Microbiological and molecular characteristics of carbapenemase-producing *Klebsiella pneumoniae* endemic in a tertiary Greek hospital during 2004-2010

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We report 570 carbapenemase-producing *Klebsiella pneumoniae* (CPKP) clinical isolates in a 1,040-bed Greek tertiary hospital during 2004 to 2010. The first CPKP (VIM-producing) was isolated in September 2004. Despite initial containment, VIM producers have become endemic since 2006. KPC-producing *K. pneumoniae* was first isolated in August 2007 from a patient who came from Israel, spread rapidly, and outcompeted VIM. Overall, 267 (47%) VIM-producing and 301 (53%) KPC-producing strains were isolated, including 141 (24.7%) from patients with bacteraemia. Two isolates carrying both VIM and KPC were isolated in two consecutive months in 2009, but not since. The prevalence of CPKP increased from 0% in 2003 to 38.3% in 2010 ($p<0.0001$). All genotyped KPC producers harboured bla_{KPC-2} and belonged to two clones, among which the hyperepidemic Greek clone, related to those from the United States and Israel, predominated. Most metallo-beta-lactamase (MBL) producers carried the bla_{VIM-1} gene and belonged to several clones, whereas all but one isolate with bla_{VIM-12} were clustered within a five-month period, arising from one clone. Resistance to non-beta-lactam antibiotics was also increased among CPKP. They were almost invariably resistant to ciprofloxacin and trimethoprim-sulfamethoxazole. Resistance to colistin increased from 3.5% (4/115) in 2008 to 20.8% (25/120) in 2010, and resistance to tigecycline also increased. Following reinforcement of infection control measures, prevalence of CPKP (mainly KPC) has been reduced since mid-2009 (from 46% in 2009 to 38.3% in 2010). In view of the exhaustion of available therapies, investment in infection control resources and optimal antibiotic use is urgently required.

Introduction

Carbapenems are important therapeutic agents for treating infections caused by multi-drug resistant Gram-negative bacteria. Their efficacy, however, is threatened by the emergence of resistant isolates. In Greece (and elsewhere in Europe) a common mechanism is acquisition of hydrolytic enzymes (carbapenemases) inactivating beta-lactams [1,2]. The genes encoding carbapenemases are located on mobile genetic elements, allowing them to spread. Other mechanisms of resistance to carbapenems include the combination of extended spectrum beta-lactamase (ESBL) production with porin changes and/or upregulated efflux pumps (the latter particularly common among carbapenem-resistant *Pseudomonas aeruginosa*) [3,4].

Carbapenemases were initially found in non-fermenting bacteria; however, among *Enterobacteriaceae*, *Klebsiella pneumoniae* strains carrying acquired carbapenemases are increasingly reported [5]. The most prevalent carbapenemases are the molecular class B metallo-beta-lactamases (MBLs), mainly of VIM- and IMP-type, and the (class A) *K. pneumoniae*-carbapenemases (KPCs) [5]. More recently, outbreaks have been described of *K. pneumoniae* carrying the carbapenemases OXA-48 (Ambler class D) [6] and the New Delhi MBL (NDM-1) [7]. Carbapenemase-producing *K. pneumoniae* (CPKP) have been isolated worldwide, including most European countries [8]; CPKP are nowadays endemic in Greece (both VIM and KPC) and Israel (KPC). The presence of KPC in Israel was first reported in 2005 [9] and in Greece in 2007 [10].

Hippokration is a 1,040-bed, tertiary-care hospital in northern Greece, with all medical, surgical and paediatric subspecialties, a solid-organ transplantation unit and four intensive care units. The first CPKP in Hippokration Hospital was isolated in September 2004. Herein, we describe a seven-year study of the microbiological and molecular characteristics of *K. pneumoniae* producing different MBL- and KPC-type carbapenemases, endemic in this institution since 2006.

