metallo-β-lactamases (MBLs) at two Belgian university hospitals located in the Brussels area.

Between January 2004 and May 2007, multidrug-resistant \( P. \) putida strains originating from 10 inpatients hospitalized at Saint-Luc (hospital 1) and Erasme (hospital 2) university hospitals were characterized for resistance mechanisms to β-lactams. All the isolates were high-level resistant to imipenem and meropenem by disk diffusion testing (no inhibition zone). The 10 patients presented with severe underlying diseases (Table 1) had been hospitalized more than 9 days in ICUs and had all previously received broad-spectrum antimicrobial therapy. All but one of the isolates had been recovered from urine specimens. Bacterial identification to the species level was achieved with Vitk2-GN (bioMérieux) and control growth at 42°C on trypticase soy agar complemented with sheep blood. MICs determined by Etest (AB Biodisk) showed that all isolates were resistant to piperacillin/tazobactam, ceftazidime, aztreonam, imipenem and meropenem and all but one were resistant to cephalosporins (Table 1). Isolates recovered from hospital 1 were resistant to amikacin, whereas isolates from hospital 2 remained susceptible to this aminoglycoside. Resistance to ciprofloxacin was variable but all isolates remained susceptible to colistin. The MBL screening test was positive both by double-disc method (imipenem versus imipenem-EDTA; Rosco Diagnostica A/S) and by MBL double-sided Etest (imipenem/imipenem-EDTA; AB Biodisk) for all isolates (data not shown). PCR targeting \( \text{bla}^{\text{IMP}} \) (FW, 5'-GGC GTT TAT GTT CAT ACT TCG TT; RV, 5'-ATT CGG CCA CTC TAT TCC), \( \text{bla}^{\text{VIM}} \) (FW, 5'-TGG CGT ATC CCT; RV, 5'-TGG AAC CAC CAC CA; FW, 5'-TGG AAA CGG GAA AAC GTT GG; RV, 5'-CAG CCG ACC CAT AAC CA; \( \text{bla}^{\text{PER}} \) to 4 and 6; FW, 5'-GGA TTA CCA TGG CAA TCA GC; FW, 5'-TGT CGT ATC CCT CAA ATC ACC) was only positive for the \( \text{bla}^{\text{VIM}} \) gene in all 10 isolates and for the \( \text{bla}^{\text{PER}} \) gene in a single isolate (no. 6). Sequencing of the variable region of class I integrons obtained for the different strains revealed two distinct integrons. The first one, isolated from all five isolates from hospital 1, harboured an \( \text{aacA}^4 \) allele coding for the AAC(6’)-Ib aminoglycoside-modifying enzyme explaining the resistance to amikacin, followed by the \( \text{bla}^{\text{VIM-4}} \) gene. The same integron has already been identified in \( Pseudomonas aeruginosa \) isolates reported from Poland and Hungary\(^{2,3} \) and presents a specific 170 bp 3’-terminal repeat of the \( \text{bla}^{\text{VIM-4}} \) gene. The second class I integron, obtained from the five strains isolated in hospital 2, revealed a \( \text{bla}^{\text{VIM-2}} \) gene cassette, following an unidentified open reading frame of 318 nucleotides named \( \text{orf}_{\text{okr}} \). This last sequence is referenced in GenBank under number EU284133. PCR sequencing confirmed that the \( \text{bla}^{\text{PER}} \) gene detected in isolate no. 6 was a \( \text{bla}^{\text{PER-1}} \) allele. The co-presence of \( \text{bla}^{\text{PER}} \) and \( \text{bla}^{\text{VIM-2}} \) has been reported in \( P. \) aeruginosa\(^{4,5} \) and \( Providencia \),\(^6 \) but to the best of our knowledge, this is the first description in \( P. \) putida. PFGE analysis revealed five PFGE types among the 10 \( P. \) putida isolates. Types A and B were recovered from hospital 1, whereas types C, D and E were found in hospital 2. A cluster of four patients showing PFGE type B was found in hospital 1 and another cluster of three patients with PFGE type C was present in hospital 2. Further, the content of the gene cassettes of the \( P. \) putida strains also clearly differed between the two centres.
# Table 1: Case history, MIC data for selected antimicrobial agents, and integron structure of *P.* putida clinical isolates expressing VIM MBL

