False extended-spectrum β-lactamase detection in Acinetobacter spp. due to intrinsic susceptibility to clavulanic acid

A. Beceiro, F. Fernández-Cuenca, A. Ribera, L. Martínez-Martínez, A. Pascual, J. Vila, J. Rodríguez-Baño, J. M. Cisneros, J. Pachón and G. Bou

1Servicio de Microbiología, Complejo Hospitalario Universitario Juan Canalejo, Xubias de Arriba s/n, 15006 La Coruña, Spain; 2Servicios de Microbiología, Hospital Virgen Macarena, Avda. Sánchez Pizjuan, s/n 41071 Sevilla, Spain; 3Servei de Microbiología, Hospital Clinic, Villarroel 170, 08036 Barcelona, Spain; 4Enfermedades Infecciosas, Hospital Virgen Macarena, Avda. Sánchez Pizjuan, s/n 41071 Sevilla, Spain; 5Servicio de Enfermedades Infecciosas, Hospital Universitario Virgen del Rocio, Avda. Manuel Siurot, s/n 41013 Sevilla, Spain

Received 7 June 2007; returned 10 September 2007; revised 11 October 2007; accepted 29 October 2007

Background: There are some reports showing the susceptibility of some strains of Acinetobacter baumannii to the β-lactamase inhibitor clavulanic acid. To address this issue, we determined the MIC of clavulanic acid for a broad collection of Acinetobacter spp. isolates collected in a multicentre study. In addition, we showed the consequences of this susceptibility to yield false extended-spectrum β-lactamase (ESBL) detection in this genus.

Methods: The strains used were 244 isolates of Acinetobacter (226 A. baumannii, 15 Acinetobacter genomic species 3 and 3 unidentified Acinetobacter spp.) and several A. baumannii as positive controls. The isolates were subjected to molecular typing. One isolate of each genotype was subjected to clavulanic acid MIC analysis. As no breakpoints for clavulanic acid are available, we arbitrarily established three categories of susceptibility: /C20 <16, 32–128 and /C21 ≥256 mg/L. The presence of ESBL in Acinetobacter spp. was analysed by using microdilution, double disc diffusion, combined discs, Etest and isoelectric focusing.

Results: A total of 100 different genotypes were detected. Among them, 44, 26 and 30 genotypes were inhibited by /C20 <16, 32–128 and ≥256 mg/L clavulanic acid, respectively. Representative isolates of each group were tested for ESBL production. Only those with the lower clavulanic acid MICs yielded a false-positive ESBL test with all methods tested with the exception of the double disc diffusion assay.

Conclusions: Forty-four per cent of the genotypes tested were inhibited by ≤16 mg/L clavulanic acid and these Acinetobacter isolates yielded a false ESBL-positive test. These results may have implications for susceptibility testing in routine microbiology laboratories.

Keywords: β-lactamase inhibitors, PBP alterations, disc diffusion

Introduction

Acinetobacter spp. are opportunistic pathogens with increasing relevance in nosocomial infections. They can be associated with a wide range of clinical complications, such as pneumonia, sepsicaemia, urinary tract infections, wound infections and meningitis, especially in immunocompromised patients. Antimicrobial treatment of these clinical infections, particularly those caused by Acinetobacter baumannii, may be compromised by multiple resistances to antibiotics. To treat infections caused by A. baumannii isolates, the use of the β-lactamase inhibitor sulbactam with or without ampicillin has provided good results, especially with multiresistant A. baumannii strains. While A. baumannii is highly susceptible to sulbactam,
it is less susceptible to other β-lactamase inhibitors, such as clavulanic acid and tazobactam. Additionally, polymyxin B remains active against multiresistant strains of A. baumannii.

Among A. baumannii strains, resistance to β-lactams is often mediated by a naturally occurring AmpC-type cephalosporinase, which may confer a noticeable resistance when overexpressed. In addition, TEM-1, TEM-2, CARB-5, PER-type, CTX-M-type, VEB-1, OXA derivatives and several metalloenzymes have been found in A. baumannii. Among Acinetobacter genomic species 3 (AG3), only VIM-2 and IMP-4 have been detected in addition to the class C chromosomal cephaporphinase. Also, the poor outer membrane permeability (OMP) of A. baumannii and PBP alterations are implicated in β-lactam resistance.

