Introduction

Extended-spectrum β-lactamase (ESBL)-producing Enterobacteriaceae have become a major problem in recent decades especially in nosocomial infections 1–3 with a number of outbreaks having been reported worldwide.4–6 Several reports have documented the risk of mortality from mis-identification of ESBL producers.3,7,8 In 1999, the NCCLS suggested a new testing and reporting strategy for the identification of ESBL-producing Klebsiella pneumoniae, Klebsiella oxytoca and Escherichia coli.9 However, this suggestion has only recently been implemented in routine laboratory practice. False reporting may occur as a result of other factors. In K. pneumoniae, two major porins have been implicated in antibiotic resistance. Modification of outer membrane proteins (Omps) OmpK35 (the homologue of OmpF in E. coli) and OmpK36 (the homologue of OmpC in E. coli) combined with production of ESBL have been associated with increased cephalosporin and quinolone MICs.7,10–12 Loss of the porin OmpK36 combined with production of AmpC-like β-lactamase has also been described as a cause of increased MICs of carbapenems.13 In this report, eight outbreak isolates of K. pneumoniae from a neonatal ward were incorrectly designated as ESBL producers due to the loss of an outer membrane protein.

Materials and methods

Patients and bacterial strains

Nine isolates of K. pneumoniae were collected during an outbreak in a neonatal ward from June 1997 to April 1998. Isolates were obtained from eight premature infants with bacteraemia and from one colonized (rectal swab culture) premature infant. All the infants were hospitalized on the day of birth except one who was hospitalized on day 64 after birth. Cefotaxime or ceftazidime were the main antibiotics used for treatment during and after bacteraemia. All the infants recovered and were discharged.
Susceptibility testing

Antimicrobial susceptibility was determined by both agar dilution and disc diffusion tests according to NCCLS guidelines. The following antimicrobial agents were obtained as standard reference powders of known potency for susceptibility testing by the agar dilution method: ampicillin and cephalothin (Sigma Chemical Co., St Louis, MO, USA); clavulanic acid (SmithKline Beecham, Brockham Park, Surrey, UK); cefamandole (Eli Lilly, Taipei, Taiwan); cefotaxime (Hoechst Marion Roussel, Frankfurt, Germany); ceftazidime (Glaxo Group Research Ltd, Greenford, UK); ceftizoxime (Fujisawa, Osaka, Japan); amikacin and aztreonam (Bristol-Myers-Squibb Laboratories, Princeton, NY, USA); ciprofloxacin (Bayer Co., Leverkusen, Germany). All drugs were incorporated into Mueller–Hinton agar (BBL Microbiology Systems, Cockeysville, MD, USA) in serial two-fold concentrations from 0.03 to 64 mg/L. Two control strains, E. coli ATCC 35218 and ATCC 25922, were included in each test run. Inoculated plates were incubated at 35°C for 16–18 h. The MIC of each antimicrobial agent was defined as the lowest concentration that inhibited visible growth of the organism.

Confirmatory tests for ESBLs

The double-disc synergy test, Etest for ESBLs and agar diffusion tests were used as screening tests to detect ESBL-producing strains. In the double-disc synergy test, cefotaxime (30 μg), ceftazidime (30 μg) and aztreonam (30 μg) discs were placed on Mueller–Hinton agar adjacent to a co-amoxiclav disc (20 μg of amoxicillin plus 10 μg of clavulanate). All discs were purchased from Becton Dickinson Microbiology System (Sparks, MD, USA). The procedures and interpretation of the double-disc synergy test were as described previously.

The Etest ESBL screen (PDM Epsilometer; AB Biodisk, Solna, Sweden), based on the recognition of a reduction of ceftazidime or cefotaxime MIC in the presence of clavulanic acid, was performed according to the manufacturer’s instructions. The agar diffusion test was performed according to NCCLS guidelines. A ≥5 mm increase in a zone diameter for either ceftazidime/clavulanic acid (30 μg/10 μg) or cefotaxime/clavulanic acid (30 μg/10 μg) versus its zone when tested alone was taken as indicative of ESBL production.

Pulsed-field gel electrophoresis

Total DNA was prepared and pulsed-field gel electrophoresis (PFGE) was performed as described previously. The restriction enzyme XbaI (New England Biolabs, Beverly, MA, USA) was used. Restriction fragments were separated by PFGE in 1% agarose gels (Bio-Rad, Hercules, CA, USA) in 0.5 × TBE buffer (45 mM Tris, 45 mM boric acid, 1.0 mM EDTA, pH 8.0) using a Bio-Rad CHEF-Mapper apparatus (Bio-Rad Laboratories, Richmond, CA, USA). Gels were stained with ethidium bromide and photographed under UV light. Band patterns were compared visually and classified as indistinguishable (clonal), closely related (clonal variants, three or less band differences), possibly related (four to six band differences) and unrelated (more than six band differences) according to criteria described previously.

