Epidemiology of the Acinetobacter-derived cephalosporinase, carbapenem-hydrolysing oxacillinase and metallo-β-lactamase genes, and of common insertion sequences, in epidemic clones of Acinetobacter baumannii from Spain

Pilar Villalón1*, Sylvia Valdezate1, Maria J. Medina-Pascual1, Gema Carrasco1, Ana Vindel2 and Juan A. Saez-Nieto1

1Laboratorio de Taxonomı´ a, Servicio de Bacteriologı´ a, Centro Nacional de Microbiologı´ a, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain; 2Laboratorio de Infecciones Intrahospitalarias, Servicio de Bacteriologı´ a, Centro Nacional de Microbiologı´ a, Instituto de Salud Carlos III, Majadahonda, Madrid, Spain

*Corresponding author. Tel: +34-91-822-3733; Fax: +34-91-509-7919; E-mail: pvillalon@isciii.es

Received 7 June 2012; returned 1 August 2012; revised 27 September 2012; accepted 12 October 2012

Objectives: To study the distribution, diversity and activity of Acinetobacter-derived cephalosporinase (ADC)-, carbapenem-hydrolysing oxacillinase (CHO)- and metallo-β-lactamase (MBL)-encoding genes, and of the most common insertion sequences (ISs), in the genome of nosocomial, epidemic, multidrug-resistant Acinetobacter baumannii (MDRAB) clones from Spain.

Methods: The studied population included 59 MDRAB strains previously genotyped by PFGE and multilocus sequence typing. The search for the ADC (blaADC), CHO (blaOXA-51-like, blaOXA-23-like, blaOXA-40-like and blaOXA-58-like) and MBL (blaIMP, blaVIM, blaTIM, blaOXA-11-like and blaOXA-18-like) genes, and for the ISs (ISAb1, ISAb2, ISAb3, ISAb4 and IS18) was done by PCR assays. The phenotypic presence of MBL enzymes was examined using imipenem/imipenem+EDTA strips.

Results: The most prevalent IS, ISAb1 (93.2%), was detected upstream of blaADC and blaOXA-51-like. These genes showed ample diversity (10 and 8 alleles, respectively). Four ADC sequences (ADC1-like, ADC2-like, ADC11-like and ADC11-like) are described here for the first time. blaOXA-58-like was carried by 20.3% of strains, in association with ISAb2, ISAb3 or IS18. blaOXA-40-like was the most prevalent acquired CHO gene (57.6%), and was associated with none of the studied ISs. Neither blaOXA-23-like nor ISAb4 was detected in any strain. Some 67.8% of strains with MBL activity showed no corresponding gene in PCR; these results were more common in strains with a highly active CHO, such as OXA-40.

Conclusions: All the studied genes and their related ISs showed a clonal distribution. Imipenem resistance was probably provided by OXA-40 for the most part, while MBL- and OXA-23-encoding genes were absent in the studied population.

Keywords: multidrug resistance, β-lactamases, carbapenemases, imipenem

Introduction

Multidrug-resistant Acinetobacter baumannii (MDRAB) is one of the main pathogens involved in outbreaks in hospitals.1 Carbapenemases are the first choice in the treatment of severe A. baumannii infections. The study of resistance to this group of antimicrobials is therefore essential if we are to continue to successfully treat infected patients.

The production of β-lactamases is a mechanism of resistance to β-lactams of particular importance. Unfortunately a wide range of β-lactamases has now been detected in A. baumannii, including intrinsic Acinetobacter-derived cephalosporinas (ADCs), main carbapenem-hydrolysing oxacillinases (CHOs; OXA-51, OXA-23, OXA-40 and OXA-58) and metallo-β-lactamases (MBLs).1-4 The transcription of these β-lactamase genes is enhanced by different insertion sequences (ISS) located in their proximity. The ISSs most commonly associated with the carbapenemase genes in MDRAB are ISAb1, ISAb2, ISAb3, ISAb4 and IS18.5-6 The aim of the present work was to determine the distribution, diversity and activity of the most important β-lactamase
genes, and their related ISs, in a well-characterized population of nosocomial, epidemic MDRAB clones from Spain.6

