Predominance of carbapenem-resistant *Pseudomonas aeruginosa* isolates carrying *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> metallo-β-lactamases in a major hospital in Costa Rica

Francisco Toval,1† Anel Guzmán-Marté,1‡ Vivian Madriz,1§ Teresita Somogyi,1,2 César Rodríguez1 and Fernando García1

1Centro de Investigación en Enfermedades Tropicales, Facultad de Microbiología, Universidad de Costa Rica, San José, Costa Rica

2Laboratorio Clínico, Hospital Mexico, San José, Costa Rica

This study aimed to assess the molecular basis of the resistance to carbapenems in clinical isolates of *Pseudomonas aeruginosa* recovered from a tertiary-level health facility in San José, Costa Rica. A total of 198 non-duplicated isolates were evaluated for their susceptibility to β-lactams, aminoglycosides and fluoroquinolones. The production of metallo-β-lactamases (MBLs), the presence of MBL encoding genes (*bla*<sub>IMP</sub>, *bla*<sub>VIM</sub> and *bla*<sub>GIM-1</sub>) and the occurrence of these genes within class 1 integrons were investigated. In addition, an ERIC2 PCR fingerprinting method was used to elucidate the distribution of the detected MBL genes within the strain collection. Of the 198 isolates tested, 125 (63.1 %) were categorized as carbapenem-resistant. The majority (88.8 %) of the carbapenem-resistant isolates also showed resistance to ceftazidime, cefepime, aztreonam, ticarcillin/clavulanic acid, amikacin, gentamicin, tobramycin, ciprofloxacin and gatifloxacin. Among the carbapenem-resistant isolates, 102 (81.6 %) showed MBL activity. Strikingly, both *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes were simultaneously detected in most (94.1 %) of the 102 MBL producers. Five carbapenem-resistant MBL producers were positive only for *bla*<sub>IMP</sub> genes. Almost 70 % of the isolates examined harboured the *intI1* gene, accompanied by the *sul1* and *qacE.D1* genes in 136 (99 %) and 122 (89 %) isolates, respectively. The majority (94.4 %) of the carbapenem-resistant isolates carried the *intI1* gene, in contrast to 26 % of the carbapenem-susceptible isolates. Ninety-three out of 96 (96.9 %) isolates carrying both *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes also harboured the *intI1*, *sul1* and *qacE.D1* genes. Gene cassettes from carbapenem-susceptible and MBL-negative carbapenem-resistant isolates encoded aminoglycoside-resistance enzymes (*aadA2*, *aadA4* and *aadA6*) as well as *orfD* and *qacF* genes. RAPD analysis distributed 126 of the isolates in 29 clusters. Eighty of the 90 *bla*<sub>IMP</sub><sup>+</sup> *bla*<sub>VIM</sub><sup>+</sup> isolates were sorted into 16 different clusters, suggesting that the *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes detected were located within a genetic element capable of lateral transfer. Carbapenem-resistant MBL-positive isolates were recovered from almost all hospital wards and were over-represented in samples obtained from the surgical emergency and intensive care therapy units. Remarkably, three carbapenem-resistant isolates, exhibiting MBL activity and carrying both *bla*<sub>IMP</sub> and *bla*<sub>VIM</sub> genes, were recovered from outpatients. Sequence analysis of both *bla* genes in various isolates revealed that they correspond to the alleles *bla*<sub>IMP</sub>-18 and *bla*<sub>VIM</sub>-2. To our knowledge, this is the first report of the combination of two metallo-β-lactamases encoded by the *bla*<sub>IMP</sub>-18 and *bla*<sub>VIM</sub>-2 genes in *P. aeruginosa.*

†Present address: Institute for Hygiene, University of Münster, Münster, Germany.
‡Present address: Hospital General de la Plaza de la Salud, Santo Domingo, Dominican Republic.
§Present address: Boston Scientific, Heredia, Costa Rica.