Transfer of ampicillin resistance by conjugation

Conjugation was carried out by both broth mating and plate mating. A rifampicin-resistant strain of E. coli (JP-995) was used as the recipient. Recipient and donor strains were separately inoculated into brain–heart infusion (BHI) broth and incubated at 37°C for 4 h. For broth mating, recipient and donor strains were mixed at a volume ratio of 1:1 for overnight incubation at 37°C. The next morning, 0.01 mL of the mixture was spread on a MacConkey agar plate containing rifampicin (100 mg/L) and ampicillin (100 mg/L). As for plate mating, 0.2 mL of the donor and 1.8 mL of the recipient culture were mixed and passed through a filter with a diameter of 2.5 cm and a pore size of 0.45 μm. The filter was then placed on a pre-warmed Mueller–Hinton agar plate with the cells uppermost and incubated at 37°C for 4 h. The filter was then immersed in 2 mL of broth and the cells resuspended. A 0.1 mL aliquot of the suspension was then plated on an ampicillin/rifampicin selection plate.

Isoelectric focusing and PCR for blaTEM and blaSHV

Isoelectric focusing (IEF) was performed as described previously. Bacteria were harvested from 20 h BHI broth cultures by centrifugation and the pellet was resuspended in 1 mL of phosphate buffer (0.05 M, pH 7). Enzymes were released by two cycles of freezing (−70°C) and thawing (room temperature), and sonication for 5 min in ice-cold water. IEF was performed in ampholine gel (pH 3.0–10.0; Pharmacia, Uppsala, Sweden). Preparations from standard strains known to harbour TEM-1, SHV-1 and SHV-5 were used as standards. After IEF, β-lactamases were detected by spreading nitrocefin (50 mg/L) on the gel surface. For detection of ESBL, molten Mueller–Hinton agar, containing 0.6% (w/v) cefotaxime, 6% (w/v) potassium iodide and 0.6% (w/v) iodine, was poured on to the IEF gel and allowed to solidify. The bands corresponding to ESBLs produced clear halos in the black background of agar within 30 min.

Oligonucleotide primers (Gibco-BRL, Taipei, Taiwan) used for PCR assay were as follows: 5′-ATAAAAATTCTTGAGACGAAA-3′ (primer A), 5′-GACAGTTTCAATGCTTAACTCA-3′ (primer B), 5′-GGGATTCTTATTTGTGCCC-3′ (primer C) and 5′-TTCAGGTTGCCACTGCTC-3′ (primer D). Primers A and B were
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specific for \( \text{bla}_{\text{TEM}} \). Primers C and D were specific for \( \text{bla}_{\text{SHV}} \). Reactions were performed in 50 \( \mu \)L mixtures containing 2.5 U Taq polymerase (Promega, Madison, WI, USA), 1 \times \) buffer consisting of 10 mM Tris–HCl pH 8.3, 1.5 mM MgCl\(_2\), 50 mM KCl, 0.01 \( \mu \)g gelatin, 200 \( \mu \)M of each deoxynucleoside triphosphate and 2 \( \mu \)M of each oligonucleotide primer. Thirty-five cycles were performed for each reaction, with the following temperature profile: 94°C, 1 min; 58°C, 1 min and 72°C, 1 min.

For direct DNA sequencing, PCR products were purified with microspin S-300 HR PCR purification columns (Pharmacia). Sequencing was done with corresponding primers specific for the \( \text{bla}_{\text{TEM}} \) and \( \text{bla}_{\text{SHV}} \) genes according to the method of Sanger et al. An automated sequencer (377, ABI Prism, Perkin-Elmer, CT, USA) was used.

Isolation and analysis of Omp

Bacteria were cultured in Mueller–Hinton broth and low osmolality nutrient broth for 16 h separately. Cell membrane proteins were obtained by disrupting the culture with a VCX 600 sonicator (Sonic and Materials Inc., CT, USA). Cell debris was removed by centrifugation at 6000 g for 10 min at 4°C, and the supernatant was subjected to ultracentrifugation at 50 000 g for 30 min at 4°C to collect the membranes. Omps were then solubilized in 1.66% sodium lauryl sarcosinate for 30 min at room temperature. The suspension was centrifuged at 5000 g for 45 min at 4°C, and the pellet containing the Omp was resuspended in 100 \( \mu \)L of double distilled water. The Omps were separated on a SDS–polyacrylamide gel (acylamide:bisacrylamide ratio 3:0.27; 5% stacking gel; 10% running gel) and were visualized by Coomassie Blue staining (Gibco-BRL).

