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Increased Resistance to Carbapenems in *Proteus mirabilis* Mediated by Amplification of the bla_{VIM-1}-Carrying and IS*26*-Associated Class 1 Integron

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Objective: The aim of the study was to decipher the mechanisms and associated genetic determinants responsible for increased carbapenem resistance among *Proteus mirabilis* clinical isolates.
Methods: The entire genetic structure surrounding the β-lactam resistance genes was characterized by PCR, gene walking, and DNA sequencing.
Results: A series of clinical *P. mirabilis* isolates were consecutively recovered from different patients at the Military hospital of Sofia, Bulgaria. They showed variable levels of resistance to carbapenems. All isolates produced the same carbapenemase VIM-1 that was chromosomally encoded. We showed that increased resistance to carbapenems was related to an increased number of bla_{VIM-1} gene copies.
Conclusion: We showed here that increased carbapenem resistance in *P. mirabilis* may result from increased expression of the bla_{VIM-1} carbapenemase gene through multiplication of its copy number.

Keywords: *Proteus mirabilis*, VIM-1, carbapenem resistance, IS*26*

Introduction

Reduced susceptibility to imipenem is intrinsic in *Proteus mirabilis*, and this feature is mainly related to decreased expression of penicillin-binding protein PBP1a or reduced binding of imipenem to PBP2. However, high-level resistance to carbapenem may be observed in that species due to loss of outer membrane porin.[1] The emergence of multidrug-resistant *P. mirabilis* isolates producing acquired extended-spectrum β-lactamase (ESBL), AmpC, and carbapenemases has also been described.[2] In *P. mirabilis*, few isolates producing the carbapenemases KPC-2 (class A)[3] or OXA-23 (class D)[4] have been reported, along with some NDM (class B) producers.[5] However, the most common carbapenemase identified in that species is the metallo-β-lactamase VIM-1.[6–8]

Interestingly, a previous study showed that variable levels of carbapenem resistance observed among different VIM-1-producing *Klebsiella pneumoniae* isolates might result from additional porin deficiency, particularly due to loss of OmpK36.[9] In that study, it was shown that some isolates might possess two copies of the bla_{VIM-1} gene, giving rise to higher hydrolysis rates of imipenem.[9]

We investigated here the genetic bases of the variable carbapenem resistance levels of a series of bla_{VIM-1}-positive *P. mirabilis* isolates.

Materials and Methods

Bacterial strains, antibiotic susceptibility testing, plasmid extraction, and conjugation assays

Proteus mirabilis isolates were identified at the species level using the API 20E system (bioMérieux, La-Balme-les-Grottes, France). *Escherichia coli* TOP10 (Life Technologies, Cergy-Pontoise, France) and *E. coli* J53 reference strain were used in cloning and conjugation experiments, respectively.[10] Minimal inhibitory concentrations (MICs) of carbapenems were determined by Etest (AB bioMérieux, Solna, Sweden), and were interpreted according to Clinical and Laboratory Standards Institute breakpoints.[11] Mating-out assays were attempted using the VIM-1-producing *P. mirabilis* isolate PM5 as donor (representing the clonal

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strain) and *E. coli* J53 (azide resistant) as recipient. Selection was made using trypticase-soy plates supplemented with amoxillin 100 µg/mL and sodium azide (100 µg/mL). In addition, extraction of natural plasmids was attempted by using the Kieser method.[12]

PCR, cloning, and sequencing

PCR approach was used to detect carbapenemase encoding genes, as previously described.[10] Cloning experiments were performed using plasmid pTOPO as vector, and expression in *E. coli* TOP10, and selection on trypticase-soy agar supplemented with ticarcillin (50 mg/L) and kanamycin (30 mg/L). Plasmid DNAs were extracted by using Qiagen columns (Qiagen, Courtaboeuf, France). Sequencing of the amplicons and recombinant plasmids was performed by the Microsynth company (Balgach, Switzerland). PCR mapping of the $bla_{\text{VIM-1}}$-surrounding sequences was performed with primers localized in the class 1 integron.[9] PCR fragments generated with primers on which the *Xba*I restriction site was added were cloned in pTOPO-PCR Blunt vector (Invitrogen, Thermo Fisher).

Quantification of carbapenemase activity

The production of carbapenemase was first evaluated by using the Carba NP test, as previously described.[13] In addition, specific activities with imipenem as substrate were measured. In brief, a 10-mL broth culture of each tested isolate was centrifuged and submitted to sonication. Then, the pellets were, respectively, resuspended in 500 µL of 100 mM sodium phosphate buffer (pH 7) and submitted to sonication. Supernatants were then used for kinetic measurements.[14] Hydrolysis was measured by UV spectrophotometry using 100 µM imipenem as substrate and 10 µL of the bacterial crude extract. The protein content was measured using the Bio-Rad DC protein assay.

