KPC-Like carbapenemase-producing enterobacteriaceae colonizing patients in Europe and Israel

KPC-like carbapenemase-producing *Enterobacteriaceae* colonizing patients across Europe and Israel

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In a 2008-11 survey, 17,945 patients in 18 hospital units in Europe and Israel were screened for carriage of KPC-producing *Enterobacteriaceae*, resulting in identification of 124 positive patients. The isolates were dominated by *Klebsiella pneumoniae* ST258 KPC-2 and ST512 KPC-3, mainly from Greece and Italy, respectively, whereas Israeli isolates were of diverse species, clones and KPC variants. Various bla_{KPC} platforms were observed, among which $IncFII_K$+FIB_K plasmids with $bla_{KPC-2/-3}$ genes in the Tn*4401*a transposon prevailed.

Carbapenemase-producing *Enterobacteriaceae* (CPE) constitute an urgent epidemiological issue (1). One of their major, globally-spread mechanisms are the *Klebsiella pneumoniae* carbapenemases (KPCs), hydrolyzing most of β-lactams (1, 2). KPC-2 and -3 are the most prevalent variants, while *K. pneumoniae* is their predominant host species (3, 4). KPCs have occurred in many *K. pneumoniae* clones (sequence types, STs) (5-8), but ST258 and its close relative ST512 are key players in the pandemic spread (2-4, 6, 9-11). bla_{KPC} genes are located in Tn*4401* transposon variants (12-15), inserted into plasmids of various replicon types and transmission potential (5, 7, 16-20). One type of these, pKpQIL, found first in KPC-3-producing *K. pneumoniae* ST258 in Israel, has two specific replicons, FII_K and FIB_K, and low conjugation efficiency (21-23). Later KPC-2- or -3-encoding pKpQIL-like molecules were observed in other countries, usually in *K. pneumoniae* ST258 (2, 3, 10, 24, 25).

During the EU project MOSAR patients in ICUs and rehabilitation units (RUs) in Europe and Israel were screened for *Enterobacteriaceae* resistant to expanded-spectrum cephalosporins (ESCs) (26). Since KPCs and metallo-β-lactamases (MBLs) confer resistance to ESCs (1), the project allowed performing a large-scale comparative study of the KPC and MBL CPE carriage. A previous report concerned MBL CPE (27), while here we present the KPC data.

Between mid-2008 and mid-2011 all patients in 13 ICUs and five RUs in nine countries (n=17,945) were screened for ESC-resistant (ESC-R) *Enterobacteriaceae* (Table 1). Rectal swabbing was performed regularly from admission until discharge. Swabs were plated onto Brilliance™ ESBL Agar (Oxoid, Basingstoke, UK); enterobacterial colonies were stored for definite analysis. Species were identified with Vitek 2 (bioMérieux, Marcy l'Etoile, France). All isolates were tested for extended-spectrum β-lactamases (ESBLs) and AmpC-type cephalosporinases by the ESBL double-disk synergy test (DDST) without and with 250µg/ml cloxacillin (28), and for susceptibility to ertapenem, imipenem and meropenem. Carbapenemase screening breakpoints were from EUCAST (http://eucast.org). All suspected

CPE isolates were subjected to KPC, MBL and OXA-48 phenotypic detection, using the combined disk test with phenylboronic acid (PBA CDT) (29), DDST with EDTA (30), and temocillin disk (31), respectively. All non-duplicate PBA CDT-positive organisms were tested by PCR for bla_{KPC} genes (32), performed also for putative MBL producers (27).

A total of 124 patients carrying 127 unique KPC CPE organisms were identified in six of 18 clinical sites, located in Greece (centers AT, n=44, and LA, n=35), France (RP, n=1), Israel (LH, n=6, and TA, n=16) and Italy (FS, n=22) (Table 1). They were 59.0% of all patients with CPE. Four Greek patients had *K. pneumoniae* co-producing KPC and MBL (VIM-1) and were reported previously too (27). The results for individual countries concurred with other reports. After the nation-wide outbreak in 2006-07, the KPC situation in Israel has been endemic at a lower level since 2008 (2, 33, 34). Consistently, the KPC cases in the Israeli RUs were scattered during the study, being ~1% of all patients screened and ~2% of those with ESC-R organisms (Table 1). The KPC spread in Greece commenced in 2007 and was much advanced by the mid-2008 (2, 34-36). Both Greek ICUs recorded KPC cases from the survey start and their contribution to all patients screened and to ESC-R *Enterobacteriaceae* carriers was ~6% and ~35%, respectively. Italy reported the first KPC case in 2008, followed by an outbreak progressing rapidly from 2010 (2, 34, 37). The RU FS, screening patients from February 2009 to February 2011, had first two cases late in 2009, then 12 in 2010, and eight in the first two months of 2011, being ~3% of all patients and ~6% of those with ESC-R organisms.