Results

Genomic fingerprinting

The eight blood culture isolates and one rectal isolate showed identical PFGE patterns (Figure 1), indicating that an outbreak of \( K. \) pneumoniae infection had occurred in the neonatal ward.

Susceptibility testing and confirmatory tests for ESBLs

All isolates were susceptible to co-amoxiclav (MIC 8/4 mg/L), cefoxitin (MIC 1 mg/L), ceftazidime (MIC 0.25 mg/L), cefotizoxime (MIC 0.25 mg/L), cefotaxime (MIC 1 mg/L), aztreonam (MIC 0.5 mg/L), amikacin (MIC 4 mg/L) and ciprofloxacin (MIC 0.25 mg/L), but resistant to piperacillin (MIC \( \geq 128 \) mg/L), cephalothin (MIC \( \geq 64 \) mg/L), cefamandole (MIC 32 mg/L) and gentamicin (MIC 16 mg/L) (Table). Initial disc test and MIC results revealed that only cefotaxime met the NCCLS criteria (zone size \( \leq 27 \) mm) for further ESBL confirmatory testing among the four indicator antibiotics, aztreonam, ceftazidime, cefotaxime and ceftriaxone. Agar diffusion testing for ESBL production showed significant values (an increased zone size of \( 5 \) mm) for cefotaxime/clavulanic acid (30 \( \mu \)g/10 \( \mu \)g) versus cefotaxime alone. On the other hand, an increase of only \( 2 \) mm in the zone diameter (from 29 to 31 mm) was recorded for ceftazidime/clavulanic acid compared with ceftazidime (Figure 2). All tests were repeated twice in two different laboratories to confirm the reproducibility.

![Figure 1. PFGE profiles from XbaI macrorestriction fragments of K. pneumoniae isolates.](image)

![Figure 2. Etest for ESBLs and agar diffusion tests for detecting ESBL-producing strains. CTX, cefotaxime; CTX/CA; cefotaxime/clavulanic acid (30 \( \mu \)g/10 \( \mu \)g); CAZ, ceftazidime; CAZ/CA, ceftazidime/clavulanic acid (30 \( \mu \)g/10 \( \mu \)g); ATM, aztreonam; CRO, ceftriaxone.](image)
A phantom phenomenon was observed with cefotaxime Etest strips but not with ceftazidime (Figure 2). According to the manufacturer’s recommendations, if either agent gave a positive test result, the isolate should be interpreted as an ESBL producer. Double disc tests showed synergy for all isolates on co-amoxiclav with cefotaxime or ceftriaxone but not with ceftazidime or aztreonam.

Transferability of $\beta$-lactam resistance and susceptibility testing of transconjugant

The $\beta$-lactam resistance of isolates was found to be conjugatively transferable. Transconjugants were susceptible to co-amoxiclav (MIC 8/4 mg/L), cefoxitin (MIC 1 mg/L), ceftazidime (MIC 0.5 mg/L), ceftizoxime (MIC 0.25 mg/L), cefotaxime (MIC 0.5 mg/L), aztreonam (MIC 0.5 mg/L), amikacin (MIC 4 mg/L), gentamicin (MIC 8 mg/L) and ciprofloxacin (MIC 0.25 mg/L) but resistant to piperacillin (MIC ≥ 128 mg/L), cephalothin (MIC 32 mg/L) and cefamandole (MIC 32 mg/L). Initial tests with cefotaxime revealed a zone size of 29 mm and an MIC of 0.5 mg/L, respectively, for transconjugants, which did not meet the NCCLS criteria (zone size < 27 mm) for further ESBL confirmatory testing (data not shown). A non-significant phantom phenomenon was observed with cefotaxime Etest strips.

IEF, PCR amplification and sequencing of PCR products for $bla_{TEM}$ and $bla_{SHV}$

IEF of crude extracts identified two $\beta$-lactamases at pI 5.4 and 7.6, which were similar to TEM-1 and SHV-1 $\beta$-lactamases, respectively. ESBL detection using a cefotaxime overlay gel failed to show evidence of ESBL activity. All nine K. pneumoniae isolates were positive for both $bla_{TEM}$ and $bla_{SHV}$ amplification. The entire $bla_{TEM}$ and $bla_{SHV}$ sequences including the promoter region for K. pneumoniae were found to be identical to the $bla_{TEM-1}^{15}$ encoding Tn2 and the published SHV-1 gene sequence.24,25 No mutation was found among those strains. No SHV-1 or TEM-1 was detected by PCR amplification in transconjugants. IEF of transconjugants identified one $\beta$-lactamase corresponding to TEM-1. Insertion sequences between the $bla_{TEM}$ amplification sequences region or gene rearrangement during the conjugation were proved by using new $bla_{TEM}$-specific primers which were not on the transposon (Tn2) but the structural gene of TEM-1. Positive $bla_{TEM}$ amplification was observed using new primers within the structural gene of TEM-1 (data not shown).

