

NOTES

In Vivo Acquisition of a Plasmid-Mediated *bla*_{KPC-2} Gene among Clonal Isolates of *Serratia marcescens*[∇]

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Three patients admitted to a Greek hospital were infected with *Serratia marcescens* isolates that exhibited reduced susceptibility to carbapenems and harbored *Klebsiella pneumoniae* carbapenemase (KPC) enzymes. In two of these cases, the patients were initially infected by carbapenem-susceptible *S. marcescens* isolates. Molecular typing and plasmid analysis suggested that all three patients had clonally indistinguishable isolates of *S. marcescens* that acquired a plasmid-mediated *bla*_{KPC-2} gene during the hospitalization.

The emergence of organisms producing class A β-lactamases of *Klebsiella pneumoniae* carbapenemase (KPC) types is a major clinical and public health concern (2, 10). They are typically transposon-encoded determinants and therefore have the potential to disseminate between plasmids and across bacterial species (9). *K. pneumoniae* remains the species most likely to harbor *bla*_{KPC} genes (2, 8, 10, 13). Nevertheless, other species of *Enterobacteriaceae*, as well as species of nonfermenters, have been occasionally reported to exhibit this pattern of resistance (3, 15, 16, 19). In *Serratia marcescens* carbapenemase production is mostly attributed to class B metallo-β-lactamases (MBLs) as well as to the class A SME family of carbapenemases (14). Only recently has carbapenem-hydrolyzing activity in *S. marcescens* been attributed to the production of a KPC in China and the United States (3, 17, 23). We report the spread of three *bla*_{KPC}-possessing *S. marcescens* isolates in a Greek intensive care unit and give *in vitro* and *in vivo* evidence of the potential acquisition of such plasmid-borne resistance genes.

In December 2008 a 77-year-old woman was admitted to the unit following a neurosurgical procedure. Ampicillin-sulbactam was administered postoperatively. Two months after her admission, the patient developed pneumonia, and bronchial lavage samples grew a *S. marcescens* isolate (S53) that exhibited reduced carbapenem susceptibility. The patient was successfully treated with tigecycline and inhaled colistin.

Approximately 5 months later, in April 2009, a 49-year-old man was admitted following the surgical removal of a subcranial hematoma. He remained febrile while receiving empirical

prophylactic antibiotic treatment with ampicillin-sulbactam, vancomycin, and amikacin. Bronchial lavage samples produced a carbapenem-susceptible *S. marcescens* isolate (S51) and a carbapenem-resistant *K. pneumoniae* isolate (K72). Antibiotic therapy was changed to meropenem and colistin. A second episode of pneumonia occurred approximately 2 weeks later, and a new *S. marcescens* isolate (S54) with reduced susceptibility to carbapenems was recovered from the bronchial lavage cultures. The patient was successfully treated with tigecycline and colistin.

Finally, in April 2009 a 33-year-old woman was admitted following extensive surgery to the spine. The patient received ampicillin-sulbactam postoperatively. Approximately a week after her admission she presented with bacteremia due to a carbapenem-susceptible *S. marcescens* isolate (S52) and was treated with ciprofloxacin. Three weeks later the patient had an episode of pneumonia. Bronchial lavage sample cultures produced a new *S. marcescens* isolate (S55) that exhibited reduced susceptibility to carbapenems. Administration of ciprofloxacin in combination with gentamicin led to the successful treatment of this episode.

The isolates that were recovered from the aforementioned patients were evaluated. Species identification was performed with the Vitek 2 system (bioMérieux, Marcy l'Étoile, France) and confirmed with API 20E (bioMérieux). MICs for several β-lactams, aminoglycosides, ciprofloxacin, tigecycline, and colistin were further determined by agar dilution according to CLSI recommendations (4). The MBL Etest (AB Biodisk, Solna, Sweden) and the combined disk test with imipenem and EDTA (5) were used to screen for MBL production. The phenotypic detection of KPC-possessing isolates was evaluated with the boronic acid potentiation disk test using meropenem as an antibiotic substrate (20). Extended-spectrum β-lactamase (ESBL) production was tested with the CLSI confirmatory test and in the KPC-possessing isolates with the modified CLSI

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TABLE 1. Antimicrobial susceptibility patterns of the study's clinical isolates, their transconjugants, and the recipient strain, *E. coli* 26R764

