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Contribution of β-Lactamases to β-Lactam Susceptibilities of Susceptible and Multidrug-Resistant *Mycobacterium tuberculosis* Clinical Isolates

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The β-lactamases in 154 clinical *Mycobacterium tuberculosis* strains were studied. Susceptibilities to β-lactam antibiotics, their combination with clavulanate (2:1), and two fluoroquinolones were determined in 24 *M. tuberculosis* strains susceptible to antimycobacterial drugs and in nine multiresistant strains. All 154 *M. tuberculosis* isolates showed a single chromosomal β-lactamase pattern (pI 4.9 and 5.1). *M. tuberculosis* β-lactamase hydrolyzes cefotaxime with a maximum rate of 22.5 ± 2.19 IU/liter (strain 1382). Neither amoxicillin, carbenicillin, cefotaxime, ceftriaxone, nor aztreonam was active alone. Except for aztreonam, β-lactam combinations with clavulanate produced better antimycobacterial activity.

The rise in the incidence of tuberculosis and the increasing resistance to antimycobacterial drugs (2, 3), particularly in human immunodeficiency virus-infected patients, underline the urgent need for new compounds which overcome this resistance. Reevaluation of old compounds, such as the β-lactam group, a widely used, nontoxic and extensively developed group, could be one way to address this problem.

Mycobacterium tuberculosis has been historically considered intrinsically resistant to β-lactam antibiotics, owing to its β-lactamase and impermeability. Reports of susceptibility to this group of antibiotics and the role of β-lactamase in resistance are documented; however, they are scarce and do not study the β-lactam activity in multidrug-resistant strains (7–10, 21). Systematic study of *M. tuberculosis* β-lactamases and their correlation with β-lactam and inhibitor susceptibility data could be interesting in the search for effective compounds.

We studied β-lactamases in 154 *M. tuberculosis* individual nonreplicate strains isolated from clinical specimens between 1991 and 1993 at the Laboratori de Referència de Catalunya. Susceptibility tests were performed on 22 of these strains. Furthermore, 11 strains (8 multidrug resistant) from Trías i Pujol University Hospital of Badalona were included in the susceptibility assays.

Standard biochemical tests and nucleic acid hybridization tests (Accuprobe; Gen-Probe Inc., San Diego, Calif.) were employed for the identification of clinical isolates (12, 13, 23).

β-Lactamase characterization. The qualitative spectrum of hydrolysis of the β-lactamases was screened by using an acidimetric method (20). Determination of the isoelectric point was carried out in crude extracts by analytical isoelectric focusing (17) with commercially prepared polyacrylamide gel plates (pH 3.5 to 9.5 and 4.0 to 6.5) and LKB instrumentation. Crude extracts were obtained by ultrasonication in an ice bath (Branson 2000 sonicator for 60 min) of a 9-McFarland (27×10^8 bacteria/ml) bacterial suspension in distilled water. Cell debris was removed by centrifugation (Ultracentrifuge TL100; Beckman Instruments Inc.) at 20,000 rpm and 4°C for 20 min. The β-lactamase activity of extracts was determined with a 50-μg/ml nitrocefin solution. Hydrolysis of nitrocefin and cefotaxime was determined spectrophotometrically for a crude extract obtained from the susceptible strain 1382 (4). Wavelengths selected were 495 nm for nitrocefin and 264 nm for cefotaxime (Shimadzu UV-160 spectrophotometer). Various concentrations of antibiotics in 0.1 M sodium phosphate buffer (pH 7) were tested with 0.1 ml of crude extract (total volume, 1.6 ml). The ε value used for these calculations was that reported by Amicosante et al. (1). K_m values and maximum rates (V_{max}) were obtained according to Michaelis-Menten plots by using the Fig P program (Fig. P Software Corp., Durham, N.C.). Enzymatic activity is reported in international units/liter of crude extract, where an international unit is defined as the amount of enzyme hydrolyzing 1 μmol of substrate per min.