Materials and methods

Bacterial strains

This work examined 59 MDRAB strains representative of a total of 729 epidemic isolates, most of them isolated from respiratory samples in intensive care unit facilities, and all collected during outbreaks in 19 public hospitals located in 17 Spanish provinces between 1997 and 2007. These 729 isolates were previously genotyped by ApaI PFGE, returning 59 pulsotypes that grouped into 16 epidemic PFGE clones.6 These 59 strains were also subjected to multilocus sequence typing (MLST) (http://www.pasteur.fr/recherche/genopole/PF8/mlst/Abaumannii.htm), giving seven sequence types (STs): ST2, ST3, ST15, ST32, ST79, ST80 and ST81 (see Figure S1, available as Supplementary data at JAC Online).

Antimicrobial susceptibility

Antimicrobial susceptibility testing, also previously undertaken,6 showed all 59 strains to have a multidrug-resistant phenotype.

In the present work, Etest strips (AB Biodisk, Solna, Sweden) were used to detect MBL production, comparing the MICs obtained for imipenem and imipenem + EDTA. A difference between the imipenem and imipenem + EDTA ratio of ≥2 was interpreted as indicative of MBL activity, according to the manufacturer’s instructions. The control strains were Escherichia coli ATCC 25922 and Pseudomonas aeruginosa ATCC 27853. The Etest results were interpreted using the CLSI criteria for MBL-positive strains with phenotypic carbapenem resistance. A suggested explanation is that, in the presence of EDTA, oxacillinases change to a less active state, leading to a drastic reduction in MICs.15 Table 1 shows the MICs of imipenem and imipenem + EDTA, and their distribution according to the CHO genes. Carrying the blaOXA-51-like gene, moderate in those with the blaOXA-40-like gene and high in those carrying the blaOXA-60-like gene. The more efficacious the hydrolytic activity of the CHO, the more evident the synergic activity of imipenem + EDTA.

IS detection

blaOXA-1, ISblaOXA-2, ISblaOXA-3 and ISblaOXA-4 were amplified in 55 (93.2%), 15 (25.4%), 12 (20.3%) and 3 (5.1%) strains, respectively. In three cases, the ISblaOXA-40-like gene was detected. Two copies of ISblaOXA-1 were detected upstream of both blaADC and blaOXA-51-like.16

ADC analysis

blaADC was amplified in all strains, with 10 allelic variants detected. Analysis of these 10 ADC partial sequences resulted in 55 polymorphic sites (41 synonymous and 14 non-synonymous mutations). Five sequences were exclusively identical to ADC-1, ADC-2, ADC-5, ADC-11 and ADC-25. The sequence ADC-29/30/57 was consistent with ADC-29, ADC-30 and ADC-57. Finally, four newly detected ADC sequences ( provisionally named ADC-1-like(II), ADC-2-like(II), ADC-3-like(II) and ADC-11-like(III) were assigned the GenBank accession numbers JQ765381, JQ765383, JQ765382 and JQ765380, respectively. These new sequences differed from the respective ADC types in the amino acid substitutions indicated in subscripts. Figure S1 and Table 2 show the clonal distribution and diversity of the ADC sequences detected. The only IS associated with blaADC was ISblaOXA-1. However, no phenotypic differences were observed between the strains (n=54) with ISblaOXA-1 upstream of blaADC and those that did not have ISblaOXA-1 (n=5), and between the different ADC types detected; all were resistant to wide-spectrum cephalosporins.

