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Article

Extended-Spectrum TEM- and SHV-Type β-Lactamase-Producing *Klebsiella pneumoniae* Strains Causing Outbreaks in Intensive Care Units in Italy

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Abstract The aim of the present study was to investigate the production of extended-spectrum β-lactamases (ESβLs) and the epidemiological correlations in a total of 107 *Klebsiella pneumoniae* strains resistant to third- and fourth-generation cephalosporins. The strains were collected from patients in four intensive care units (3 neonatal and 1 general) in three hospitals in Italy between March 1996 and July 1997. All strains were found to produce ESβLs. Phenotypic (antibiotyping and ESβL patterns) and genotypic (plasmid profile and pulsed-field gel electrophoresis) analyses showed that a single strain had been responsible for each outbreak in each of the four intensive care units. Isoelectric focusing, activity on substrates and gene sequencing showed that the strains produced SHV-5, SHV-2a, SHV-12 and TEM-52 β-lactamases. This is not only the first time that ESβL-producing *Klebsiella pneumoniae* strains have been reported as causing epidemics in Italian hospitals, it is also, to the best of our knowledge, the first time that an outbreak caused by a TEM-52 ESβL-producing *Klebsiella pneumoniae* strain has been reported. The data presented here illustrate the complexity of determining the epidemiological pattern of ESβL producers in large hospitals that do not have an ESβL-monitoring program.

Introduction

Klebsiella pneumoniae is often implicated in hospital-acquired infections and is estimated to account for 3–7% of all nosocomial bacterial infections in the USA and in Europe. According to statistics from the Centers for Disease Control and Prevention, Atlanta, USA, *Klebsiella* spp. cause 3% of all epidemic outbreaks [1–3].

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Multidrug-resistant *Klebsiella pneumoniae* strains that produce extended-spectrum β-lactamases (ESβLs) of the TEM and SHV types have often been implicated in cases of nosocomial infection, and many outbreaks caused by these strains have been reported worldwide in the last few years [4–10]. Endemic and epidemic nosocomial infections caused by ESβL-producing *Klebsiella pneumoniae* strains, especially in intensive care units (ICUs), represent a persistent problem in many parts of the world, and epidemics caused by *Klebsiella pneumoniae* strains resistant to third-generation cephalosporins (3GCs) have been associated with increased morbidity and mortality in hospitalized patients.

The fourth-generation cephalosporins (4GCs) cefepime and cefpirome have been found to be more active than cefotaxime against ESβL producers, but an inoculum effect has been observed with these agents, as well as with the oxyimino-cephalosporins [11, 12]. Since ESβL production is frequently accompanied by resistance to multiple antibiotics, therapeutic options are often limited; this makes the first recovery of ESβL-

producing *Klebsiella pneumoniae* strains showing additional resistance to imipenem very disturbing. The development of multidrug resistance in these strains should, therefore, be monitored closely [13–15].

Hospital colonization by ESβL-producing *Klebsiella pneumoniae* strains is usually a complex phenomenon involving different mechanisms, including dissemination of several epidemic strains, dissemination of plasmids and resistance genes, or concurrent dissemination of genes, plasmids and strains [6, 8–10, 16–18]. Understanding the epidemiology of infections caused by these organisms requires the use of accurate epidemiological markers that are able to differentiate between the spread of resistance plasmids and strain dissemination. In Italy, clinical isolates of *Klebsiella pneumoniae* have been reported to produce plasmid-mediated ESβLs, mainly of the SHV type, but no outbreaks have been previously reported [19, 20].

The aims of the present study were as follows: (i) to characterize the ESβLs produced by 107 *Klebsiella pneumoniae* strains, which were resistant to 3GCs, had reduced susceptibility to 4GCs, were involved in epidemics and were responsible for therapeutic failures in four ICUs in three Italian hospitals; and (ii) to delineate the possible clonal relationships and transmission patterns of these strains.

