A New Plasmidic Cefotaximase from Patients Infected with Salmonella typhimurium

Summary: Salmonella typhimurium strains resistant to most β-lactams, co-trimoxazole, tobramycin and gentamicin were isolated from patients in two hospitals in Buenos Aires, Argentina, beginning in August 1990. The patients were suffering from meningitis, septicaemia or enteritis. Therapy including ampicillin, ceftriaxone and gentamicin failed. The strains produced a plasmidic (pMVP-4) extended broad-spectrum β-lactamase which is more active against cefotaxime than against ceftazidime (V_{max} for cefotaxime 350 times higher than for ceftazidime). This cefotaximase demonstrates similarity to the previously described CTX-ase-M-1 from Escherichia coli, but it is distinctly different from CTX-ase-M-1 by its isoelectric point (7.9 for CTX-ase-M-2 in comparison with 8.9 for CTX-ase-M-1) as well as in its lower susceptibility to the β-lactamase inhibitors sulbactam, clavulanic acid, tazobactam and BRL 42715. Thus, the β-lactamase produced by S. typhimurium strains from Argentina appears to represent a new member (CTX-ase-M-2) of a novel group of plasmidic extended broad-spectrum β-lactamases designated as cefotaximases.

Materials and Methods

Strains: The resistant wild-type strain used was Salmonella typhimurium CAS-5, isolated from feces of a 16-month-old child suffering from enteritis. The recipient strain used was Escherichia coli A 15 R-resistant to nalidixic acid (MIC = 1.024 mg/l). Reference strains used for isoelectric focusing were E. coli A 15 R + SHV-5 [6]; Klebsiella pneumoniae 197 SHV-4 [7]; and E. coli GRI CTX-M-1 [3].

Minimal inhibitory concentrations (MICs): MICs were determined by an agar dilution technique using Mueller-Hinton agar (Difco, Detroit, USA). The inoculum was 10^4 CFUs per spot deposited on the agar by a Denley multipoint inoculator. MICs were read after 16 h of incubation at 35°C. E. coli ATCC 25922 was used as a quality reference strain.

Antimicrobial agents: Clavulanic acid, BRL 42715 (SmithKline Beecham Pharma); ceftazidime (Glaxo Pharmaceuticals Ltd.); carumonam, ceftriaxone, cefetamet, trimethoprim and sulfamethoxazole (Hoffmann-La Roche Inc.); meropenem (ICI

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Purification of β-lactamases: Identification of the cefotaxime-specific β-lactamase containing cefotaxime (1 mg/l) and incubated for 2 h at 35°C. A isoelectric focusing gel was covered with an agarose overlay second layer with growth at the spot on the gel where cefotaxime had been hydrolysed allowed specific localization of the cefotaximase band. 

Transfer of resistance determinants: The donor strain and recipient strain (10⁶ CFUs/ml per strain) were suspended in Mueller-Hinton broth (Difco, Detroit, USA) and incubated for 18 h at 35°C. Transconjugants were selected on MacConkey agar (Oxoid, Wesel, FRG) supplemented with nalidixic acid (64 mg/l) to inhibit the growth of the donor strain and co-trimoxazole (16 mg/l) to inhibit the growth of the recipient strain.

Isoelectric focusing of β-lactamases: Crude homogenates of bacterial cells (4x10¹¹ CFUs) were sedimented and resuspended in 4.5 ml of 1% glycine, sonicated and centrifuged. A volume of 5.5 ml of the sonicate was loaded onto a Multiphor preparative flatbed electrofocusing unit (LKB). Ultrodex granulated gel (LKB) containing 5% ampholytes pH 7-11 (LKB) was used. Focusing was performed with 5 μl of the supernatant applied to polyacrylamide gels (pH gradient from 3--10) that had been prepared in our own laboratory. They were run at 1,000 V, 14 mA, at 4°C for 2.5 h. Second separated by intervals of one second on a Branson Sonifier B 15, Geneva, Switzerland). Isoelectric focusing was performed with 5 μl of the supernatant applied to polyacrylamide gels (pH gradient from 3--10) that had been prepared in our own laboratory. They were run at 1,000 V, 14 mA, at 4°C for 2.5 h. β-lactamase bands were visualized by the chromogenic cephalosporin nitrocefin [8].

Identification of the cefotaxime-specific β-lactamase: After isoelectric focusing the gel was covered with an agarose overlay containing cefotaxime (1 mg/l) and incubated for 2 h at 35°C. A second layer with E. coli susceptible to cefotaxime (MIC 0.03 mg/l) was then applied. Following overnight incubation, visible growth at the spot on the gel where cefotaxime had been hydrolysed was allowed specific localization of the cefotaximase band.