**Abbreviations:** ERIC, enterobacterial repetitive intergenic consensus; MBL, metallo-β-lactamase.

One supplementary table is available with the online Supplementary Material.
INTRODUCTION

Pseudomonas aeruginosa is a Gram-negative bacterium with extraordinary physiological and metabolic versatility. This organism has a wide distribution in nature and colonizes various ecological niches, such as aquatic and terrestrial plants and animals, including humans. Moreover, it tolerates a variety of physical conditions, and is able to persist in both community and hospital settings (Klockgether et al., 2011; Maschmeyer & Braveny, 2000; Rajan & Saiman, 2002). Although this bacterium is a relatively rare component of the normal human microbiota, the colonization rate of P. aeruginosa is relatively common in some major hospitals in San José, Costa Rica and represents an important threat to the local health-care system. The aim of this study was to determine the susceptibility of a collection of P. aeruginosa isolates recovered at one tertiary-level hospital in Costa Rica to β-lactams, aminoglycosides and fluoroquinolones, to clarify whether these isolates carry IMP, VIM, or GIM-1 MBLs, and to evaluate the association of these genes with class 1 integrons.

METHODS

Bacterial strains. A total of 198 independent clinical isolates were randomly selected among 708 P. aeruginosa isolates obtained between November 2004 and October 2005 at the Hospital Mexico (San José, Costa Rica), a tertiary-level health facility with 519 beds. These isolates were recovered from patients hospitalized in the intensive care unit \((n=46)\) or the general wards \((n=140)\), or from outpatients \((n=12)\). The selected isolates were recovered from a variety of clinical specimens including respiratory tract \((n=62)\), urine \((n=59)\), secretion \((n=48)\) and other \((n=29)\) specimens. This latter category comprises a diversity of clinical samples with low numbers. P. aeruginosa ATCC 27853, Escherichia coli ATCC 25922, and E. coli ATCC 35218 were used as susceptible controls. P. aeruginosa A5386 (blaIMP-1), P. aeruginosa A3486 (blaIMP-1b), P. aeruginosa 75-3636C (blaVIM-1), P. aeruginosa 81-11963A (blaVIM-1) and P. aeruginosa 73-5671 (blaIM-1), kindly provided by Dr Ana C. Gales (Laboratório Especial de Microbiologia Clínica, Brazil), were used as positive controls for PCR amplification of metallo-β-lactamase genes. E. coli DH5α (F- endA1 glnV44 thi-1 recA1 gyrA96 deoR supG43 thr-1 leu-1 metB5 lacZΔM15 procF1 temperature-sensitive F’ traD36 proAB lacY1) and E. coli JM107 (endA1 glnV44 thi-1 recA1 gyrA96 Δ(lac-proAB) F’ traD36 proAB lacY1) were used as host strains to propagate recombinant plasmids.

In vitro susceptibility tests. The isolates were identified at the species level using GNI cards (bioMérieux Vitek). This automated system and AST-GN cards were also employed to determine their susceptibility to cefazidime, cefepime, imipenem, meropenem, ticarcillin/clavulanic acid, piperacillin/tazobactam, amikacin, gentamicin, tobramycin, ciprofloxacin and gatifloxacin. Some of the P. aeruginosa isolates showing intermediate susceptibility were considered resistant in the statistical analysis. Metallo-β-lactamases were phenotypically detected using E-test MBL strips following the manufacturer’s instructions (AB Biodisk).

Detection of genes encoding metallo-β-lactamases by multiplex PCR. DNA isolation was performed as described previously (Speijer et al., 1999). Multiplex PCR analysis for detection of bladIM, bladVIM and bladIM-1 was performed as described by Mendes et al. (2007). Amplification products were separated by electrophoresis on 1.5 % (w/v) agarose gels and visualized using ethidium bromide. A Mass Ruler DNA Ladder (Fermentas) was used as a size marker.

Detection and nucleotide sequence analysis of class 1 integrons. All isolates were initially screened for the integrase gene intI1 with primers IntA and IntB. Thereafter, primers suffix and sufr rev, and orf4 and orf4 rev were used to detect suffix and sacAE1 genes contained in 3′-conserved segments (Lévesque et al., 1995; Rosser & Young, 1999). Primers 5′CS and 3′CS were used to amplify gene cassettes while primers int2F and 3′CS were used to amplify variable segments comprising a fragment of the integrase gene and the
inserted cassettes (Lévesque et al., 1995). All amplification reactions were performed as previously described (Lévesque et al., 1995; Rosser & Young, 1999) and PCR products were visualized by gel electrophoresis as indicated above.