Materials and Methods

Bacterial Strains. From March 1996 to July 1997 we collected a total of 107 3GC-resistant *Klebsiella pneumoniae* strains that had been isolated from infected and/or colonized patients in one general ICU and three neonatal ICUs in three hospitals located in three different Italian cities. The patients were considered infected and/or colonized based on their biological and clinical data.

The strains were isolated from routine clinical specimens submitted to the laboratories of the three hospitals. Fourteen *Klebsiella pneumoniae* strains were recovered from eight patients admitted to the neonatal ICU of the Varese university hospital from September to November 1996. Seventy-eight strains were isolated from 66 patients at Pavia university hospital; specifically, 53 strains were isolated from 43 patients in a general ICU and 25 strains were isolated from 23 patients in the neonatal ICU from May 1996 to May 19997 and from February 1997 to June 1997, respectively. Finally, 15 isolates were obtained from eight patients at the neonatal ICU of Trento hospital from September to December 1996. At Varese university hospital, two strains were isolated individually from two patients, while from six patients, two different strains were isolated from two separate sites in each patient. At Pavia university hospital, 54 strains were found individually in as many patients, while in 12 patients a different strain was found in two separate sites. At Trento hospital, 14 of the 15 strains were isolated from multiple sites in different patients.

All patients were severely debilitated and, thus, highly susceptible to infection; they had all undergone invasive procedures (e.g., surgery, insertion of tubes for parenteral nutrition, tracheostomy and urinary catheterization) and had been hospitalized for prolonged periods of time. All patients had been receiving β-lactam antibiotics (including oxyimino-cephalosporins in monotherapy) prior to the isolation of *Klebsiella pneumoniae*.

Antimicrobial Susceptibility Testing. Minimum inhibitory concentrations (MICs) of ampicillin, amoxicillin plus clavulanate, piperacillin, piperacillin plus tazobactam, ticarcillin, ticarcillin plus clavulanate, cephalothin, cefoxitin, cefotaxime, ceftazidime, ceftriaxone, cefepime, aztreonam, imipenem, amikacin, gentamicin, netilmicin, tobramycin and ciprofloxacin were determined using the broth macrodilution technique in Mueller-Hinton medium. Clinical isolates were classified as susceptible, intermediately susceptible or resistant in accordance with the criteria of the National Committee for Clinical Laboratory Standards [21].

Conjugation. Conjugal transfer of 3GC resistance determinants was attempted using both liquid and plate mating methods. The wild-type strains and the recipient *Escherichia coli* K12 strains (J53–2 *met*$^-$, *pro*$^-$, *rif*^{r}; J62 *pro*$^-$, *his*$^-$, *trp*$^-$, *lac*$^-$ and *Sm*^{r}) were cultured in L-broth (Difco Laboratories, USA). Transconjugants were selected in McConkey agar and Mueller-Hinton agar (Difco Laboratories) supplemented with rifampicin (100 mg/l) or streptomycin (1000 mg/l) to inhibit growth of donor strains and with ampicillin (50 mg/l) or ceftazidime (2 mg/l) to inhibit growth of recipient *Escherichia coli* K12.

Plasmid DNA Analysis. Plasmid DNA was extracted and purified using the alkaline lysis method. In order to repress capsular polysaccharides, which interfere with the isolation of plasmid DNA, clinical strains were grown in Luria broth containing 0.5 mM bismuth nitrate and 2.5 mM sodium salicylate (Sigma Chemicals, USA) [22]. Plasmids were subjected to electrophoresis on a 0.7% agarose minigel at 80–100 V for 2–3 h, stained with ethidium bromide and photographed under UV light. The sizes of the plasmids were determined by comparing their distances of migration with those of plasmids of known size from *Escherichia coli* 39R861 and V517 strains (National Collection of Type Cultures, London, UK).

