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Predominance of KPC-3 in a Survey for Carbapenemase-Producing *Enterobacteriaceae* in Portugal

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Among the 2,105 *Enterobacteriaceae* tested in a survey done in Portugal, 165 were nonsusceptible to carbapenems, from which 35 (26 *Klebsiella pneumoniae*, 3 *Escherichia coli*, 2 *Enterobacter aerogenes*, and 3 *Enterobacter cloacae* isolates and 1 *Klebsiella oxytoca* isolate) were confirmed to be carbapenemase producers by the presence of 30 *Tn*4401d-bla_{KPC-3}, 4 *intI*3-bla_{GES-5}, and one *intI*1-bla_{VIM-2} gene, alone or in combination with other *bla* genes. The dissemination of bla_{KPC-3} gene carried by an IncF plasmid suggests lateral gene transfer as a major mechanism of dissemination.

Carbapenem-nonsusceptible *Enterobacteriaceae* are increasingly reported worldwide, mainly because of the acquisition of carbapenemase-encoding genes located on highly mobile genetic elements (e.g., plasmids and/or integrons) that facilitate their horizontal spread (1, 2). In Portugal, little is known regarding the molecular epidemiology of carbapenemase-producing *Enterobacteriaceae* (CPE). Indeed, only sporadic isolates or single hospital cases have been described (3), namely, a VIM-2-producing *Klebsiella oxytoca* isolate (4), 2 VIM-34-producing *Klebsiella pneumoniae* isolates (5), and 6 *K. pneumoniae* carbapenemase (KPC)-3-producing *Enterobacteriaceae* isolates (6). The objectives of this study were to (i) characterize a large number of CPE isolates recovered from different Portuguese health care institutions, (ii) determine the potential diversity of these isolates, and (iii) describe the respective plasmids harboring carbapenemase-encoding genes in an international context.

This study included 2,105 consecutive and nonrepetitive clinical *Enterobacteriaceae* isolates that were collected between April 2006 and February 2013 and sent to the National Reference Laboratory of Antimicrobial Resistances at the National Institute of Health, as an integrative part of the Antimicrobial Resistance Surveillance Program in Portugal (ARSIP) laboratory. ARSIP is a voluntary Portuguese surveillance program that continually monitors the susceptibilities of pathogens of clinical importance against several antibiotics. Thirteen Portuguese hospitals located in five different regions (North, Central, Lisbon and Tagus Valley, South, and Autonomous Region of Madeira) participated in this survey.

The screening of carbapenem susceptibility, performed by the disk diffusion method, according to the French Society for Microbiology (http://www.sfm-microbiologie.org/), revealed that 165 (7.8%) isolates were ertapenem-nonsusceptible, and these were selected for further analysis. In addition, clinical isolates showing an inhibition of carbapenemase, as evidenced by the synergy between carbapenems (imipenem, meropenem, and/or ertapenem) and 3-aminophenylboronic acid or EDTA, were considered presumptive carbapenemase producers from class A or B, respectively (7).

Thirty-five isolates suspected to produce CPE (35/168 [20.8%]) demonstrated a positive synergy between carbapenems and 3-aminophenylboronic acid ($n = 34$) or with EDTA ($n = 1$), indicative of serine or metallo-β-lactamase production, respectively. The majority of those isolates were collected from urine samples (54.3%) from elderly (≥65 years old) male patients (54.3%), admitted to the emergency room/ambulatory (22.9%), internal medicine (17.1%), or surgery (17.1%) wards. The diminished susceptibilities to carbapenems among the remaining 130 ertapenem-nonsusceptible isolates (59 *Klebsiella* spp., 28 *Enterobacter* spp., 23 *Escherichia coli*, 11 *Morganella morganii*, 8 *Proteus mirabilis*, and 1 *Serratia marcescens*) suggested a decrease in the outer membrane permeability associated with the weak hydrolytic activity of carbapenems due to the expression of a β-lactamase, such as extended-spectrum β-lactamases (ESBLs), and/or AmpC overexpression (7).

The MICs of the 35/168 isolates were obtained by the reference microdilution broth method (8, 9), according to the EUCAST guidelines (http://www.eucast.org/clinical_breakpoints/). Tigecycline and ciprofloxacin were potent against 57.1% and 28.6% of the isolates, respectively, but colistin was the only antibiotic effective against all CPE isolates; all isolates were multidrug resistant (MDR), i.e., presented reduced susceptibility to three or more structurally unrelated antibiotics (10) (Table 1).