The bla_{KPC} amplicons were digested by RsaI (Fermentas, Vilnius, Lithuania), distinguishing bla_{KPC-2} and bla_{KPC-3} (38), followed by sequencing for representative isolates. KPC-producing isolates were typed by pulsed-field gel electrophoresis (PFGE), as described (39). PFGE types and subtypes were distinguished visually according to Tenover et al. (40). Selected isolates were analyzed also by multi-locus sequence typing (MLST) (41-44); databases available at http://pubmlst.org/cfreundii/ (*Citrobacter freundii*), http://pubmlst.org/ecloacae (*Enterobacter*

cloacae), http://mlst.warwick.ac.uk/mlst/dbs/Ecoli (*Escherichia coli*) and http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html (*K. pneumoniae*) were used for assigning STs. *E. cloacae* STs and β-lactamases were shown previously (45).

K. pneumoniae isolates, being the predominant species (n=110; 86.6%), were classified into 10 STs (Table 2). ST258 prevailed (n=76; 69.1%) and was observed in all but one of the sites (FS, Italy), dominating in Greece with bla_{KPC-2} (n=73; 93.6%). The next prevalent clone, ST512 (n=21; 19.1%), was originally identified in this study in an Israeli isolate from 2008 (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html). This SLV of ST258 carried bla_{KPC-3} and dominated in the Italian RU FS (n=19; 86.4%), being sporadic in Israel. Four KPC-2+VIM-1-positive Greek isolates belonged to ST147, the major VIM producer in Greece (27), while the remaining STs represented single isolates with KPC-2 or -3 in individual sites. *C. freundii*, *E. cloacae* and *E. coli*, usually producing KPC-2, were identified vastly in Israel and were clonally diverse, except for *E. coli* ST131 with three KPC-2 or -3 isolates. Most of the *E. cloacae*, *E. coli* and *K. pneumoniae* isolates represented international clones (45, 46). For *C. freundii* the clonality data are scarce (27, 41, 47) but KPC-producing *C. freundii* ST14, originally identified here, was found in 2015 in Malaysia [http://pubmlst.org/cfreundii/]. In general the clonality plus KPC type data were congruent with national reports. The high KPC CPE diversity in the Israeli centers corresponds to the endemic situation, following the polyclonal outbreak of KPC-2 and clonal spread of *K. pneumoniae* ST258 KPC-3 (7, 19, 22, 48, 49), even if other studies still indicate importance of *K. pneumoniae* ST258/ST512 (50). In contrast, the high prevalence of ST258 KPC-2 in Greece and ST512 KPC-3 in Italy reflected their clonal dissemination in real time (35-37). This study is also a yet another report on KPC-producing *E. coli* ST131, repeatedly identified in Israel (4, 49, 51, 52).

Location of bla_{KPC} genes within Tn*4401*-like transposons and polymorphism of these was analyzed by PCR mapping (12). For the Tn*4401*g variant (15) an additional primer was

designed (5'-GTTCCACTGAGCGTCAGAC-3') for use with primer 3781L (12) (expected product size, 370bp). All *bla*$_{KPC}$ genes were located in Tn*4401* variants (12). The main type was Tn*4401*a (12), observed in all isolates from Greece, Italy and France, and in 9/22 Israeli isolates, including most of *K. pneumoniae* with *bla*$_{KPC-2}$ or *bla*$_{KPC-3}$ (Table 2). Tn*4401*c (14) and Tn*4401*g (15) were found only in Israel in various species and clones, always containing *bla*$_{KPC-2}$. Tn*4401*a has been the main type of Tn*4401*, strongly associated with *K. pneumoniae* ST258 worldwide (6, 10, 18, 21, 36), while Tn*4401*c has been observed in diverse KPC-2-producing organisms in Israel (15, 49). Interestingly, the Tn*4401*c-derived Tn*4401*g was identified only recently in a single *K. pneumoniae* KPC-2 isolate recovered in Israel in 2008 (15), whereas here it occurred frequently in *C. freundii*, *E. coli* and *K. pneumoniae*.