Molecular Typing of Bacterial DNA by Pulsed-Field Gel Electrophoresis (PFGE). Genomic DNA was analyzed using the Bio-Rad Gene Path Procedure (Bio-Rad Laboratories, Italy). The Θ1 pathogen Group reagent kit was used, but the DNA was cleaved overnight with restriction endonuclease *Xba*I. DNA fragments were separated by electrophoresis in 1% agarose gels in 0.5X Tris-borate-EDTA buffer (45 mM Tris-borate, 1 mM EDTA [pH 8]) with the CHEF-DRIII system (Bio-Rad). Electrophoresis conditions were 14 °C at 6 V/cm for 20 h, and the program used was that suggested for *Klebsiella pneumoniae* (i.e., 50–700 Kb; pulse times ranging from 5 to 5.1 s)

DNA fragments from bacteriophage lambda concatemers were used as size markers. The gels were stained with ethidium bromide and an image was acquired with the Gel Doc 1000 System (Bio-Rad). Restriction patterns were interpreted according to the criteria proposed by Tenover et al. [23].

Beta-Lactamase Characterisation. Supernatants (5 µl) of crude bacterial lysates from clinical isolates and related transconjugants, obtained after sonication, were subjected to isoelectric focusing (IEF) on polyacrylamide gels containing Pharmalyte (pH range 3.5 – 10; pH 4 – 6; pH 7 – 9; Pharmacia, Sweden).

Gels were run for 3 h at 10 W at 4 °C using the Multiphor II system (Pharmacia) and visualized with nitrocefin. Preparations of TEM-1, TEM-8, TEM-9, SHV-2, SHV-5 and MIR-1 β-lactamases were included as standards in gels. Activities against β-lactams of crude cell sonicates from clinical strains and transconjugants were detected by the substrate overlaying procedure, as reported elsewere [24].

Polymerase Chain Reaction (PCR) Amplification of β-Lactamase Genes. The oligonucleotides used in this study were designed

according to the nucleotide sequences of extended-spectrum β-lactamases for SHV types deposited in EMBL and/or GeneBank. The SHV gene was amplified by PCR with the primers 5′– d [GCC TTT ATC GGC CCT C] 3′ and 5′ – d [GTT GCC AGT GCT CGA TCA] 3′. The SHV-type primers were the same as those used by Nüesch-Inderbinen et al. [25]. Primers for TEM-derived β-lactamases were obtained according to the methods described by Mabilat et al. [26, 27].

Polymerase chain reaction was performed according to the methods described by Kidd and Ruano [28]. The following reagents were added in a 100 μl reaction volume: 200 μM of each dNTP, 80 pmol of each primer, 1 μg plasmid DNA, and 2.5 U of Taq DNA polymerase (Boeringher Mannheim, Germany). The samples were subjected to 30 amplification cycles in a Perkin-Elmer automated thermal cycler apparatus (Perkin-Elmer, Italy) with 30 s denaturation at 94 °C, 1 min annealing at 53 °C or 42 °C when utilizing SHV- or TEM-type primers, respectively, and 1 min extension at 72 °C. A final DNA extension step at 72 °C for 7 min was used at the end of the thermal cycling procedure.

Beta-Lactamase Production and Purification. Overnight cultures of transconjugants obtained in brain heart infusion broth (Difco) from the clinical isolates chosen to represent each outbreak (see Results) were diluted tenfold in 5 l of the same medium containing ampicillin (50 μg/ml). Bacteria were grown for 18 h at 37 °C with orbital shaking (180 rpm) and harvested by centrifugation at 6000 × g for 10 min at 4 °C. The pellet was washed twice with 50 mM sodium phosphate buffer at pH 6.5, and the cells were disrupted by ultrasound (4 cycles of 1 min at 60 W). Cell debris was removed by centrifugation at 105,000 × g for 30 min in a preparative ultracentrifuge, and the cleared supernatant was loaded onto a Sephadex G-75 column (Pharmacia) equilibrated with 50 mM sodium phosphate buffer at pH 6.5. The fractions, active with 100 μM nitrocefin, were pooled and loaded onto a Sepharose S fast-flow column (Pharmacia) equilibrated with the same phosphate buffer. The enzyme was eluted with a linear gradient of NaCl from 0 to 1 M in the phosphate buffer. This procedure was repeated for each enzyme. The enzyme preparations were at least 70% pure, as analysed by SDS-PAGE (data not shown).