Amplified DNA was purified using a Wizard SV gel and PCR clean-up kit according to the manufacturer’s instructions (Promega). The purified DNA was ligated into the positive selection cloning vector pJET (Fermentas) and transformed by heat shock into purified DNA was ligated into the positive selection cloning vector pJET (Fermentas) and transformed by heat shock into E. coli DH5α and/or E. coli JM107. Transformants were cultured at 37 °C in Lysogeny Broth (LB) or in LB agar plates containing ampicillin (100 µg ml⁻¹).

Plasmid DNA for restriction analysis and sequencing was isolated with a small-scale boiling lysis method (Sambrook & Russell, 2001) and restriction enzymes BglII and XhoI were used to linearize the plasmids in accordance with the manufacturer’s instructions (Fermentas). Inserts were sequenced with primers specific for pJET (forward sequencing primer: 5’-GGCTGAAACCATATCCATCC-3’; reverse sequencing primer: 5’-GCAGCTGAAATATGTGGAGATC-3’). When necessary, primers were designed to complete the sequence of the variable region of the cassette arrays by primer walking. DNA sequencing was done with the dyeex-chains-termination method in a Genetic Analyzer 3130 Autosampler (Applied Biosystems). Sequence assembly was performed with DNA-Man v.6.0 (Lynnon Biosoft). Details of nucleotide sequences deposited in GenBank (http://www.ncbi.nlm.nih.gov) (accession numbers FN824507.1, FN824508.1, FN824509.1 and FN827339.1) are given in Table 1.

ERIC2 fingerprinting. Genotyping by ERIC2 (enterobacterial repetitive intergenic consensus) PCR was performed to analyse the clonal relatedness of all 198 P. aeruginosa. These reactions were carried out as previously described (Speijer et al., 1999). Gel electrophoresis patterns were analysed with the GelCompar II software (Applied Maths) using a cut-off of ≥80 % level of similarity to establish clusters.

**RESULTS**

**Prevalence of resistance to carbapenems and other antibiotics**

Out of 198 isolates, 125 (63.1 %) were categorized as carbapenem-resistant using the Vitek system, including 103 isolates showing resistance to both imipenem and meropenem. By contrast, 13 isolates were imipenem-resistant and meropenem-susceptible and nine isolates were imipenem-susceptible and meropenem-resistant. Isolates displaying resistance to imipenem could be divided into three groups: a major group with MICs above 256 µg ml⁻¹ (n=79, 68.1 %), a group with MICs of between 16 µg ml⁻¹ and 128 µg ml⁻¹ (n=27, 23.7 %), and a minor group comprising isolates with MICs of more than 8 µg ml⁻¹ but less than 16 µg ml⁻¹ (n=10, 8.6 %). As shown in Table 2, the isolates analysed in this study showed a high percentage of resistance to other β-lactams, aminoglycosides and fluorquinolones. The percentages of resistance were even higher among the carbapenem-resistant isolates, most of which showed resistance to antibiotics of different structural classes. In fact, 111 (88.8 %) of the carbapenem-resistant isolates also showed resistance to cefazidime, cefepime, aztreonam, ticarcillin/clavulanic acid, amikacin, gentamicin, tobramycin, ciprofloxacin and gatifloxacin. Forty-seven (23.7 %) isolates were resistant to the combination piperacillin/tazobactam.

**Detection of metallo-β-lactamase activity and MBL genes**

We found 102 MBL producers (81.6 %) among the carbapenem-resistant isolates. None of the carbapenem-susceptible isolates gave positive results in the MBL assays. Strikingly, both blaIMP and blaVIM genes were simultaneously detected in 96 out of the 102 MBL producers (94.1 %). Five carbapenem-resistant MBL producers were positive only for blaIMP genes and another isolate with the same phenotype did not yield amplification products for either blaIMP or blaVIM genes (PAE-029). Both blaIMP and blaVIM genes were also detected in the imipenem-resistant meropenem-resistant isolate PAE-171 (MICIMP 12 µg ml⁻¹) and in the imipenem-susceptible meropenem-resistant isolate PAE-046 (MICIMP <4 µg ml⁻¹), both of which showed no MBL activity in the E-test assay. The blaGIM-1 gene was not detected.