Plasmid profiling and identification of *bla*$_{KPC}$-carrying plasmids was done by the nuclease S1 (New England Biolabs, Beverly, MA) analysis (53) and hybridization with the *bla*$_{KPC}$ probe, using ECL Random-Prime Labeling and Detection system (Amersham Pharmacia Biotech, Little Chalfont, United Kingdom). The analysis comprised 44 isolates of all species, STs and pulsotypes (15 *K. pneumoniae* ST258/ST512 isolates), revealing highly variable plasmid profiles, with *bla*$_{KPC}$-carrying plasmids ranging in size from ~60 to ~320kb (Table 2). Plasmid DNA of 27 isolates of various species, STs and S1 profiles was purified with the QIAGEN Plasmid Midi Kit (QIAGEN, Hilden, Germany) and electroporated into *E. coli* DH5α, with the transformant selection by 0.5μg/ml imipenem or 1μg/ml cefotaxime. Subsequently, plasmids of the transformants were purified and subjected to PCR-based replicon typing (PBRT) (54-57). KPC-positive transformants were obtained for 22 isolates (Table 2). PBRT revealed that 12 of these had plasmids with FII$_K$ and FIB$_K$ replicons (alternating in two cases) of ~90-~140kb. PCR mapping, performed as proposed by Baraniak et al. (10), showed that all these were of the pKpQIL type (21), and molecules positive in that assay were identified also in selected isolates for which no transformants were available (Table 2). The pKpQIL-like

plasmids carried *bla*$_{KPC-2}$ or $_{-3}$ (Tn*4401*a), and were mainly hosted in *K. pneumoniae* ST258 & ST512; however, these occurred also in other organisms (10, 22, 23). The other group were IncN plasmids of ~60-~150kb, identified in various *C. freundii*, *E. coli* and *K. pneumoniae* Israeli strains, usually carrying *bla*$_{KPC-2}$ (Tn*4401*g). These plasmids have been observed among diverse KPC-2-producing *E. coli* and non-CG258 *K. pneumoniae* in Israel (15, 49). However, some of our isolates fell beyond this pattern, like *K. pneumoniae* ST833 (SLV of ST258) with *bla*$_{KPC-2}$ on a pKpQIL-like plasmid or *K. pneumoniae* ST512 with *bla*$_{KPC-3}$ on an IncN molecule. Finally, the *bla*$_{KPC-2}$ gene in the Tn*4401*c variant was observed in *C. freundii* and *E. cloacae* in large plasmids (~300-~320kb) that could not be separated by transfer despite repeated attempts; their replicon types thus remained not determined.

The KPC CPE isolates were analyzed for other acquired β-lactamase genes, namely *bla*$_{SHV-5/-12}$, *bla*$_{CTX-M}$, *bla*$_{CMY-2}$, *bla*$_{TEM}$, and *bla*$_{OXA-1}$ types by PCR and sequencing (32, 58-60). The isolates had various β-lactamase combinations, including SHV- and CTX-M-like ESBLs, AmpCs of the CMY-2 type and broad-spectrum enzymes TEM-1 and OXA-1 (Table 2).

We assessed the KPC CPE carriage among ICU and RU patients on a large international scale, using the same time frame and methodology. Not surprisingly, KPC producers were found mainly in the countries which reported their wide spread, *i. e.* Greece, Italy and Israel (2, 33-37). Considering the study period, 2008-2011, the rhythm of occurrence of cases in individual centers and characteristics of the organisms reflected the situation in the countries, *i. e.* the onset and advanced stage of nation-wide outbreaks in Italy and Greece, respectively, and the post-outbreak endemicity in Israel (33, 35-37). The analysis provided a comparative snapshot of the geographic and quantitative distribution of species/clones, Tn*4401* transposon variants and *bla*$_{KPC}$-carrying plasmids, often observed in national reports. Also, this has been one of the first studies of *C. freundii* and *E. cloacae* that included MLST data.