Methods

Between September 2004 and December 2010, we collected all *K. pneumoniae* isolates from clinical specimens (one per patient) that had a minimum inhibitory concentration (MIC) of >1 mg/L imipenem and stored them at -74 °C in 1% proteose-peptone containing 7% glycerol for further evaluation. Bacterial identification to species level and initial antibiotic susceptibility testing were performed with the VITEK2–automated system (bioMérieux, Marcy l'Etoile, France). Isolates were tested for tigecycline and colistin using the Etest (AB Biodisk, Solna, Sweden). For tigecycline, the breakpoints recommended by the United States Food and Drug Administration were used (susceptible: MIC ≤2 mg/L; resistant: MIC ≥8 mg/L). For colistin, the breakpoints recommended by the Clinical Laboratory Standards Institute (CLSI) for *Acinetobacter* spp. were used (susceptible: MIC ≤2 mg/L; resistant: MIC ≥4 mg/L) because there are no established CLSI MIC breakpoints against colistin for *Enterobacteriaceae*. All isolates were phenotypically screened for MBL- and KPC-type carbapenemases, using the imipenem/EDTA double-disk synergy test [11] and the imipenem/boronic acid combined-disc test [12], respectively.

Following phenotypic identification, MBL- and KPC-producing isolates were grouped according to their susceptibility profile (data not shown). However, no clear relationship between specific susceptibility profiles and clones could be identified with certainty. We selected 152 strains randomly (one of four) from each group, spanning all study years, for PCR amplification and sequencing, using primers specific for bla_{VIM}, bla_{IMP} and bla_{KPC}, as previously described [13,14]. The MICs of imipenem, meropenem and ertapenem for those 152 isolates were confirmed with the CLSI broth microdilution method [15], using *Escherichia coli* ATCC 25922 as control. The relatedness of isolates was determined by enterobacterial repetitive intergenic consensus (ERIC) PCR using the primer ERIC-2 [16].

Results

During the study period, 570 CPKP were isolated from clinical samples: blood (n=141; 24.7%), urine (n=166; 29.1%), surgical wounds (n=94; 16.5%), bronchial aspirates (n=35; 6.2%), central venous catheter tips (n=44; 7.7%), drainage sites (n=43; 7.5%), abscesses (n=17; 3.0%) and other sites (n=30; 5.3%, including cerebrospinal fluid, pleural or peritoneal tap, etc).

CPKP were isolated in all departments and 46.1% of isolates derived from two units: the eight-bed intensive care unit (ICU) (154 isolates; 27.0%) and the 10-bed organ transplant unit (109 isolates; 19.1%). The remaining CPKP were isolated in the hospital's surgical wards (146 isolates; 25.6%), the medical wards (139 isolates; 24.4%) and the paediatric/neonatal wards (22 isolates; 3.9%). The overall prevalence of CPKP among *K. pneumoniae* in the hospital increased from 0% in 2003 to 38.3% in 2010 ($p<0.0001$).

VIM-producing *Klebsiella pneumoniae*

In our hospital, the first CPKP was isolated in September 2004 from an infected wound of a patient who had been transferred from the ICU to the orthopedic ward; The MIC of imipenem and meropenem were 4 and 2 mg/L, respectively. Phenotypic testing revealed synergy between imipenem and EDTA, and the presence of the bla_{VIM-1} gene was identified. A further seven VIM-1 producing CPKP were isolated in the following three months: four in the ICU, two in surgical ward and one in the transplantation unit. Following rigorous infection control measures, the outbreak temporarily ceased, and only five sporadic cases occurred over the following 14 months. A new wave started in March 2006; since then CPKP have been endemic in the hospital. The outbreak trend is depicted in Figure 1.