Most (n=77, 80.2 %) of the isolates carrying both blaIMP and blaVIM genes displayed a MIC to imipenem of ≥256 µg ml⁻¹, although the isolates carrying only the blaIMP gene showed a similar phenotype. Carbapenem-resistant isolates lacking both MBL activity and blaIMP and blaVIM genes showed MIC values to imipenem between 6 and 24 µg ml⁻¹.

**Detection of integrons and sequencing of gene cassettes**

Almost 70 % (n=137) of the 198 isolates examined harboured the int1 gene. This element was accompanied by the sul1 and qacEAl genes in 136 (99 %) and 122 (89 %) isolates, respectively. One hundred and eighteen out of 125 (94.4 %) carbapenem-resistant isolates carry int1, in contrast to 26.0 % of the carbapenem-susceptible isolates. Ninety-three out of 96 (96.8 %) isolates carrying both blaIMP and blaVIM genes also harboured the int1, sul1 and qacEAl genes. Three blaIMP⁺ blaVIM⁺ isolates also carried the int1 and sul1 but lacked qacEAl.

Analysis of gene cassettes amplified from 19 int1⁺ carbapenem-susceptible and MBL-negative carbapenem-resistant isolates generated single products ranging in size

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**Table 1. Gene cassettes of carbapenem-susceptible P. aeruginosa int1⁺ isolates from a major Costa Rican hospital**

<table>
<thead>
<tr>
<th>Gene cassette (arrays)</th>
<th>Number of isolates</th>
<th>Accession numbers</th>
</tr>
</thead>
<tbody>
<tr>
<td>orfD – aadA6</td>
<td>7</td>
<td>FN824508.1</td>
</tr>
<tr>
<td>aacA4</td>
<td>6</td>
<td>FN824507.1</td>
</tr>
<tr>
<td>aacA4 – aadA1</td>
<td>3</td>
<td>FN824509.1</td>
</tr>
<tr>
<td>qacF</td>
<td>1</td>
<td>FN823739.1</td>
</tr>
</tbody>
</table>

http://jmm.sgmjournals.org
from 800 bp to 2500 bp. Cloning and DNA sequencing analysis of 17 of these amplicons revealed five different gene cassettes that appeared alone or in combination (Table 1). Three of these gene cassettes encoded aminoglycoside-resistance enzymes (aadA2, aadA4 and aadA6). In addition, we found a gene encoding a protein that confers resistance to quaternary ammonium compounds (qacF) and one open reading frame of unknown function (orfD). The most prevalent gene cassette was aacA4, followed closely by aadA6 and orfD (Table 1). In contrast, amplification analysis of gene cassettes using the primers 5′CS and 3′CS of intI1′ blaIMP′ blaVIM′ isolates revealed a predominant 180 bp product, which probably represented empty integron(s), and several discrete faint bands of higher molecular mass, which may indicate the presence of multiple gene cassettes (data not shown). In an attempt to determine the gene cassette sequences of blaIMP and blaVIM, and the structure of the corresponding integrons, we attempted several times to clone these multiple amplification products in pJET and to select for E. coli DH5α and E. coli JM107 colonies with imipenem-resistance. These efforts were unsuccessful.

**Allelic variants of blaIMP and blaVIM**

Cloning and sequence analysis of MBL amplification products (Mendes et al., 2007) obtained from one representative carbapenem-resistant isolate, *P. aeruginosa* PAE-006, indicated that blaIMP corresponds to blaIMP-18 and blaVIM to blaVIM-2. The open reading frame sequence of blaIMP-18 is 100 % identical to those present in In706 and In707, two class 1 integrons recently described in Puerto Rico (GenBank No. JN596991.2, Martinez et al., 2012). The sequence of the blaVIM-2 gene showed 100 % identity to those of the gene cassettes present in In56, In58, In59 and In72, which were the first descriptions of the nucleotide sequence of this blaVIM variant (Pallecchi et al., 2001; Poirel et al., 2000, 2001; Yatsuyanagi et al., 2004).