Determination of Kinetic Parameters. Substrate hydrolysis was monitored by following absorbance variations with a lambda 2 spectrophotometer (Perkin Elmer). Km and V_{max} values were determined by analysing initial rate conditions and using the Hanes' linearisation of the Michaelis-Menten equation. Kinetic experiments were performed in triplicate at 30 °C in sodium phosphate buffer (50 mM, pH 6.5). Ki = Km values for aztreonam were determined using nitrocefin as reporter substrate in a competitive assay. The wavelengths and absorbance variations are shown in Table 1.

Table 1 The wavelengths and absorbance variations of substrates used in the study

Antibiotic substrate	Wavelengths (nm)	Δ (M^{-1} cm^{-1})
Piperacillin	235	−820 ± 70
Nitrocefin	482	+15000 ± 300
Cephaloridine	255	−9000 ± 500
Cefazolin	260	−7400 ± 200
Cefotaxime	260	−7500 ± 200
Ceftazidime	260	−9000 ± 300
Cefepime	260	−10000 ± 500
Cefpirome	260	−4500 ± 300
Aztreonam	318	−750 ± 50

Results

Susceptibility of Clinical Isolates. All 107 isolates studied were uniformly resistant to ampicillin (MICs >512 mg/l), piperacillin (MICs >256 mg/l) and ticarcillin (MICs ≥256 mg/l); they were also resistant to the combinations amoxicillin plus clavulanate (MICs ≥128 mg/l), ticarcillin plus clavulanate (MICs ≥128 mg/l) and gentamicin alone (MICs ≥8 mg/l), but they were susceptible to cefoxitin, imipenem and amikacin. All strains isolated from the neonatal ICU in Varese were also resistant to cefotaxime (MICs = 64 mg/l), ceftazidime (MICs = 64 mg/l) and aztreonam (MICs >128 mg/l), and they were intermediately susceptible or fully resistant to cefepime (MICs 16–32 mg/l) and piperacillin-tazobactam (MICs 32–128 mg/l); four isolates were also resistant to tobramycin.

The patterns of resistance to β-lactam antibiotics and aminoglycosides distinguished two sets of isolates among the *Klebsiella pneumoniae* strains collected at Pavia university hospital. The isolates from the general ICU were characterized by the same resistance pattern, including resistance to cefotaxime (MICs ≥64 mg/l), ceftazidime (MICs >64 mg/l), cefepime (MICs ≥64 mg/l), aztreonam (MICs = 128 mg/l), tobramycin (MICs ≥16 mg/l) and netilmicin (MICs ≥32 mg/l), while they were found to be susceptible or intermediately susceptible to piperacillin-tazobactam (MICs 8–64 mg/l). The isolates from the neonatal ICU were intermediately susceptible to cefotaxime (MICs 16–32 mg/l), intermediately susceptible or resistant to ceftazidime (MICs 16–64 mg/l) and piperacillin plus tazobactam (MICs 64–128 mg/l) and resistant to aztreonam (MICs ≥64 mg/l); they also showed biological resistance to cefepime (MICs = 4 mg/l) but were susceptible to netilmicin (MICs ≤ 4 mg/l) and tobramycin (MICs = 2 mg/l).

Finally, the isolates from the neonatal ICU of Trento hospital were all characterized by resistance to cefotaxime (MICs = 64 mg/l), intermediate susceptibility or resistance to ceftazidime (MICs 16–32 mg/l), aztreonam (MICs 16–32 mg/l) and piperacillin plus tazobactam (MICs 64–128 mg/l), biological resistance to cefepime (MICs = 8 mg/l), and resistance to netilmicin (MICs = 32 mg/l) and tobramycin (MICs = 16 mg/l). The results of susceptibility testing are listed in Table 2.