Currently there is a relative paucity of data regarding the effectiveness of in vitro antibiotic susceptibility testing methods for Acinetobacter spp. A similar situation may be considered regarding extended-spectrum β-lactamase (ESBL) detection in Acinetobacter spp. CLSI only report criteria to detect ESBL in Escherichia coli, Klebsiella spp. and Proteus mirabilis. In addition, some guidelines raise the question of ESBL detection (ESBLs) which may confer a noticeable resistance when overexpressed. Among Acinetobacter spp. as ESBLs are not the main cause of cephalosporin resistance in this genus and should be sought routinely.

This study aimed to assess the susceptibility of a broad collection of Acinetobacter spp. isolated from a multicentre study from 28 different hospitals of Spain to clavulanic acid, and to show how susceptibility to clavulanic acid relates to a false ESBL detection phenotype in this genus.

Materials and methods

**Bacterial strains**

In November 2000, all phenotypically and preliminary identified A. baumannii isolates from clinical samples were collected from 28 hospitals in Spain, as part of a nationwide multicentre study. A total of 244 strains of Acinetobacter spp. were collected: 226 A. baumannii, 15 AG3 and 3 unidentified Acinetobacter strains. These species were identified by amplified ribosomal DNA restriction analysis (ARDRA) and sequencing of nucleotides of 16S rRNA.

Clinical strains of A. baumannii producing PER-1 and CTX-M-2 were used as ESBL-positive controls in the experiments.

**Antibiotic susceptibility tests and screening of β-lactamases (ESBLs)**

Antibiotic susceptibility profiles were determined by microdilution following CLSI criteria. As no breakpoints for clavulanic acid are available, we arbitrarily established three categories of susceptibility: ≤16, 32–128 and ≥256 mg/L.

The compounds tested were: cefotaxime, ceftazidime and piperacillin (Sigma-Aldrich, Madrid, Spain), cefepime (Bristol-Myers Squibb Co., Wallingford, CT, USA), clavulanic acid (GlaxoSmithKline, Madrid, Spain) and sulbactam (Pfizer Inc., Central Research Division, Sandwich, UK). The range of clavulanic acid concentrations for MIC analysis was 1–512 mg/L.

ESBL detection was performed using the following methods.

(i) Microdilution for determining cefotaxime, ceftazidime and cefepime MICs with and without clavulanic acid (at a fixed concentration of 4 mg/L). Following the CLSI criteria for Enterobacteriaceae, a ≥3 doubling concentration decrease in the MIC was considered as an ESBL-positive result.

(ii) Etests (AB Biodisk, Solna, Sweden) with cefotaxime, ceftazidime and cefepime in the presence and absence of clavulanic acid were used. Results were interpreted according to the criteria of the manufacturer for E. coli and Klebsiella spp. whereby a ≥3 doubling concentration decrease in the MIC for every cephalosporin in combination with clavulanic acid compared with the MIC tested alone was considered as a positive result.

(iii) Double disc diffusion method with cefotaxime, ceftazidime and cefepime discs (30 μg) (BD, Meylan, France) at three different distances (1, 2 and 3 cm centre to centre) from an amoxicillin/clavulanic acid disc (20:10 μg) in the centre of the plate as previously described.

(iv) Combined discs with cefotaxime, ceftazidime and cefepime discs (30 μg) with and without 10 μg of clavulanic acid. Following the CLSI criteria and those previously reported for Enterobacteriaceae, a ≥5 mm increase in a zone diameter for every cephalosporin tested in combination with clavulanic acid compared with its zone when tested alone was considered as indicative of ESBL production.

**Molecular typing experiments**

The epidemiological relationship of the 244 Acinetobacter isolates was determined by PFGE with SmaI following the method of Gautoy and confirmed by repetitive extragenic palindromic (REP)-PCR as previously described.