CHO analysis

The intrinsic blaOXA-51-like gene was detected in all strains, with eight allelic variants obtained. Figure S1 shows the clonal distribution of the different CHOs and the diversity of the OXA-51 sequences. The different allelic variants of blaOXA-51-like showed a biased distribution in the three international clones of A. baumannii that had been previously observed.11,12 The most common, OXA-65/66/76/79 (45.8%), was consistent with OXA-65, OXA-66, OXA-76 and OXA-79, and was mainly distributed among ST2 (international clone II). OXA-69/92/112 (10.2% of strains) was associated with ST81, which is included in the
An association was also observed for OXA-71 and ST3 (international clone III).

**bla**OXA-40-like was PCR-amplified in 34 (57.6%) strains and showed just one allele distributed among ST2, ST3, ST32, ST79 and ST80. The sequenced fragment took in 511–699 nucleotide positions, according to the DBL (class D \(\beta\)-lactamases) numbering system. No IS was detected close to **bla**OXA-40-like. A high level of resistance to imipenem was detected in all those strains.

Twelve strains (20.3%) harboured a single **bla**OXA-58-like allele. The sequenced fragment took in 52–579 nucleotide positions (DBL numbering system). This gene was always associated with some IS. The most common combinations were upstream-IS\(\text{Aba}_2\) and downstream-IS\(\text{Aba}_3\) (eight pulsotypes distributed among ST15, ST79 and ST81), downstream-IS\(\text{Aba}_3\) (one pulsotype in ST79) and upstream-IS18 (in three pulsotypes of clone D6 of ST2). All strains that carried **bla**OXA-58-like were resistant to imipenem, except for the susceptible Ab3 strain (Figure S1). The absence in Ab3 of any IS upstream of **bla**OXA-58-like may be an indirect indicator of the enhancing effect of IS\(\text{Aba}_2\) and IS18 on the expression of OXA-58.

**bla**OXA-23-like was detected in no member of the studied population.

**Table 1.** Distribution of IPM and IPI MICs correlated with the presence of CHO genes in MDRAB

<table>
<thead>
<tr>
<th>MDRAB population (no. of strains)</th>
<th>Antimicrobial agent</th>
<th>Number of pulsotypes inhibited at each concentration (mg/L)(^a)</th>
<th>MIC(_{50}) (mg/L)</th>
<th>MIC(_{90}) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population (n=59)</td>
<td>IPM</td>
<td>0 2 2 9 5 7 0 4 14 5 11 128 &gt;256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Only <strong>bla</strong>OXA-51-like(^b) (n=13)</td>
<td>IPI</td>
<td>10 7 14 18 9 0 1 0 0 0 0 4 8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><strong>bla</strong>OXA-58-like (n=12)</td>
<td>IPM</td>
<td>0 0 0 1 5 6 0 0 0 0 0 8 16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td><strong>bla</strong>OXA-40-like (n=34)</td>
<td>IPM</td>
<td>0 0 0 0 0 0 0 4 14 5 11 128 &gt;256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>IPI</td>
<td>0 0 6 18 9 0 1 0 0 0 0 4 8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

IPM, imipenem; IPI, imipenem + EDTA.

\(^a\)IPM/IPI Etest strips were only used for MBL detection. Imipenem susceptibility was tested by standard Etest strips (data shown in Figure S1). CLSI imipenem breakpoints for Acinetobacter spp. are susceptible (\(\leq\) 4 mg/L) and resistant (\(\geq\) 16 mg/L).

\(^b\)Strains that only carry the CHO **bla**OXA-51-like gene.

