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

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Received 13 September 2004; received in revised form 13 October 2004; accepted 7 December 2004

First published online 27 December 2004

Edited by Dr. R.A. Bonomo

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.

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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 immunocompromised 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 (Zárnayová 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 b-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 (bioMérieux, 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, cephalexin, cefoxitin, moxalactam, ceftazidime, cefotaxime, cefepime, cefpirome, imipenem; kanamycin, tobramycin, amikacin, gentamicin, netilmicin; nalidixic acid, ofloxacin, ciprofloxacin; trimethoprim/sulfamethoxazole; 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 b-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]. b-Lactamase activities were detected by an iodine–starch procedure in agar gel containing different b-lactam antibiotics as a substrate according to the type of b-lactamase: – penicillin G (400 µg ml⁻¹) for the detection of all b-lactamases [12]; – ceftriaxone (800 µg ml⁻¹) for ESBL; – and cefoxitin (800 µg ml⁻¹) for AmpC b-lactamase. b-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 b-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 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 x 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 Rif³) was carried out by a
<table>
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<th>Pulsotype</th>
<th>Isolate</th>
<th>Ward</th>
<th>Source</th>
<th>Date</th>
<th>Age/Sex of patient</th>
<th>Previous therapy</th>
<th>Omp</th>
<th>( \beta )-Lactamase&lt;sup&gt;a&lt;/sup&gt;</th>
<th>MIC (( \mu )g ml&lt;sup&gt;-1&lt;/sup&gt;)</th>
<th>Other determinants of resistance&lt;sup&gt;b&lt;/sup&gt;</th>
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<td>23/06/02</td>
<td>89/F</td>
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<td>+</td>
<td>SHV-2a + TEM-1</td>
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<td>Sxt&lt;sup&gt;R&lt;/sup&gt;</td>
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<td>07/09/03</td>
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<td>Ofloxacine</td>
<td>–</td>
<td>SHV-2a</td>
<td>128 64 1024 64 32 &gt;2048 4 2 32 16 8</td>
<td>Mox&lt;sup&gt;R&lt;/sup&gt;</td>
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<sup>a</sup> TEM-1 identification is presumptive, based upon the pI 5.4 and substrate profile in IEF.

<sup>b</sup> Screening by disk diffusion test, all isolates were susceptible to imipenem and fosfomycin.

**Abbreviations:** Fox, cefoxitin; Mox, moxalactam; Ctx, cefotaxime; Caz, ceftazidime; Atm, aztreonam; Fep, cefepime; Imp, imipenem; Mer, meropenem; Cip, ciprofloxacin; Cmp, chloramphenicol; Tet, tetracycline; Amo, amoxicillin; Amc, amoxicillin + acid clavulanic; Tic, ticarcillin; Tcc, ticarcillin + acid clavulanic; Pip, piperacillin; Trp, piperacillin + acid clavulanic; Cf, cephaplatin; Cpo, cefpirome; Kn, kanamycin; Tm, tobramycin; An, amikacin; Gm, gentamicin; Nt, netilmicin; Sxt, trimetoprim + sulfamethoxazole; Do, doxycyclin; Na, acid nalidixic; Of, ofloxacine; Rif, rifampicin.
broth mating procedure in LB medium and by mating on membrane (Millipore, USA) on Mueller–Hinton agar. Transconjugants were selected on Mueller–Hinton agar containing rifampin (200 μg ml⁻¹) and ceftazidime (4 μg ml⁻¹) or gentamicin (10 μg ml⁻¹).

2.7. Plasmid DNA analysis

The size of plasmid DNA was determined using extraction method described by Kieser [16], plasmid DNA for PCR and cloning was extracted and purified using the Qiafilter Kit (Qiagen, Germany). The visualization was performed on a 0.6% agarose gel.

2.8. Characterization of β-lactamase-encoding (bla) genes

Detection and sequencing of the bla BSHVB gene coding for SHV-type enzymes were performed as described previously [17]. Detection of ampC genes was performed with primers specific for E. coli (COL-A, 5’ AC-GACGCTCTGCGCTTATA 3’, COL-B, 5’ AAAGATTCTGTCAAGCGGC 3’), Enterobacter cloacae (ECL-A, 5’ CCCCCTTGCTGCCCCTGCTA 3’, ECL-B, 5’ TGCC-GGCTCAACCCGTGC 3’), Citrobacter freundii (CF-A, 5’ ATTCGGGATATGCGCTGTG 3’, CF-B, 5’ GGGGTTACCTCAACGGC 3’), and for the FOX group of AmpC β-lactamases (FOX-A, 5’ ATGCCAACACGCGCG 3’, FOX-B, 5’ TCATGCACGCGTGACT 3’). The PCR products were sequenced using an Applied Biosystems sequencer ABI prism model 3100.

2.9. Cloning experiments

Plasmid DNA of K. pneumoniae was extracted as described above, partially digested by Sau3A I, (New England Biolabs, Hertfordshire, UK) and ligated into the BamHI-digested pK18 plasmid carrying resistance to kanamycin. E. coli DH5α treated with CaCl₂ was transformed by the recombinant plasmid and transformants were selected on Mueller–Hinton containing kanamycin (30 μg ml⁻¹) and cefoxitin (8 μg ml⁻¹) or moxalactam (4 μg ml⁻¹).