**Isoelectric focusing (IEF) assay and β-lactam hydrolysis**

β-Lactamases were characterized by IEF of ultrasonic bacterial extracts as previously reported.

The same bacterial extracts from the Acinetobacter isolates yielding an ESBL-positive test were analysed by spectrophotometry for cefotaxime and ceftazidime hydrolysis with and without 4 mg/L clavulanic acid as previously described.

**β-Lactamase identification**

Several primer pairs hybridized with previously reported β-lactamase genes were used in an amplification reaction to detect some β-lactamase genes. The primer pairs used were AMPC1 5'-ATGGCGATTAAAAAAAATTC and AMPC2 5'-TTATTTCTTTAT TTGACATC as well as AMPC3 5'-ACTTTACTTACACTCGCA CG and AMPC4 5'-AATTTCTTATAAGTTT, which amplify the cephalosporinase ampC gene from AG3 and A. baumannii, respectively.

Chromosomal DNA from Acinetobacter spp. isolates was purified according to standard protocols (MasterPure DNA purification kit; Epicenter, Madison, WI, USA). Five hundred nanograms of the Acinetobacter spp. chromosomes were used as templates to be amplified by PCR with the two primer pairs: AMPC1/AMPC2 and AMPC3/AMPC4. The PCR was performed under the following conditions: denaturation, 10 min at 94°C; amplification, 30 cycles of 1 min at 94°C, 1 min at 55°C and 2 min at 72°C; and elongation, 16 min at 72°C. An aliquot...
False ESBL detection in Acinetobacter spp

(20 μL) of the sample was subjected to electrophoresis in a 1.0% agarose gel. The gel showed an amplified product, detected by ethidium bromide staining (50 mg/L), of ~1150 and 550 bp with combinations AMPC1/AMPC2 and AMPC3/AMPC4, respectively, which were the expected sizes of the ampC gene in relation to that in AG3 and A. baumannii, respectively.

Expression of AmpC cephalosporinase and RT–PCR analysis

Detection of the expression of the naturally occurring cephalosporinase AmpC enzymes in the Acinetobacter spp. isolates was carried out by comparing piperacillin and ceftazidime MICs with and without clavulanic acid at 250 mg/L.

Also, and with the same aim, total RNA from Acinetobacter isolates at identical exponential phase of growth was purified by TRIRizol Max Bacterial RNA Isolation Kit (Invitrogen, Carlsbad, CA, USA), following the manufacturer’s instructions. Two A. baumannii isolates in which an ESBL phenotype was not suggested by Etest or anyotypic method were used as controls.

AmpC expression of Acinetobacter spp. isolates was monitored in a semi-quantitative manner by RT–PCR (Promega, Madison, WI, USA), following the manufacturer’s instructions.

To semi-quantify AmpC expression, the transcription of the gyrA gene was analysed and used as an internal control. Band intensity was compared between the ampC and gyrA amplicons. The oligonucleotides used to retrotranscribe and amplify ampC and gyrA genes were as follows: AMPCfor 5'-ACTTACCTCAACTCGCGACG and AMPC2rev 5'-AATTACTGTCTAATAAAGTTT; and GYRAfor 5'-AATCTGCCCCTGTCGTTG and GYRArev 5'-GCCATACTC ACGGCGA. Aliquots (10 μL) taken at 17, 20 and 23 cycles of the amplification procedure were subjected to electrophoresis in a 1.0% agarose gel. The gel showed two amplified products, the amplification procedure were subjected to electrophoresis in 1.0% agarose gel. The gel showed an amplified product, and 550 bp, which were the expected sizes of the ampC and gyrA genes, respectively, in relation to those in A. baumannii.

Purification of AmpC enzymes

The ampC gene (GenBank accession no. AM283527) of a representative clavulanic acid-susceptible Acinetobacter (strain 65 in Tables 2 and 3) was cloned into the expression vector pGEX-6P-1, which allows a fusion protein between glutathione S-transferase (GST) and the AmpC enzyme. The β-lactamase was purified to homogeneity with the GST gene fusion system (Amersham Pharmacia Biotech, Europe GmbH) according to the manufacturer’s directions. IC_{50} values were determined as described previously.7

Results

Genotypes and clavulanic acid susceptibility

A total of one hundred different genotypes were obtained among the 244 Acinetobacter spp. isolates of this study. Eighty-two different genotypes were obtained among the 226 A. baumannii isolates, and 18 different genotypes were obtained among the 15 AG3 isolates and the 3 unidentified Acinetobacter spp. isolates.