Susceptibility tests. MICs were determined by agar dilution with 7H10 agar supplemented with 10% oleic acid-albumin-dextrose-catalase. Mycobacteria were grown in 7H9 broth at 37°C and diluted with distilled water to match the turbidity of a 1-McFarland standard (approximately 2×10^8 CFU/ml) (7). The plates were inoculated by using a Steer's replicator with a 1/100 dilution in distilled water of the above suspension. The results were read after 3 weeks of incubation in a CO_2 atmosphere at 37°C. The MIC was defined as the lowest concentration of antibiotic which completely inhibited visible bacterial growth (one colony was disregarded). *M. tuberculosis* H37Rv (ATCC 25618), *Staphylococcus aureus* ATCC 25923, and *Escherichia coli* ATCC 25922 were used as controls. The antibiotics evaluated, kindly provided as standard powders by the manufacturers, were lithium clavulanate, amoxicillin, carbenicillin, cefotaxime, ceftriaxone, aztreonam, rifampin, isoniazid, streptomycin, ciprofloxacin, and ofloxacin. Combinations with clavulanate were made at a 2/1 ratio (β-lactam/inhibitor ratio). The stabilities of the antibiotics during the incubation period were monitored by inoculation of the plates with the *E. coli* control strain (ATCC 25922) on days 1 and 11.

The 154 strains and the type strain H37Rv expressed β-lactamases which hydrolyzed benzylpenicillin and cephaloridine in the qualitative acidimetric assay, and all strains showed the same pattern of β-lactamase bands by analytical isoelectric

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TABLE 1. MICs of antimycobacterial agents for susceptible and multidrug-resistant *M. tuberculosis* isolates

Susceptibility and antimycobacterial agent[a]	No. of strains susceptible at the following MIC (μg/ml):										
	0.06	0.12	0.25	≤1	2	4	8	16	>16	32	>32	≥128
Susceptible (n = 24)												
Rifampin				24								
Streptomycin				13	10						1	
Isoniazid	5	15	4									
MDR[b] (n = 9)												
Rifampin									1			8[d]
Streptomycin				7		1					1	
Isoniazid							2	2		4	1[c]	

[a] Breakpoints used are 1.0 μg/ml for rifampin, 2.0 μg/ml for streptomycin, and 0.2 μg/ml for isoniazid.
[b] MDR, multidrug resistant.
[c] The MIC was 64.
[d] For seven strains the MIC was 128, and for one strain the MIC was 256.

focusing, with two major bands with isoelectric points (pIs) of 4.9 and 5.1.

The susceptibility data are presented in two groups according to the results of the antimycobacterial agents tested. Multidrug resistance was reported when resistance was observed for at least two drugs (Table 1).

The fluoroquinolones, rifampin, streptomycin, and all the β-lactam antibiotics except carbenicillin and its combination with clavulanate appeared to be stable during the extended incubation period. MICs of the agents tested against *E. coli* (ATCC 25922) on days 1 and 11 were the same. Carbenicillin and its combination with clavulanate were the only antibiotics for which some degradation was observed; that is, the MIC on day 11 was 2 dilutions higher than that on day 1. As seen in Table 2, neither amoxicillin, carbenicillin, cefotaxime, ceftriaxone, nor aztreonam was active against the *M. tuberculosis* strains studied. For all of them except aztreonam, the combination with the β-lactamase inhibitor produced better antimycobacterial activity.

TABLE 2. MICs of several β-lactam antibiotics alone and in combination with clavulanate (2:1 ratio) and two fluoroquinolones

Antibiotic(s)	MIC (μg/ml) for:				
	Susceptible strains (n = 24)			MDR[a] strains (n = 9)	
	Range	MIC_{50}[b]	MIC_{90}[c]	Range	MIC_{50}
Amoxicillin	64–>256	128	>256	64–128	64
Amoxicillin + CA[d]	≤16–32	≤16	≤16	≤16–128	32
Carbenicillin[e]	128–>256	>256	>256	>256	>256
Carbenicillin + CA[e]	8–64	32	64	8–16	8
Cefotaxime	16–128	128	128	32–128	64
Cefotaxime + CA	4–32	16	32	8–32	16
Ceftriaxone	64–256	128	256	128	128
Ceftriaxone + CA	4–32	16	16	2–16	4
Aztreonam[f]	>64	>64	>64	NT[g]	NT
Aztreonam + CA[f]	>64	>64	>64	NT	NT
Ciprofloxacin	0.25–1	1	1	0.12–2	0.25
Ofloxacin	0.5–1	1	1	0.5–1	0.5

