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Detection of the new metallo-β-lactamase VIM-19 along with KPC-2, CMY-2 and CTX-M-15 in *Klebsiella pneumoniae*

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Objectives: To report the identification of the metallo-β-lactamase (MBL) variant VIM-19 in a *Klebsiella pneumoniae* clinical strain co-producing KPC-2 carbapenemase, CMY-2 cephalosporinase and CTX-M-15 extended-spectrum β-lactamase.

Methods: MICs were determined by agar dilution. Phenotypic tests were performed to detect carbapenemase production. PCR and nucleotide sequencing were used for the identification of *bla* gene types and mapping of the integron carrying the MBL gene. The location of the MBL and KPC alleles was investigated by mating experiments, plasmid analysis and PCR assays.

Results: Imipenem, meropenem and ertapenem MICs for the study strain were 32, 16 and 64 mg/L, respectively. The strain carried bla_{TEM-1}, bla_{CMY-2}, bla_{KPC-2} and $bla_{CTX-M-15}$ genes along with the gene bla_{VIM-19}, which was located in a class 1 integron as the first gene cassette, followed by *aacA6*, *dfrA1* and *aadA1* cassettes. Mating experiments, plasmid analysis and PCR assays revealed that bla_{VIM-19} and bla_{CMY-2} were carried on an ~150 kb self-transferable plasmid, while bla_{KPC-2} and bla_{TEM-1} were on an ~70 kb self-transferable plasmid; $bla_{CTX-M-15}$ was non-transferable.

Conclusions: The detection of the new MBL, VIM-19, which has enhanced carbapenemase activity, along with KPC-2, CMY-2 and CTX-M-15 is of concern. Further spread of the respective strains or plasmids may have serious consequences for antimicrobial chemotherapy.

Keywords: bla_{VIM-19}, bla_{KPC-2}, plasmids, transferable, conjugation, carbapenemases, MBLs

Introduction

During the last few years, carbapenem resistance has been increasingly reported in *Klebsiella pneumoniae* and is largely attributed to the production of carbapenem-hydrolysing enzymes, such as metallo-β-lactamase (MBL) and *K. pneumoniae* carbapenemase (KPC) types.[1,2]

K. pneumoniae isolates producing KPC enzymes have become increasingly prevalent on the East Coast of the USA since 2001.[2] They have also caused outbreaks in Israel, and have recently become emerging public health concerns in several regions worldwide, such as China, Latin America and Greece.[2] The dissemination of KPC producers poses a significant threat for carbapenem activity, considering the increasing rates also of MBL-producing *K. pneumoniae*.

MBLs of the VIM group have been identified in different countries as a source of several nosocomial *K. pneumoniae* outbreaks.[1,2] The VIM group currently includes 23 variants, clustered into three evolutionary lineages, represented by VIM-1, VIM-2 and VIM-7 (www.lahey.org/studies). In 2009, a new bla_{VIM} gene variant, designated bla_{VIM-19}, was detected in enterobacterial pathogens from Algiers and characterized by two research groups.[3,4] In both descriptions, the gene bla_{VIM-19} was located as the first gene cassette in a class 1 integron with an unidentified 3′ extremity.[3,4] In addition, Rodriguez-Martinez *et al.*[4] have shown that the amino acid substitutions by which VIM-19 differs from the closely resembled VIM-1 and VIM-4 enzymes confer increased carbapenem hydrolytic activity. During the same year, *K. pneumoniae* producing this potent VIM-19 carbapenemase, along with KPC-2, CMY-2 and CTX-M-15 enzymes, was identified in another Mediterranean region, Greece (GenBank accession number FJ 915116). The present report documents this worrisome evolution.

Materials and methods

Bacterial isolates and phenotypic testing

K. pneumoniae strain KP1935 was recovered in May 2008 from a urinary tract infection of a 64-year-old female patient hospitalized at Serres General Hospital, Greece. MICs for the clinical and transconjugant strains of several β-lactams, aminoglycosides, ciprofloxacin, trimethoprim, tetracycline, tigecycline and colistin were determined by agar dilution. The isolate was phenotypically screened for carbapenemase production by performing combined tests using four discs of meropenem, one without and the other three with EDTA, phenylboronic acid (PBA) or both EDTA and PBA.[5] *K. pneumoniae* ATCC 13883, *Pseudomonas aeruginosa* ATCC 27853 and isolates from our laboratory collection that produce KPC, VIM and extended-spectrum β-lactamases (ESBLs) were used as controls in susceptibility assays.