Table 1. Clavulanic acid MIC distribution among the 100 different genotypes of Acinetobacter isolates included in this study

<table>
<thead>
<tr>
<th>MIC of clavulanic acid (mg/L)</th>
<th>No. of isolates</th>
<th>Cumulative no. of isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>17</td>
<td>20</td>
</tr>
<tr>
<td>8</td>
<td>9</td>
<td>29</td>
</tr>
<tr>
<td>16</td>
<td>15</td>
<td>44</td>
</tr>
<tr>
<td>32</td>
<td>12</td>
<td>56</td>
</tr>
<tr>
<td>64</td>
<td>12</td>
<td>63</td>
</tr>
<tr>
<td>128</td>
<td>7</td>
<td>70</td>
</tr>
<tr>
<td>256</td>
<td>7</td>
<td>77</td>
</tr>
<tr>
<td>512</td>
<td>17</td>
<td>94</td>
</tr>
<tr>
<td>&gt;512</td>
<td>6</td>
<td>100</td>
</tr>
</tbody>
</table>

Clavulanic acid MICs were determined using a single representative of each of the 100 different genotypes. The Acinetobacter spp. isolates were classified into three different phenotypic categories based upon their clavulanic acid MICs: (i) n = 44 (44%) with ≤16 mg/L (group I); (ii) n = 26 (26%) with 32–128 mg/L (group II); and (iii) n = 30 (30%) with ≥256 mg/L (group III) (Table 1). The A. baumannii controls expressing PER-1 and CTX-M-2 showed clavulanic acid MICs of 32 and 128 mg/L, respectively. The sulbactam MIC_{50} value was 8 mg/L for all Acinetobacter isolates tested and no differences were observed among the Acinetobacter isolates with different susceptibilities to clavulanic acid.

Phenotypic detection of ESBL in Acinetobacter spp. clinical strains

To determine whether or not the clavulanic acid susceptibility was related to a false-positive ESBL test, 15 genotypically distinct strains with different clavulanic acid MIC values were chosen for further studies: 5 categorized within group I (MIC ≤16 mg/L) (strains 11, 34, 65, 73 and 91), 5 within group II (MIC 32–128 mg/L) (strains 40, 48, 58, 155 and 183) and 5 within group III (MIC ≥256 mg/L) (strains 26, 71, 104, 108 and 167) (Table 2).

A microdilution assay was performed with the 15 clinical strains of Acinetobacter spp. as well as with A. baumannii controls (Table 2). Clear synergy was observed among the group of isolates that were susceptible to clavulanic acid (more than three doubling concentration dilutions reduction in the presence of the inhibitor in most of the isolates). Synergy was more pronounced with cefotaxime and ceftazidime. In contrast, no synergy was detected with clavulanic acid-intermediate or -resistant isolates (Table 2). A. baumannii positive control isolates producing CTX-M-2 and PER-1 showed clear synergy, which was more evident with cefotaxime (CTX-M-2), and ceftazidime and cefepime (PER-1), respectively.

The same 15 Acinetobacter isolates were studied for ESBL detection by the double disc diffusion method with cefotaxime, ceftazidime and cefepime discs at three different distances from the centre of a disc containing 10 μg of clavulanic acid (Figure 1a). None of the cephalosporins and clavulanic acid combinations showed apparent synergy against Acinetobacter spp. isolates. On the contrary, synergy (mainly with cefepime) was obtained with the ESBL-positive A. baumannii controls.