Purification of β-lactamases: Cells grown in 900 ml of tryptic soy broth (Oxoid, Wesel) for 6 h at 35°C to late logarithmic phase were sedimented and resuspended in 4.5 ml of 1% glycine, sonicated and centrifuged. A volume of 5.5 ml of the sonicate was loaded onto a Multiphor preparative flatbed electrofocusing unit (LKB). Ultrodex granulated gel (LKB) containing 5% ampholytes pH 7-11 (LKB) was used. Focusing was performed lengthwise at a constant power of 16 W at 9°C. After 16 h the gel-bed was fractionated in equally large compartments. The fractions containing β-lactamases were loaded on polypropylene columns with a nylon net and eluted with 6 ml of 0.05 M phosphate buffer pH 7.0. β-lactamase activity was estimated spectrophotometrically using cefotaxime as substrate. The cefotaxime-hydrolysing fraction was used for molecular weight determination and kinetic parameters.

Molecular weight determination of β-lactamases: Enzyme molecular weight was determined by sodium dodecyl sulfate (SDS) polyacrylamide gel electrophoresis. A low molecular weight calibration kit (LKB) with marker proteins from 14 to 94 kD was used. Proteins were boiled for 3 min in 2% SDS (Sigma) in 0.5% DTT (dithiothreitol, Sigma) before application.

Determination of kinetic parameters: Hydrolysis of β-lactam antibiotics was determined spectrophotometrically at 20°C in 0.1 M phosphate buffer pH 7.0 using a Gilford 240 spectrophotometer. Wavelengths used were 260 nm for both cephaloridine and ceftazidime and 264 nm for cefotaxime. Kinetic parameters of Vₘₐₓ and Kₘ were determined by using Lineweaver-Burke plots [9].

Determination of I₅₀ values: Inhibition studies were conducted with clavulnate, sulbactam, tazobactam and BRL 42715. Amounts of enzyme found to totally hydrolyse 0.1 mM cephaloridine in 15 min were preincubated with a range of concentrations of the inhibitor for 5 min at 20°C. After adding 0.1 mM cephaloridine the protective effect of the inhibitor was monitored by following the substrate hydrolysis photometrically at 260 nm. The concentration required to inhibit the β-lactamase activity by 50% after 15 min was determined graphically.

Plasmid DNA preparation: Cells were lysed in 200 mM NaOH and 1% sodium dodecyl sulfate SDS (Sigma, St. Louis, USA) at room temperature for 5 min. Potassium acetate (2.55 M, pH 4.8) was added to separate proteins and chromosomal DNA by centrifugation (15,000 x g). Purification of the plasmidic DNA in the supernatant was performed with an anion exchange column eluted by a solution containing 1.2 M NaCl, 50 mM morpholinopropanesulfonic acid MOPS (Sigma, St. Louis, USA) and 15% ethanol adjusted to pH 8.0.

Separation of plasmid DNA: Samples of the lysates (7.5 μl) were loaded onto a 1% agarose gel (Seakem, FMC Corporation Marine Colloids Div., Rockland, USA) and run for 13 h at 4 V/cm in an LKB 2297 macrodrive 5. The gel was stained for 30 min with a 0.1% solution of ethidium bromide in tris-borate-buffer (90mM tris-acetate, 2.55mM EDTA, 89mM boric acid), examined by UV transillumination and photographed.

Determination of plasmid size: Purified plasmid DNA was restricted with endonuclease Dra I (Boehringer Mannheim) according to the manufacturer's recommendations. Digested samples were loaded onto a 1% agarose gel. Lambda DNA digested with EcoR1 was used as molecular size marker (Boehringer Mannheim, Mannheim, FRG).

Results

Comparative Antibiotic Susceptibility of Wild-Type and Transconjugant Strains

The resistance phenotype of a broad variety of β-lactam antibiotics was determined for S. typhimurium CAS-5, the wild-type strain E. coli GRI producing cefotaximase M-1 and their E. coli R⁺ transconjugants (Table 1). The β-lactam susceptibility profile of S. typhimurium CAS-5 as well as of the Escherichia coli R⁺ strain harbouring the plasmid pMVP-4 is closely related to the profile of the CTX-ase-M-1-producing strains (Table 1). MIC values for the CTX-ase-M-2 producers are mostly four times higher than those for the CTX-ase-M-1 producers. This is reflected by the observation that crude homogenates of β-lactamase CTX-ase-M-2 producers from 10⁴ cells hydrolyse nitrocefin about four times as fast as CTX-ase-M-1 producers. After isoelectric focusing, the same strength of colorization on the polyacrylamide gel is achieved by only one- fourth of the volume of homogenate of CTX-ase-2 producers in comparison with CTX-ase-M-1.