Resistance Transfer and Susceptibility Testing of Transconjugants. Experiments to determine the transfer of ceftazidime or cefotaxime resistance were carried out on all clinical isolates included in the study. Transconjugants selected on the Mueller-Hinton agar supplemented with ceftazidime or cefotaxime were obtained for all strains. The β-lactam resistance patterns of the transconjugant strains were similar to those of the

Table 2 Susceptibility of outbreak *Klebsiella pneumoniae* strains

Place of isolation	MIC (mg/l)															
	AMP	PIP	TRC	AMC	TCL	GNT	FOX	IPM	AMK	CTX	CAZ	ATM	FEP	TZP	TOB	NET
Neonatal ICU of Varese	>512	>256	≥256	≥128	≥128	≥8	≤8	≤4	≤16	64	64	>128	16–32	32–128	2–16	NT
General ICU of Pavia	>512	>256	≥256	≥128	≥128	≥8	≤8	≤4	≤16	≥64	>64	128	≥64	8–64	≥16	≥32
Neonatal ICU of Pavia	>512	>256	≥256	≥128	≥128	≥8	≤8	≤4	≤16	16–32	16–64	≥64	4	64–128	2	≤4
Neonatal ICU of Trento	>512	>256	≥256	≥128	≥128	≥8	≤8	≤4	≤16	64	16–32	16–32	8	64–128	16	32

AMP, ampicillin; PIP, piperacillin; TRC, ticarcillin; AMC, amoxicillin plus clavulanate; TCL, ticarcillin plus clavulanate; GNT, gentamicin; FOX, cefoxitin; IPM, imipenem; AMK, amikacin; CTX, cefotaxime; CAZ, ceftazidime; ATM, aztreonam; FEP, cefepime; TZP, piperacillin-tazobactam; TOB, tobramycin; NET, netilmicin; NT, not tested

corresponding donor strains, although some of them had a lower degree of resistance.

Plasmid analysis was carried out on all clinical strains and related transconjugants. All isolates recovered from patients in the same ward possessed a plasmid of the same size. The plasmids in the transconjugants were large and cotransferred resistance to aminoglycoside substrates, except to tobramycin in the resistant strains from Varese hospital. All clinical isolates from Varese possessed a further nonconjugative plasmid of approximately 39 Kb in size.

Four isolates were chosen as representatives of the four outbreaks: Kl 6 VA from Varese neonatal ICU, Kl 2 SM from Pavia general ICU, Kl 146 SM from Pavia neonatal ICU and Kl 2 TN from Trento neonatal ICU. Results are listed in Table 3.

Typing of Genomic DNA by PFGE Analysis. All clinical isolates collected were subjected to digestion of genomic DNA with X*ba*I, producing 15 fragments of different sizes in all isolates. Evaluated according to the interpretative criteria for DNA restriction patterns of Tenover et al. [23], all isolates had the same number of bands and the corresponding bands were of the same apparent size. In addition, the respective isolates from the four ICUs were genetically unrelated, so four definite outbreak strains were found. Figure 1 presents the results of a few macrorestriction genomic DNA analyses carried out with strains isolated from the four ICUs.