---

**Table 2.** Diversity and distribution of ADC types in epidemic MDRAB strains

<table>
<thead>
<tr>
<th>ADC type</th>
<th>Amino acid position and changes(^a)</th>
<th>Frequency (%)</th>
<th>ST distribution</th>
<th>GenBank accession number</th>
</tr>
</thead>
<tbody>
<tr>
<td>ADC-1</td>
<td>K K S P G H N A R N N R</td>
<td>26/59 (44.1)</td>
<td>2, 79, 80</td>
<td>AJ009979</td>
</tr>
<tr>
<td>ADC-1-like P240S(^b)</td>
<td>— — — — — — — — G</td>
<td>5/59 (8.5)</td>
<td>2</td>
<td>JQ763581</td>
</tr>
<tr>
<td>ADC-2</td>
<td>Q — P — D N T T — — — G</td>
<td>1/59 (1.7)</td>
<td>15</td>
<td>AF117427</td>
</tr>
<tr>
<td>ADC-2-like N260H/T264N(^b)</td>
<td>Q — P — D — — — — G</td>
<td>3/59 (5.1)</td>
<td>3</td>
<td>JQ763583</td>
</tr>
<tr>
<td>ADC-5</td>
<td>Q Q P — D — T T F G — — G</td>
<td>4/59 (6.8)</td>
<td>79</td>
<td>AJ575184</td>
</tr>
<tr>
<td>ADC-11</td>
<td>Q Q P — D — — — F — — G</td>
<td>1/59 (1.7)</td>
<td>81</td>
<td>CP0017112</td>
</tr>
<tr>
<td>ADC-11-like Q342R(^b)</td>
<td>Q Q P — D — — — F — — G</td>
<td>5/59 (8.5)</td>
<td>81</td>
<td>JQ763580</td>
</tr>
<tr>
<td>ADC-11-like Q163K(^b)</td>
<td>Q Q P — D — — — F — — G</td>
<td>1/59 (1.7)</td>
<td>32</td>
<td>JQ763582</td>
</tr>
<tr>
<td>ADC-25</td>
<td>— — — — — — — — — — — T</td>
<td>9/59 (15.3)</td>
<td>2</td>
<td>EF016355</td>
</tr>
<tr>
<td>ADC-29/30/57(^c)</td>
<td>— — — — — — — — — — — T</td>
<td>4/59 (6.8)</td>
<td>2</td>
<td>CP002522</td>
</tr>
</tbody>
</table>

With reference to the 1152 bp ADC-1 sequence (GenBank accession number AJ009979). The analysis takes in nucleotide positions 445–1039, which correspond to amino acid positions 149–346. No change is indicated by a dash.

\(^a\)ADC-1-like, ADC-2-like and ADC-11-like sequences differ from ADC-1, ADC-2 and ADC-11, respectively, in the amino acid substitutions indicated in subscripts.

\(^b\)ADC-29/30/57 corresponds to a partial ADC sequence consistent with ADC-29, ADC-30 and ADC-57 sequences.

---

---

**Villalon et al.**

---

**Table 2.** Distribution of IPM and IPI MICs correlated with the presence of CHO genes in MDRAB

<table>
<thead>
<tr>
<th>MDRAB population (no. of strains)</th>
<th>Antimicrobial agent</th>
<th>Number of pulsotypes inhibited at each concentration (mg/L)(^a)</th>
<th>MIC(_{50}) (mg/L)</th>
<th>MIC(_{90}) (mg/L)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total population (n=59)</td>
<td>IPM</td>
<td>0 2 2 9 5 7 0 4 14 5 11 128 &gt;256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>Only <strong>bla</strong>OXA-51-like(^b) (n=13)</td>
<td>IPI</td>
<td>10 7 14 18 9 0 1 0 0 0 0 4 8</td>
<td>8</td>
<td>2</td>
</tr>
<tr>
<td><strong>bla</strong>OXA-58-like (n=12)</td>
<td>IPM</td>
<td>0 0 0 1 5 6 0 0 0 0 0 8 16</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td><strong>bla</strong>OXA-40-like (n=34)</td>
<td>IPM</td>
<td>0 0 0 0 0 0 0 4 14 5 11 128 &gt;256</td>
<td>256</td>
<td>256</td>
</tr>
<tr>
<td>IPI</td>
<td>0 0 6 18 9 0 1 0 0 0 0 4 8</td>
<td>8</td>
<td>8</td>
<td></td>
</tr>
</tbody>
</table>

IPM, imipenem; IPI, imipenem + EDTA.

