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Emerging *Klebsiella pneumoniae* Isolates Coproducing KPC-2 and VIM-1 Carbapenemases▽

VIM-1-producing *Klebsiella pneumoniae* strains, increasingly isolated in Greece since 2002, constitute the majority of multiresistant clinical isolates of this species found in most Greek hospitals (9). Moreover, following reports on *Klebsiella pneumoniae* carbapenemase (KPC)-producing isolates from Greek patients transferred to other European countries (2, 10), a nationwide surveillance revealed a parallel epidemic of KPC-2 producers (4). Consequently, clinical laboratories were required to screen all *K. pneumoniae* isolates displaying reduced susceptibility to carbapenems (MIC of imipenem ≥ 1 μg/ml) by both EDTA- and boronic acid-imipenem combined disk tests (3, 8). Potential carbapenemase producers were sent to the Microbiology Laboratory, National School of Public Health (NSPH), Athens, Greece, for confirmation and further study.

Here, we report on the emergence of *K. pneumoniae* isolates coproducing KPC-2 and VIM-1.

Clinical isolates H-1406, A-1797, and T-1780 had been submitted to the reference laboratory (NSPH) as metallo-β-lactamase (MβL) positive (based on EDTA-imipenem synergy) from hospitals located in Crete (Heraklion), Athens, and Thessaly (Trikala), Greece, during December 2008 to April 2009. All three isolates were derived from severely ill patients who had been hospitalized for prolonged time periods (two were in intensive care units) and had received multiple courses of antibiotics, including carbapenems. MβL production was phenotypically confirmed, while boronic acid-based tests using either imipenem, meropenem, or ertapenem discs appeared negative (Table 1). PCR and sequencing showed carriage of $bla_{\text{VIM-1}}$ by all three isolates. High-level resistance to penicillins, penicillin-inhibitor combinations, cefotaxime, and ceftazidime and decreased susceptibility or resistance to cefepime, imipenem, meropenem, and ertapenem determined by a microdilution technique were consistent with VIM-1 production (Table 1). To explain resistance to aztreonam, we applied isoelectric focusing of β-lactamase extracts; PCR assays specific for various class A and C *bla* genes, including the bla_{TEM}, bla_{SHV}, $bla_{\text{CTX-M}}$, bla_{GES}, bla_{VEB}, bla_{KPC}, bla_{CMY}, bla_{DHA}, and bla_{ACC} types (6, 7); and sequencing of PCR products. It was documented that isolates H-1406, A-1797, and T-1790 carry $bla_{\text{KPC-2}}$ and produce the respective enzyme, with an isoelectric point of 6.7. To our knowledge, this is the first report of coproduction of VIM and KPC carbapenemases by clinical isolates.

Pulsed-field gel electrophoresis of XbaI-digested genomic material from H-1406, A-1797, and T-1780 produced patterns with significant similarity (>80%), suggesting a genetic relationship. These patterns were distinct from those of four KPC-2-producing strains identified so far, including the prevalent one (represented by T-1504) (Table 1 and Fig. 1). On the other hand, the genomic type of a VIM-1-producing *K. pneumoniae* strain occurring sporadically since 2003 in Athens hospitals (represented by isolate A-1760) was highly similar (>95%) to that of the *K. pneumoniae* VIM-1 and KPC-2 coproducer T-1780 (Fig. 1). Notwithstanding the small number of isolates and the potentially frequent changes in plasmid content, a plausible hypothesis is that the *K. pneumoniae* VIM-1 and KPC-2 coproducer evolved through acquisition of a $bla_{\text{KPC-2}}$-carrying plasmid by an established VIM-1-producing *K. pneumoniae* strain. Plasmid content analysis did not contradict the clonal and evolutionary hypotheses regarding *K. pneumoniae* VIM-1 and KPC-2 coproducer isolates. The S1 nuclease method (1) showed that H-1406, A-1797, and T-1780, as well as the KPC-2-producing *K. pneumoniae* isolate T-1504, all harbored similarly sized plasmids (approximately 100 kb) that hybridized strongly with a $bla_{\text{KPC-2}}$-specific probe (data not shown). Conjugation experiments by a mixed broth culture method using a rifampin (rifampicin)-resistant *Escherichia coli* K-12 laboratory strain as a recipient and isolates H-1406 and A-1780 as donors were successful. KPC-2-producing *E.*