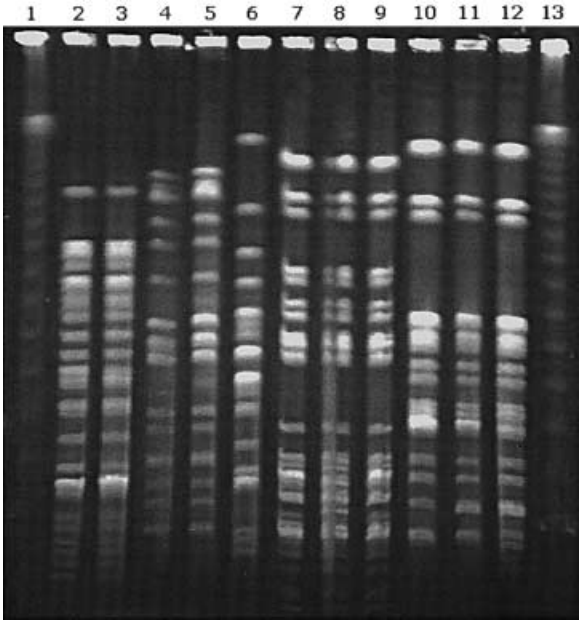

Figure 1 PFGE of *Xba*I-digested genomic DNA fragments from ESβL-producing *Klebsiella pneunoniae* isolates. *Lanes 1* and *13* contain a lambda ladder (Bio-Rad), which served as a molecular size marker; *lanes 2* and *3* contain DNA digest of isolates from the neonatal ICU of Varese hospital; *lanes 4* and *5* contain DNA digest of isolates from the neonatal ICU of Trento hospital; *lane 6* contains DNA digest of one ESβL-producing *Klebsiella pneumoniae* strain isolated from the neonatal ICU of Pavia hospital 3 months before the outbreak; *lanes 7–9* and *lanes 10–12* contain DNA digest of isolates from the neonatal ICU and a general ICU of Pavia hospital, respectively

Table 3 MICs of β-lactam antibiotics and sizes of β-lactamase-encoding plasmids for *Klebsiella pneumoniae* outbreak strains and their *Escherichia coli* transconjugants

Strain	β-lactamase-encoding plasmids (kb)	MICs (mg/l)			
		CTX	CAZ	FEP	ATM
Kl 6 VA	39, 46	64	>64	32	128
Kl 6 VA transconjugant	46	16	64	16	128
Kl 2 SM	47	64	64	64	128
Kl 2 SM transconjugant	47	32	32	16	128
Kl 146 SM	48	32	64	4	64
Kl 146 SM transconjugant	48	4	32	2	32
Kl 2 TN	42	64	16	8	16
Kl 2 TN transconjugant	42	16	8	4	8

CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam

Isoelectric Focusing of Beta-Lactamases and Detection of Substrate Activities. The pI values of β-lactamases, determined by analytical IEF in crude cellular extracts of all clinical isolates and related transconjugants, are reported in Table 4. Isolates from the neonatal ICU of Varese produced two β-lactamases with pIs of 7.6 and

Table 4 Characterization of β-lactamases from outbreak isolates of *Klebsiella pneumoniae*

Organism	pI	PCR results		Hydrolysis[a]			
		TEM	SHV	CTX	CAZ	FEP	ATM
Kl 6 VA	7.6+8.2						
Kl 6 VA transconjugant	8.2		+	+	+	±	+
Kl 2 SM	7.6+6.0						
Kl 2 SM transconjugant	6.0	+		+	+	+	+
Kl 146 SM	7.6+8.2						
Kl 146 SM transconjugant	8.2		+	+	±	±	+
Kl 2 TN	7.1+7.6						
Kl 2 TN transconjugant	7.6		+	+	±	±	±

[a] +, evidence of substrate hydrolysis by overlaying procedure; ±, evidence of slight hydrolysis.
CTX, cefotaxime; CAZ, ceftazidime; FEP, cefepime; ATM, aztreonam

8.2. The R+ transconjugants were found to express an enzyme with a pI of 8.2, which was demonstrated by the substrate overlaying procedure to possess activity on cefotaxime, ceftazidime, cefepime and aztreonam. The focusing of isolates from Pavia hospital showed the production of two β-lactamases from the general ICU with pIs of 7.6 and 6 and two from the neonatal ICU with pIs of 7.6 and 8.2; however, only those with pI 6 and pI 8.2 were detected in the transconjugants and were found able to hydrolyze oxyimino-cephalosporins, cefepime and aztreonam in the bioassay experiments. Finally, in the extracts from the Trento isolates, two β-lactamase bands were revealed, focusing at pI 7.1 and pI 7.6, but only the latter was detected in the transconjugants.

Kinetic Parameters. The enzymes produced by transconjugants R2TN, R6VA and R146SM were also analysed by means of a series of substrates. The results for the three SHV-type enzymes were heterogeneous, and the only enzyme that significantly hydrolysed aztreonam was that encoded by R2TN (Table 5).