\(^a\)IPM/IPI Etest strips were only used for MBL detection. Imipenem susceptibility was tested by standard Etest strips (data shown in Figure S1). CLSI imipenem breakpoints for Acinetobacter spp. are susceptible (\(\leq\) 4 mg/L) and resistant (\(\geq\) 16 mg/L).

\(^b\)Strains that only carry the CHO **bla**OXA-51-like gene.

---

---

international complex 1. An association was also observed for OXA-71 and ST3 (international clone III).

**bla**OXA-40-like was PCR-amplified in 34 (57.6%) strains and showed just one allele distributed among ST2, ST3, ST32, ST79 and ST80. The sequenced fragment took in 511–699 nucleotide positions, according to the DBL (class D \(\beta\)-lactamases) numbering system. No IS was detected close to **bla**OXA-40-like. A high level of resistance to imipenem was detected in all those strains.

Twelve strains (20.3%) harboured a single **bla**OXA-58-like allele. The sequenced fragment took in 52–579 nucleotide positions (DBL numbering system). This gene was always associated with some IS. The most common combinations were upstream-IS\(\text{Aba}_2\) and downstream-IS\(\text{Aba}_3\) (eight pulsotypes distributed among ST15, ST79 and ST81), downstream-IS\(\text{Aba}_3\) (one pulsotype in ST79) and upstream-IS18 (in three pulsotypes of clone D6 of ST2). All strains that carried **bla**OXA-58-like were resistant to imipenem, except for the susceptible Ab3 strain (Figure S1). The absence in Ab3 of any IS upstream of **bla**OXA-58-like may be an indirect indicator of the enhancing effect of IS\(\text{Aba}_2\) and IS18 on the expression of OXA-58.

**bla**OXA-23-like was detected in no member of the studied population.

**Imipenem susceptibility**

Susceptibility to imipenem was seen in 25.4% of the studied strains, distributed among three of the seven STs: ST2, ST79 and ST81 (Figure S1). Heteroresistance to this agent was
recorded for 27 (45.8%) strains and was widely distributed among the STs. The high imipenem MICs recorded may have been due to the activity of CHOs other than OXA-51. As in a study performed by Héritier et al., the present results showed the blaOXA-58-carrying strains to be less resistant to imipenem (MIC range from 4 to >32 mg/L) than the highly resistant blaOXA-40-carrying strains (MICs >32 mg/L).

**Distribution of ADCs, CHOs and ISSs in the MLST STs**

ST2, which corresponds to international clone II, was the most common ST in the studied population (accounting for 47.5% of all strains). It was involved in many outbreaks in 10 Spanish provinces during the study period (1997–2007). It was also the most frequent ST in the control strains. It was the most frequent ST in the control strains (accounting for 47.5% of all strains). It was involved in many outbreaks in 10 Spanish provinces during the study period (1997–2007).

**Conclusions**

In conclusion, the present analysis of carbapenem resistance in an epidemic MDRAB population in which ST2 is widely represented showed MBL genes to be absent, ISAb1 to be ubiquitous and vary within a strain in Acinetobacter baumannii. Antimicrob Agents Chemother 2006; 54: 351–3.

**Acknowledgements**

We thank Dr Germán Bou (La Coruña, Spain) for providing the control strains positive for blaOXA-23-Blac and blaVIM, the clinical microbiologists involved in the isolation and submission of the A. baumannii strains to the Taxonomy Laboratory at the CNM, and Adrian Burton for editing (Physical Evidence Scientific Translations; http://physicoevidence.es/english/welcome).

**Funding**

This work was partially supported by the Instituto de Salud Carlos III (MPy 1116/07).

**Supplementary data**

Figure S1 is available as Supplementary data at JAC Online (http://jac.oxfordjournals.org/).

**References**