Biochemical characterization of a cefotaxime-hydrolysing β-lactamase encoded by a conjugative plasmid

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Accepted 27 March, 2009

During the nosocomials infections occurring in the intensive care unit of the military hospital of Tunis in 2005, the Enterobacter cloacae BW 1150 strain was isolated from a stool culture. This strain was found to have a high level resistance to broad-spectrum β-lactams. Resistance profile against the various families of antibiotics was determined using the disc diffusion test. The minimal inhibitory concentrations values showed that this strain was resistant to the β-lactams such as ampicillin and the extended spectrum cephalosporins (cefotaxime, ceftriaxon and cefpirome). Analysis of this strain by the disk diffusion test revealed synergies between amoxicillin-clavulanate (AMX-CA) and ceftriaxon, ceftazidime and cefotaxime. Cell sonicate of this isolate is very active against cefotaxime and showed a specific activity (AS) of 7.54 U/mg for the same antibiotic. This activity was inhibited by the sulbactam and the clavulanic acid. Isoelectrofocusing methods revealed that the crude extract of the E. cloacae BW 1150 strain showed 1 β-lactamase activity with an isoelectric piont (pI) of about 8. This activity was transferred by conjugation and was highly expressed in the transconjugant.

Key words: β-lactams, β-lactamases, cefotaximase, Enterobacter cloacae.

INTRODUCTION

Enterobacter species are a common cause of several human diseases and are predominantly associated with nosocomial infections. They are responsible for 5% of all nosocomial septicaemia cases in the United States and can be found as the third most common pathogens recovered from the respiratory tracts of patients in intensive care units (Stock et al., 2001). Among the variety of Enterobacter species, E. cloacae is a well-reconized nosocomial pathogen that causes significant infections, especially in last recent years (Conceição et al., 2004). This microorganism is the most commonly isolated member of the Enterobacteriaceae that possess a chromosomally encoded ampC β-lactamase that plays an important role in resistance to antibiotics (Bell et al., 2003). However, several reports have demonstrated that this species can acquire and express genes encoding extended spectrum β-lactamase (ESBLs), especially for the multidrug isolates (Hall et al., 2006). In fact, it has been reported that the wild-type strains of E. cloacae can express the bush group 2f carbapenemases Nmc-A, VIM-2, IMI-2 and VIM-4 (Pottumarthy et al., 2003; Jeong et al., 2003; Yu et al., 2006; Luzzaro al., 2004). The resistance to broad-spectrum penicillins and extended-spectrum cephalosporins via the acquisition of plasmids encoding TEM and SHV derivatives BLSEs has also been reported (Liu et al., 2004). Various extended-spectrum β-lactamases not derived from TEM or SHV enzymes have been described in this species, such as SFO type in Japan (Matsumoto et al., 1999), IBC-1 type in Greece (Giakkoupi et al., 2000), VEB-3 type in Thailand (Jiang et al., 2005) and CTX-M class that have been des-
cribed more recently (Doucet et al., 2000; Chanawong et al., 2002; Kim et al., 2005; Touati et al., 2006).

In this work, we report the biochemical analysis of cefotaximase producing clinical isolate of *E. cloacae* from the military hospital of Tunis.

**MATERIALS AND METHODS**

**Bacterial strains**

One isolate of *E. cloacae* BW 1150 is included in this study. This strain was isolated from a stool culture in intensive care unit of the military hospital of Tunis in 2005. This isolate was identified using the API 20 E identification system (bioMérieux, Marcy l’Etoile, France). E. coli HB101 (F, Δ(gpt-proA) 62, leuB6, supE44, ara-14, galK2, lac Y1, Δ(mcr-mrr), rps, L26, Xyl-rtm1 t, thi-1, IncFI, rec AB, str) is resistant to streptomycin and is used for the conjugaison experiences.

**Susceptibility testing and extended-spectrum β-lactamase detection**

The antibiotics susceptibilities of the *E. cloacae* BW 1150 strain was determined by Mueller-Hinton agar by the standard disk diffusion procedure as described by the antibiogram committee of the French society for microbiology (www.sfm.asso.fr) (Cavallo et al., 2007). The following antibiotics were tested, ampicillin, ticarcillin, cloxacillin, cefotaxime, ceftazidime, ceftriaxone, cefpirome, streptomycin, ciprofloxacin, aztreonam, imipenem, tetracycline, cloramphenicol, acid nalidixique (Biorad, Marnes-la-Coquette, France).

The ESBL detection was based on the double-disk synergy test (DDST) as described previously (Ben et al., 2007). DDST was performed as follows: the surface of a Mueller-Hinton (MH) agar plate was inoculated with an overnight culture suspension of clinical isolate. After inoculation, disks containing 30 µg of cefazidime, cefotaxime, ceftriaxone, cefpirome and amoxicillin-clavulanic acid (20/10 µg) were placed at distances of 20 mm (centre to centre).

Enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime, ceftriaxone, ceftazidime or cefpirome indicated the presence of ESBL production.

