Multiplex PCR for rapid detection of genes encoding oxacillinases and metallo-\(\beta\)-lactamases in carbapenem-resistant Acinetobacter spp.

Acinetobacter spp. resistant to carbapenems have become common in hospitals worldwide (Dijkshoorn et al., 2007). Carbapenem resistance mechanisms described in Acinetobacter spp. include hydrolysis by \(\beta\)-lactamas, alterations in outer-membrane proteins and penicillin-binding proteins, and increased activity of efflux pumps (Peleg et al., 2008). However, carbapenemases, such as metallo-\(\beta\)-lactamase (MBL) or oxacillinases, cause the most concern due to the chance of rapid dissemination (Lee et al., 2005; Poirel & Nordmann, 2006; Brown & Amyes, 2006). Four families of oxacillinases have been described in Acinetobacter baumannii so far: OXA-23-like, OXA-24-like and OXA-58-like enzymes, and OXA-51-like, a chromosomal oxacillinase enzyme present in A. baumannii that when overexpressed can be associated with carbapenem resistance (Turton et al., 2006; Poirel & Nordmann, 2006).

Acinetobacter spp. is one of the most frequent agents of health-care associated infection in Brazil (Mendes et al., 2005; Girão et al., 2008); however, data regarding the prevalence of carbapenemases in Brazilian hospitals is scant (Tognim et al., 2006; Carvalho et al., 2009).

The aim of the present study was therefore to develop a multiplex PCR assay for detecting alleles encoding oxacillinases and MBL to evaluate the presence of carbapenemase genes among nosocomial carbapenem-resistant Acinetobacter spp. isolated from four hospitals in the state of São Paulo, Brazil, during a 7 year period (2002 to 2008).

The study included a total of 68 isolates of A. baumannii, 64 carbapenem-resistant and 4 carbapenem-susceptible strains; 50 (78 \%) of the resistant strains were isolated from bloodstream infections. The following reference strains were used in this study: P. aeruginosa producing IMP-1 and VIM-1, Acinetobacter spp. producing SIM-1, OXA-23 and OXA-24 enzymes as positive controls, and A. baumannii ATCC 19606 was used as a negative control for all carbapenemase except OXA-51. The isolates were identified by API (NE) test (bioMérieux). MICs of carbapenemases were determined by broth microdilution and interpreted using Clinical and Laboratory Standards Institute breakpoints (CLSI, 2005).

Both multiplex PCRs were performed at the same time with seven pairs of specific primers, one pair for each of the seven gene families (Table 1). The PCR template was obtained at the concentration of 20 ng \(\mu\)l\(^{-1}\) using a genomicPrep cell and tissue DNA isolation kit (Amersham Pharmacia Biotech). The PCR mixture used was as follows: 1 \(\mu\)l DNA template in a 49 \(\mu\)l mixture containing 10 mM Tris/\(\mathrm{HCl}\) (pH 8.8), 4 mM \(\mathrm{MgCl}_2\), 50 mM KCl, 0.1 \% Triton X-100, 200 \(\mu\)M each dNTP, 30 nM oxacillinase primers, 200 nM IMP primers, 100 nM VIM primers, 50 nM SIM primers and 1 U Taq DNA polymerase. The PCR conditions were as follows: initial denaturation at 94 \(^\circ\)C for 5 min, 33 cycles of 94 \(^\circ\)C for 25 s, 53 \(^\circ\)C for 40 s and 72 \(^\circ\)C for 50 s, followed by a single, elongation step at 72 \(^\circ\)C for 6 min. The PCR products were then purified using GFX PCR DNA and gel band purification kit (GE Healthcare) and subjected to automated DNA sequencing. The aligned sequences were then analysed with the BioEdit sequence program and similarity searches for the nucleotide sequences were performed with the BLAST program (http://www.ncbi.nlm.nih.gov).