2.10. Omp analysis

Bacteria were grown in nutrient broth (0.5% peptone, 0.3% beef extract) without or with 20% sorbitol (low and high osmolarity conditions) to A₆₀₀nm of 0.5B, pelleted, washed and resuspended in 50 mM phosphate buffer pH 7. Cells were disrupted by sonication and unbroken cells and cell debris were pelleted and removed. Cell envelopes (cytoplasmic and outer membranes) were recovered from supernatant by centrifugation at 100,000g for 1 h at 15 °C. Outer membrane proteins (Omp) were isolated as sodium-lauroyl sarcosinate (2%)-insoluble material from cell envelopes [18]. Electrophoresis of Omps was carried out in 12% acrylamide – 0.16% (low) or 0.41% (high) bisacrylamide – 0.1% SDS resolving gels and stained by Coomassie blue.

3. Results

3.1. Genotyping

All 11 ESBL-producing K. pneumoniae isolated from different patients showed similar PFGE profiles (Fig. 1) with five minor variations (subtypes a–e) and were considered clonally related.

3.2. Susceptibility testing

Table 1 presents antibiotic resistance profiles of the isolates determined by disk diffusion method and MICs of antibiotics relevant for porin characterization. All isolates were highly resistant to penicillins and β-lactamase inhibitors partially restored their activity as judged by the diameters of inhibition zones (not showed). They were also resistant to first- and third-generation cephalosporins, but remained susceptible to cefoxitin and moxalactam, except for one isolate – K. pneumoniae 9.747. The MICs of this isolate for cefoxitin, moxalactam, meropenem, cefepime, ciprofloxacin, and aztreonam and imipenem were 16–64-fold, 16-fold, 16->64-fold, >4–16-fold, 4–8-fold 4–16-fold and 2–16-fold, respectively, higher than the cefoxitin-susceptible isolates. All isolates were resistant to aminoglycosides and fluoroquinolones (except for ciprofloxacin) but differed in their susceptibility to trimethoprim/sulfamethoxazole, chloramphenicol and...
tetracyclines. There were large differences in MICs of tetracycline and chloramphenicol among the isolates, but these were not dependent on the porin status.

3.3. \(\beta\)-Lactamase study

The ESBL production was detected in all the strains by the double-disk synergy test. For the cefoxitin resistant isolate synergy was observed only when the disks were placed 2 cm apart. A single ESBL of pI 7.6 was detected in all the isolates, presumably SHV-1, in addition to a non-ESBL of pI 5.4 in subtypes a, b and c, presumably TEM-1. The transfer of ESBL by conjugation was unsuccessful in spite of the presence of two large plasmids of about 120 and 140 kb in three randomly chosen isolates. However, the ESBL was identified as SHV-2a by sequencing a 926 bp PCR product amplified by SHV-specific primers using plasmid extract as a template. No enzyme with other pI was detected by isoelectric focusing after induction with cefoxitin or moxalactam. The possibility of the presence of AmpC-type \(\beta\)-lactamase was excluded for the following reasons: no PCR products were obtained by primers specific for AmpC of \(E.\ coli\), \(C.\ freundii\) or \(E.\ cloacae\); FOX-group \(\beta\)-lactamases were not detectable by Masuda test using cefoxitin as a substrate (Fig. 2); and the failure of cloning the cefoxitin resistance determinant.

3.4. Omp profiles

SDS–PAGE analysis showed that all the isolates expressed an OmpA-like protein of about 32 kDa and a protein of about 45 kDa corresponding to LamB maltoporin (Fig. 3). In all cefoxitin-susceptible isolates a single additional band of about 35 kDa corresponding to porins was observed in both low (0.16%)- and high (0.41%)-bisacrylamide resolving gels. The 35 kDa band was absent in the cefoxitin-resistant isolate. The culture of cefoxitin-susceptible isolates in different osmolarity conditions did not modify the porin expression, as judged by SDS–PAGE.

4. Discussion

Nosocomial outbreaks due to ESBL-producing enterobacteria have become a serious problem worldwide. In our case, a breakout of an epidemic strain of pulsotype, named Kp10, was detected over more than one-year in the long term facility suggesting the potential of dissemination to other wards. All 11 tested isolates of this pulsotype produced SHV-2a ESBL that resulted in resistance to oxyimino-cephalosporins, but one of them was also resistant to cefoxitin (isolate 9.747, MIC 128 \(\mu\)g ml\(^{-1}\)).

As the cefoxitin resistance in \(K.\ pneumoniae\) is often caused by acquisition of plasmid-encoded AmpC \(\beta\)-lactamases [19], the first step of the study was to determine the presence of this enzyme. The fact that AmpC-type \(\beta\)-lactamase could not be detected by IEF, PCR or Masuda test and the failure of cloning the cefoxitin resistance determinant lead us to conclude that the resistance to cefoxitin in isolate 9.747 was not of an enzymatic nature.