Also, we tested whether or not the combined disc method was able to detect ESBL production in the Acinetobacter spp. isolates. For this, the diameters of zones of inhibition of cephalosporins with and without 10 μg of clavulanic acid were measured in these isolates and two A. baumannii ESBL-positive
controls (Figure 1b and Table 3). The results showed that whereas the isolates included in the clavulanic acid-susceptible group (isolates 11, 34, 65, 73 and 91) showed some degree of synergy in the presence of cephalosporin plus clavulanic acid (in some cases and with specific antibiotics higher than 5 mm in the presence of clavulanic acid and mainly due to its intrinsic clavulanic acid susceptibility), the remaining intermediate and resistant *Acinetobacter* isolates did not show any synergy in the combined disc method tests (no more than 1–2 mm inhibition zone difference in the presence of clavulanic acid). The *A.

**Table 2.** Clavulanic acid microdilution MICs (mg/L) for the *Acinetobacter* clinical isolates for the indicated antibiotic combinations

<table>
<thead>
<tr>
<th>Strain</th>
<th>CTX</th>
<th>CTX + CLA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CAZ</th>
<th>CAZ + CLA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>FEP</th>
<th>FEP + CLA&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CLA&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>32</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>256</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>34</td>
<td>128</td>
<td>*</td>
<td>32</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>32</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>65</td>
<td>32</td>
<td>*</td>
<td>32</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>73</td>
<td>128</td>
<td>*</td>
<td>128</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>4</td>
</tr>
<tr>
<td>91</td>
<td>4</td>
<td>*</td>
<td>4</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1</td>
<td>*&lt;sup&gt;c&lt;/sup&gt;</td>
<td>2</td>
</tr>
<tr>
<td>40</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>16</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>48</td>
<td>64</td>
<td>32</td>
<td>16</td>
<td>8</td>
<td>8</td>
<td>8</td>
<td>64</td>
</tr>
<tr>
<td>58</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>32</td>
<td>32</td>
<td>64</td>
</tr>
<tr>
<td>155</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>256</td>
<td>64</td>
<td>64</td>
<td>128</td>
</tr>
<tr>
<td>183</td>
<td>128</td>
<td>128</td>
<td>16</td>
<td>8</td>
<td>32</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>26</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>71</td>
<td>256</td>
<td>128</td>
<td>256</td>
<td>128</td>
<td>32</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>104</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;512</td>
</tr>
<tr>
<td>108</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>&gt;256</td>
<td>16</td>
<td>16</td>
<td>&gt;512</td>
</tr>
<tr>
<td>167</td>
<td>64</td>
<td>32–64</td>
<td>64</td>
<td>64</td>
<td>32</td>
<td>32</td>
<td>512</td>
</tr>
<tr>
<td>PER-1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>128</td>
<td>64</td>
<td>256</td>
<td>16</td>
<td>128</td>
<td>16</td>
<td>128</td>
</tr>
<tr>
<td>CTX-M-2</td>
<td>256</td>
<td>&lt;2</td>
<td>16</td>
<td>&lt;2</td>
<td>128</td>
<td>16</td>
<td>128</td>
</tr>
</tbody>
</table>

<sup>a</sup>CLA at fixed concentration of 4 mg/L.
<sup>b</sup>CLA alone.
<sup>c</sup>No growing at lower antibiotic concentrations tested (0.25/4 mg/L in all cases).
<sup>d</sup>*A. baumannii* controls expressing the indicated β-lactamases.

**Table 3.** Diameters of inhibition zones (mm) of the *Acinetobacter* spp. clinical isolates for the indicated antibiotics