An MBL Etest (AB Biodisk) was carried out following the manufacturer’s recommendations to confirm the phenotypic expression of MBL in the strains. Amplification by PCR with oligonucleotide primers specific for ISAbal region was performed as described elsewhere (Stoeva et al., 2008). The MIC\(_{50}\) values were 64 and 128 \(\mu\)g ml\(^{-1}\), and the MIC\(_{90}\) values were 128 and >128 \(\mu\)g ml\(^{-1}\) for imipenem and meropenem, respectively. All clinical isolates were positive for blaoxa-51-like genes. Twenty-two (34 \%) of these isolates had

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Length (bp)</th>
<th>Reference</th>
</tr>
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<tbody>
<tr>
<td>Imp-F</td>
<td>5’-GAAATGAGATGGTTAATCCTTC-3’</td>
<td>188</td>
<td>Mendes et al. (2007)</td>
</tr>
<tr>
<td>Imp-R</td>
<td>5’-CCTAAACCATAGTTATC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>VIM-F</td>
<td>5’-GCTTGTGTCATCTGCAAC-3’</td>
<td>382</td>
<td>Mendes et al. (2007)</td>
</tr>
<tr>
<td>VIM-R</td>
<td>5’-AATCCGGACAGCAGATAG-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>SIM-F</td>
<td>5’-GTCAAGGGATTCGCCATGC-3’</td>
<td>569</td>
<td>Mendes et al. (2007)</td>
</tr>
<tr>
<td>SIM-R</td>
<td>5’-GTCAAGGGATTCGCCATGC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Oxa-51-like-F</td>
<td>5’-TAATGCTTTTGATGCGCTTG-3’</td>
<td>353</td>
<td>Woodford et al. (2006)</td>
</tr>
<tr>
<td>Oxa-51-like-R</td>
<td>5’-TGATTGCTACATCTCTTG-3’</td>
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<tr>
<td>Oxa-24-like-F</td>
<td>5’-GTTTGTGTTGCCCCCCTAAA-3’</td>
<td>246</td>
<td>Woodford et al. (2006)</td>
</tr>
<tr>
<td>Oxa-24-like-R</td>
<td>5’-AGTGGACGAAAAAGGGATT-3’</td>
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<td>Oxa-58-like-F</td>
<td>5’-AAGTATGATGGGATCTGCT-3’</td>
<td>599</td>
<td>Woodford et al. (2006)</td>
</tr>
<tr>
<td>Oxa-58-like-R</td>
<td>5’-CCGCTCTGCGATTCACATAC-3’</td>
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Table 1. Sequences of primers used for multiplex PCR for detection of genes encoding MBLs and oxacillinases in isolates of Acinetobacter spp.
**Fig. 1.** Detection of genes encoding oxacillinases and MBLs in carbapenem-resistant *Acinetobacter* spp. by multiplex PCR. Lane 1 and 15, negative control; lane 2, control *bla*<sub>OXA-23</sub>-like gene and *bla*<sub>OXA-24</sub>-like gene; lane 3, control *bla*<sub>OXA-51</sub>-like gene and *bla*<sub>OXA-24</sub>-like gene; lane 4, control *bla*<sub>IMP</sub> gene; lane 5, control *bla*<sub>TEM</sub>; lane 6, control *bla*<sub>SIM</sub> gene; lanes 7–8 and 14 isolates carrying *bla*<sub>OXA-51</sub>-like gene; lanes 9–11 isolates carrying *bla*<sub>OXA-23</sub>-like gene; lane 12 and 13 isolates carrying *bla*<sub>OXA-51</sub>-like and *bla*<sub>IMP</sub> genes. M, Molecular mass markers (100 bp DNA ladder; Invitrogen).

bla<sub>OXA-23</sub>-like genes and four (6 %) had the gene *bla*<sub>IMP</sub> (Fig. 1). The imipenem MIC among *bla*<sub>OXA-23</sub>-like positive strains ranged from 16 to 128 μg ml<sup>−1</sup> and was lower compared with IMP-1 positive strains (MIC >128 μg ml<sup>−1</sup>). Insertion element ISA<sub>ba1</sub> was detected in all carbapenem-resistant strains except one. Phenotypic expression of MBL was confirmed by MBL Etest in all four IMP-1 positive strains.