Analysis of Omp

SDS–PAGE analysis of the Omps showed that the clinical isolates and the control strain ATCC 13883 expressed...
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OmpK36 but not OmpK35 in Mueller–Hinton broth. Although OmpK35 was highly expressed in control strain ATCC 13883 grown in low osmolality nutrient broth, only extremely low-level expression of OmpK35 was noted in the clinical isolates (Figure 3). This result indicates a deficiency of OmpK35 in the clinical isolates.

Discussion

In the current study, two \( \beta \)-lactamases were identified in each of eight outbreak isolates. IEF followed by PCR and sequencing showed that the \( \beta \)-lactamases were SHV-1 and TEM-1. No promoter mutation was observed. Recent data suggest that chromosomally encoded SHV-1 in \( K. pneumoniae \) produces relatively low levels of enzyme, resulting in moderate resistance to ampicillin. Although previous studies showed that hyperproduction of SHV-1 enzyme is one of the explanations for an increased MIC (2 mg/L) of ceftazidime in \( K. pneumoniae \), the combination of low-level production of SHV-1 and normal production of TEM-1 is unlikely to cause an increase in the MIC of ceftazidime. We have tested the susceptibility of two TEM-1-hyperproducing strains of \( E. coli \) and one clinical isolate of \( K. pneumoniae \) that harboured SHV-1 and TEM-1. Increased MICs of ceftazidime and ceftazidime were not observed as compared with the isolates in this study (data not shown).

Our results suggest that the false designation of isolates as ESBL producers may result from a combination of factors. Several investigators have observed that the loss or reduction of outer membrane proteins in clinical isolates is one of the factors resulting in an increased MIC of ceftazidime. Previous analyses have shown that the major resistance proteins are the outer membrane doublet, OmpK35 and OmpK36. Recently, OmpK37 and OmpK45 have also appeared to be involved in resistance to \( \beta \)-lactam antibiotics. Our results suggest that the combination of low-level expression of OmpK35 and production of SHV-1 and TEM-1 has caused a false designation of ESBL production.

One study showed that OmpF (homologous to OmpK35) is a wider channel than OmpC (homologous to OmpK36), theoretically allowing larger molecules to pass through the outer membrane. Lack of OmpF should presumably play a more important role in resistance than lack of OmpC. However, a highly antibiotic-resistant clinical isolate that lacked OmpC but retained OmpF was isolated from a patient during cephalaxin therapy. Subsequent in vitro experiments showed that the synthesis of OmpF was strongly repressed during growth in media of high osmolality, a repression mimicking that seen in human tissues and body fluid. The authors concluded that as OmpF is strongly repressed by high osmolarity such as that in body fluid, the predominant expression of the OmpC porin should play an important role in drug permeability. This observation is in line with one study showing that alteration of Omps, especially the loss of OmpK35 when OmpK36 is present, can result in an increase in the MIC of cefotaxime to 4 mg/L. Thus, the reduced expression of OmpK35 seen in this study probably makes only a marginal contribution to the slightly increased MIC of cefotaxime because OmpK36 still allows \( \beta \)-lactams to pass through the outer membrane. Patients may, therefore, be successfully treated with third-generation cephalosporins as monotherapy.

Another intriguing finding in the present study was the phantom phenomenon in the Etest and the positive disc augmentation in the agar diffusion test. Possibly the combination of decreased outer membrane permeability and the hydrolytic effect of TEM-1 and SHV-1 \( \beta \)-lactamases increased the MIC of cefotaxime slightly. The addition of clavulanic acid inhibited TEM-1 and SHV-1 \( \beta \)-lactamase production and the reduction in MIC was due to the inhibition of SHV-1 and TEM-1. This caused the Etest phantom phenomenon and the augmentation of the zone size diameter. Since the loss of outer membrane proteins selectively slowed down the penetration of cefotaxime but not ceftazidime, no significant effect was observed with ceftazidime/clavulanate. However, this hypothesis needs further study.

We conclude that the loss of OmpK35 combined with SHV-1 and TEM-1 \( \beta \)-lactamases caused false designations of ESBL-producing \( K. pneumoniae \). Reports of ESBL producers should be studied carefully if inconclusive susceptibility test results are obtained. Screens for ESBL producers should be re-evaluated to avoid such false designations. The results from this study suggest that the loss of OmpK35 is not related to third-generation cephalosporin resistance.
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References


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