions and five of the KP-KPC+ strains investigated in that paper have been analyzed in the present study (Gaiarsa et al., 2014). Finally, the main aim of the study was to assess the strains distribution for local epidemiological purposes. In our experience, RAPD typing proved to be more discriminatory compared to the ERIC-PCR technique. In order to minimize any potential problem related to RAPD analysis and particularly to reduce the variables due to the evaluation of different strains in different tests, we performed the experiments every 2 weeks and in every new session we included those strains which gave different profiles in previous experiments (starting from the DNA stored from the first isolation). The variability in the fingerprints we obtained was indeed expected for strains carrying the bla_{VIM} gene, whereas more surprising considering the bla_{KPC} strains. All the fingerprints we obtained were highly similar, although with some polymorphic bands that allowed to differentiate the strains. The major group of KPC-KP consisted of 54 isolates and the clusters obtained were congruent with the temporal distribution of the cases (i.e., in the two outbreaks we documented the presence of the same RAPD-type).

Nonetheless, our molecular typing results clearly emphasize the idea that, for surveillance protocols, every setting should perform this kind of molecular analysis to trace the microorganisms, in order to better understand if the control procedures in place are adequate or ineffective.

Italy is a country where the problem of CPE has a major impact – a situation which some Authors correctly describe as the epidemiological "Italian scenario" (Nordmann, 2014). The present study provides information about the magnitude of the problem in a relatively small area, covering different aspects of the problem from the microbiological point of view. The variability in the resistance genes found, as well as the genetic variability of the fingerprint patterns obtained, and the problems in the correct evaluation of MICs values for carbapenems revealed a multifaceted reality, which should be known and correctly managed in the different settings, especially in endemic situations or during outbreaks.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.meegid.2014.

11.025. These data include Google maps of the most important areas described in this article.

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