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Outer membrane protein profiles of clonally related *Klebsiella pneumoniae* isolates that differ in cefoxitin resistance

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Abstract

Eleven genotypically related *Klebsiella pneumoniae* isolates were obtained from 11 patients. All isolates were resistant to third-generation cephalosporins due to the production of SHV-2a extended-spectrum β-lactamase. Comparison of the outer membrane protein profiles revealed one isolate that lacked porins. This porin-deficient isolate was also resistant to cefoxitin (MIC 128 μg ml^{-1}) and moxalactam (MIC 64 μg ml^{-1}) and had elevated MIC of meropenem (2 μg ml^{-1}) when compared to porin-expressing isolates (2–8, 4 and <0.06–0.125 μg ml^{-1}, respectively). Higher MICs, associated with loss of porins in outer membrane, were also observed for cefotaxime (4–8-fold), cefepime (>2–16-fold), ciprofloxacin (4–16-fold), imipenem and aztreonam (2–16-fold), but there was no significant difference among MICs of ceftazidime. The porin-deficient mutant was probably selected in vivo during ofloxacin therapy.

Keywords: *Klebsiella pneumoniae*; Bacterial porin; Multiresistance; β-Lactam antibiotic; ESBL; Hospital-acquired infection

1. Introduction

Klebsiella pneumoniae is an important opportunist human pathogen associated with hospital-acquired infections such as pneumonia, urinary tract infections or bacteraemias. It attacks particularly immunocompromized patients, therefore an adequate antimicrobial treatment is critical for management of these infections. β-Lactam antibiotics are currently used in the treatment of infections with *K. pneumoniae*. This species naturally produces a broad-spectrum β-lactamase SHV-1 or penicillinase LEN that confer resistance to penicillins and to first-generation cephalosporins [1]. Resistance to third-generation cephalosporins is mainly due to the production of extended-spectrum β-lactamase (ESBL) or AmpC-type β-lactamases. When the strain produces an ESBL it remains susceptible to cephamycins, whereas an AmpC-type β-lactamase production confers resistance even to these antibiotics. Decreased susceptibility to cephamycins is also encountered in porin deficient mutants [2].

Klebsiella pneumoniae synthesizes two major porins, OmpK35 [3] and OmpK36 [4] and a quiescent porin

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OmpK37 [5]. OmpK35, an OmpF homologue of *Escherichia coli*, has reduced expression under high osmolarity conditions, while OmpK36, an *E. coli* OmpC homologue, is expressed under both low and high osmolarity conditions [6]. OmpK35 allows more efficient penetration of cephalosporins than OmpK36 [7]. Most clinical isolates of *K. pneumoniae* without ESBL production express both OmpK35 and OmpK36, while the majority of ESBL-producing strains synthesize only OmpK36 [6]. The porin OmpK37 is similar to *E. coli* OmpN and *Salmonella typhi* OmpS2, both quiescent porins whose expression in enterobacteria is downregulated in standard laboratory conditions [5].

The first detailed study dealing with the problem of nosocomial outbreaks due to ESBL-producing enterobacteria in Slovakia has been performed recently (Žarnayová et al., unpublished data). One ESBL-producing *K. pneumoniae* isolate described in that study was highly resistant to cefoxitin. We report here an investigation of possible mechanisms of resistance to cefoxitin in that isolate and compare results with genotypically related cefoxitin-susceptible isolates.

2. Materials and methods

2.1. Bacterial isolates

Nine isolates of ESBL-producing *K. pneumoniae* (positive in double-disc diffusion test) were recovered from different patients hospitalized in the Ružinov hospital (900 beds) in Bratislava (Slovakia) in the period from May to October 2002. In September 2003, a further two isolates were obtained. One of these showed a reduced susceptibility to cefoxitin, probably due to AmpC β-lactamase production. Nine patients were hospitalized in a long term care facility, one in a surgical ward and one in a general ICU (Table 1). The isolated were identified using the API 20 E system (bioMerieux, France).

2.2. Susceptibility study

Susceptibilities to antimicrobial agents were determined by a disk-diffusion method on Mueller–Hinton agar according to CA-SFM recommendations [8]. The following antibiotics (BioRad, France) were tested: amoxicillin, amoxicillin/clavulanic acid, ticarcillin, ticarcillin/clavulanic acid, piperacillin, piperacillin/tazobactam, cephalothin, cefoxitin, moxalactam, ceftazidime, cefotaxime, cefepime, cefpirome, imipenem; kanamycin, tobramycin, amikacin, gentamicin, netilmicin; nalidixic acid, ofloxacin, ciprofloxacin; trimethoprim/sulfametoxazole; doxycycline; chloramphenicol; rifampin; fosfomycin. MICs were determined by a microdilution method in Mueller–Hinton broth according to NCCLS recommendations [9]. ESBL production was studied by double-disk synergy test [10].

2.3. Isoelectric focusing of β-lactamases (IEF)

Isolates were grown overnight in Tryptic-soy broth (bioMérieux, France). For induction studies, moxalactam or cefoxitin were added to final concentration of 16 μg ml^{-1} in the middle of the exponential phase of growth. Crude enzyme extracts, obtained by sonication, were separated by isoelectric focusing in pH 3.5–9.5 Ampholine (Amersham Pharmacia Biotech, UK) polyacrylamide gel using a LKB 2117 Multiphor apparatus as described by Matthew et al. [11]. β-Lactamase activities were detected by an iodine–starch procedure in agar gel containing different β-lactam antibiotics as a substrate according to the type of β-lactamase: – penicillin G (400 mg l^{-1}) for the detection of all β-lactamases [12]; – ceftriaxone (800 mg l^{-1}) for ESBL; – and cefoxitin (800 mg l^{-1}) for AmpC β-lactamase. β-Lactamases with known pI values were used as standards for determination of pIs: TEM-1 (pI 5.4), TEM-2 (pI 5.6), TEM-3 (6.3), TEM-24 (6.5), SHV-1 (pI 7.6), SHV-2 (pI 7.8) and CTX-M-1 (pI 8.4).

2.4. Masuda test [13]

A Mueller–Hinton agar plate was inoculated with a cefoxitin susceptible strain (*E. coli* DH5α). A disk containing cefoxitin was placed in the center and disks with 10, 15, 20 and 25 μl of analyzed enzyme extract were placed 17 mm from the central disk, where the edge of the inhibition zone was expected. Enhanced growth of the susceptible strain into the zone of inhibition due to the enzyme extract was considered to be evidence of β-lactamase activity against cefoxitin. Cefotaxime was used as a substrate for SHV-2a for a positive control of enzyme extract.

2.5. Genotyping

Agarose plugs were prepared as described previously [14]. After digestion of whole cell DNA by 40 U of *Xba* I (BioLabs, USA), the fragments were separated in a 1% agarose gel (Appligene, France) by pulsed-field gel electrophoresis (PFGE) using a contour-clamped homogeneous electric field apparatus (CHEF-DRII; BioRad, France). The migration was run in 0.5 × TBE buffer, at 5.4 V cm^{-1} and 14 °C for 24 h with the time ramp from 50 to 5 s. The restriction patterns were interpreted according to Tenover's criteria [15].

2.6. Conjugation

Conjugation assay between donor (clinical strain) and recipient (*E. coli* C600 Rif^r) was carried out by a