PCR amplification and DNA sequencing were applied to the 165 ertapenem-nonsusceptible isolates to identify the presence or confirm the absence of the carbapenemase-encoding genes bla_{KPC} and bla_{GES} (class A), bla_{IMP}, bla_{VIM}, and bla_{NDM} (class B), and bla_{OXA-48} (class D) (11). Among the 165 isolates, only the 35 identified as carbapenemase-producing isolates were confirmed to be

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TABLE 1 MIC data and susceptibilities of 35 CPE isolates

	All isolates (n = 35)					KPC-3-producing isolates (n = 30)					GES-5-producing isolates (n = 4)						VIM-2-producing isolates (n = 1)	
				Susceptibility[b]					Susceptibility[b]		MIC (μg/ml) for strain:				Susceptibility[b]		MIC for Ko9864 (μg/ml)	
Antibiotic[a]	MIC_{50}	MIC_{90}	MIC range	%R	%IR	MIC_{50}	MIC_{90}	MIC range	%R	%IR	Kp15587	Kp15743	Kp17060	Kp17061	%R	%IR		Susceptibility[d]
AMX	>256	>256	>256	100.0	100.0	>256	>256	>256	100.0	100.0	>256	>256	>256	>256	100.0	100.0	>256	R
AMC	>256	>256	>256	100.0	100.0	>256	>256	>256	100.0	100.0	>256	>256	>256	>256	100.0	100.0	>256	R
TIC	>256	>256	>256	100.0	100.0	>256	>256	>256	100.0	100.0	>256	>256	>256	>256	100.0	100.0	>256	R
CXM	>256	>256	64 to >256	100.0	100.0	>256	>256	64 to >256	100.0	100.0	>256	>256	>256	>256	100.0	100.0	>256	R
FOX[c]	128	>128	16 to >128	100.0	100.0	128	>128	16 to 128	100.0	100.0	>128	128	>128	>128	100.0	100.0	128	NWT
CAZ	128	512	64 to 512	100.0	100.0	128	512	128 to >512	100.0	100.0	256	256	128	256	100.0	100.0	128	R
CAZ-CLA	128	256	16 to 512			128	256	16 to 512			32	32	16	32			16	
CTX	256	>256	64 to >512	100.0	100.0	256	>256	64 to >256	100.0	100.0	64	64	64	64	100.0	100.0	256	R
CTX-CLA	64	256	8 to >256			64	256	8 to >256			64	64	32	64			32	
ATM	256	512	32 to >512	100.0	100.0	256	512	64 to 512	100.0	100.0	256	256	128	32	100.0	100.0	128	R
FEP	32	>256	8 to >256	100.0	100.0	32	>256	16 to >256	100.0	100.0	8	16	16	8	100.0	100.0	8	R
IPM	8	64	0.5 to >512	48.6	85.7	8	32	1 to 128	46.7	90.0	>512	512	1	0.5	50.0	50.0	16	R
MEM	32	64	0.25 to >128	85.7	97.1	32	64	0.25 to >128	90.0	96.7	128	128	8	4	50.0	100.0	64	R
DOR	8	32	0.5 to 64	82.9	91.4	8	16	1 to 64	86.7	96.7	64	64	1	0.5	50.0	50.0	8	R
ERT	32	64	1 to >64	91.4	100.0	32	64	1 to >64	90.0	100.0	32	>64	16	8	100.0	100.0	64	R
CIP	32	>32	≤0.06 to >32	62.9	71.4	4	16	≤0.06 to >32	56.7	66.7	>32	>32	>32	>32	100.0	100.0	32	R
GEN	32	512	4 to 512	97.1	100.0	32	256	4 to 256	96.7	100.0	512	512	512	512	100.0	100.0	256	R
TMP	>1,024	>1,024	>1,024	100.0	100.0	>1,024	>1,024	>1,024	100.0	100.0	>1,024	>1,024	>1,024	>1,024	100.0	100.0	>1,024	R
CST	0.25	1	≤0.03 to 2	0.0	0.0	0.25	1	≤0.03 to 2	0.0	0.0	0.25	0.25	0.25	0.25	0.0	0.0	0.5	S
TGC	1	4	≤0.03 to 8	20.0	42.9	1	4	≤0.03 to 8	23.3	33.3	2	2	2	2	0.0	100.0	2	I