<table>
<thead>
<tr>
<th>Isolate</th>
<th>site of isolation</th>
<th>duration of stay before isolation (days)</th>
<th>underlying disease</th>
<th>PCR-sequencing-typing</th>
<th>MIC (mg/L)</th>
<th>resistance mechanisms</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>endotracheal aspirate</td>
<td>9</td>
<td>liver cirrhosis</td>
<td>0.5</td>
<td>256</td>
<td>aacA4</td>
</tr>
<tr>
<td>2</td>
<td>urine</td>
<td>10</td>
<td>vascular stroke</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 A</td>
</tr>
<tr>
<td>3</td>
<td>urine</td>
<td>39</td>
<td>meningitis</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>4</td>
<td>urine</td>
<td>31</td>
<td>meningitis</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>5</td>
<td>urine</td>
<td>54</td>
<td>pyelonephritis</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>6</td>
<td>urine</td>
<td>32</td>
<td>cancer</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>7</td>
<td>urine</td>
<td>12</td>
<td>renal graft</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>8</td>
<td>urine</td>
<td>18</td>
<td>prostatectomy</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>9</td>
<td>urine</td>
<td>62</td>
<td>cancer</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
<tr>
<td>10</td>
<td>urine</td>
<td>96</td>
<td>cancer</td>
<td>0.38</td>
<td>256</td>
<td>blaVIM-4 B</td>
</tr>
</tbody>
</table>

*Note:* MIC data for selected antimicrobial agents include TZP (tizapenem), CAZ (ceftazidime), FEP (cefepime), ATM (aztreonam), IPM (imipenem), MER (meropenem), AMK (氨卡林), CIP (卡巴联素), COL (科林). Resistance mechanisms include aacA4, blaVIM-4 A, blaVIM-4 B, and orf MRSA-1. PFGE: pulsed-field gel electrophoresis.

Excluding the possibility of strain transfer or horizontal gene transfer between the two hospitals. The data, instead, suggest that clonal dissemination of *P.* putida strains as well as horizontal *blaVIM-4* and *blaVIM-5* gene transfer through different clones independently occurred at both centres. Indeed, the same variable sequence of integron was found in isolates with different PFGE types in one centre. The fact that geographical and time clustering of these multidrug-resistant *P.* putida isolates were recorded in both centres also sustains the hypotheses of either patient-to-patient cross-transfer of strains or a common source acquisition. Although no epidemiological investigations were performed, it is striking that nearly all *P.* putida isolates originated from urine obtained from patients with bladder catheters, four of whom had undergone invasive urinary tract examinations. In some cases, long periods (e.g. isolate nos 5 and 7) elapsed between the isolation of genotypically similar resistant *P.* putida strains from ICU patients. This observation raises the possibility that these strains may have persisted unnoticed in the ICU environment, which served as a source of patient contamination.

Among the 10 patients, 5 recovered clinically and 5 died. No direct link could be established between the isolation of *P.* putida and death in any of these patients. The *P.* putida isolates were considered as colonizing organisms in eight patients (none of whom received any specific antimicrobial therapy) and as clinically significant in the remaining two patients. The latter two patients received intravenous colistin therapy and were considered as cured from their urinary tract infection following this treatment.

Overall, our data document the emergence of multidrug-resistant *P.* putida isolates producing VIM-2 and VIM-4 MBLs, presumably arising by independent horizontal transfer of integron-associated resistance genes into distinct epidemic *P.* putida clones in two hospitals. This observation also underscores that *P.* putida, although infrequently isolated, may occasionally cause difficult-to-treat nosocomial infections in severely ill patients.

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## Transparency declarations

None to declare.

## References

Activity of doripenem and comparator β-lactams against US clinical isolates of *Streptococcus pneumoniae* with defined mutations in the penicillin-binding domains of *pbp1a*, *pbp2b* and *pbp2x*

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Sir,

Doripenem, a parenteral carbapenem, was recently approved in the USA for the treatment of complicated intraabdominal infections (cIAIs) and complicated urinary tract infections (cUTIs) including pyelonephritis. In Europe, a marketing authorization application has been filed for the treatment of cIAIs, cUTIs and nosocomial pneumonia. Doripenem has a broad spectrum of activity against clinically important pathogens including *Staphylococcus spp.*, group A streptococci and pneumococci.1

β-Lactam resistance in *Streptococcus pneumoniae* is caused by mutations in the penicillin-binding domains of one or more of its six penicillin-binding proteins (PBPs) resulting from mosaic genes or point mutations.2,3 Altered *PBP1a*, *PBP2b* and *PBP2x* are most important for β-lactam resistance in clinical isolates.2,3