**Table 2. Resistance to various antibiotics among carbapenem-resistant and carbapenem-susceptible *P. aeruginosa* isolates assessed by Vitek**

<table>
<thead>
<tr>
<th>Resistance to</th>
<th>Carbapenem-resistant (n=125, 63.1 %)</th>
<th>Carbapenem-susceptible (n=73, 36.9 %)</th>
<th>Total (n=198)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>%</td>
<td>n</td>
</tr>
<tr>
<td>Ceftazidine</td>
<td>123</td>
<td>98.4</td>
<td>12</td>
</tr>
<tr>
<td>Cefepime</td>
<td>120</td>
<td>96.0</td>
<td>5</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>120</td>
<td>96.0</td>
<td>28</td>
</tr>
<tr>
<td>Tiacarclillin-clavulanate</td>
<td>120</td>
<td>96.0</td>
<td>22</td>
</tr>
<tr>
<td>Pipercillin-tazobactam</td>
<td>40</td>
<td>32.0</td>
<td>7</td>
</tr>
<tr>
<td>Amikacin</td>
<td>112</td>
<td>89.6</td>
<td>9</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>120</td>
<td>96.0</td>
<td>22</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>120</td>
<td>96.0</td>
<td>14</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>118</td>
<td>94.4</td>
<td>17</td>
</tr>
<tr>
<td>Gatifloxacin</td>
<td>118</td>
<td>94.4</td>
<td>21</td>
</tr>
</tbody>
</table>

**Diversity of *P. aeruginosa* isolates assessed by ERIC2 analysis**

One hundred and eighty isolates yielded appropriate ERIC2 patterns to be analysed using the GelCompar II software (Table S1, available in the online Supplementary Material). Of these, 126 isolates were classified in 29 clusters, each of which included 2–12 isolates. These data reflect a high level of diversity in the *P. aeruginosa* isolates analysed. The 126 isolates included 90 blaIMP+ blaVIM+ isolates, five blaIMP+ blaVIM isolates and 85 blaIMP blavIM isolates. Eighty of the 90 blaIMP+ blavIM+ isolates were sorted in 16 different clusters, and 10 of these isolates showed a level of similarity below 80 %, suggesting that the blaIMP and blavIM genes detected are probably linked in a genetic structure susceptible to lateral transfer.

**Association of carbapenem resistance to hospital epidemiology**

There was no statistically significant difference between the recovery of carbapenem-resistant MBL-positive and carbapenem-susceptible isolates and the sex of the patients or the type of specimen, with the exception of the specimens in the category ‘other’ (79.3 % versus 20.7 % P=0.061). Carbapenem-resistant MBL-positive isolates were recovered from almost all hospital wards and were over-represented in samples obtained from the surgical emergency (92.3 %) and intensive care therapy (91.3 %) units. Remarkably, seven carbapenem-resistant isolates were recovered from outpatients, of which three displayed MBL activity and carried both blaIMP and blavIM genes.

**DISCUSSION**

Resistance to carbapenems is a predominant feature among *P. aeruginosa* isolates recovered from a major tertiary-level health facility in San José, Costa Rica. This finding is quite
alarming because the vast majority of these isolates also showed resistance to other β-lactams, aminoglycosides and fluoroquinolones. The percentage of the \textit{P. aeruginosa} isolates exhibiting resistance to carbapenems observed in this study (63.1\%) is much higher than the frequencies observed in previous studies (reviewed in Andrade \textit{et al.}, 2003; Lister \textit{et al.}, 2009). The low number of therapeutic alternatives available to treat infections caused by this pathogen, particularly those occurring in immunocompromised patients with underlying conditions, poses a serious threat to public health. Patients infected with MBL-producing \textit{P. aeruginosa} are treated in this hospital with high doses of carbapenems (either imipenem or meropenem), along with an aminoglycoside or piperacillin-tazobactam or with colistin/polymixin B and rifampicin (A. A. Avilés-Montoya, personal communication).

Since production of metallo-β-lactamas is a major mechanism of resistance to carbapenems, detection of MBL activity in most of the carbapenem-resistant isolates was not unexpected. However, resistance to carbapenems could not be explained by the production of MBLs in 23 isolates, indicating that other mechanisms should be involved. Interestingly, in most of the strains, resistance to carbapenems could be explained by the presence of not just one, but two metallo-β-lactamas encoded by \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} genes, while the \textit{bla}_{\text{GIM-1}} gene was not detected. Expression analyses of \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} should be performed to precisely define the contribution of each gene to the resistance to carbapenems in selected isolates from our collection. Additional mechanisms, including other serine- and metallo-β-lactamas, porins and efflux pumps were not analysed and their contribution to the phenotype of resistance to carbapenems in our \textit{P. aeruginosa} isolates cannot be disregarded.