**Determination of minimum inhibitory concentration**

Minimum inhibitory antibiotics concentrations were determined by the serial dilution method. The minimum inhibitory concentration results were interpreted according to the recommendations of the antibiogram committee of the French society for microbiology (ACFSM) (Cavallo et al., 2007).

**Preparation of crude enzyme extracts**

Bacterial strain was grown overnight with shaking in 50 ml of trycase soy broth (TSB) (Diagnostics, Pasteur, France) at 37°C. The cells were harvested by centrifugation and washed once in 25 mM potassium-sodium phosphate buffer (pH 7) and resuspended in 1 ml of the same buffer.

For preparation of cell-free extract, the cells were ruptured by ultrasonic treatement in a UP 400 S sonicator at 4°C. Cell debris was removed by centrifugation at 10000 rpm for 10 min in a hettich centrifuge R 32 rotor. The supernatant was used for all β-lactamases enzyme tests (Ben et al., 2007).

**β-Lactamase assay**

β-Lactamase activities were determined by spectrophotometric method of O’calloghan et al. (1975). Briefly, the decrease in absorbance of the antibiotics at an appropriate concentration and wavelength was measured in a temperature controlled spectrophotometer (Varian® Cary 50 Bio UV-visible) at 37°C (Ross et al., 1975).

**Inhibitors effects (IC50 determination)**

For determination of inhibitor effects, rates of hydrolysis of 1 mM cephalotin were determined in the presence of various concentrations of clavulanic acid and sulbactam. EDTA (Ethylenediamine tetraacetic acid) was used for 1 mM as concentration. In these experiments the protein extract was preincubated with the inhibitors for 10 min before the addition of cephalotin. The inhibitory concentration that allowed the reduction of β-lactamases activities of 50% was determined from the curve: AS = f (inhibitors concentration) (Réjiba et al., 2002).

**Conjugation and resistance transfer**

Conjugation experiment using *Escherichia coli* HB101 str® as the recipient strain was performed. Transconjugants were selected on Miller-Hinton agar plates containing streptomycin (20 µg/ml), ticarcillin (2 µg/ml) and cefotaxime (2 µg/ml). To select the plasmid-encoded resistance, the transjuncts growing on the selection plates were subjected to DDST to confirm the presence of ESBL (Ben-Hammouda et al., 2004).

**Analytical isoelectric focusing (IEF)**

IEF was performed as follows, bacterium growing exponentially at 37°C in trycase soy broth was harvested at 10000 rpm for 10 min (Beckman centrifuge, FO650 Rotor) and cell-free lysate was prepared by sonicat (sonicator UP 400S cycle: 25 and amplitude 50 Hz). Crude sonic extract was centrifuged at 10000 rpm for 10 min and the supernatant was collected and subjected to IEF in broad range (Ph 3 - 10) polyacrylamide gel 7% at 4°C. The β-lactamase activity was detected by the iodine procedure in gel by using benzylpenicillin (30 µg/ml) as the substrate (Ross et al., 1975).

**RESULTS**

**Susceptibility testing and extended-spectrum β-lactamase detection**

Antibiotics susceptibility testing revealed that this isolate was resistant to all penicillins and cephalosporins tested except ceftazidime. For its resistance to other antibiotics, this strain was resistant to cloramphenicol, tetracycline, nalidixic acid. Whereas, this isolate remain susceptible to imipenem and streptomycin (Table 1). The enhancement of the inhibition zone between the disks containing amoxicillin-clavulanate and cefotaxime, ceftriaxone and cefpirome indicated the presence of ESBL production (data not shown).

**Minimal inhibitory concentration**

The resistance to β-lactams of *E. cloacae* BW 1150 strain
Table 1. Antibiotics susceptibility of the *E. cloacae* BW 1150 strain, transconjugant and *E. coli* recipient.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>E. cloacae</em> BW 1150</th>
<th><em>E. coli</em> HB 101 x <em>E. cloacae</em> BW 1150</th>
<th><em>E. coli</em> HB 101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazidime</td>
<td>22</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>Cefotaxime</td>
<td>12</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>13</td>
<td>14</td>
<td>26</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>14</td>
<td>14</td>
<td>23</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>27</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>Ciproflaxacin</td>
<td>15</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>6</td>
<td>6</td>
<td>25</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>8</td>
<td>6</td>
<td>30</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>6</td>
<td>6</td>
<td>32</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>18</td>
<td>16</td>
<td>35</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>6</td>
<td>28</td>
<td>32</td>
</tr>
<tr>
<td>Cloramphenicol</td>
<td>6</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>6</td>
<td>24</td>
<td>29</td>
</tr>
<tr>
<td>Imipinem</td>
<td>25</td>
<td>32</td>
<td>35</td>
</tr>
</tbody>
</table>