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424 TABLE 1. Occurrence of patients colonized by KPC CPE in study centers.

425

country	centre	unit type	patients enrolled in the study (n)[a]	patients colonized by *Enterobacteriaceae* producing acquired ESC-hydrolyzing β-lactamases (n)[b,c,d]	patients colonized by CPE (n)[b,d,e]	patients colonized by KPC CPE (n)[d]
France	HM	ICU	2,373	256 (10.8%)	1 (0.04%) [e]	-
France	RP	ICU	1,328	85 (6.4%)	1 (0.08%) [f]	1 (0.08%)
France	SJ	ICU	1,049	51 (4.9%)	4 (0.4%)	-
Greece	AT	ICU	796	117 (14.7%)	53 (6.7%) [g]	44 (5.5%)
Greece	LA	ICU	558	99 (17.7%)	83 (14.9%) [h]	35 (6.3%)
Italy	CA	ICU	788	49 (6.2%)	2 (0.3%)	-
Latvia	RI	ICU	1,464	526 (35.9%)	10 (0.7%)	-
Luxemburg	LU	ICU	1,823	54 (3.0%)	-	-
Portugal	PO	ICU	910	18 (2.0%)	-	-
Portugal	VR	ICU	628	24 (3.8%)	1 (0.2%)	-
Slovenia	GO	ICU	919	32 (3.5%)	-	-
Slovenia	LJ	ICU	685	115 (16.8%)	-	-
Spain	BA	ICU	1,069	41 (3.8%)	-	-
France	BM	RU	410	76 (18.5%)	-	-
Israel	LH	RU	564	177 (31.4%)	6 (1.1%)[i]	6 (1.1%)
Israel	TA	RU	1,650	870 (52.7%)	16 (1.0%)[i]	16 (1.0%)
Italy	FS	RU	704	340 (48.3%)	28 (4.0%)	22 (2.8%)
Spain	GI	RU	227	104 (45.8%)	5 (2.2%)	-
Total			17,945	3,034 (16.9%)	210 (1.2%)	124 (0.7%)

426

427 [a] – all patients that were swabbed at least once at a clinical centre, regardless the length of
428 hospitalization
429 [b] – these numbers were shown also in the report on colonization by MBL CPE in MOSAR
430 centers (27)
431 [c] – acquired ESC-hydrolyzing β-lactamases include ESBLs, AmpC-type cephalosporinases,
432 MBLs and KPCs
433 [d] – patients in this column include both those who were colonized at admission and those who
434 were colonized due to in-hospital transmission
435 [e] – carbapenemases include KPCs and MBLs except for one patient in the French ICU HM
436 who was colonized by *E. coli* co-producing OXA-48 carbapenemase and ESBL
437 [f] – this patient was colonized by KPC-producing *K. pneumoniae* and MBL-producing *E. coli*
438 [g] – one patient was colonized by KPC-producing *E. coli* and MBL-producing *K. pneumoniae*
439 [h] – four patients were colonized by *K. pneumoniae* co-producing KPC and MBL
440 [i] – one patient was colonized by KPC-producing *E. coli* and *K. pneumoniae*
441 [j] – two patients were colonized by two different KPC producers: *C. freundii* and *E. coli*, or *E.*
442 *coli* and *K. pneumoniae*, respectively
443

444 TABLE 2. KPC CPE isolates: geographic distribution, species, clones, pulsotypes, S1 plasmid profiles, plasmids and Tn*4401* transposons with
445 bla_{KPC} genes, and other acquired β-lactamases (MBLs, ESBLs, AmpCs, broad-spectrum β-lactamases).
446