KPC-producing *Klebsiella pneumoniae*

In August 2007, a *K. pneumoniae* isolate resistant to imipenem (MIC>16 mg/L) was recovered from a central venous catheter tip of a Dutch tourist, who was admitted to the ICU of our hospital after a stay in Israel. Unlike the previous CPKP isolates, the isolate from this patient was negative in the imipenem-EDTA test. This strain was resistant to aztreonam, and synergy was demonstrated between amoxicillin/clavulanic acid and cefotaxime. A positive imipenem/boronic acid test suggested the presence of KPC, which was confirmed by bla_{KPC} sequencing. KPC-producing organisms were initially confined to the ICU and organ transplant unit. In October 2007, they were isolated in surgical wards (orthopedic) and later in the same month in medical wards (renal, neurology).

KPC spread rapidly in the hospital, becoming increasingly prevalent (from 21 isolates in 2007 to 134 in 2009), while VIM-producing isolates declined (from 77 isolates in 2007 to 25 in 2009) (Table 1).

Since mid-2009, the prevalence of KPC isolates has been gradually declining, whereas the much lower rate of VIM producers has slightly increased (Figure 2). In May and June 2009, two (to date unique) isolates were identified that carried both bla_{KPC} and bla_{VIM} [17]. Table 2 summarises the CPKP isolated in the different wards/departments of the hospital over the study period.

Phenotypic and genotypic analysis of isolates

Overall, 267 (47%) isolates were phenotypically characterised as MBL- and 301 (53%) as KPC-producing (Table 1). All 70 genotypically tested KPC producers harboured the bla_{KPC-2} gene. Among MBL producers, molecular analysis revealed the presence of 72 bla_{VIM-1} and 10 bla_{VIM-12} genes. The latter were clustered between November 2006 and April 2007, with the exception of one sporadic case in June 2007. ERIC analysis revealed several different patterns among VIM producers, including a distinct clone comprising all VIM-12 carrying strains. KPC producers belonged to two different clones, one being predominant (data not shown).

Resistance to imipenem and meropenem, as well as of MBL producers to ertapenem, was variable (MIC ranging from 2 to >32 mg/L). KPC producers had invariably a MIC of >32 mg/L to ertapenem. Notably, 87 (32.6%) VIM-producing isolates were resistant to aztreonam (an antibiotic stable to the hydrolytic activity of MBLs). PCR analysis revealed that these 87 isolates also contained an extended-spectrum beta-lactamase (ESBL) gene, bla_{SHV-12} or bla_{SHV-5}.

Antimicrobial susceptibilities to non-beta-lactam agents are reported in Table 3. Almost all CPKP isolates were resistant to ciprofloxacin and trimethoprim-sulfamethoxazole. Gentamicin was more active than amikacin in vitro, particularly among KPC producers. Of note, 18.6% (56/301) of KPC-producing isolates were also resistant to colistin. Among CPKP, colistin resistance increased from 3.5% (4/115) in 2008 to 20.8% (25/120) in 2010. Tigecycline resistance, although less frequent than colistin, also increased among KPC producers (Table 3).