<table>
<thead>
<tr>
<th>Strain&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CLA&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CTX</th>
<th>CTX + CLA</th>
<th>CAZ</th>
<th>CAZ + CLA</th>
<th>FEP</th>
<th>FEP + CLA</th>
</tr>
</thead>
<tbody>
<tr>
<td>11</td>
<td>22</td>
<td>19</td>
<td>26</td>
<td>22</td>
<td>26</td>
<td>25</td>
<td>27</td>
</tr>
<tr>
<td>34</td>
<td>15</td>
<td>19</td>
<td>22</td>
<td>21</td>
<td>23</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>65</td>
<td>14</td>
<td>22</td>
<td>17</td>
<td>15</td>
<td>17</td>
<td>20</td>
<td>21</td>
</tr>
<tr>
<td>73</td>
<td>18</td>
<td>24</td>
<td>27</td>
<td>24</td>
<td>26</td>
<td>28</td>
<td>31</td>
</tr>
<tr>
<td>40</td>
<td>10</td>
<td>20</td>
<td>21</td>
<td>25</td>
<td>25</td>
<td>27</td>
<td>27</td>
</tr>
<tr>
<td>58</td>
<td>9</td>
<td>19</td>
<td>21</td>
<td>21</td>
<td>22</td>
<td>22</td>
<td>19</td>
</tr>
<tr>
<td>155</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>9</td>
<td>11</td>
</tr>
<tr>
<td>183</td>
<td>(−)</td>
<td>14</td>
<td>14</td>
<td>12</td>
<td>12</td>
<td>18</td>
<td>19</td>
</tr>
<tr>
<td>26</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>71</td>
<td>(−)</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>12</td>
<td>14</td>
<td>16</td>
</tr>
<tr>
<td>104</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>108</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>(−)</td>
<td>12</td>
<td>13</td>
</tr>
<tr>
<td>167</td>
<td>(−)</td>
<td>10</td>
<td>19</td>
<td>19</td>
<td>19</td>
<td>17</td>
<td>17</td>
</tr>
<tr>
<td>PER-1</td>
<td>10</td>
<td>(−)</td>
<td>11</td>
<td>(−)</td>
<td>14</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>CTX-M-2</td>
<td>8</td>
<td>(−)</td>
<td>22</td>
<td>21</td>
<td>25</td>
<td>11</td>
<td>27</td>
</tr>
</tbody>
</table>

<sup>a</sup>*A. baumannii* expressing PER-1 and CTX-M-2 β-lactamases.
<sup>b</sup>CLA, clavulanic acid; CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime.
<sup>c</sup>Growing until the edge of the disc.
baumannii ESBL-positive controls showed clear synergy (the exception is CTX-M-2 with ceftazidime for its lower hydrolytic activity towards this antibiotic).

Lastly, we attempted to determine whether Etests for ESBL detection in Enterobacteriaceae can detect ESBL activity in Acinetobacter spp. and the impact of clavulanic acid susceptibility in the Etest analysis. With this aim, the same 15 Acinetobacter isolates were studied for ESBL detection with the above mentioned Etest strips. Those isolates included in the clavulanic acid-susceptible group (isolates 11, 34, 65, 73 and 91) yielded a positive test for ESBL production (Figure 1c). Those Acinetobacter isolates that were intermediate or resistant to clavulanic acid (MICs 16 to >256 mg/L) yielded a negative ESBL Etest result. The ESBL-producing positive control Acinetobacter isolates were also negative for ESBL activity by Etest.

**β-Lactamase identification and analysis**

To elucidate the number of β-lactamases produced by each of the 15 isolates, protein extracts were analysed by IEF, as described in the Materials and methods section. The 15 Acinetobacter spp. isolates yielded a unique band of pI > 9.0. This strongly suggested the presence of the previously reported chromosomal cephalosporinase4,7 that corresponded to the chromosomal ampC gene of Acinetobacter, which we identified in these isolates by PCR (data not shown).

Cefotaxime and ceftazidime hydrolysis was detected by spectrophotometric analysis to different degrees using protein extracts from the 15 Acinetobacter spp. isolates. This activity was not inhibited by 4 mg/L clavulanic acid. When protein extracts from positive controls were used, some degree of inhibition was observed (data not shown). Together the data showed no ESBL production occurred in the 15 Acinetobacter spp. isolates.