Centers	Species	ST (CC or CG)[a,b,c,d]	n isolates	n pulsotypes (subtypes)	S1 profiles [a,e,f]	plasmids with bla_{KPC} genes [a,g]	bla_{KPC} [h]	Tn*4401* variant [i]	MBLs, ESBLs, AmpCs (n)[j]
AT (Greece)	*E. coli*	ST10 (CC10)	1	1	Eco1	**~130kb**; **FII_K+FIB_K**	bla_{KPC-2}	Tn*4401*a	TEM-1
	K. pneumoniae	ST258 (CG258)	43	2 (18)	Kpn1	**~120kb**; **FII_K+FIB_K**	bla_{KPC-2}	Tn*4401*a	SHV-12+TEM-1
					Kpn2	**~115kb**; **FII_K+FIB_K**	bla_{KPC-2}	Tn*4401*a	SHV-12+TEM-1
LA (Greece)	*K. pneumoniae*	ST17 (CG17)	1	1	Kpn4	**~115kb**; **FII_K+FIB_K**	bla_{KPC-2}	Tn*4401*a	SHV-5+TEM-1
	K. pneumoniae	ST147 (CC147)[k]	4	1 (2)	Kpn6	~115kb; *FII_K+FIB_K*	bla_{KPC-2}	Tn*4401*a	VIM-1+TEM-1
					Kpn9	~100kb; *FII_K+FIB_K*			
	K. pneumoniae	ST258 (CG258)	30	1 (12)	Kpn2	~115kb; *FII_K+FIB_K*	bla_{KPC-2}	Tn*4401*a	SHV-12+TEM-1
					Kpn10	**~70kb**; **nt**	bla_{KPC-2}	Tn*4401*a	SHV-12
FS (Italy)	*K. pneumoniae*	ST16 (CG17)	1	1	Kpn7	**~90kb**; **nt**	bla_{KPC-3}	Tn*4401*a	CTX-M-15
	K. pneumoniae	ST45 (CG485)	1	1	Kpn3	~115kb; *FII_K+FIB_K*	bla_{KPC-3}	Tn*4401*a	TEM-1
	K. pneumoniae	ST383 (CC42)	1	1	Kpn8	**~100kb**; **FII_K**	bla_{KPC-2}	Tn*4401*a	CMY-4+TEM-1
	K. pneumoniae	ST512 (CG258)	19	1 (9)	Kpn2/6	**~115kb**; **FII_K+FIB_K**	bla_{KPC-3}	Tn*4401*a	TEM-1 (19); SHV-12+CMY-2 (1); OXA-1 (1)[l]
RP (France)	*K. pneumoniae*	ST258 (CG258)	1	1	nd	nd	bla_{KPC-2}	Tn*4401*a	CTX-M-15+ SHV+TEM-1
LH (Israel)	*E. cloacae*	ST78 (CC74)[m]	1	1	Ecl1	nd	bla_{KPC-2}	Tn*4401*c	SHV-12+TEM-1[m]
	E. coli	ST131 (CC131)	2	1 (2)	Eco2	**~75kb**; **N**	bla_{KPC-2}	Tn*4401*g	TEM-1