PCR assays and DNA sequencing

The detection of broad-spectrum β-lactamase genes (KPC, CMY and other plasmidic AmpC-like genes, OXA-48 carbapenemase, MBLs, and ESBLs of TEM, SHV and CTX-M types) was sought by PCR using consensus primers that were specific for each enzyme group.[6] Isolates from our collection that were previously characterized were used as controls in the PCR assays. For integron mapping, PCR assays combining primers specific for conserved 5′-CS and 3′-CS sequences with primers for bla_{VIM}, *aacA*, *dfr*, *aadA*, *qacEΔ1* and *sul* genes were performed. PCR products of the genes tested and also of the bla_{VIM}-carrying integron were sequenced on both strands. The bla_{KPC}-flanking region PCR was mapped using a series of successive primers.[7]

Conjugation assays and plasmid analysis

Filter mating experiments were performed using *Escherichia coli* 26R793 (lac^-, rif^r) as recipient. Selection of the transconjugants was made on MacConkey agar plates containing rifampicin (100 mg/L) and either ampicillin (40 mg/L) or ertapenem (0.5–1 mg/L). Plasmid isolation was performed with the QIAfilter Plasmid Maxi Kit (Qiagen) and with a standard alkaline lysis protocol, using *E. coli* 39R861 as the standard plasmid control. The plasmid DNA bands of the transconjugants were extracted from the agarose gel, and used as templates in PCR for bla_{KPC}, bla_{VIM}, bla_{CMY} and bla_{TEM} genes.

Results

The strain KP1935 was resistant or intermediate to all β-lactams, including carbapenems, with imipenem, meropenem and ertapenem MICs being 32, 16 and 64 mg/L, respectively. It also exhibited resistance to almost all alternative antimicrobials, including colistin (MIC 16 mg/L), and was susceptible to only tigecycline (MIC 2 mg/L) (Table 1). The simultaneous presence of both MBL and KPC carbapenemases was indicated by a positive combined-disc test using meropenem with both EDTA and PBA, while meropenem discs containing EDTA or PBA[6] were negative.

PCR for β-lactamase genes showed that KP1935 was positive for bla_{TEM}, bla_{CTX-M}, bla_{CMY}, bla_{VIM} and bla_{KPC} genes, and negative for the remaining genes tested, including all bla_{SHV} variants. Sequencing analysis identified bla_{TEM-1}, $bla_{CTX-M-15}$, bla_{CMY-2}, bla_{VIM-19} and bla_{KPC-2} alleles. PCR and sequencing using primers 5′-CS and 3′-CS in various combinations with primers for bla_{VIM}, *aacA*, *aadA* and *dfrA1* genes revealed a new class 1 integron with a structure similar to that carrying the close variant gene bla_{VIM-4} in an *Enterobacter cloacae* isolate from Greece.[8] The bla_{VIM-19} gene cassette is located downstream of the *attI1* recombination site, followed by an *aacA6* cassette, a *dfrA1* cassette, an *aadA1* cassette and the 3′-CS, containing *qacEΔ1* and *sulI* (Figure 1). PCR mapping of the bla_{KPC}-flanking region showed that this gene was located in a Tn*4401* transposon similar to that found in *K. pneumoniae* isolates previously.[7]

Table 1. MICs of antibiotics for KP1935 and the respective transconjugants

	MICs (mg/L) of antibiotics for:			
Antibiotic	KP1935 (VIM-19+KPC-2+CTX-M-15)	Tcs *E. coli* 26R793 (pVIM-19+CMY)	Tcs *E. coli* 26R793 (pKPC-2)	*E. coli* 26R793
Imipenem	32	8	2	0.25
Meropenem	16	2	1	0.06
Ertapenem	64	8	2	0.06
Ampicillin	>32	>256	>256	4
Amoxicillin+CLA	>256	256	64	4
Piperacillin+TZB	>256	>256	256	1
Aztreonam	>256	4	32	0.12
Ceftazidime	>256	>256	16	1
Cefotaxime	>32	>32	16	0.12
Cefepime	128	64	8	0.06
Cefoxitin	>256	>256	16	4
Ciprofloxacin	>32	0.03	0.03	0.03
Gentamicin	64	1	1	1
Amikacin	16	16	2	2
Trimethoprim	>256	>256	0.25	0.25
Tetracycline	>256	128	1	1
Tigecycline	2	0.25	0.12	0.12
Colistin	16	0.06	0.06	0.06

CLA, clavulanic acid (2 mg/L); TZB, tazobactam (4 mg/L); Tcs, transconjugant strain.

