ORIGINAL ARTICLE

Transferable plasmid mediating resistance to multiple antimicrobial agents in *Klebsiella pneumoniae* isolates in Greece

I. Galani[1], *E. Xirouchaki*[1], *K. Kanellakopoulou*[1], *G. Petrikkos*[2] *and H. Giamarellou*[1]

[1]4th Department of Internal Medicine, Molecular Biology Section, and [2]1st Department of Propedeutic Medicine, University of Athens Medical School, Athens, Greece

Objective To investigate the underlying resistance mechanisms in 10 *Klebsiella pneumoniae* isolates.

Methods Ten *K. pneumoniae* strains according to distinct bacteriocin typing and REP-PCR, were examined for their plasmid content, their ability to transfer their resistance to aminoglycosides and third-generation cephalosporins, and their production of aminoglycoside-modifying enzymes and β-lactamases.

Results Transfer of resistance to the above-mentioned antibiotics as well as to co-trimoxazole and tetracycline in *Escherichia coli* strain RC 85 at a frequency of $5–10^6$ was achieved for all strains by conjugation. Similar strains harbor a self-transferable multiresistant plasmid (80 kb) with similar *Eco*RI and *Hind*III restriction patterns. This plasmid encodes an extended-spectrum β-lactamase which confers high-level resistance to third-generation cephalosporins and aztreonam. It produces SHV-5 β-lactamase, as demonstrated by isoelectric focusing and DNA sequencing. Aminoglycoside resistance was co-transferred, and AAC(6′)-I, mediating resistance to gentamicin, tobramycin, netilmicin and amikacin, and AAC(3)-I, mediating resistance to gentamicin and sisomycin, were encoded in all isolates and their transconjugants, while APH(3′)-I, mediating resistance to kanamycin and neomycin, was encoded in seven strains.

Conclusions It appears that a multiresistant transferable plasmid encoding the SHV-5 β-lactamase, causing unusually high resistance to ceftazidime and aztreonam, and the combination AAC(6′)-I + AAC(3)-I of acetylating enzymes causing, also resistance to all clinically available aminoglycosides, is established in *K. pneumoniae* in Greece.

Keywords *Klebsiella pneumoniae*, transferable plasmid, resistance mechanisms

Accepted 22 November 2001

Clin Microbiol Infect 2002; 8: 579–588

INTRODUCTION

Klebsiella pneumoniae strains, multiresistant to aminoglycosides, ureidopenicillins, broad-spectrum cephalosporins and monobactams, pose a serious epidemiologic and therapeutic problem in Greek hospitals. Since their recognition, isolates of *K. pneumoniae* producing extended-spectrum β-lactamases (ESBLs) have been a major cause of concern worldwide [1]. Most ESBLs are plasmid-encoded enzymes derived from TEM- or SHV-type β-lactamases by one or more amino acid substitutions which confer resistance to broad-spectrum cephalosporins [2]. SHV ESBLs have been termed SHV-2 through SHV-26, including an SHV-2 variant, SHV-2a [3]. An inhibitor-resistant enzyme was described, termed SHV-10, derived from an SHV-5 variant (SHV-9) [4,5], which retained its ability to hydrolyze penicillins, although its activity against cephalosporins was drastically reduced [5]. Data from 55 hospitals in Greece showed high resistance rates in *K. pneumoniae* not only to

H. Giamarellou, 4th Department of Internal Medicine, Sismanoglion General Hospital, 151 26 Maroussi, Greece
Tel: +30 1 08039542, 080338173
Fax: +30 1 08039543
E-mail: hgiama@ath.forthnet.gr

ceftazidime (31%) and aztreonam (30%) but also to aminoglycosides (30% to netilmicin) [6]. These findings, as well as the fact that an extended broad-spectrum β-lactamase (SHV-5 type) responsible for ceftazidime resistance was discovered 11 years before in *K. pneumoniae* isolates from Greece [7], stimulated our interest in searching for resistance mechanisms in *K. pneumoniae* strains multiresistant to all aminoglycosides and third-generation cephalosporins.

This was very important in order to deal with the difficult therapeutic problem of *K. pneumoniae* infections, since empirical treatment of nosocomial infections in most Greek tertiary hospitals is usually initiated with a combination of a cephalosporin plus an aminoglycoside.

MATERIALS AND METHODS

Bacterial strains

The ten *K. pneumoniae* clinical iso1ates used in the present study were isolated from patients with urinary, lung or abdominal infections hospitalized in four different Greek tertiary hospitals located in Athens. The strains were identified as *K. pneumoniae* by the API20E system (Merieux, Marcy-l'Etoile, France). *K. pneumoniae* strain 160, producing β-lactamase SHV-5 (pI 8.2), was used as reference strain for β-lactamase determination [8]. *Escherichia coli* RC85 R^- K12 strain resistant to rifampicin (MIC > 128 mg/L) was used as recipient strain in mating experiments. *E. coli* strain MXR containing plasmid pULB113 (68 kb) was used as reference strain for plasmid DNA isolation [9].

Antibiotics

Disks for agar diffusion tests were purchased from Diagnostics Pasteur, Marnes la Coquette, France. Antibiotic powders were provided as follows: ceftazidime, Glaxo Wellcome, UK; cefotaxime, Hoechst-Roussel, Greenford, Puteaux, France; ceftriaxone, Hoffman-La Roche, Basel, Switzerland; cefuroxime, Glaxo Wellcome UK; aztreonam, Bristol-Myers Squibb, Syracuse, NY, USA; imipenem, Merck Research Laboratories, Elkton, VA, USA; meropenem, Zeneca Pharmaceuticals, Macclesfield, UK; clavulanic acid, SmithKline Beecham, Worthing, UK; sulbactam, Pfizer, New York, NY, USA; amikacin, Bristol-Myers Squibb, USA; gentamicin, Schering Plough, Manati, PR, USA; tobramycin, Eli-Lilly, Indianapolis, IN, USA; netilmicin, Schering Plough, USA; rifampicin, Gruppo Lepetit SpA, Milano, Italy.