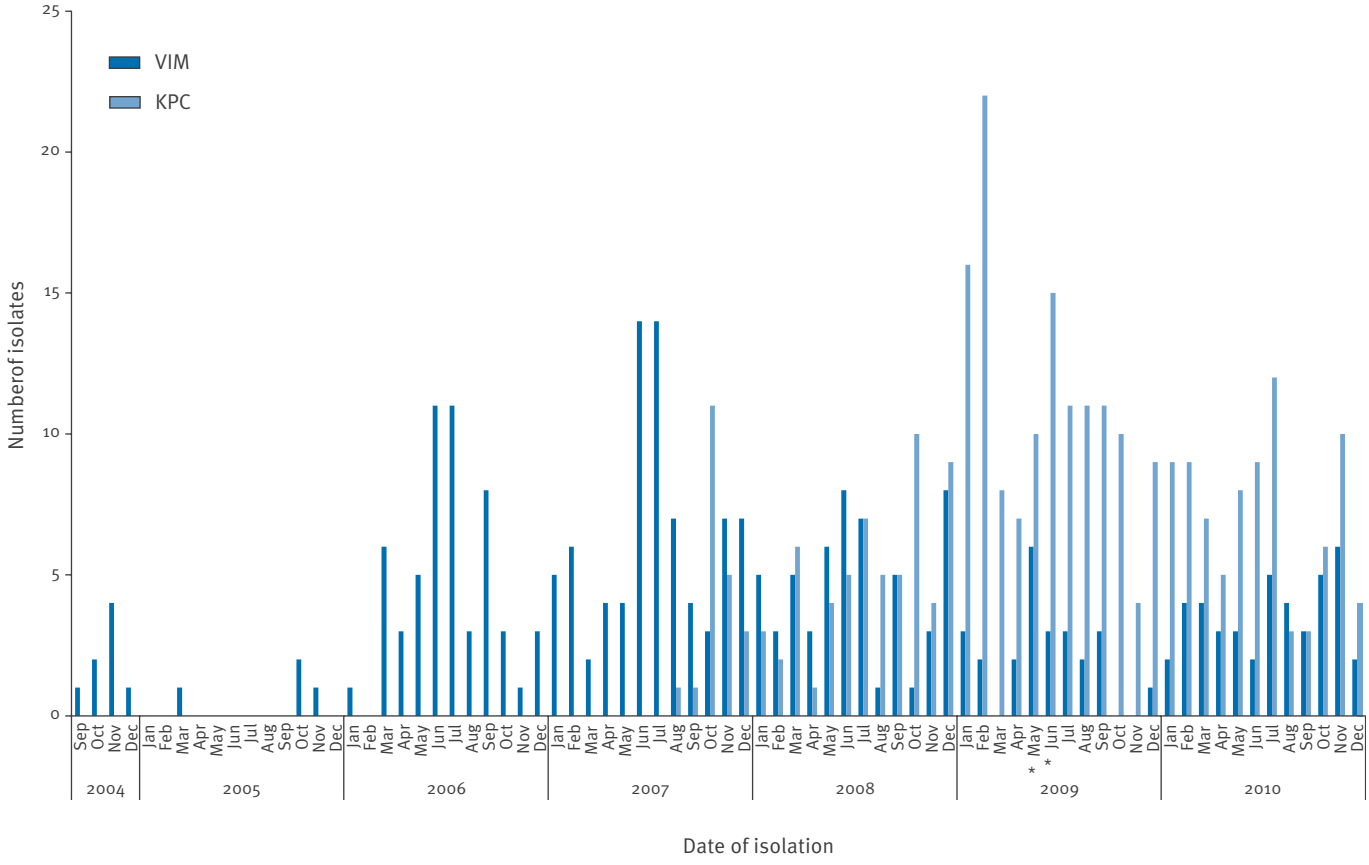
Discussion and conclusion

This study describes the, to our knowledge, largest outbreak of CPKP in a healthcare institution. As only clinical specimens from unique patients were included, and the MIC threshold for carbapenemase testing (imipenem >1 mg/L) was higher than the subsequently defined epidemiological cut-off values [18], it is possible that the prevalence of CPKP was underestimated. We observed on the one hand the dynamic co-existence of VIM- and KPC-producing strains, where KPC producers outcompeted pre-existing VIM producers, and on the other hand the rare emergence of strains co-producing VIM and KPC appearing late in this epidemic.

ERIC results suggest both clonal expansion and horizontal transmission of resistance determinants. Although all early VIM-1 producing isolates belonged to the same clone [14], subsequent VIM producers belonged to multiple distinct clones; multi-clonality of VIM-producing CPKP circulating in Greek hospitals was supported by previous reports [19]. All KPC producers belonged to two clones, the predominant of which likely corresponded to the hyperepidemic Greek clone, related to those from the United States and Israel; this has also been shown previously for some of our samples, using pulsed-field gel electrophoresis (PFGE) [13]. Notably, our index KPC strain was isolated from a patient who had been to Israel. To the best of

Figure 1

Clinical isolates of *Klebsiella pneumoniae* producing VIM or KPC, in Hippokration hospital, Thessaloniki, Greece, September 2004–December 2010 (n=570)



KPC: *Klebsiella pneumoniae* carbapenemase; VIM: Verona integron-encoded metallo-beta-lactamase.