This study reports the activity of doripenem against 30 *S. pneumoniae* US clinical isolates. β-Lactam MICs were determined using panels from Trek Diagnostic Systems (Cleveland, OH, USA) using CLSI recommendations.4 Breakpoints (non-meningitis) for all drugs except doripenem were those approved by CLSI.5 Currently, there are no approved doripenem breakpoints for *S. pneumoniae*. PBP gene sequencing and competition assays were performed as described previously.5

Seven penicillin-susceptible isolates had β-lactam MICs ≤0.03 mg/L (genotype wild-type) (Table 1). These isolates had no mutations in the penicillin-binding motifs of *pbp1a*, *pbp2b* and *pbp2x*. Of the eight penicillin-intermediate isolates (genotypes 1 and 2), three had penicillin MICs of 0.12 mg/L and MICs for the other β-lactams were ≤0.12 mg/L (genotypes 1A and 1B) (Table 1). They had a T446A substitution in the *PBP2b* SSNT motif; one isolate (genotype 1B) also had *PBP2b* substitutions (T338A and R384G). Five penicillin-intermediate isolates with penicillin MICs of 1 mg/L additionally had *PBP2b* substitutions (T371T and L546V) (genotype 2, Table 1). The carbapenem MICs were the lowest of the agents tested, with imipenem having 2- and 4-fold lower MICs than doripenem and meropenem, respectively (genotype 2A). One isolate (genotype 2B) had substitutions of T371S in *PBP1a* and D623G in *PBP2b*. These substitutions were associated with 2–4-fold increases in carbapenem MICs (Table 1).

Among the penicillin-resistant isolates (genotypes 3–5), seven had penicillin MICs of 2–4 mg/L and were nonsusceptible to imipenem and meropenem, three were nonsusceptible to ceftriaxone and one was non-susceptible to amoxicillin/clavulanic acid; doripenem MICs were 0.5–1 mg/L (Table 1). These seven isolates (genotype 3) had *PBP1a* substitutions of T371A and P432T in addition to the *PBP2x* and *PBP2b* changes mentioned previously (Table 1).

Two isolates with genotype 4 had additional substitutions of A619G and D623G in or near the KTGTA motif of *PBP2b* and a substitution of T371S in the STMK motif of *PBP1a* (Table 1). These changes corresponded with increased penicillin MICs (8 mg/L) and resistance to amoxicillin/clavulanic acid, imipenem and meropenem (Table 1). Doripenem MICs were 1–2 mg/L.

Six isolates with genotype 5 had the same PBP substitutions as genotype 4 isolates, with the addition of the M339F change in *PBP2x*; in four isolates (genotypes 5A, 5B and 5C), there was also a Y595F substitution (Table 1). These changes were associated with a 2–16-fold increase in ceftriaxone MICs leading to resistance, whereas the MICs of the other drugs were within one doubling dilution when compared with genotype 4 isolates (Table 1).

PBP binding studies with a wild-type isolate indicated that the carbapenems had good affinity for all six PBPs with IC₅₀ ≤0.06 mg/L (Table 1). Ceftriaxone bound tightly to all PBPs (IC₅₀ ≤0.1 mg/L) except *PBP2b* (Table 1) as expected, as cephalosporins do not use *PBP2b* as a primary target.6 In a genotype 5C isolate, the carbapenem binding affinities for all PBPs were reduced: *PBP2b* and *PBP2x* had the highest increase in IC₅₀ (2–4 and 3.7–8 mg/L, respectively) (Table 1). The ceftriaxone IC₅₀ for *PBP2x* was increased at least 200-fold when compared with strain 8865 (Table 1).

β-Lactam MIC increases correlated with increases in the number of *PBP1a*, 2x and 2b substitutions. In this study, the *PBP1a*, 2b and 2x substitutions found in, and adjacent to, the penicillin-binding motifs SXKK, SXN and KTGTA were similar to substitutions reported by others.2,5 Carbapenems, like penicillins, are thought to have *PBP2b* as their primary target.1 In a study examining clinical isolates from Japan, a T624G *PBP2b* substitution was associated with carbapenem resistance.2 No isolates in our study had this mutation; however, a substitution at the adjacent amino acid (D623G) was found in seven of the eight genotype 4 and 5 isolates, all of which had elevated β-lactam MICs. This substitution was reported in a β-lactam-resistant *S. pneumoniae* recombinant that resulted from transformation with DNA from a β-lactam-resistant *Streptococcus mitis* strain.2

In summary, doripenem and imipenem (MIC₅₀ 1 mg/L) were 2-fold more active than meropenem (MIC₅₀ 2 mg/L) against the pneumococcal isolates in this study, including ceftriaxone-resistant...