Quantification of the bla_{VIM-1} gene by real-time PCR amplification

Real-time experiments were performed at least three times in different PCR runs, using a Rotor-Gene Q (Qiagen, Hilden, Germany) with a KAPA SYBR FAST qPCR Kit (Kapabiosystems, Wilmington, MA).[15] The *hcaT* gene was chosen as the normalization control. The qPCR assays included primers VIM-1-qF1 (5′-GAG GTC CGA CTT TAC CAG ATT G-3′) and VIM-1-qR1 (5′-ATC ACC ATC ACG GAC AAT GAG-3′) for the $bla_{\text{VIM-1}}$ gene, and included primers hcaT-Pm-qF1 (5′-CAC TGT GGC TTG CCT TAG AT-3′) and hcaT-Pm-qR1 (5′-CTC GCC CTT TAA CCA GAT AGA C-3′) for the *hcaT* gene.

The quantity of each target in each isolate was expressed as the difference in Ct values between the *hcaT* and the $bla_{\text{VIM-1}}$ genes. PCR efficiency was ~90% for both targets. Real-time PCR was performed by an initial denaturation at 95°C for 60 sec, then rounds were repeated at 95°C for 3 sec, 60°C for 20 sec, and 72°C for 10 sec using primers VIM-1-qF1 and VIM-1-rR1 on one hand, and hcaT-Pm-qF1 and hcaT-Pm-qR1 on the other hand. The Real-Time PCR reaction mix was prepared in a volume of 20 µL containing 20 ng of purified genomic DNA, using the KAPA SYBR FAST qPCR Kit Master Mix (2×) Universal, and 0.25 µM of each primer. Analysis was performed using the Rotor-Gene Q machine (Qiagen).

Table 1. MICs of Carbapenems for *Proteus mirabilis* PM1 (Carbapenemase Negative), and VIM-1-Producing *Proteus mirabilis* PM2, PM3, PM4, and PM5, Being Representatives of the Different Carbapenem Resistance Patterns

	MIC (mg/L) for respective isolates[a]				
β-Lactam(s)	*PM1*	*PM2*	*PM3*	*PM4*	*PM5*
Imipenem	0.25	12	>32	>32	>32
Meropenem	0.047	0.25	8	16	>32
Ertapenem	0.04	0.032	1.5	1.5	4

[a]Respective $bla_{\text{VIM-1}}$ copy numbers are 0, 1, 1, 2, and 4 for PM1, PM2, PM3, PM4, and PM5.
MICs, minimal inhibitory concentrations.

Clonal relationship

Genotyping was performed by pulsed-field gel electrophoresis (PFGE), using restriction enzyme *Not*I, as previously described.[16]

Results and Discussion

Susceptibility testing and carbapenemase identification

During the March–June 2013 period, a total of 23 *P. mirabilis* isolates were recovered at the Military hospital of Sofia from urine of patients hospitalized in three different units, namely two intensive care units and a neurosurgical ward. They exhibited variable susceptibility patterns to carbapenems, with four isolates remaining susceptible, and the other showing resistance at variable levels. While MICs of imipenem ranged from 0.25 to >32 mg/L, those of ertapenem ranged from 0.004 to 4 mg/L, and those of meropenem from 0.047 to >32 mg/L (Table 1). This prompted us to evaluate whether these isolates might be corresponding to different clones. PFGE analysis showed that the four imipenem-susceptible isolates corresponded to a single clone, and the 19 other isolates to a second clone (data not shown). Results of the Rapid Carba NP test showed that all the 19 imipenem-resistant isolates produced a carbapenemase, but no carbapenemase activity was detected in the susceptible isolates. PCR and sequencing identified the carbapenemase gene $bla_{\text{VIM-1}}$ in all imipenem-resistant isolates. Five isolates were retained for further analysis, with the $bla_{\text{VIM-1}}$-negative isolate being PM1, and four $bla_{\text{VIM-1}}$-positive isolates PM2 to PM5 exhibiting distinct resistance levels to carbapenems (Table 1). Those isolates all showed an identical susceptibility pattern to non-β-lactam antibiotics, being susceptible to fluoroquinolones, tetracycline, chloramphenicol, amikacin, tobramycin, gentamicin, and fosfomycin, but showing resistance to kanamycin and rifampicin.