Table 2. MIC values (µg/ml) of the *E. cloacae* BW 1150 strain, transconjugant and *E. coli* recipient.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>E. cloacae</em> BW 1150</th>
<th><em>E. coli</em> HB 101 x <em>E. cloacae</em> BW 1150</th>
<th><em>E. coli</em> HB 101</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cefazidime</td>
<td>8</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Céfotaxime</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>1</td>
</tr>
<tr>
<td>Cefpirome</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>2</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>&gt;512</td>
<td>&gt;512</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>4</td>
<td>&gt;256</td>
<td>&gt;256</td>
</tr>
<tr>
<td>Ciproflaxacin</td>
<td>&gt;512</td>
<td>&gt;256</td>
<td>2</td>
</tr>
<tr>
<td>Ticarcillin</td>
<td>&gt;512</td>
<td>&gt;256</td>
<td>2</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>512</td>
<td>&gt;256</td>
<td>1</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>&gt;512</td>
<td>&gt;256</td>
<td>4</td>
</tr>
<tr>
<td>Aztreonam</td>
<td>128</td>
<td>128</td>
<td>4</td>
</tr>
<tr>
<td>Cefoxitin</td>
<td>512</td>
<td>4</td>
<td>2</td>
</tr>
<tr>
<td>Cloramphénicol</td>
<td>256</td>
<td>4</td>
<td>&lt;1</td>
</tr>
<tr>
<td>Tetracyclin</td>
<td>512</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Imipinem</td>
<td>4</td>
<td>4</td>
<td>1</td>
</tr>
</tbody>
</table>

is confirmed by the Minimal inhibitory concentration of the penicillins (ampicillin and ticarcillin), the extended spectrum cephalosporins (cefoxitin, cefotaxime, ceftriaxone, cefpirom except ceftazidime) which exceed 256 µg/ml. This strain is also resistant to others antibiotic, in addition to the β- lactams. Indeed, this strain is highly resistant to cloramphenicol, tetracyclin and quinolones (Nalidixic acid) with a value that exceed 256 µg/ml. Nevertheless, it remains sensitive to imipinem and streptomycin (Table 2).

**β-Lactamase essay and inhibitor susceptibility**

Analysis of the crude extract by the starch, 7% polyacrylamide gel electrophoresis revealed one band in the presence of benzylpenicillin; the same band was visualized with cefotaxime (Figure 1).

*E. cloacae* BW 1150 hydrolyzed oxyimino-cephalosporins in addition to benzylpenicilline and ticarcillin. Cefotaxime was the most stable among the cephalosporins tested. The hydrolysis of imipinem cloxacillin and
aztreonam by *E. cloacae* BW 1150 was not detectable (Table 3). *E. cloacae* BW 1150 activity was inhibited by clavulanic acid and sulbactam but was not inhibited by EDTA (Table 4). These characteristics resembled those of class A enzymes reported previously (Bush et al., 1995).

**Conjugation and resistance transfer**

Conjugation from *E. cloacae* BW 1150 to *E. coli* HB 101 was successful. Only the resistance to ampicillin, ticarcillin, cefotaxime and ceftipime was transferred, suggesting that the genes of resistance to these antibiotics are on a conjugable R plasmid (Table 1). This resistance is confirmed by the minimal inhibitory concentration (Table 2). The enhancement of the inhibition zone between the disks containing clavulanic acid and cefotaxime and ceftipime indicated the presence of ESBL production (data not shown).

**Analytical isoelectric focusing (IEF)**

The supernatants of *E. cloacae* BW 1150 and the transconjugant were collected and subjected to IEF in broad range (pH 3 - 10) polyacrylamide gel 7% at 4°C. The β-lactamase activity was detected according to the iodo-metric method in the presence of the benzylpenicillin as substrate. The *E. cloacae* BW 1150 and its transconjugant produce the same β-lactamase activity with a pi of about 8 (Figure 2).

**DISCUSSION**

The clinical isolate of *E. cloacae* BW 1150 included in this study was isolated in the intensive care unit at the military hospital in Tunisia in 2005. This isolate showed a multidrug resistance phenotype including resistance to the extended spectrum β-lactams except the ceftazidime, chloramphenicol, tetracyclin and quinolone (Nalidixic acid). Whereas, this isolate was found susceptible to imipinem.
the ICM of various antibiotics was determined, which showed a high level of resistance for this isolate. The double disk synergy gave a positive result for this isolate, suggesting the presence of extended spectrum β-lactamase. This activity was strongly active against cefotaxime and it was inhibited by the clavulanic acid which confirm that it belong to serine active β-lactamase. Conjugation experiment using E. coli HB 101 S^R as the recipient strain was performed. Penicillins and cephalosporins resistance was transferred by conjugation. Isoelectric focusing analysis of the supernatant of the specific activity of this isolate was restored in the pre-

<table>
<thead>
<tr>
<th>Organism</th>
<th>(IC_{90} μM)</th>
<th>Clavulanic acid</th>
<th>Sulbactam</th>
<th>EDTA</th>
</tr>
</thead>
<tbody>
<tr>
<td>E. cloacae BW 1150</td>
<td>0.76</td>
<td>1.34</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

(−) without effect.

### ACKNOWLEDGEMENT

This work was funded by grants from the Tunisian Ministry of Scientific Research And Technology.

### REFERENCES


Table 4. Inhibitor effects on β-lactamase activity produced by E. cloacae BW 1150.