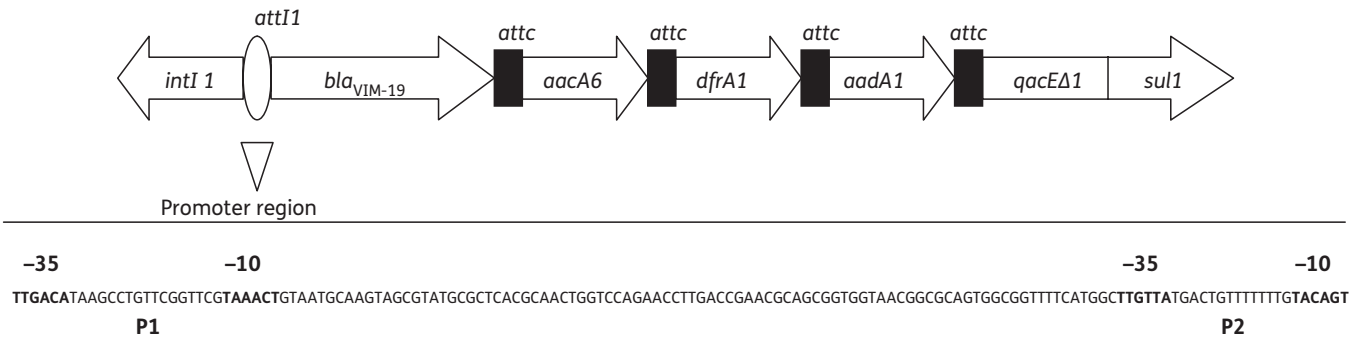

Figure 1. Schematic representation of the class 1 integron structure of the strain KP1935.

Repeated mating experiments yielded transconjugant colonies with MICs that, compared with the MICs for the recipient strain *E. coli* 26R793, were elevated for penicillins, cephalosporins and carbapenems. Phenotypic testing of many transconjugant colonies showed that some of them were positive in the combined-disc test using meropenem and boronic acid, while others were positive using meropenem and EDTA. PCR assays showed that all boronic acid-positive transconjugant colonies tested were positive for bla_{KPC} and bla_{TEM} genes and negative for bla_{VIM}, bla_{CMY} and bla_{CTX-M}, while all EDTA-positive colonies tested were positive for bla_{VIM} and bla_{CMY} and negative for bla_{KPC}, bla_{CTX-M} and bla_{TEM}. Transconjugant colonies that were PCR-positive for bla_{CTX-M} were not detected after repeated mating experiments and testing numerous colonies. Plasmid analysis of the clinical strain KP1935 revealed two plasmids of ~70 and 150 kb, while the bla_{VIM}- and bla_{CMY}-bearing transconjugants had a single plasmid of ~150 kb and the bla_{KPC}- and bla_{TEM}-bearing transconjugants had a single plasmid of ~70 kb. PCR, using as template the gel-extracted plasmid DNA band of the transconjugants, confirmed the carriage of the bla_{KPC} and bla_{TEM} genes on the ~70 kb plasmid, and of the bla_{VIM} and bla_{CMY} genes on the ~150 kb plasmid. The MICs for the bla_{KPC}-positive and the bla_{VIM}-positive transconjugant colonies of the antibiotics tested are shown in Table 1.

Discussion

In the present report, the novel MBL bla_{VIM-19} was detected in a clinical strain of *K. pneumoniae* that also harboured bla_{TEM-1}, bla_{CMY-2}, bla_{KPC-2} and $bla_{CTX-M-15}$. The gene bla_{VIM-19} differs from bla_{VIM-4} by one amino acid residue at position 215 (Asn215Lys, standard class B β-lactamase numbering scheme) and from bla_{VIM-1} by two amino acid residues (Asn215Lys and Ser228Arg) that increase the carbapenemase activity of the VIM-19 enzyme compared with VIM-1.[4] It could be postulated that VIM-19 has arisen under carbapenem pressure in our hospitals, where VIM-1- or VIM-4-producing microorganisms are common.[8,9] Alternatively, this novel variant may have arisen independently and be common in our regions. The latter possibility is supported by the detection of bla_{VIM-19} in a distantly located Mediterranean country[3,4] and also by the wide occurrence of the close variant gene bla_{VIM-4} in remote countries.[8,10] Indeed, the emergence of bla_{VIM-19} in a class 1 integron with a gene content similar to that of an integron detected previously in Greece, but including bla_{VIM-4} instead of bla_{VIM-19},[8] underlines the continuous evolution of these important determinants. It should be noted that a similar integron carrying bla_{VIM-4} in an *E. cloacae* isolate was harboured in a different plasmid (40 kb) that also included the ESBL gene bla_{SHV-2a},[8] in contrast with the bla_{VIM-19}-carrying integron, indicating that the dissemination of a single plasmid is probably not the cause of VIM-4 or VIM-19 spread.