Antibiotic(s)	MIC ($\mu\text{g/ml}$)						
	Clinical isolates			Transconjugants			
	<i>S. marcescens</i> S51 and S52	<i>S. marcescens</i> S53, S54, and S55	<i>K. pneumoniae</i> K72	<i>E. coli</i> 26R764 S53, S54, and S55 (pTEM-1)	<i>E. coli</i> 26R764 S54, and S55 (pKPC-2 and pTEM-1)	<i>E. coli</i> 26R764 K72 (pKPC-2/ TEM-1)	<i>E. coli</i> 26R764
Imipenem	0.5	2–4	16	0.25	1–2	1	0.12
Meropenem	0.12	2–4	16	0.12	1	0.5	0.06
Ertapenem	0.12–0.25	8–16	>32	0.12	2–4	0.5	0.06
Aztreonam	0.12	128–256	>256	0.12	128	8	0.06
Cefotaxime	0.12	>32	>32	1	16	2	0.12
Cefepime	0.5	8–16	32	0.5	4–8	2	0.5
Ceftazidime	0.12	8–16	32	1	4–8	2	0.12
Cefoxitin	0.25–0.5	16–32	32	1	16	2	1
Amoxicillin	>256	>256	>256	>256	>256	>256	4
Amoxicillin- clavulanate	64–128	64	>256	8	32–128	32	4
Piperacillin- tazobactam	2–4	>256	>256	2–4	128	32	2
Tigecycline	1–2	1	1	0.12	0.25	0.5	0.12
Colistin	>16	>16	0.5	0.5	0.5	0.5	0.5
Ciprofloxacin	0.12	0.06–0.12	16	0.03	0.03	0.015	0.03
Amikacin	2–8	16–32	32	16–32	16–32	2	2
Tobramycin	2–4	16–32	32	16	16	1	1
Gentamicin	1	1	2	1	2	1	1

ESBL confirmatory test, using clavulanate in combination with boronic acid (21).

Isolates were screened for β -lactamase genes by PCR amplification using a panel of primers for the detection of all types of MBLs (6), KPCs (8), plasmid-mediated AmpCs in single PCRs for each gene (11), and ESBLs (22). PCR products were subjected to direct sequencing. Pulsed-field gel electrophoresis (PFGE) of SpeI- and of XbaI-digested genomic DNA of the *S. marcescens* isolates was performed with a CHEF-DRIII system (Bio-Rad, Hemel Hempstead, United Kingdom), and PFGE patterns were compared visually following previously described criteria (18). The potential for conjugational transfer of carbapenem resistance was examined in biparental matings using LB broth cultures and *Escherichia coli* 26R764 (lac^+ Rif^r) as the recipient strain. Transconjugant clones were screened on MacConkey agar plates containing rifampin (150 $\mu\text{g/ml}$) and amoxicillin (40 $\mu\text{g/ml}$) or ertapenem (0.5 $\mu\text{g/ml}$). MICs were determined by agar dilution (4). All β -lactamase genes were detected by PCR amplification. Plasmid extraction was performed by using an alkaline lysis protocol with *E. coli* 39R861 as a control strain (7).

The susceptibility patterns of the *S. marcescens* and *K. pneumoniae* isolates are shown in Table 1. *S. marcescens* isolates S51 and S52 were susceptible to all carbapenems and to most β -lactam antibiotics. Phenotypic tests were negative for carbapenemase and ESBL production, amplification of the β -lactamase genes confirmed the presence solely of the bla_{TEM} gene, and DNA sequencing identified the gene in both isolates as $\text{bla}_{\text{TEM-1}}$.

S. marcescens isolates S53, S54, and S55 exhibited reduced susceptibility to imipenem and meropenem and were resistant to ertapenem. They were also resistant to various other β -lactam antibiotics and tobramycin; they also exhibited reduced susceptibility to amikacin but remained susceptible to genta-

micin, ciprofloxacin, and tigecycline. The phenotypic tests were negative for MBL production, but the boronic acid potentiation disk test yielded a positive result for KPC production. The modified confirmatory test for ESBL production was negative. Amplification of the β -lactamase genes confirmed the presence of bla_{KPC} and bla_{TEM} genes, which were identified by sequencing analysis as $\text{bla}_{\text{KPC-2}}$ and $\text{bla}_{\text{TEM-1}}$, respectively.

K. pneumoniae isolate K72 was resistant to all carbapenems, ciprofloxacin, and tobramycin and exhibited reduced susceptibility to amikacin. The isolate remained susceptible to gentamicin, colistin, and tigecycline. Phenotypic tests showed KPC production. PCR assays and subsequent DNA sequencing confirmed the presence of $\text{bla}_{\text{KPC-2}}$ and $\text{bla}_{\text{TEM-1}}$ genes.

Analysis of the PFGE patterns of SpeI- and of XbaI-digested genomic DNA revealed that all five *S. marcescens* isolates, irrespective of their susceptibility patterns, were genetically indistinguishable (Fig. 1). For comparison we also tested five carbapenem-susceptible *S. marcescens* isolates that were recovered from separate patients hospitalized in the intensive care unit (ICU) before or during the period of the study (October 2008 to May 2009) and three historical *S. marcescens* isolates from our collections. All five isolates from the ICU belonged to the same pulsotype as the ones in our cases, whereas the historical isolates belonged to different pulsotypes (data not shown).