Note: Two strains that produced both VIM and KPC are not included in the graph itself. However, their isolation dates (May and June 2009) are indicated by asterisks.

our knowledge, this is the first report of a documented transfer of KPC from Israel to Greece. Remarkably, the timing of this transfer coincided with the peak of the KPC outbreak in Israel [20]. These data are in accordance with previous findings on the similarities of KPC-producing clones reported from those two countries in 2007 [13]. It should be noted that KPC-2 strains had been reported from another area in Greece (Crete) earlier in the same year [21], but the origin of those isolates was unknown.

Standard infection control measures (including contact precautions) were implemented at the beginning of the outbreak in Hippokration hospital. However, adherence to the measures seemed to subside after the first months and CPKP were transmitted more widely. The highest incidence of CPKP was monitored in 2009, and since then infection control policy has been reassessed and intensified by the infection control committee (infectious disease physicians, the head of the microbiology department, infection control nurses and physicians from other hospital departments) according to guidelines from the Centers for Disease Control and Prevention [22].

The number of hospital infection control nurses was increased from one to three, and they actively monitored adherence and effectiveness of intensified interventions under the guidance of an infectious disease physician. Staffing levels in all hospital departments, including the ICUs, were reassessed. Moreover, the accurate identification of CPKP was verified and the presence of carbapenemase-producing bacteria was communicated in written reports to all physicians. Affected areas received quality visits from infection

Table 1

Klebsiella pneumoniae isolates carrying VIM and/or KPC in Hippokration hospital, Thessaloniki, Greece, September 2004–December 2010 (n=570)

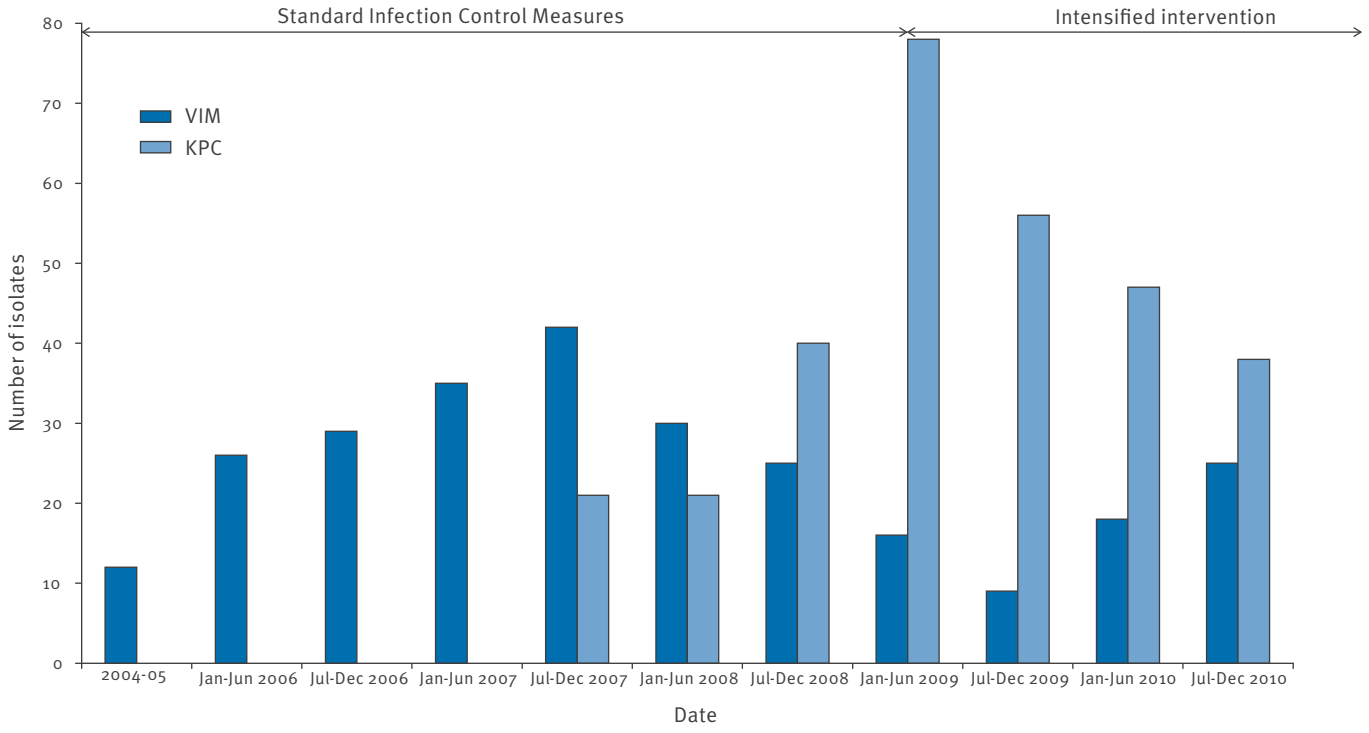
Year	All CPKP	VIM (% of all CPKP that year)	KPC (% of all CPKP that year)
2004	8	8	0
2005	4	4	0
2006	55	55	0
2007	98	77 (78.6)	21 (21.4)
2008	116	55 (47.4)	61 (52.6)
2009	161[a]	25 (15.7)	134 (84.3)
2010	128	43 (33.6)	85 (66.4)
Total	**570[a]**	**267 (47.0)**	**301 (53.0)**