Carbapenemase activities of the P. mirabilis isolates

To precisely determine the capacity of the different isolates to compromise the efficacy of carbapenems upon hydrolysis, β-lactamase activities were measured using

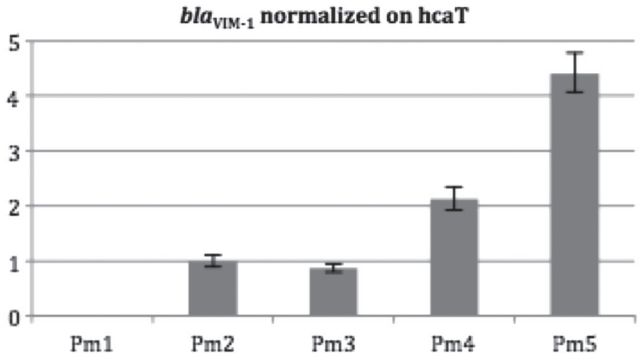

FIG. 1. Copy numbers of the *bla*$_{\text{VIM-1}}$ genes measured by quantitative PCR. Isolate PM1 (*bla*$_{\text{VIM-1}}$ negative) was used as negative control. Isolates PM2, PM3, PM4, and PM5 (*bla*$_{\text{VIM-1}}$ positive) were used for testing. Vertical axis; *bla*$_{\text{VIM-1}}$ copy number normalized on *hcaT*. Horizontal axis; distribution of the different isolates. Error bars represent the standard deviation between the three experiments.

imipenem as substrate. While no hydrolysis could be detected with a crude extract of isolate PM1, enzymatic activities of 43 ± 3, 46 ± 5, 82 ± 3, and 196 ± 8 U/mg of protein (1 U of enzyme activity being defined as the activity that hydrolyzed 1 μmol of imipenem per min) were obtained for isolates PM2, PM3, PM4, and PM5, respectively.

Copy number of the bla$_{VIM-1}$ *gene*

To assess whether the increased expression of the *bla*$_{\text{VIM-1}}$ gene could be related to an increased copy number, quantitative PCR was performed. Isolate PM1 used as control gave a negative result, as expected. For isolates PM2 and PM3, a single copy of the *bla*$_{\text{VIM-1}}$ gene was detected. Two copies were detected in isolate PM4 and finally, four copies were detected in isolate PM5. Interestingly, the *bla*$_{\text{VIM-1}}$ gene copy number very well correlated with the MIC data (Fig. 1; Table 1).

Genetic structures surrounding the bla$_{VIM-1}$ *gene*

Recombinant plasmid pPM2, recovered after *Xba*I cloning experiments using DNA of *P. mirabilis* PM2, possessed an ~8-kb insert and harbored the *bla*$_{\text{VIM-1}}$ gene into a class 1 integron structure. In fact, the *bla*$_{\text{VIM-1}}$ gene was the first cassette in the integron structure, followed by *aacA7* encoding resistance to aminoglycosides, *dfrA1* (resistance to trimethoprim), and *aacA1* (resistance to aminoglycosides) (Fig. 2). The 3′-extremity of this class 1 integron was truncated by an IS*1* element, and consequently lacked the usual *qacEΔ1* and *sul1* genes. The whole class 1 integron was bracketed by two copies of insertion sequences IS*26*, as previously identified in some *bla*$_{\text{VIM-1}}$-positive strains.[9] Here, a likely IS*26*-mediated homologous recombination was at the origin of this integron acquisition. In fact, IS*26* is very often identified in association with class 1 integron structures. It has been previously shown to be a key element in homologous recombination processes, leading to acquisition of some antibiotic resistance genes, such as *bla*$_{\text{BES-1}}$.[17] In fact, such integron structure is very similar to the one (named In-e541) reported from a *bla*$_{\text{VIM-1}}$-positive *E. coli* isolate recovered in Greece, but differed in its 3′-extremity with the insertion of IS*1*.[18]

By PCR mapping, we showed that isolate PM2 possessed the same unique structure. A second copy of this whole *bla*$_{\text{VIM-1}}$-positive integron was identified in isolates PM3 and PM4, bracketed by another IS*26* element at its 3′-end extremity. Finally, four copies of the same structure were identified in isolate PM5, with IS*26* elements always bracketing those different copies (Fig. 2). This structure was likely the result of an IS*26*-mediated recombination process, leading to multicopies of the *bla*$_{\text{VIM-1}}$-positive integron.

In all isolates, sequencing showed that the promoter sequences located in the 5′-extremity of the class 1 integrons