More than 30 allelic variants in diverse bacterial species have been reported for both \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}}, the most frequent MBL genes in \textit{P. aeruginosa} isolates (Cornaglia \textit{et al.}, 2011; Walsh \textit{et al.}, 2005; Nicolau & Oliver, 2010). Sequence analysis of the MBL genes from one isolate (PAE-006) indicated that the \textit{bla}_{\text{IMP}} corresponds to \textit{bla}_{\text{IMP}-18} and the \textit{bla}_{\text{VIM}} to \textit{bla}_{\text{VIM}-2}. Since allelic variants of the corresponding \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} genes were not determined in other \textit{P. aeruginosa} isolates from the same hospital, we cannot assume that both alleles are predominant among the isolates carrying both genes. However, we analysed carbapenem-resistant \textit{P. aeruginosa} isolated in other hospitals in San José, specifically, \textit{P. aeruginosa} AG1 (GenBank No. KC907377.1, KC907378.1) from the Hospital San Juan de Dios (general hospital, 720 beds) and \textit{P. aeruginosa} GRI from the Hospital Nacional de Niños (paediatric hospital, 330 beds). Strikingly, both isolates also carry \textit{bla}_{\text{IMP}-2} and \textit{bla}_{\text{VIM}-2} genes showing 100\% sequence identity to the sequences obtained from \textit{P. aeruginosa} PAE-006 (A. Guzmán-Martí and others, unpublished). These data provide evidence for inter-hospital dissemination of \textit{P. aeruginosa} carrying both \textit{bla}_{\text{IMP}-18} and \textit{bla}_{\text{VIM}-2}.

Whereas the \textit{bla}_{\text{VIM}-2} gene has been reported to be more than 29 countries or regions around the world in over 50 different integron structures and is considered the most important VIM-type MBL in clinical settings (Zhao & Hu, 2011a, b), the distribution of the \textit{bla}_{\text{IMP}-18} gene is more limited. The \textit{bla}_{\text{IMP}-18} gene was first described in a \textit{P. aeruginosa} isolate in the United States in 2006 (Borgianni \textit{et al.}, 2011; Hanson \textit{et al.}, 2006), and has since been identified in Mexico and France (Garza-Ramos \textit{et al.}, 2008; Hocquet \textit{et al.}, 2010; Wolter \textit{et al.}, 2009). More recently, \textit{bla}_{\text{IMP}-18} has been associated with two class 1 integrons, In706 and In707, in \textit{P. aeruginosa} isolates recovered in Puerto Rico (Martínez \textit{et al.}, 2012).

To our knowledge, this is the first report of the combination of two metallo-β-lactamas encoded by the \textit{bla}_{\text{IMP}-18} and \textit{bla}_{\text{VIM}-2} genes in \textit{P. aeruginosa}. Recently, \textit{P. aeruginosa} isolates carrying both \textit{bla}_{\text{IMP}-2} and \textit{bla}_{\text{VIM}-11} have been described in Mexico (Castillo-Vera \textit{et al.}, 2012). Other combinations of MBL genes have also been described in \textit{Serratia marcescens} (\textit{bla}_{\text{IMP}-5} and \textit{bla}_{\text{VIM}-11} encoded by a class 1 integron) (Lee \textit{et al.}, 2008), \textit{Klebsiella pneumoniae} (\textit{bla}_{\text{IMP}} and \textit{bla}_{\text{SIM-1}}, \textit{bla}_{\text{VIM}} and \textit{bla}_{\text{SIM-1}}), \textit{Enterobacter aerogenes} (\textit{bla}_{\text{IMP}} and \textit{bla}_{\text{SIM-1}}) (Dwivedi \textit{et al.}, 2009) and \textit{Acinetobacter} genospecies 10 isolates (\textit{bla}_{\text{IMP}-1} and \textit{bla}_{\text{VIM}-2}, \textit{bla}_{\text{IMP}-1} and \textit{bla}_{\text{SIM-1}}, \textit{bla}_{\text{VIM}-2} and \textit{bla}_{\text{SIM-1}} separately encoded each by class 1 integrons) (Lee \textit{et al.}, 2010).