**AmpC expression**

To explain the basis for the increased susceptibility to clavulanic acid in the Acinetobacter isolates (or genotypes) under study, we considered that if somehow the AmpC enzyme affects the amount of clavulanic acid present in the cell, a reduction in the expression of the enzyme may increase susceptibility to the β-lactamase inhibitor. To test the hypothesis that increasing clavulanic acid susceptibility may be due to underexpression of the chromosomal cephalosporinase, we used two experimental approaches:

(i) Piperacillin and ceftazidime MICs were determined with and without cloxacillin (250 mg/L) for the 15 Acinetobacter isolates. The results showed that piperacillin MICs were reduced in the presence of cloxacillin in most isolates, indicating that the isolates overexpress the AmpC enzyme and no differences were detected regardless of their susceptibility to clavulanic acid (data not shown).
(ii) A RT–PCR was performed using as a source total RNA from the 15 Acinetobacter spp. isolates. PCR samples collected at 17, 20 and 23 cycles of the amplification procedure showed no difference in amplicon band intensity among all of the isolates (clavulanic acid-susceptible and -non-susceptible isolates).

We also considered whether inhibition of the AmpC enzyme by clavulanic acid may occur in response to the nucleotide sequences of the ampC genes in these isolates. The AmpC enzyme from isolate 65 (clavulanic acid MIC of 4 mg/L and false ESBL producer) was cloned, expressed and purified by affinity chromatography. Kinetic experiments revealed an IC₅₀ (μM) of clavulanic acid of >250, indicating non-susceptibility.

Discussion

Nosocomial outbreaks of infection by strains of A. baumannii have been reported worldwide⁶,³³ and most strains are resistant to many of the antibiotics usually used in clinical practice. Sulbactam and polymyxin B drugs are sometimes the only therapeutic alternatives for the treatment of these pandrug-resistant A. baumannii isolates.¹³,¹⁵ In this study, we found that 44 out of 244 Acinetobacter clinical isolates (mostly A. baumannii and AG3) belonging to 44 different genotypes showed clavulanic acid susceptibility (MICs of 2–16 mg/L). Further, we demonstrated that this phenomenon was related to a false ESBL test result when a synergistic method between cephalosporin and clavulanic acid was tested (microdilution, combined disc and Etest). However, none of the Acinetobacter isolates with clavulanic acid susceptibility (clavulanic acid MICs ≤16 mg/L) showed synergy between cephalosporins and clavulanic acid when they were tested by the double disc diffusion method, thus confirming the reliability of this test for ESBL detection in Acinetobacter (A. baumannii ESBL producers tested yielded a positive result with this technique). The remaining Acinetobacter isolates (with higher clavulanic acid MICs, groups II and III) yielded a negative ESBL test result with all methods used.

We found no evidence of ESBLs in the 15 Acinetobacter spp. isolates and concluded that clavulanic acid susceptibility in the strains under study was not related to any β-lactamase (ESBL or naturally occurring AmpC enzyme). Outer membrane protein (OMP) profiles did not vary between Acinetobacter spp. isolates with different degrees of clavulanic acid susceptibility (groups I, II and III) (data not shown). MIC values for a more resistant spontaneous revertant from group I were observed in the presence of a specific efflux pump inhibitor (data not shown). No differences in MIC values were detected when the efflux pump inhibitor was added. Together the data suggest that clavulanic acid susceptibility is most likely associated with PBP alterations in these isolates.

The susceptibility of Acinetobacter spp. to clavulanic acid has been described previously.¹⁴ Pandey et al.³⁴ studied 100 consecutive isolates of A. baumannii obtained from various clinical samples. They found that 11% of isolates showed an amoxicillin/clavulanic acid MIC of <8/4 mg/L, therefore being categorized as susceptible to the antibiotic combination. Also, Sturenburg et al.³⁵ reported that two out of six Acinetobacter strains tested for ESBL production with MicroScan ESBL plus confirmation panel yielded a positive ESBL result although PCR analysis showed that these strains were lacking an ESBL. In another study, Higgins et al.³⁶ studied in vitro activities of the β-lactamase inhibitors alone or in combination with β-lactams against 115 epidemiologically characterized multidrug-resistant A. baumannii strains. They showed clavulanic acid MIC₅₀ and MIC₉₀ values of 16 and 64 mg/L. Interestingly, 40% of the strains showed clavulanic acid MIC values lower than 16 mg/L and one strain had a clavulanic acid MIC of 2 mg/L. Overall, these results may indicate that, for unknown reasons, some Acinetobacter isolates may show an increased clavulanic acid susceptibility, but not with sulbactam, and these strains may yield a false ESBL test result when they are tested by an inhibitor-based method.