OXA-23 carbapenemase has been reported in Brazil during an outbreak of multiresistant *Acinetobacter* spp. that occurred in two hospitals in the south of the country (Dalla-Costa et al., 2003), and more recently in a study of 110 carbapenem-resistant *A. baumannii* isolates from eight hospitals in Rio de Janeiro that showed that 87 % of strains had the gene encoding carbapenemase OXA-23 (Carvalho et al., 2009). The present study showed that all strains had *bla*<sub>oxa-51</sub>-like genes and 34 % had *bla*<sub>oxa-23</sub>-like genes. However, IMP-1 has been identified in several Brazilian hospitals since 2003 (Gales et al., 2003; Sader et al., 2005; Tognim et al., 2006). A SENTRY Antimicrobial Surveillance Program study of the dissemination and diversity of MBL in Latin America showed that the greatest concentration of carbapenemase-containing strains was in Brazil, and that only IMP-like was identified among isolates of carbapenem-resistant *Acinetobacter* spp. (Sader et al., 2005). IMP-1 was less frequent in the present study, being identified only in four strains of *A. baumannii*.

In conclusion, the present study showed that *bla*<sub>OXA-23</sub>-like was the most frequent carbapenemase identified among carbapenem-resistant *A. baumannii* strains isolated from four Brazilian hospitals, and that ISA<sub>ba1</sub> was present in all carbapenem-resistant strains except one; IMP-1 was present only in four strains. The multiplex PCR assay results were consistent with previous single PCR assays, and the multiplex assay could be a useful tool for the rapid detection of genes encoding both oxacillinases and MBLs, and could help in the implementation of measures for the control of the dissemination of carbapenem resistance in the hospital setting.

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**Anna Karina Mostachio, 1,2**

**Inneke van der Heijden, 1,2**

Flavia Rossi, 1,2 Anna Sara Levin 1,2 and Silvia F. Costa 1,2

1LIM-54, Departamento de Doenças Infecciosas e Parasitárias, Faculdade de Medicina, Universidade de São Paulo, Brazil

2Laboratório de Microbiologia, Hospital das Clínicas da Universidade de São Paulo, São Paulo, Brazil

Correspondence: Silvia F. Costa (costasilvi@ig.com.br)


Antimicrobial susceptibility in intensive care units: MYSTIC program Brazil 2002. 
Braz J Infect Dis 9, 44–51.

Mendes, R. E., Kiyota, K. A., Monteiro, J., 
Castanheira, M., Andrade, S. S., Gales, A. C., 
detection and identification of metallo-β-
lactamase-encoding genes by multiplex real-time 
PCR assay and melt curve analysis. J Clin 
Microbiol 45, 544–547.

Acinetobacter baumannii: emergence of a 
successful pathogen. Clin Microbiol Rev 21, 
538–582.

resistance in Acinetobacter baumannii: 
mechanisms and epidemiology. Clin Microbiol 
Infect 12, 826–836.

Sader, H. S., Castanheira, M., Mendes, R. E., 
Dissemination and diversity of metallo-β-
lactamas in Latin America: report from the 
SENTRY Antimicrobial Surveillance Program. 

Stoeva, T., Higgins, P. G., Bojkova, K. & 
carbapenem-resistant OXA-23-positive 
Acinetobacter baumannii in a Bulgarian 
university hospital. Clin Microbiol Infect 14, 
723–727.

Tognim, M. C. B., Gales, A. C., Penteado, A. P., 
of IMP-1 metallo-β-lactamase-producing 
Acinetobacter species in a Brazilian teaching 
hospital. Infect Control Hosp Epidemiol 27, 742– 
747.

Turton, J. F., Woodford, N., Glover, J., Yarde, S., 
Identification of Acinetobacter baumannii by 
detection of the blaoxa-51-carbapenemase 
gene intrinsic to this species. J Clin Microbiol 44, 
2974–2976.

Woodford, N., Ellington, M. J., Coelho, J. M., 
Turton, J. F., Ward, M. E., Brown, S., Amyes, 
PCR for genes encoding prevalent OXA 
carbapenemases in Acinetobacter spp. Int J 