Most of the \textit{P. aeruginosa} isolates examined harboured the \textit{intI1} gene accompanied by the \textit{sul1} and \textit{qacEA1} genes. Occurrence of class 1 integron-like structures was more predominant among carbapenem-resistant isolates, particularly in those carrying both \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} than among carbapenem-susceptible isolates. However, although \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} genes have been separately associated with class 1 integrons in other studies, we cannot directly demonstrate this physical association in our isolates. Carbapenem-resistant isolates carrying both \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} genes generated multiple faint bands of diverse sizes in our PCR approach for the amplification of the gene cassettes, strongly suggesting the occurrence of multiple integrons in those isolates. Carbapenem-susceptible and MBL-negative carbapenem-resistant isolates instead yielded single amplification products of gene cassettes encoding mostly for aminoglycoside-resistance enzymes. Thus, MBL-producing \textit{P. aeruginosa} isolates carrying both \textit{bla}_{\text{IMP}} and \textit{bla}_{\text{VIM}} genes seem to differ from carbapenem-susceptible and MBL-negative carbapenem-resistant isolates with regard to the structure of their integrons. Preliminary data derived from a current genomic study in our lab using a genomic approach indicate that the \textit{bla}_{\text{IMP}-18} and \textit{bla}_{\text{VIM}-2} genes of \textit{P. aeruginosa} AG1 are separately associated with different class 1 integron-like structures, each of which being, in turn, a part of distinct transposons (A. Guzmán-Martí and others, unpublished). It has been recently reported that class 1 integrons/transposons seem to be undergoing successful dispersal by lateral gene transfer in \textit{P. aeruginosa} clinical isolates (Martínez \textit{et al.}, 2012). This fact could explain the occurrence of both \textit{bla}_{\text{IMP}}
and \( \text{bla}_{\text{VIM}} \) genes among isolates belonging to diverse ERIC2 clusters or lineages of \( \text{P. aeruginosa} \).

In order to determine if the \( \text{bla} \) genes are localized in plasmids, several assays were performed in selected MBL-positive \( \text{P. aeruginosa} \) isolates, including plasmid purification by diverse protocols and gel electrophoresis analysis, curing experiments under several conditions, as well as conjugation experiments with or without a helper plasmid (pUB307) using various carbapenem-sensitive \( \text{E. coli} \) and \( \text{P. aeruginosa} \) strains as receptors cells (data not shown). All results were negative suggesting that \( \text{bla}_{\text{IMP}} \) and \( \text{bla}_{\text{VIM}} \) are probably located on the chromosome of the carbapenem-resistant \( \text{P. aeruginosa} \) isolates. Since carbapenem-resistant \( \text{P. aeruginosa} \) isolates carry several lysogenic phages on the chromosome (C. Rodriguez and F. Garcia, unpublished results), we are currently testing the hypothesis that horizontal transfer of \( \text{bla}_{\text{IMP}} \) and \( \text{bla}_{\text{VIM}} \) might occur by transduction.

Carbapenem-resistant MBL-positive \( \text{P. aeruginosa} \) isolates carrying both \( \text{bla}_{\text{IMP}} \) and \( \text{bla}_{\text{VIM}} \) genes were recovered from almost all hospital services, particularly from the intensive care therapy and surgical emergency units, as well as from outpatients, and from two other major hospitals in San José. These data suggest that community-acquired infections as well as inter-hospital dissemination of \( \text{bla}_{\text{IMP}} + \text{bla}_{\text{VIM}} + \text{P. aeruginosa} \), probably by the transfer of colonized or infected patients between hospitals, have been occurring.

In conclusion, we describe the predominance of carbapenem-resistant MBL-producing \( \text{P. aeruginosa} \) in a hospital setting in San José, Costa Rica. Our isolates exhibited high percentages of resistance to other \( \beta \)-lactams, fluoroquinolones and aminoglycosides, and were also recovered from outpatients as well as from other hospitals, suggesting community-acquired infections as well as inter-hospital dissemination. We provide evidence that the majority of those isolates carry both \( \text{bla}_{\text{IMP}} \) and \( \text{bla}_{\text{VIM}} \) genes, probably linked in an integron-like structure susceptible to lateral transfer. Finally, we report the simultaneous presence of the allelic variants \( \text{bla}_{\text{IMP-18}} \) and \( \text{bla}_{\text{VIM-2}} \) encoding metallo-\( \beta \)-lactamas in \( \text{P. aeruginosa} \).

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