The present results have clear implications for routine clinical practice in microbiology laboratories, as they suggest that manual or automatic procedures focused on inhibitory-based methods for ESBL detection may yield false results with Acinetobacter isolates. Any indication of ESBL production in Acinetobacter spp. by these methods must be confirmed by another method, e.g. double disc diffusion method or combined disc method (if a disc of clavulanic acid alone is also used) with ceftazidime, and by a reference laboratory. These findings will help in the monitoring of the epidemiology of infections caused by Acinetobacter spp., allowing better control of the diagnosis and detection of ESBL-producing Acinetobacter spp. isolates.

Acknowledgements

We thank Drs Vahaboglu and Nagano for the gift of Acinetobacter harbouring PER-1 and CTX-M-2 ESBL.

Members of the Spanish Group for Nosocomial Infection (GEIH) from the Spanish Society on Infectious Diseases and Clinical Microbiology included: Javier Ariza, Mª Angeles Domínguez, Miquel Pujol and Fe Tubau (Ciutat Sanitaria i Universitaria de Bellvitge, Barcelona); Juan Pablo Horcajada, Anna Ribera and Jordi Vila (Hospital Clinic i Provincial, Barcelona); Jordi Cuquet, Carmina Martí and Dolors Navarro (Hospital General de Granollers, Barcelona); Francisco Alvarez Lerma and Margarita Salvadó (Hospital del Mar, Barcelona); Irene Planells and Oscar del Valle-Ortiz Maestu (Hospital de la Vall d’Hebron, Barcelona); Fernando Chaves and Antonio Sánchez Porto (Hospital del SAS de la Línea de la Concepción, Cádiz); Fernando Rodríguez López and Elisa Vidal (Hospital Universitario Reina Sofía, Córdoba); Alejandro Beceiro and Germán Bou (Hospital Juan Canalejo, Ourense); Alfonso March and Jose María Jesús Martínez Beltrán (Hospital Ramón y Cajal, Madrid); Paloma García Hierro and Josefa Gómez Castillo (Hospital Universitario de Getafe, Madrid); Belén Padilla (Hospital Universitario Gregorio Marañón, Madrid); Jesús Martínez Beltrán (Hospital Ramón y Cajal, Madrid); Manuel López Brea and Lucía Pérez (Hospital Universitario de la Princesa, Madrid); Manuel Causse and Pedro Mancho (Complejo Hospitalario Carlos Haya, Málaga); Inés Dorronsoro and José Javier García Irure (Hospital de Navarra, Pamplona); Almudena Tinajas (Hospital Santo Cristo de Piñor, Ourense); Gloria Esteban and Begoña Fernández (Hospital Santa María Nai, Ourense); Nuria Borrell and Antonio Ramírez (Hospital Universitario Son Dureta, Palma de Mallorca); Isabel Alamo and Diana García Bardec (Hospital de Gran Canaria Dr.
False ESBL detection in *Acinetobacter* spp

Negrín, Las Palmas de Gran Canaria}; José Angel García Rodríguez (Hospital Universitario de Salamanca); Carmen Fariñas and Carlos Fernández Mazarrasa (Hospital Universitario Marqués de Valdecilla, Santander); Eduardo Varela and Mercedes Treviño (Hospital Universitario de Santiago de Compostela, Santiago de Compostela); Luis Martínez, Alvaro b

baumannii (REIPI RD06/0008).

4BTF916028PR) and also by Ministerio de Sanidad y Consumo, None to declare.

Transparency declarations

Funding

A. B. is in receipt of a scholarship from SEIMC. This study was partially supported by FIS (PI040514, PI061368), Consellería de Innovación, Industria y Comercio, Xunta de Galicia (PGIDT0-4BTF916028PR) and also by Ministerio de Sanidad y Consumo, ISCIII, Spanish Network for Research in Infectious Diseases (REIPI RD06/0008).

References