TABLE 1. β-Lactam resistance phenotypes and β-lactamase-related characteristics of carbapenemase-producing isolates of *K. pneumoniae*

Isolate	β-Lactamase(s)[b]	MIC of β-lactam (μg/ml)[c]:										Carbapenemase activity[d]		EDTA test result	Increase in diam (mm) of boronic acid test zone (interpretation)
		PIP	PTZ	TIM	CTX	CAZ	FEP	ATM	IPM	MEM	ETP	Total (U)	Reduction by EDTA (%)		
H-1406	VIM-1, KPC-2	>256	>256	>256	256	>256	128	64	32	8	64	55	57	+	3 (−)
A-1797	VIM-1, KPC-2	>256	>256	>256	>256	>256	16	64	2	2	8	42	80	+	2 (−)
T-1780	VIM-1, KPC-2	>256	>256	>256	>256	>256	>256	64	32	64	>128	76	64	+	3 (−)
A-1760[a]	VIM-1	>256	>256	>256	>256	>256	256	0.12	32	64	64	80	100	+	0 (−)
T-1504[a]	KPC-2	128	64	256	8	16	16	32	2	16	32	31	<2	−	9 (+)

[a] Isolate used for control purposes.
[b] The intrinsic penicillinase produced by all isolates is not indicated.
[c] PIP, piperacillin; PTZ, piperacillin plus 4 μg/ml tazobactam; TIM, ticarcillin plus 2 μg/ml clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime, FEP, cefepime; ATM, aztreonam; IPM, imipenem; MEM, meropenem; ETP, ertapenem.
[d] Determined as described in reference 5. EDTA was used at a final concentration of 100 μM. One unit was the amount of enzyme hydrolyzing 1 nmol of imipenem per min per mg of protein. Values are the means of three measurements not differing more than 10%.