CPKP: Carbapenemase-producing *Klebsiella pneumoniae*; KPC: *Klebsiella pneumoniae* carbapenemase; VIM: Verona integron-encoded metallo-beta-lactamase.

[a] Two strains producing both VIM and KPC were isolated in 2009 and are not included in the columns of VIM- and KPC-expressing isolates.

Figure 2

VIM- and KPC-producing *Klebsiella pneumoniae* isolates, Hippokration hospital, Thessaloniki, Greece, September 2004–December 2010 (n=568)



KPC: *Klebsiella pneumoniae* carbapenemase; VIM: Verona integron-encoded metallo-beta-lactamase.
Data shown by half year, except for the period 2004-2005, when VIM producers occurred only sporadically.

control nurses. Feedback from those visits,as well as resistance rates were communicated to the infectious disease physicians. Due to the endemic situation only infections with CPKP were monitored and no active surveillance took place. A database of all patients with CPKP was generated and distributed to the microbiology department, infection control nurses and infectious disease physicians. Incidence rates of CPKP were reported weekly to all hospital departments as well as the hospital's administration. Patients' location and transfer between departments and/or hospitals were monitored daily. Infection control precautions to prevent patient-to-patient transmission were intensified and targeted patients with CPKP. Contact precautions were put in place for all patients with a positive CPKP test: Where feasible, a single patient room was used for isolation. However, cohorting of patients was also used in departments where single patient rooms were not available (including all ICUs). Environmental measures were also implemented including dedicated use of non-critical equipment. Surface cleaning and disinfection was reinforced as well as final cleaning after a patient was moved from a department.

In February 2009, the infection control committee and hospital administration decided not to accept new admissions to adult ICU for 10 days. Education of healthcare personnel was intensified using audits, posters and video presentations about hand hygiene, contact precautions and severity of infections due to CPKP. Judicious use of antimicrobials was encouraged and daily quality rounds and audits of antimicrobial prescriptions were implemented. An antimicrobial restriction policy was in place and all antimicrobials with extended spectrum (especially carbapenems) were closely monitored by an infectious disease physician in cooperation with the hospital's pharmacy. Given the magnitude of the problem in Greece, 'Procrustes', a nationwide action plan for the containment of carbapenem-resistant bacteria has been implemented as of November 2010; its main features have been outlined elsewhere [2]. With these measures in place, a reduction in the prevalence of CPKP in the hospital was recorded (from 46% of *K. pneumoniae* strains isolated in 2009 to 38.3% in 2010, see Figures 1 and 2).