	E. coli	ST167 (CC10)	1	1	Eco4	**~90kb**; **FII_K+FIB_K**	bla_{KPC-3}	Tn*4401*a	SHV-12+TEM-1
	E. coli	ST1571	1	1	Eco3	nd	bla_{KPC-2}	Tn*4401*c	SHV-12+TEM-1
	K. pneumoniae	ST258 (CG258)	1	1	Kpn5	~115kb; *FII_K+FIB_K*	bla_{KPC-3}	Tn*4401*a	TEM-1
	K. pneumoniae	ST512 (CG258)	1	1	Kpn11	**~150kb**; **N**	bla_{KPC-3}	Tn*4401*a	-
TA (Israel)	*C. freundii*	**ST14**	1	1	Cfr1	**~80kb**; **N**	bla_{KPC-2}	Tn*4401*g	TEM-1+OXA-1
	C. freundii	**ST12**	1	1	Cfr2	**~80kb**; **N**	bla_{KPC-2}	Tn*4401*g	CTX-M-15+TEM-1
	C. freundii	nd	1	1	Cfr3	**~80kb**; **N**	bla_{KPC-2}	Tn*4401*g	SHV-12+TEM-1+OXA-1
	C. freundii	**ST10**	1	1	Cfr4	~300kb; nd	bla_{KPC-2}	Tn*4401*c	TEM-1+OXA-1
	C. freundii	**ST15**	1	1	Cfr4	~300kb; nd	bla_{KPC-2}	Tn*4401*c	TEM-1+OXA-1
	E. cloacae	**ST118**[m]	1	1	Ecl3	~320kb; nd	bla_{KPC-2}	Tn*4401*c	CTX-M-27+SHV-12+TEM-1[m]
	E. cloacae	**ST146**[m]	1	1	Ecl2	~300kb; nd	bla_{KPC-2}	Tn*4401*c	TEM-1[m]
	E. coli	ST69 (CC69)	1	1	Eco8	**~70kb**; **N**	bla_{KPC-2}	nt	TEM-1
	E. coli	ST131 (CC131)	1	1	Eco5	**~115kb**; **FII_K+FIB_K**	bla_{KPC-3}	Tn*4401*a	TEM-1
	E. coli	ST216	1	1	Eco6	**~60kb**; **N**	bla_{KPC-2}	Tn*4401*g	TEM-1+OXA-1
	E. coli	**ST3541**	1	1	Eco7	nd	bla_{KPC-2}	Tn*4401*c	CTX-M-15+ SHV-12+CMY-2+TEM-1+OXA-1
	K. pneumoniae	ST17 (CG17)	1	1	Kpn12	**~140kb**; **N**	bla_{KPC-2}	Tn*4401*g	TEM-1+OXA-1
	K. pneumoniae	ST34 (CC34)	1	1	Kpn13	nd	bla_{KPC-2}	Tn*4401*c	CTX-M-15+ SHV-12+TEM-1+OXA-1
	K. pneumoniae	ST36 (CG485)	1	1	Kpn14	**~115kb**; **FII_K+FIB_K**	bla_{KPC-3}	Tn*4401*a	TEM-1
	K. pneumoniae	ST258 (CG258)	1	1	Kpn2	~115kb; *FII_K+FIB_K*	bla_{KPC-3}	Tn*4401*a	TEM-1

K. pneumoniae	ST383 (CC42)	1	1	Kpn15	**~115kb**; **FIB$_K$**	*bla*$_{KPC-2}$	Tn*4401*a	CTX-M-15+CMY-4+TEM-1
K. pneumoniae	**ST512** (CG258)	1	1	Kpn16	**~140kb**; **FII$_K$+FIB$_K$**	*bla*$_{KPC-3}$	Tn*4401*a	TEM-1+OXA-1
K. pneumoniae	ST833 (CG258)	1	1	Kpn17	**~100kb**; **FII$_K$+FIB$_K$**	*bla*$_{KPC-2}$	Tn*4401*a	SHV-12

[a] – nd, not determined; nt, non-typeable

[b] – new STs are indicated in bold; numerous reports on *K. pneumoniae* ST512 have been published since 2012 (2, 11, 34, 37); however, this ST was identified originally in this study (isolate ID 578 in the *K. pneumoniae* MLST database; http://bigsdb.web.pasteur.fr)

[c] – CC, clonal complex; CG, clonal group

[d] – in groups of four or more isolates MLST was performed for representative isolates, based on the PFGE data

[e] – in large groups of isolates of the same ST/pulsotype (*K. pneumoniae* ST258 & ST512) the S1 analysis was performed for representative isolates

[f] – S1 plasmid profiles are numbered within species groups of isolates; profiles differed from each other by number and/or size of plasmids

[g] – plasmids found in transformants are shown in bold; replicons shown in italics represent the probable types of *bla*$_{KPC}$ plasmids (PBRT and pKpQIL PCR mapping was performed on DNA of clinical isolates)

[h] – in groups of four or more isolates of the same ST/pulsotype the *bla*$_{KPC}$ sequencing was performed for representative isolates; for the remaining isolates the RsaI PCR-RFLP analysis distinguishing between *bla*$_{KPC-2}$ and *bla*$_{KPC-3}$ sequences (38) was carried out

[i] – in groups of four or more isolates of the same ST/pulsotype the PCR mapping of Tn*4401*-like elements was performed for representative isolates

[j] – in groups of four or more isolates of the same ST/pulsotype and *bla* genes' PCR profile sequencing was performed for representative isolates

[k] – these isolates were also included in the study of MBL CPE isolates identified during the MOSAR project (27)

[l] – all isolates of this group produced TEM-1; one isolate had additionally SHV-12 & CMY-2, and another one had OXA-1

[m] – STs and β-lactamases of the *E. cloacae* isolates from LH & TA were reported previously (45)