[a] AMX, amoxicillin; AMC, amoxicillin-clavulanic acid; TIC, ticarcillin; CXM, cefuroxime; FOX, cefoxitin; CAZ, ceftazidime; CAZ-CLA, ceftazidime-clavulanic acid; CTX, cefotaxime; CTX-CLA, cefotaxime-clavulanic acid; ATM, aztreonam; FEP, cefepime; IPM, imipenem; MEM, meropenem; DOR, doripenem; ERT, ertapenem; CIP, ciprofloxacin; GEN, gentamicin; TMP, trimethoprim; CST, colistin; and TGC, tigecycline.

[b] R, resistant; IR, nonsusceptible.

[c] Epidemiological breakpoint, according to EUCAST (http://www.eucast.org/mic_distributions_ecoffs/).

[d] NWT, non-wild type; S, susceptible; I, intermediate.

TABLE 2 Characteristics of 35 CPE isolates detected in this study and of the 10 transconjugant strains obtained[a]

Species (no.) encountered by carbapenemase type[b]	MLST (no.)	Hospital code/yr of isolation (no.)	Other β-lactamase(s) detected[c]
KPC-3 (*Tn*4401b)			
E. aerogenes (3)		A/2012, E/2011, H/2013	TEM-1, (OXA-30), (CTX-M-15)
E. cloacae (2)		E/2010, E/2012	ACT-type, (TEM-1), (OXA-30)
E. coli ($n = 3$)	ST58	G/2013	TEM-1
K. pneumoniae (22)	ST11 (2)	E/2012, E/2013	TEM-1, SHV-11, (OXA-30), (CTX-M-15)
	ST14 (3)	F/2010, G/2012, G/2013	TEM-1, SHV-1
	ST15 (4)	G/2012 (2), G/2012 (1), G/2013 (1)	TEM-1, SHV-1, CTX-M-15
	ST34 (1)	E/2011	TEM-1, SHV-26
	ST59 (1)	E/2011	TEM-1
	ST147 (4)	E/2012 (2), E/2012 (2)	TEM-1, SHV-11
	ST416 (1)	B/2011	TEM-1, SHV-14, OXA-30, CTX-M-15
	ST698 (1)	G/2012	TEM-1, SHV-11, CTX-M-15
	ST960 (1)[d]	I/2011	TEM-1, SHV-164
	ST1138 (4)[d]	D/2011 (1), D/2011 (1), E/2011 (2)	TEM-1, SHV-36
GES-5 (*intI3*)			
K. pneumoniae (4)	ST231	C/2012 (2), C/2013 (2)	TEM-1, SHV-12, SHV-1
VIM-2 (*intI1*)			
K. oxytoca (1)		H/2010	SHV-12

[a] Isolates that successfully transferred the KPC-3 to recipient strains are underlined. All clinical isolates and their respective transconjugant isolates contained KPC-3- and TEM-1-harboring IncF(FII) plasmids.
[b] Parentheses indicate the mobile genetic element associated with the respective carbapenemase-encoding gene.
[c] Parentheses indicate the variable presence of an antibiotic resistance determinant.
[d] MLST first described here.

CPE by the presence of 30 bla_{KPC-3} and 4 bla_{GES-5} genes and 1 bla_{VIM-2} gene, alone or in combination with other *bla* genes (Table 2) (11).

An analysis of the genetic elements associated with the CPE-encoding genes showed that all the bla_{KPC-3} genes were flanked by IS*Kpn7* on the right and IS*Kpn6* on the left (12) and a Tn*3*-like Tn*4401* transposon, with a 68-bp deletion upstream of bla_{KPC-3}, corresponding to the isoform d. This uncommon isoform has been described so far only in KPC-producing *K. pneumoniae* strains with high-level carbapenem resistance in the United States and Canada (13–15).