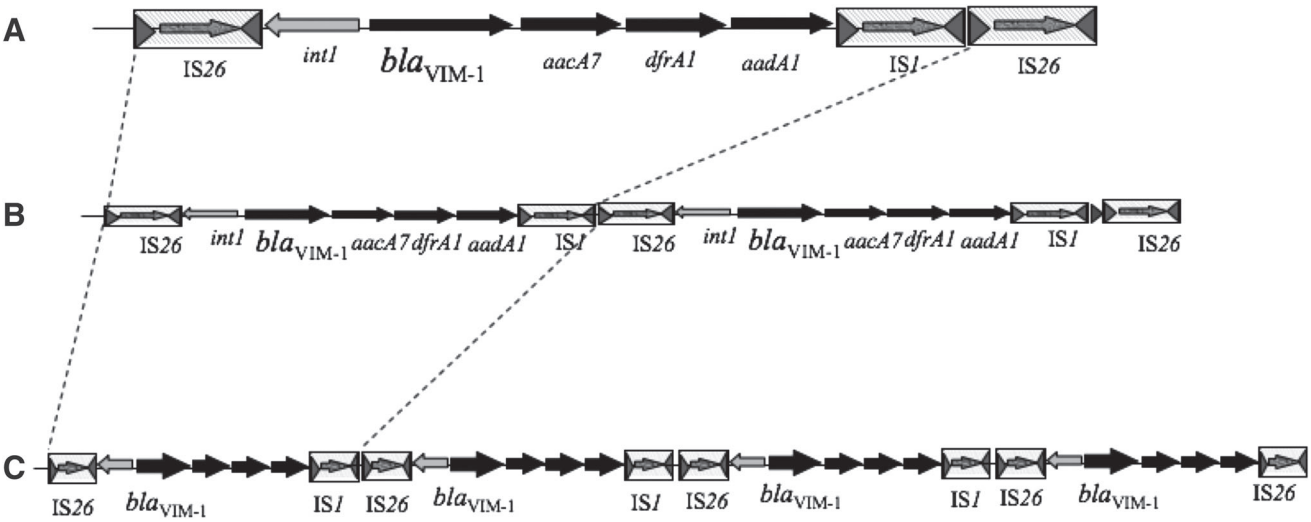

FIG. 2. Schematic representation of the different *bla*$_{\text{VIM-1}}$ integron structures identified in isolates PM2 and PM3 **(A)**, PM4 **(B)**, and PM5 **(C)**. Insertion sequences IS*26* and IS*1* are represented. The *int1* integrase gene is followed by the following gene cassettes, namely *bla*$_{\text{VIM-1}}$, *aacA7*, *dfrA1*, and *aadA1*. *Dashed arrows* indicate the boundaries of the repeated fragments. Respective structures are not to scale between each other, to better represent the evolution of the whole content.

identified that are known to drive the expression of the right-end located gene cassettes were conserved. Therefore, the overall variability in bla_{VIM-1} expression level could not be attributed to heterogeneity of the corresponding promoter sequences.

Mating-out assays remained unsuccessful despite repeated attempts, further correlating with a chromosomal location of the bla_{VIM-1} gene in all positive isolates. In addition, repeated plasmid extractions did not reveal any plasmid band on gel electrophoresis, which is another correlating feature.

Conclusion

This study identified a series of multiresistant *P. mirabilis* clinical isolates, exhibiting variable susceptibility patterns to carbapenems. Here, we showed that the increased resistance patterns to imipenem observed among those clonally related isolates were explained by an increased bla_{VIM-1} copy number. Notably, the different bla_{VIM-1} genes identified were all chromosomally located, thus showing that the process of gene multiplication was not related to a plasmid mobilization. Our data showed that up to four copies of the bla_{VIM-1} gene could be identified, and that the multiplication process was actually related to transposition-independent IS*26*-related recombination events. The likely scenario might be that overtime selective pressure by β-lactams (and particularly carbapenems) might have led to the selection of isolates possessing those multiple bla_{VIM-1} copies. However, we could not establish a direct link with antibiotic usage in that hospital, due to difficulties to retrospectively trace the clonal dissemination within patients.

Tandem amplification of antibiotic resistance genes related to IS*26* has been previously reported for the ESBL gene bla_{SHV-5} in *E. coli.*[19] In addition, chromosomal amplification of the bla_{OXA-58} carbapenemase gene has been identified in a *P. mirabilis* isolate.[20] In the latter case, this genetic event was related to a phage-type recombinase. Nonetheless, the occurrence of bla_{OXA-58} gene in *Proteus* spp. (and more generally in Enterobacteriaceae) remains exceptional, while that of the bla_{VIM-1} is frequently reported.[21] However, the multiplication of the bla_{OXA-58} carbapenemase gene through IS*26*-mediated recombination event has been identified in *Acinetobacter baumannii*, leading to an overtime increased production of OXA-58 and consequently increased MIC values for carbapenems, similarly to what we have observed in this study.[22] Recent studies showed that the involvement of IS*26* in the mobilization and dissemination of antibiotic resistance genes occurs more frequently than that previously considered, by reorganizing plasmids by a replicative transposition mechanism.[23]

Our study showed that the multiplication of a clinically relevant resistance gene in *P. mirabilis* can occur and potentially lead to increased resistance. Moreover, the chromosomal location of the duplicated integron probably confers persistence of the resistance phenotype, as opposed to resistance genes located on plasmids.

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Disclosure Statement

No competing financial interests exist.

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