[a] MDR, multidrug resistant.
[b] MIC_{50}, MIC at which 50% of strains are inhibited.
[c] MIC_{90}, MIC at which 90% of strains are inhibited.
[d] CA, clavulanic acid.
[e] Twenty-two strains in the susceptible group and eight in the multidrug-resistant group were studied.
[f] Twenty-one strains were studied.
[g] NT, not tested.

Nitrocefin hydrolysis was used to verify the enzymatic activity of the extract. With nitrocefin as the substrate, a K_m of 68 ± 12 μM and a V_{max} of 10.61 ± 0.59 IU/liter were calculated. For cefotaxime, V_{max} was 22.5 ± 2.19 IU/liter and K_m was 0.46 ± 0.16 μM at the concentration ranges assayed (between 0.33 and 5 μM); when higher concentrations were employed no activity was detected, suggesting substrate inhibition like those of other β-lactamases (5, 22).

The 154 *M. tuberculosis* clinical isolates and the control strain studied showed only one pattern of chromosomal β-lactamase with two major bands, with pIs of 4.9 and 5.1, the same pattern recorded by other authors (16, 24, 25). These results agree with other genetic and phenotypic studies showing minimal heterogeneity in *M. tuberculosis* (11, 15, 19).

Chromosomal β-lactamase is the main resistance determinant observed for amoxicillin, carbenicillin, ceftriaxone, and cefotaxime. This can be deduced from the comparison of the MIC results for these compounds alone and their combination with clavulanate and is reinforced by cefotaxime hydrolysis results. Clavulanate has no antibacterial activity in *M. tuberculosis* due to the lack of affinity for penicillin-binding proteins (PBPs) (9), but it is a powerful inhibitor of its β-lactamase (21, 25). The results observed were not simply due to an additive effect unrelated to β-lactamase inhibition, since β-lactams tested bound to *M. tuberculosis* PBPs at therapeutically achievable concentrations (9), and they are also reinforced by the fact that clavulanate did not reduce aztreonam MICs at the concentrations assayed. These results concur with those reported previously for some β-lactams, although some were obtained by another MIC determination method (7–10, 21).

M. tuberculosis β-lactamase belongs to the class A β-lactamases (14); its resistance phenotype, qualitative hydrolysis spectrum, cefotaxime hydrolysis, and clavulanate inhibition indicate that *M. tuberculosis* β-lactamase could be a group 2be β-lactamase according to the classification of Bush et al. (6). Although Hackbarth et al. (14) also described the presence, in the H37Ra strain, of the *bla* C (β-lactamase class C gene), not in close proximity to *bla* A, it is not clear if the class C β-lactamase is produced by *M. tuberculosis.*

β-Lactam antibiotics could be considered for the treatment of tuberculosis caused by multidrug-resistant strains. Chambers et al. (9) evaluated positively not only the factors that determine the susceptibility of *M. tuberculosis* strains to β-lactam antibiotics (PBPs and permeability) but also the macrophage penetration. Furthermore, the amoxicillin-clavulanate combination has been successfully used to treat patients with multidrug-resistant tuberculosis when the combination was administered with second-line drugs (18).

MICs of the two fluoroquinolones assayed and of all the β-lactams except the amoxicillin-clavulanate combination were very similar in the two groups of strains studied (antimycobacterial drug susceptible and multidrug resistant). In order to determine the future role of β-lactams in the treatment of tuberculosis, it will be necessary to study additional strains and to evaluate the activities of β-lactams in combination with other antimycobacterial drugs in vitro and in an animal model.

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