The study strain KP1935 had higher carbapenem MICs compared with most *K. pneumoniae* clinical strains from Greece that produce VIM or KPC as single carbapenemases.[6,9] Furthermore, the VIM-19-possessing transconjugants had MICs of imipenem (8 mg/L) and meropenem (2 mg/L) that were similar to those of other VIM-19-producing laboratory strains,[3,4] being higher than those of VIM-1-producing laboratory strains.[4,9] This observation further supports the enhanced carbapenemase activity of VIM-19 relative to VIM-1.

In tertiary care Greek hospitals, *K. pneumoniae* isolates that produce MBLs or, more recently, KPC carbapenemases are commonly recovered from clinical infections.[6,9] The current detection of VIM-19 along with KPC-2 in our strain is only the second description of a *K. pneumoniae* strain that produced two different carbapenemases after the appearance of VIM-1- and KPC-2-producing *K. pneumoniae*.[11] Considering that the combination of a potent VIM variant along with KPC and CTX-M-15 ESBL inactivates all clinically available β-lactams, a further spread of the respective strains or plasmids may have even more serious consequences in the treatment of nosocomial infections.

Nucleotide sequence accession number

The nucleotide sequence of the gene bla_{VIM-19} has been assigned accession number FJ 915116 in the EMBL/GenBank/DDBJ databases.

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Transparency declarations

None to declare.

References

1 Poirel L, Pitout JD, Nordmann P. Carbapenemases: molecular diversity and clinical consequences. *Future Microbiol* 2007; **2**: 501–12.

2 Nordmann P, Cuzon G, Naas T. The real threat of *Klebsiella pneumoniae* carbapenemase-producing bacteria. *Lancet Infect Dis* 2009; **9**: 228–36.

3 Robin F, Aggoune-Khinache N, Delmas J *et al*. Novel VIM metallo-β-lactamase variant from clinical isolates of *Enterobacteriaceae* from Algeria. *Antimicrob Agents Chemother* 2010; **54**: 466–70.

4 Rodriguez-Martinez JM, Nordmann P, Fortineau N *et al*. VIM-19, a metallo-β-lactamase with increased carbapenemase activity from *Escherichia coli* and *Klebsiella pneumoniae*. *Antimicrob Agents Chemother* 2010; **54**: 471–6.

5 Pournaras S, Poulou A, Tsakris A. Inhibitor-based methods for the detection of KPC carbapenemase-producing Enterobacteriaceae in clinical practice by using boronic acid compounds. *J Antimicrob Chemother* 2010; **65**: 1319–1321.

6 Pournaras S, Protonotariou E, Voulgari E *et al*. Clonal spread of KPC-2 carbapenemase-producing *Klebsiella pneumoniae* strains in Greece. *J Antimicrob Chemother* 2009; **64**: 348–52.

7 Naas T, Cuzon G, Villegas MV *et al*. Genetic structures at the origin of acquisition of the β-lactamase bla_{KPC} gene. *Antimicrob Agents Chemother* 2008; **52**: 1257–63.

8 Ikonomidis A, Spanakis N, Poulou A *et al*. Emergence of carbapenem-resistant *Enterobacter cloacae* carrying VIM-4 metallo-β-lactamase and SHV-2a extended-spectrum β-lactamase in a conjugative plasmid. *Microb Drug Resist* 2007; **13**: 221–6.

9 Ikonomidis A, Tokatlidou D, Kristo I *et al*. Outbreaks in distinct regions due to a single *Klebsiella pneumoniae* clone carrying a bla_{VIM-1} metallo-β-lactamase gene. *J Clin Microbiol* 2005; **43**: 5344–7.

10 Peleg AY, Bell JM, Hofmeyr A *et al*. Inter-country transfer of Gram-negative organisms carrying the VIM-4 and OXA-58 carbapenem-hydrolysing enzymes. *J Antimicrob Chemother* 2006; **57**: 794–5.

11 Giakkoupi P, Pappa O, Polemis M *et al*. Emerging *Klebsiella pneumoniae* isolates coproducing KPC-2 and VIM-1 carbapenemases. *Antimicrob Agents Chemother* 2009; **53**: 4048–50.