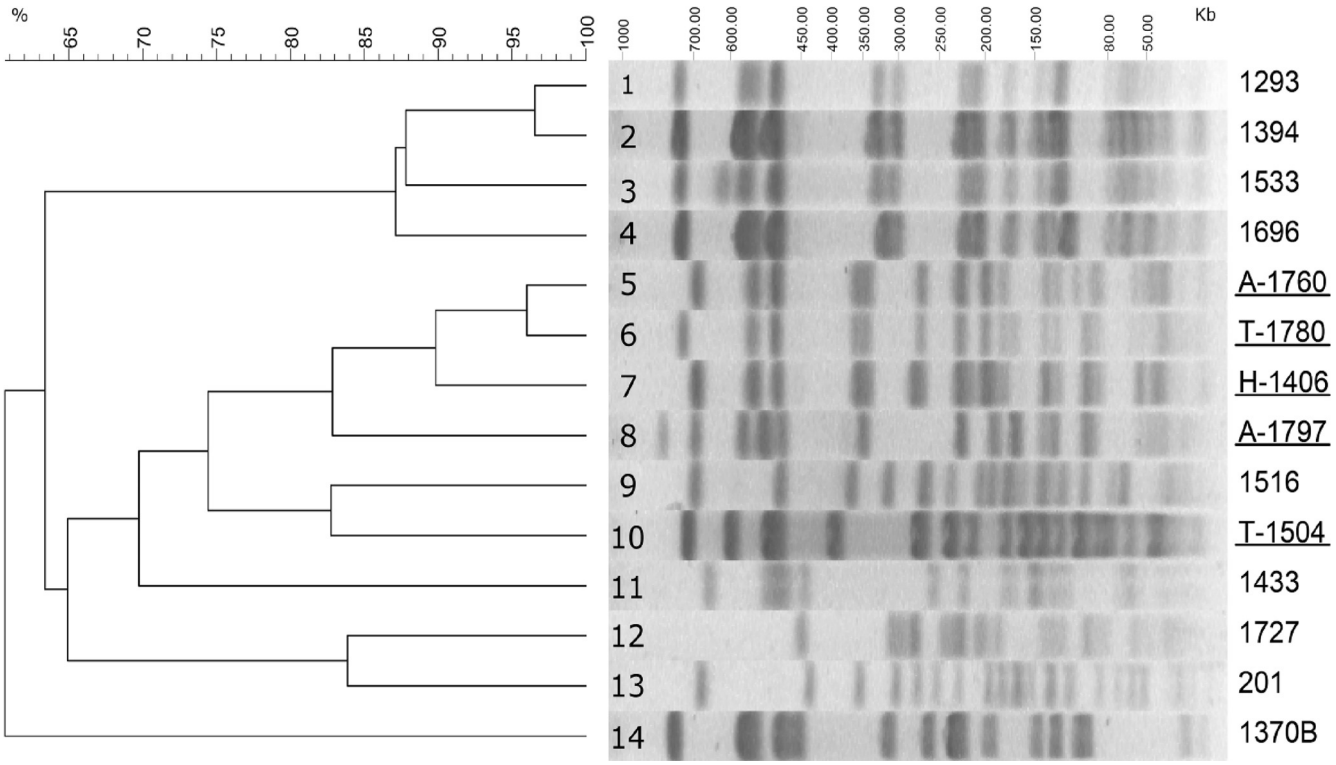

FIG. 1. Pulsed-field gel electrophoresis of XbaI-generated macrorestriction fragments of three VIM-1- and KPC-2-coproducing *K. pneumoniae* (lanes 6 to 8), five VIM-1-producing *K. pneumoniae* (lanes 1 to 5), four KPC-2-producing *K. pneumoniae* (lanes 9 to 11 and 14), and two non-carbapenemase-producing *K. pneumoniae* (lanes 12 and 13) isolates used for comparison. Underlined designations indicate isolates included also in Table 1. Preparations were run in 1% agarose under 6.0 V/cm for 20 h with a pulsing time linearly ramped from 5 to 55 s. The dendrogram was constructed using the GelCompar II v4.1 software (Applied Maths). The percentages of similarity were calculated with the Dice coefficient (position tolerance, 2.5%) and are represented by the results of the unweighted-pair group method using average linkages.

coli transconjugant clones were obtained in both cases at low frequencies (approximately 5×10^{-10} transconjugants per donor cell). Moreover, the VIM-1 MβL in the *K. pneumoniae* VIM-1 and KPC-2 coproducer isolates and the related VIM-1-producing *K. pneumoniae* isolate A-1760 was probably mediated by plasmids; these isolates were unable to transfer VIM-1 production by conjugation under the in vitro conditions employed. However, results of hybridization of S1 nuclease-treated plasmid preparations with a $bla_{\text{VIM-1}}$-specific probe indicated the existence of large (approximately 350-kb) VIM-1-encoding plasmids (data not shown).

The false-negative results of the boronic acid-based test must be attributed to a masking effect of the coproduced VIM-1. Activities of β-lactamase extracts with and without EDTA against imipenem indicated the production of meaningful relative amounts of KPC-2 (Table 1), which, nevertheless, were not adequate to produce a positive synergy image under the conditions employed in the boronic acid-based test. Identification of *K. pneumoniae* VIM-1 and KPC-2 coproducer isolates in diverse locations within a short time period along with detection difficulties may suggest a thus far unnoticed yet significant spread. Studies are under way to asses the actual prevalence of *K. pneumoniae* VIM-1 and KPC-2 coproducer isolates and the potential therapeutic impact of the coproduction of VIM-1 and KPC-2 in *K. pneumoniae*.

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