The wide difference in the affinities of the two fourth-generation cephalosporins, cefpirome and cefepime, for the three SHV-type enzymes narrowed with the enzyme produced by R146SM. Furthermore, all SHV β-lactamases hydrolysed both cefotaxime and ceftazidime significantly, although the latter was more resistant than the former. The Km values for all compounds tested also show that piperacillin, cefotaxime and ceftazidime had greater affinity with all three SHV-type enzymes than with the TEM-1 enzyme.

Identification of ESβL-Encoding Genes by Polymerase Chain Reaction and DNA Sequencing. Plasmid DNA preparations from the R+ transconjugant strains were used in a specific PCR with SHV- and TEM-type primers. PCR products of the expected size of about 960 bp, representing the part of the bla_{SHV} genes in which some of the mutations characterising the SHV family of β-lactamases occur, were obtained in three cases, i.e., when the experiments were performed with strains from the three neonatal ICUs. PCR experiments carried out with specific primers for the bla_{TEM} family genes produced products of the expected size of about 1060 bp in only one case, i.e., when the strains from the general ICU were studied. The purified amplicons were sequenced and SHV-2a, SHV-5, SHV-12 and TEM-52

Table 5 Kinetic parameters of plasmid-encoded SHV-type β-lactamases produced by outbreak strains of *Klebsiella pneumoniae* isolated from three neonatal ICUs

Substrates	R2TN		R6VA		R146SM	
	Km (μM)	Vmax (μmoli/min/mg)	Km (μM)	Vmax (μmoli/min/mg)	Km (μM)	Vmax (μmoli/min/mg)
Piperacillin	3	75	2	11	3	2
Nitrocefin	5	137	2	17	4	12
Cephaloridin	11	90	7	19	13	8
Cefepime	48	20	50	4	650	12
Cefpirome	51	36	166	8	500	18
Cefazolin	10	60	6	20	7	9
Aztreonam	60	0.3	NH	NH	NH	NH
Cefotaxime	4	0.11	16	3	6	0.3
Ceftazidime	200	0.7	13	1.4	54	0.2

NH, no hydrolysis

enzymes were produced by R2TN, R6VA, R146SM and R2SM strains, respectively.

Discussion

We investigated the extended-spectrum β-lactamases, the plasmids encoding them and the clonal relatedness among outbreak strains of *Klebsiella pneumoniae* isolated from patients in four ICUs (3 neonatal ICUs and 1 general ICU) in three Italian hospitals from 1996 to 1997. During this period, ceftazidime-intermediate or -resistant *Klebsiella pneumoniae* isolates were collected from patients hospitalised in the four ICUs. One neonatal ICU and one general ICU were in the same hospital, which indicated that the outbreak was of hospitalwide importance. All of the patients were at increased risk of infection due to their young age (1–30 days), prolonged hospitalisation (up to 60 days for patients in the general ICU), the use of various invasive diagnostic/therapeutic procedures and prior antibiotic therapy that included β-lactam agents, such as ampicillin and ceftazidime, and aminoglycosides, such as gentamicin. These factors have previously been reported to contribute to infection with ESβL-producing organisms [5, 6, 8, 10]. Susceptibility testing of the isolates revealed patterns typical for class A ESβL producers; however, a comparison of the MICs of cefotaxime, ceftazidime and aztreonam suggested that different ESβLs were being produced among the isolates. Further analysis of the β-lactamase contents of the isolates, the sequences of the determinants and the results of kinetic studies confirmed this suspicion.

Of the four outbreak *Klebsiella pneumoniae* strains analysed, one was found to produce a SHV-2a-type ESβL, one a SHV-5 and one a SHV-12. The fourth *Klebsiella pneumoniae* strain, Kl 2 SM, which was obtained from the general ICU, produced TEM-52; although this type has been described for klebsiellae by Poyart et al. [29], this is the first time it has been detected in *Klebsiella pneumoniae* in Italy. With the exception of aztreonam, for which, under our experimental conditions, spectrophotometry detected no hydrolysis by SHV-5- and SHV-12-producing strains, the three partially purified SHV-type enzymes hydrolysed all members of a group of antibiotics widely used in clinical therapy, including the wide-spectrum β-lactams. It should be noted that, worldwide, SHV-2a and SHV-12 have not often been found in clinical isolates, although a recent study (Amicosante et al., International Congress on β-lactamases, "β-lactamase Mediated Resistance: Molecular Aspects and Clinical Implications", June 17–20, 1999, L'Aquila, Italy) found that SHV-12 is one of the most amply diffused wide-spectrum enzymes in Italy [9]. Nevertheless, it has a limited distribution and has not been reported to have caused any epidemics so far.