Most ESBL-expressing \(K.\ pneumoniae\) clinical isolates lack OmpK35 porin and produce only OmpK36 [6] so the other possible mechanism of cefoxitin resistance could be the decreased production of porins. In our experiments, only one band corresponding to porins was observed in polyacrylamide gels. However, under certain conditions, these proteins may migrate as a single band [6]. That was the reason for testing migration of Omps in gels with low and high bisacrylamide content, but only one porin band was confirmed in both cases. The identity of this porin was determined by its expression under low- and high-osmolarity conditions, where the OmpK35 expression should be different. The
intensity of the porin band did not change under high-osmolarity conditions, so we can assume that OmpK36 alone was expressed in all cefoxitin-susceptible isolates tested. The quiescent porin OmpK37 is known not to be expressed or is expressed in very low levels under standard laboratory conditions. Its high-level production decreases the meropenem MICs from 2 to 0.06 μg ml⁻¹ [5]. The meropenem MIC of our porin-deficient isolate was 2 μg ml⁻¹ and there was no indication of OmpK37 expression in SDS–PAGE either, so we assume its production in this isolate is negligible.

According to the literature, OmpK36 is the only porin associated with ESBL production and provides an increased resistance to cefoxitin (MICs up to 16 μg ml⁻¹) but not to clinical resistance [20]. This was also the case in our cefoxitin-susceptible isolates, where the cefoxitin MICs ranged from 2 to 8 μg ml⁻¹. In other studies, the diminished level of expression of both porins leads to cefoxitin MICs of 64 μg ml⁻¹ and the porin deficiency was associated with high level of resistance to cefoxitin (MIC ≥ 128 μg ml⁻¹) [21]. In accordance with these observations, only the cefoxitin-resistant isolate K. pneumoniae 9,747 (MIC = 128 μg ml⁻¹) was proven to be porin-deficient.

This porin-deficient mutant versus OmpK36-producing isolates differed in resistance levels to oxyimino-cephalosporins, mainly to cefotaxime and ceftipime. There were no significant differences in resistance to ceftazidime. This could be in accordance with the suggestion that the OmpK35 is more specific for ceftazidime penetration [22]. This hypothesis was criticized and assumed to be speculative [23]. However, another recent work confirmed that ceftazidime MIC reduction caused by OmpK35 expression was 128 times higher than that caused by OmpK36 expression, whereas the difference in cefotaxime MICs was only 8 times. On the other hand, the OmpK36 expression reduced the ceftazidime MIC (from >512 to 256 μg ml⁻¹), as well, so OmpK35 can not be specific for this antibiotic [7]. The explanation can be in the size of molecule of ceftazidime, one of the limiting factors for diffusion through porins [24]. As OmpK35 has a more permeable pore than OmpK36 [25], it allows more efficient penetration of ceftazidime.

The loss of porins influences also susceptibility to other groups of antibiotics, mainly to fluoroquinolones, tetracyclines and chloramphenicol. Both OmpK36 and OmpK35 allow penetration of ciprofloxacin [7], but the contribution of their loss to resistance is significant only in the presence of topoisomerase mutations [26]. All 11 tested isolates were resistant to ciprofloxacin, probably resulting from other mechanism of resistance than porin loss. The ciprofloxacin MIC was 4-fold higher in porin-deficient than in OmpK36-expressing isolates and is comparable with the 8-fold increase documented by Doménech-Sánchez et al. [7]. As the patient colo-

nized by the porin-deficient isolate K. pneumoniae 9,747 had previously been treated by ofloxacin, it was presumably the fluoroquinolone therapy that exerted a selective pressure for loss of porins.

We did not observe any correlation between resistance and porin production in the case of chloramphenicol and tetracycline in the isolates tested. However, if we omit the isolates that evidently possess another mechanism of resistance (isolates with tetracycline MICs ≥ 512 μg ml⁻¹ and chloramphenicol MICs of 64 μg ml⁻¹), we can observe an 8-fold and a 4–16-fold decreased susceptibility of porin deficient isolate to tetracycline and chloramphenicol, respectively. The susceptibility to aminoglycosides was not analyzed in detail, because of the porin-independent penetration of these agents.

Results from this study are consistent with previous observations showing that the resistance to expanded-spectrum cephalosporins increases in K. pneumoniae strains producing ESBLs that lack the two major porins of the species (OmpK35 and OmpK36). At the same time, they point towards the possibility of increasing the resistance levels to more groups of antibiotics by decrease of porin expression, even to molecules that are not in use, as the cefoxitin resistance emerged after fluoroquinolone treatment. These data show a need for an active surveillance system for prevention of spread of nosocomial pathogens and for prevention of evolution of multiresistant strains in hospital environment. Furthermore, the accurate identification of resistance mechanism is useful for more precise aiming of infection control measures.

Acknowledgements

This research was supported by VEGA Grant No. 1/1181/04 from Slovak Research Grant Agency and by Grant of Young Scientists No. 90/2003/UK from University Comenius.

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