Not surprisingly [1], this outbreak has as yet not been contained, despite hospital-wide reinforcement of infection control measures. It is likely that its appearance and perpetuation had multiple contributors. Those included breaches in infection control practice, like low compliance with hand hygiene [23] and contact precautions. Inter-hospital transfer of carriers is favoured in Greek hospitals because there is no integrated recording system of re-admission alerts and inter-hospital communication [1]. Antibiotic overuse is an important contributor for the emergence and spread of resistance; association between carbapenem consumption and resistance has been previously documented [24]. As per institutional policy, in departments with a

Table 2

Carbapenemase-producing *Klebsiella pneumoniae* by unit/ward, Hippokration hospital, Thessaloniki, Greece, September 2004–December 2010 (n=570)

Year	Intensive care unit (%)	Transplant unit (%)	Medical wards (%)	Surgical wards (%)	Paediatric wards (%)	**Total**
2004	4	1	0	3	0	**8**
2005	1	1	1	1	0	**4**
2006	27 (49.1)	7 (12.7)	9 (16.4)	10 (18.2)	2 (3.6)	**55**
2007	25 (25.5)	23 (23.5)	21 (21.4)	26 (26.5)	3 (3.1)	**98**
2008	35 (30.2)	23 (19.8)	30 (25.9)	23 (19.8)	5 (4.3)	**116**
2009	39 (24.2)	28 (17.4)	42 (26.1)	46 (28.6)	6 (3.7)	**161**
2010	23 (18.0)	26 (20.3)	36 (28.1)	37 (28.9)	6 (4.7)	**128**
Total	**154 (27.0)**	**109 (19.1)**	**139 (24.4)**	**146 (25.6)**	**22 (3.9)**	**570**

Table 3

Susceptibility profile of carbapenem-resistant *Klebsiella pneumoniae* isolates to non-beta-lactam antimicrobial agents in Hippokration hospital, Thessaloniki, Greece, September 2004–December 2010 (n=568)

K. pneumoniae (no of isolates)	Number (%) resistant					
	GEN	AMK	CST	TGC	CIP	SXT
VIM producers (n=267)	63 (23.6)	79 (29.6)	9 (3.4)	14 (5.2)	257 (96.3)	264 (98.9)
KPC producers (n=301)	44 (14.6)	223 (74.0)	56 (18.6)	34 (11.3)	295 (98.0)	274 (91.0)

AMK: amikacin; CIP: ciprofloxacin; CST: colistin; GEN: gentamicin; KPC: *Klebsiella pneumoniae* carbapenemas; SXT: trimethoprim-sulfamethoxazole; TGC: tigecycline; VIM: Verona integron-encoded metallo-beta-lactamase.

high prevalence of CPKP, i.e. the ICU, colistin and gentamicin are used as initial empirical treatment when an infection with such an organism is suspected [25].

Notably, we observed different resistant profiles within clones in this study. Reasons for this may include the presence of additional mechanisms contributing to resistance patterns (typically, the frequent co-existence of an ESBL-type enzyme and VIM), the concurrent existence of several clones of VIM-producing strains, but also the increased rates of non-susceptibility to tigecycline and/or colistin, probably as a result of increasing use of those antibiotics for the treatment of infections with carbapenem-resistant organisms. Of particular concern are our results showing frequent aztreonam resistance among VIM producers, due to the additional carriage of an ESBL, as well as the high rates of resistance to non-beta-lactam agents, particularly among KPC producers. In agreement with recent reports [26, 27], increasing colistin resistance underlines a real threat from the emergence of multi- or pandrug-resistant bacteria. In view of the exhaustion of available therapeutic options, investment in infection control resources and optimal antibiotic use, along with co-ordinated efforts from all involved parties is urgently required.

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