SHV-5 is one of the most frequently encountered ESβLs produced by epidemic *Klebsiella pneumoniae* strains worldwide. In Italy, it has not previously been associated with outbreaks, but it has occurred in sporadic cases [8, 19, 20, 30]. In the present study, SHV-group ESβLs were more common than TEM-group enzymes; with TEM-type β-lactamase being found exclusively in the outbreak strains obtained from the general ICU. Nevertheless, this communication represents the first time that an outbreak of TEM-52-producing *Klebsiella pneumoniae* has been reported.

Multiple ESβLs are being isolated with increasing frequency in hospital environments today [10, 31]. This was evident in the isolates obtained from Pavia, which revealed the presence of at least two different ESβLs. The β-lactamase data along with the typing and plasmid results obtained from all isolates clearly demonstrated the epidemiological situation of ESβL-producing *Klebsiella pneumoniae* in the three hospitals. PFGE analysis by X*ba*I of all isolates revealed four distinct genotypes occurring in four clusters. In each cluster, high coefficients of similarity were identified among the isolates, confirming the suspicion that an outbreak of ESβL-producing *Klebsiella pneumoniae* was present. The epidemiological results of plasmid profile analysis of the clinical isolates were compatible with those of PFGE. In fact, all strains isolated from patients in the same ICU possessed an identical plasmid profile. This result further supports the hypothesis that the spread of ESβL-producing *Klebsiella pneumoniae* in two ICUs in Pavia university hospital was not due to dissemination of an epidemic plasmid. Moreover, our results confirm that PFGE, when combined with plasmid profile analysis, is an effective tool for investigating the epidemiology of ESβL-producing *Klebsiella pneumoniae* isolates.

According to the criteria of the National Committee for Clinical Laboratory Standards [21], the strains isolated from the neonatal ICUs in Pavia and Trento were intermediately susceptible to ceftazidime and aztreonam, and they were susceptible to cefepime; however, they were less susceptible to these agents than typical klebsiellae that do not produce ESβLs, which indicates biological resistance. Since ESβL producers can readily mutate to initiate hyperproduction of the enzyme, thereby elevating the degree of resistance, we believe that β-lactam therapy is not appropriate for infections caused by such organisms. Similarly, although the strains obtained from the general ICU at Pavia hospital were susceptible or had intermediate resistance to piperacillin-tazobactam, mutation to hyperproduction may result in resistance to this combination. Most of the strains studied were multidrug resistant, and, as has been reported previously for ESβL producers, they were frequently resistant to aminoglycosides. The carbapenems were the only agents tested that were active against all of the

strains studied, but the strains isolated from the three neonatal ICUs were also susceptible to ciprofloxacin. Unfortunately, this new quinolone proved to be contraindicated for use in newborn patients.

In conclusion, four outbreaks of extended-spectrum β-lactamase-producing *Klebsiella pneumoniae* were detected in three Italian hospitals between 1996 and 1997, and epidemiological investigations revealed that each outbreak was caused by a distinct single strain. One outbreak strain produced type TEM-52 ESβLs, one produced SHV-5, one produced SHV-12 and one produced SHV-2a. These enzymes were encoded on large transferable plasmids that appeared to be distinct from each other. The organisms were variably multiresistant to other antimicrobial agents, including aminoglycosides. The results confirm that PFGE in association with plasmid profile analysis should be routinely used to identify and characterise hospital outbreaks in order to treat the infected patients correctly and to prevent the outbreaks from becoming epidemics.

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