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Biochemical characterization of chromosomal cephalosporinases from isolates belonging to the *Acinetobacter baumannii* complex

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Members of the *Acinetobacter baumannii* complex have emerged as some of the most important opportunistic pathogens within the hospital environment, being able to colonize and produce infections in most immunocompromised patients, especially in intensive care units (ICUs). Such infections are difficult to treat due to their multiple resistance to the antibiotics currently available for the treatment of nosocomial infections [1]. Various mechanisms of resistance to β-lactams have been identified in this genus, including β-lactamase production, alteration of penicillin-binding proteins, and reduced levels of penetration across the outer membrane.

β-Lactamase production is one of the main mechanisms of resistance to β-lactams in *Acinetobacter* spp. [2,3]. Such enzymes can be either plasmid-encoded penicillinases (TEM and CARB type) or chromosomal cephalosporinases. The exact nature and character of these latter enzymes are not fully clarified for *Acinetobacter* spp. They belong to the group 1 β-lactamases of the classification of Bush et al [4], but several authors have found heterogeneity among the *Acinetobacter* cephalosporinases [5,6]. In the present work, the cephalosporinases produced by 125 strains belonging to the *A. baumannii* complex were characterized by determination of their pI values, β-lactamase inhibition profiles and substrate profiles.

Clinical isolates ($N = 125$) belonging to the *A. baumannii* complex, obtained in the Hospital de la Princesa (Madrid, Spain) from January 1995 to December 1997, were studied. The isolates were identified by the API 20NE system (BioMerieux, Lyon, France) and growth at 44 °C [7]. Minimal inhibitory concentrations (MICs) and susceptibility percentages of 15 β-lactam agents were determined by an agar dilution method, as recommended by the National Committee for Clinical Laboratory Standards [8]. *A. baumannii* ATCC 19606, *Escherichia coli* ATCC 25922 and *Pseudomonas aeruginosa* ATCC 27853 were used as reference strains. β-Lactamase activity was detected in crude enzymatic extracts by the hydrolysis of nitrocefin (0.5 mg/mL). The pI values were determined by isoelectric focusing (IEF) (Multiphior II Electrophoresis System; Amersham Pharmacia Biotech, Uppsala, Sweden), on wide-range ampholine (pH range 3.5–9.5) polyacrylamide gels; in some cases a narrow-range ampholine (pH 5.5–8.5) was used.

The percentage of inhibition of the following inhibitors was studied at fixed concentrations for 43 enzymatic extracts: clavulanic acid (10 μM), sulbactam (20 μM), cloxacillin (4 mg/mL), aztreonam (1.148 μM), NaCl (140 mM) and EDTA (0.1 mM). This assay was performed as described by Papanicolaou et al [9] with several modifications. After incubating the extracts with nitrocefin solution, the changes in optical density at a wavelength of 492 nm were measured at 2, 5 and 10 min, and subsequently at 10-min intervals for 40 min. Duplicate determinations were performed for each extract.

Sixteen crude enzymatic extracts were selected to study the substrate profile of the β-lactamases. Two methods were used. First, hydrolysis of the following β-lactams by the crude extracts was studied with a microbiological assay as described by Paton et al [10]: ampicillin, cefazolin, oxacillin, carbenicillin, cefuroxime, cefotaxime, ceftazidime, imipenem and meropenem. OXA-2 and TEM-1 were used as control enzymatic extracts. *Staphylococcus aureus* ATCC 25923 and *E. coli* ATCC 25922 were used as hydrolysis control strains depending on the antibiotic tested. Second, assay UV spectrophotometry was used to study the hydrolysis rate of cephaloridine, benzylpenicillin, cefotaxime, ceftazidime, imipenem, meropenem, carbenicillin and oxacillin. Duplicate determinations were performed for each enzymatic extract, with concentrations of either 100 μM or 1 mM. Hydrolysis rates were expressed as a percentage of hydrolysis with respect to cephaloridine.

A high percentage of susceptibility was found to imipenem, meropenem and ampicillin–sulbactam (100%, 100% and 84%, respectively); however, low susceptibility was shown to most penicillins (piperacillin, 8%; ticarcillin, 18.4%), and cephalosporins (cefotaxime, 4.8%; ceftazidime, 11.2%; cefepime, 12.8%). Isoelectric focusing showed a unique band of $pI > 8$

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Table 1 Average percentage of inhibition for selected chromosomal β-lactamases found in isolates belonging to the *A. baumannii* complex

Individual strain	pI group	Clavulanic acid	Cloxacillin	Sulbactam	Aztreonam	EDTA	NaCl
44	I	15	93.6	83.8	93.2	16.5	19.8
86	II	18.2	93.4	87.7	92.9	15.5	36
90	I	20	94	82	94	11.6	10
32	I	11.5	93.4	88.4	93.9	12	16
55	II	10	90.4	91.4	91.4	15.9	15.9
144	II	42.8	93.7	90.8	94.8	10.8	14.4
50	I	8.9	82	80.4	88.8	18.4	15
98	I	13.6	96.3	82.1	94.2	10.8	14.4
107	II	12.8	94.4	87.2	95	11.6	15.8

pI group I >8.5; pI group II 8–8.5.

in all the enzymatic extracts tested. When a narrower range of ampholines (5.5–8.5) was used, two different pI groups of >8.5 and between 8 and 8.5 were observed. The β-lactamases (72%) with a pI of >8.5 were designated pI group I, while the β-lactamases (28%) with a pI between 8 and 8.5 were designated pI group II.