Conjugation experiments using individual parental KPC-possessing *S. marcescens* isolates S53, S54, and S55 showed that resistance to β -lactams was self-transferable. Transconjugants received either solely the bla_{TEM} gene or both bla_{KPC} and bla_{TEM} genes, thus presenting two distinctly different susceptibility patterns, depending on the resistance genes which they acquired (Table 1). Those positive for both bla_{KPC} and bla_{TEM} genes exhibited carbapenem MICs which were higher than those of the recipient strain. Reduced susceptibility to amika-

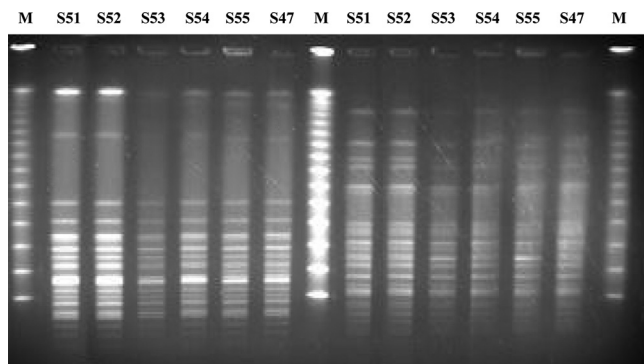


FIG. 1. PFGE profiles of SpeI-digested genomic DNAs (left side) and of XbaI-digested genomic DNAs (right side) from *S. marcescens* isolates of the study (S51, S52, S53, S54, and S55) and a contemporary carbapenem-susceptible *S. marcescens* isolate (S47) from a patient hospitalized in the ICU before the study period. Lanes M, multimers of phage lambda DNA (48.5-kb) molecular mass markers.

cin and resistance to tobramycin were transferred to all transconjugants. Plasmid analysis revealed that the parental isolates contained two separate plasmids with molecular sizes of approximately 85 kb and 35 kb, which were both transferable. The plasmid bands of the transconjugants were extracted from the gel and used as templates for the amplification of the *bla*_{KPC} and *bla*_{TEM} genes, the specific products of which were amplified from the larger and smaller plasmid, respectively, suggesting that the *bla*_{KPC} gene was located on the 85-kb plasmid while the *bla*_{TEM} gene was on the 35-kb one. EcoRI restriction analysis was performed on the purified 85-kb plasmids of the transconjugants. Restriction patterns were identical (data not shown), suggesting that all three *S. marcescens* isolates had acquired the same *bla*_{KPC-2}-bearing plasmid.

Plasmid analysis of *K. pneumoniae* isolate K72 revealed the presence of only one plasmid of approximately 110 kb, which was transmissible and conferred resistance to β -lactams including carbapenems in the recipient strain. DNA extracted from plasmid bands of K72 and its *E. coli* transconjugant yielded positive results for both *bla*_{KPC} and *bla*_{TEM} genes. These transconjugants, however, presented a different susceptibility pattern in comparison to those which also contained both the *bla*_{KPC} and *bla*_{TEM} genes yet were derived from the *S. marcescens* isolates (Table 1). They were highly resistant to penicillin and combinations of penicillin with β -lactamase inhibitors and exhibited a lower increase in cephalosporin and aztreonam MICs, while MICs of imipenem, meropenem, and ertapenem were higher than those of the recipient strain yet lower than those of the *S. marcescens* transconjugants.

The present study documents the first report of KPC production in *S. marcescens* isolates in Europe and offers evidence of the potential for acquisition of these plasmid-borne enzymes during long-term hospitalization. These KPC-positive *S. marcescens* isolates were acquired while the patients were hospitalized in the ICU, following in two cases an initial infection by a non-carbapenemase-producing *S. marcescens* isolate. The isolates were genotypically indistinguishable, indicating the dissemination of the plasmid harboring the *bla*_{KPC-2} gene within the same clone.

KPC genes along with other resistance genes are typically

located on mobile genetic elements (9, 12, 20). Previous studies have presented evidence which suggested the potential for horizontal dissemination of *bla*_{KPC} genes between different clones of the same enterobacterial species (1) or even between different genera (3, 12, 15, 17). In one of our cases the patient was simultaneously infected with a *K. pneumoniae* isolate belonging to the clonal outbreak with which we are currently dealing in our hospital (data not shown). Our results, however, suggested that the *bla*_{KPC-2} gene of *S. marcescens* isolates was located on a different plasmid from that of the *K. pneumoniae* isolate. Therefore, it can be postulated that it was the plasmid-encoded *bla*_{KPC-2} gene of the index carbapenem-nonsusceptible *S. marcescens* isolate that disseminated. It cannot be excluded, however, that through a recombination event the *bla*_{KPC-2} gene was initially transferred from the *K. pneumoniae* clone to the carbapenem-susceptible *S. marcescens* clone. Although the initial source of infection could not be verified, all KPC-producing *S. marcescens* organisms were retrieved from bronchial lavage samples. It is therefore possible that the transfer of the plasmid-encoded KPC-2 enzyme occurred in the environment of the mixed bacterial flora typically found in bronchial secretions.

Our report provides evidence that *S. marcescens* has the ability to easily acquire KPC carbapenemases. Successfully combating infections caused by these carbapenem-resistant *S. marcescens* pathogens poses a difficult challenge, given their intrinsic resistance to last-resort antibiotics, such as colistin, and their potential for dissemination.

We declare no potential conflicts of interest.

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