Regarding the bla_{GES-5} genes, the PCR results and sequence analysis, using previously described primers (11), revealed the amplification of the *intI3*-bla_{GES-5} region, where the *intI3* gene was located adjacent to the bla_{GES-5} gene in a configuration previously reported for the bla_{GES-1} gene (16, 17). *intI3* was targeted and amplified with a set of primer pairs (5′-GCAAGTGGGTGGCGA ATG-3′ and 5′-CTGAAGTCGAGGGTTTTCTG-3′) designed in this study (with initial denaturation at 94°C for 5 min, 30 cycles of denaturation at 94°C for 30 s, annealing at 58°C for 60 s, and extension at 72°C for 60 s, and a final extension at 72°C for 5 min). This study represents one of the very few reports of the acquisition of carbapenemase-encoding genes mediated by a class 3 integron (18, 19), and to our knowledge, those are the first such genes to have been found in CPE isolates. Notable is the fact that two isolates (Kp17060 and Kp17061, Table 1) were collected on the same day from patients admitted in the same hospital room, suggesting that organisms were transferred through direct contact. Also, we confirmed the location of the bla_{VIM-2} gene in a class 1 integron, as previously described (4).

In our study, among the 15 isolates with CPE-encoding genes randomly selected to be tested for transferability by broth mating-out assays using recipient rifampin-resistant (Rif^r) or streptomycin-resistant (Str^r) *E. coli* C600 and sodium azide-resistant (NaN_3^r) *E. coli* J53 strains (11), we obtained 10 (66.7%) transconjugants with bla_{KPC-3}-harboring plasmids. Using PCR-based replicon typing to type the resistance plasmids (20), we observed the transfer of an IncF group harboring the bla_{KPC-3} and bla_{TEM-1} genes within the recipient NaN3^r *E. coli* J53 ($n = 9$) and Rif^r *E. coli* C600 ($n = 1$) strains. The location of the bla_{KPC} gene has been observed in plasmids of various sizes, belonging to the IncF, IncL/M, and IncN plasmid types, and also on small rolling circle-replicating (RCR) plasmids that are not self-transmissible but can mobilized in *trans* by coresident conjugative plasmids (2). In this study, we identified the IncF plasmid in different species, such as *Klebsiella* spp. and *Enterobacter* spp., suggesting that the dissemination of the bla_{KPC-3} gene is due to lateral gene transfer rather than clonal spread. In general, the MICs of the transconjugants showed similar susceptibility profiles as those of the donor strains with nonsusceptibility to meropenem, ertapenem, cefotaxime, ceftazidime, gentamicin, and trimethoprim, as well as those with susceptibility to colistin and tigecycline. Regarding imipenem ($n = 7$), doripenem ($n = 9$), and ciprofloxacin ($n = 9$), the majority of the transconjugants were susceptible in comparison to the clinical isolates, with MICs from 8 mg/liter to 1 mg/liter for imipenem, 2 mg/liter to 0.25 mg/liter for doripenem, and 0.125 mg/liter to ≤0.06 mg/liter for ciprofloxacin.

Multilocus sequence typing of *K. pneumoniae* ($n = 26$) isolates (http://bigsdb.web.pasteur.fr/klebsiella/klebsiella.html) showed a high degree of genetic diversity, as they were from distinct sequence types (STs), namely, ST14, ST15, ST34, ST59, ST147, ST416, and ST698, and from the two novel STs ST960 and ST1138 (Table 2). Overall, 10 STs were observed to be associated with KPC production, with ST147 ($n = 4$), ST1138 ($n = 4$), and ST15 ($n =$

4) being the most prevalent types. ST147 is an important clone associated with this resistance mechanism worldwide, namely due to VIM-, KPC-, and NDM-producing *K. pneumoniae* isolates (21, 22), while ST1138 was first described here in 4 KPC-3-producing *K. pneumoniae* isolates coexpressing TEM-1 and SHV-36 (Table 2). Interestingly, isolates belonging to the well-known lineage of *K. pneumoniae* ST258, which plays a major role in the global spread of KPC carbapenemases (23), were not found. Regarding KPC-producing *E. coli* ($n = 3$) (http://mlst.warwick.ac.uk/mlst/dbs/Ecoli), all isolates belonged to ST58.

In conclusion, this study provides new data regarding the molecular epidemiology of CPE in Portugal, with the emergence of the carbapenemase KPC-3. Indeed, this study suggests that the dissemination of the bla_{KPC-3} gene occurs due to the dispersion of Tn*4401*d, carried by an IncF plasmid and spread both by polyclonal *K. pneumoniae* and by *Enterobacter aerogenes*, *Enterobacter cloacae*, and *E. coli* clinical strains.

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