Maximum inhibition of these enzymes by inhibitors was usually observed after 2 min, but occasionally after 5 min. All of the enzymes showed a maximum percentage of inhibition with clavulanic acid of <45%. All were inhibited (80–90%) by sulbactam, cloxacillin and aztreonam. The values with NaCl and EDTA were <30% for all enzymes. Examples of inhibition profiles of these enzymes, expressed as an average of the maximum percentage of inhibition, are shown in Table 1.

Hydrolysis of ampicillin, cefazolin, cefuroxime and cefotaxime could be demonstrated by a microbiological assay for all enzymatic extracts, but this test was negative for ceftazidime, carbenicillin, imipenem and meropenem. Cephaloridine hydrolysis was observed by spectrophotometric assay at concentrations of 100 μM with most β-lactamase extracts, but a concentration of 1 mM was needed to demonstrate hydrolysis with some extracts. Examples of the hydrolysis rates observed are shown in Table 2, expressed as the hydrolysis rate with respect to cephaloridine. Significant hydrolysis of cefotaxime, ceftazidime, carbenicillin, imipenem and meropenem could not be demonstrated by this method.

Cephalosporinases are considered to play a significant role in resistance to cephalosporins in *Acinetobacter* spp. Morohoshi, in 1977, reported a β-lactamase from *Acinetobacter anitratus* (syn. *calcoaceticus*) with a pI of 7.25–7.5, which was not inhibited by clavulanic acid or EDTA, and which was included in group 1 (CEP-N) of the classification of β-lactamases [11]. A new cephalosporinase, with a pI of 9.9, was characterized by Hikida et al [12]. Blechschmidt in 1992 [13] demonstrated clear inhibition of the same enzyme by cloxacillin, aztreonam, carbenicillin and sulbactam. There was no inhibition by clavulanic acid at concentrations up to 0.25 mM, nor by EDTA or pCMB. This β-lactamase was also included in the CEP-N group of cephalosporinases.

Cephalosporinases belonging to group 1 are well inhibited by cloxacillin and aztreonam but are not inhibited by clavulanic acid. Cephalosporinases from *Acinetobacter* are also inhibited by sulbactam, suggesting the inclusion of these enzymes in group 2d, but these enzymes are also inhibited by pCMB. Inhibition by sulbactam has only been observed for cephalosporinases from *Acinetobacter* spp. and in a *Citrobacter freundii* isolate [14]. The125 isolates in the present study seemed to possess cephalosporinases from group 1 of the classification of β-lactamases [15], since

Table 2 Examples of relative hydrolysis rates for chromosomal β-lactamases from isolates belonging to the *A. baumannii* complex

Individual strain	pI group	Cephaloridine	Penicillin	Cefotaxime	Ceftazidime	Carbenicillin	Imipenem	Meropenem
44	I	100[a]	17.3	< 0.03	< 0.003	11.2	0.24	0.34
90	I	100[a]	9.4	0.14	< 0.003	< 11.2	0.07	0.14
32	I	100[a]	6.2	0.03	0.003	< 11.2	0.06	0.1
55	II	100	31.8	3.7	1.5	< 11.2	0.47	1.1
86	II	100[a]	16.7	1	0.003	< 11.2	3.2	2.5
144	II	100	65.3	14.6	9.2	< 11.2	8	12.6
50	I	100[a]	12.6	2.2	0.07	< 11.2	0.04	0.01
98	I	100[a]	15.4	0.22	0.29	< 11.2	0.052	0.17
107	II	100	50	4	5	< 11.2	8.7	6.2

[a]Cephaloridine concentration of 1 mM.

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they were inhibited by cloxacillin, aztreonam and sulbactam, but not by clavulanic acid.

The separation of β-lactamases from *Acinetobacter* by isoelectric focusing is sometimes difficult because of their large molecular mass and poor enzyme solubility [5]. Some authors consider a cephalosporinase-type enzyme as having variable bands of β-lactamase activity located at $pI > 8$ [16]. In the present study, two types of cephalosporinases were characterized as the basis of a pI very close to the cathode (type I, $pI > 8.5$), and a pI between 8 and 8.5 (type II). Hood and Amyes [5] reported at least four different *Acinetobacter* cephalosporinases on the basis of different pI values always >8. The group 1 cephalosporinases produced by three *Acinetobacter* spp. clinical isolates, characterized by Perilli in 1996 [6], appeared to be different from other group 1 β-lactamases and also from each other. Our results confirm that there is a considerable heterogeneity between cephalosporinases of *Acinetobacter*.

The inhibitory profile assay described by Papanicolaou et al [9] allows β-lactamases to be characterized by determining the percentage of inhibition. The hydrolysis profile confirmed that the β-lactamases of this study were cephalosporinases. These enzymes hydrolyzed ampicillin, first- and second-generation cephalosporins and cefotaxime (only demonstrated by microbiological assay), but not ceftazidime or carbenicillin. Hydrolysis rates obtained with these substrates are usually lower by spectrophotometric assay than by other methods. Some authors have suggested that ceftazidime is less susceptible to the action of cephalosporinases than cefotaxime, as demonstrated in other studies where the hydrolysis rate of ceftazidime was even lower than the rate for cefotaxime [17,18]. Group 1 cephalosporinases of Gram-negative bacteria are defined as having preferential activity against cephalosporins, but are also able to efficiently inactivate penicillins [19]. For this reason, alternative methods, such as microbiological assay, are sometimes needed to confirm cephalosporinase activity.

In conclusion, the *A. baumannii* complex isolates investigated in this study yielded two major types of cephalosporinases, which appeared to belong in the group 1 classification of β-lactamases [15]. These enzymes showed heterogeneity on the basis of their different pI values, inhibition profiles and hydrolysis rates with several β-lactam agents. These differences in their biochemical behavior, as well as the relevance of these enzymes in the antimicrobial resistance in *Acinetobacter* spp., require further detailed studies.

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