Bacteriocin typing

Bacteriocin typing for all *K. pneumoniae* clinical isolates was performed by A. Bauernfeind according to procedures described previously [10].

Molecular typing

Molecular typing was performed by REP-PCR, a PCR-based DNA fingerprinting technique which uses oligonucleotide primers based on the highly conserved repetitive extragenic palindromic (REP) repeated DNA element [11]. Bacterial genomic DNAs were isolated from 1.5-mL samples of the overnight culture with the QIAamp DNA Mini Kit (QIAGEN GmbH, Hilden, Germany) and were used as templates for PCR. PCR was performed with REP-1 (5′-III-GCG CCG ICA TCA GGC-3′) and REP-2 (5′-ACG TCT TAT CAG GCC TAC-3′) primers derived from repetitive extragenic palindromic (REP) elements. The reaction mixture was subjected to initial denaturation (95 °C, 7 min), followed by 30 cycles of denaturation (94 °C, 1 min), annealing (40 °C, 1 min) and extension (65 °C, 8 min) and then a single final extension (65 °C, 16 min). Amplified DNA segments were visualized after running samples on 2% agarose gels for 2 h. The fingerprints were compared visually for the reproducible portion of the pattern below the 6.6-kb size marker. Patterns were considered different if they differed by one or more amplification bands, regardless of the band intensity.

Susceptibility studies

Antibiotic susceptibi1ity testing was performed by a disk diffusion method on Mueller–Hinton agar [12]. The strains were further examined by the double-disk synergy test (DDST) for the presence of extended-spectrum β-lactamases [13,14]. Clavulanic acid and sulbactam (10 μg)were spotted on filter disks (6-mm diameter) and placed at the top of antibiotic disks (ceftazidime, ceftriaxone, cefotaxime and aztreonam). A 5-mm increase in zone diameter for either antimicrobial agent, tested in combination with clavulanic acid or sulbactam, compared to its zone when tested alone, confirmed an ESBL-producing *K. pneumoniae* strain. Minimal inhibitory concentration values (MICs)

for ceftazidime, aztreonam, cefotaxime, ceftriaxone, imipenem, meropenem and aminoglycosides were determined in MH broth according to the guidelines of the National Committee for Clinical Laboratory Standards [15].

Transfer of resistance

Conjugation experiments were performed by the broth culture mating technique, with *E. coli* RC85 R^- K12 strain (rifampicin resistant, MIC > 128 mg/L) used as recipient. Recipient and donor strains were inoculated into brain–heart infusion broth and incubated at 37 °C for 4 h. They were then mixed in a ratio 10:1 (recipient/donor) and incubated at 37 °C for a further 6 h. Transconjugants were selected on MacConkey Agar, supplemented with rifampicin (128 mg/L) to inhibit the growth of the donor strain, and ceftazidime (16 mg/L) to inhibit the growth of the recipient strain.

Determination of aminoglycoside resistance mechanisms

The determination was made by disk susceptibility testing for gentamicin, amikacin, tobramycin, netilmicin, neomycin, kanamycin, SCH 21420, apramicin, fortimicin, SCH 21562, SCH 21561 and SCH 22591 (aminoglycoside resistance patterns—AGRPs) and DNA probe analysis with probes of ANT(2″)-Ia, AAC(3)-I, AAC(3)-Va, AAC(6′)-Ib, AAC(6′)-Ic, APH(3′)-I, ANT(4′)-II, APH(3′)-VIa and APH(2″) + (6′) aminoglycoside resistance genes, which were kindly provided by G. Miller [16,17].

Hybridization experiments

The DNA probes were purified twice through agarose gels after double digestion with appropriate restriction endonucleases. The fragments were then labeled with digoxigenin (Boehringer Mannheim, Mannheim, Germany). Probe labeling, DNA hybridization (dot blotting) and detection of hybridization were done using the DIG-Non Radioactive Labelling and Detection Kit (Boehringer Mannheim) as recommended by the manufacturer, on S & S Nytran-N membranes.

β-Lactamase preparation

β-Lactamases were obtained from the clinical isolates or from transconjugants after centrifugation of sonicated cells. Cultures were performed in trypticase soy broth containing subinhibitory concentrations of ceftazidime (1–2 mg/L). Cells were grown aerobically at 37 °C until the late exponential phase, collected by centrifugation, resuspended in 0.1% glycine (1/30 of the original culture volume), and disrupted by sonication (six times for 30 s each). The supernatant obtained after centrifugation at 22 000 × *g* for 45 min to remove the cell debris represented the crude extract. β-Lactamase activity was revealed by adding 5 μL of crude bacterial extract to 30 μL of a 500 mg/L solution of the chromogenic cephalosporin nitrocefin (Glaxo Wellcome).

Analytic isoelectric focusing

Sonic extracts prepared as described above were applied on filter paper strips to commercially obtained polyacrylamide gels containing ampholines with pH ranges of 3.5–10 (LKB Products, Bromma, Sweden). Electrofocusing was done by the procedure recommended by the manufacturer, using the LKB 2117 Multiphor apparatus. As reference strain for β-lactamase determination, the SHV-5 (pI 8.2) donor *K. pneumoniae* 160 was used [8]. Enzyme bands were visualized in the gels by the use of nitrocefin.

Hydrolysis of β-lactam antibiotics

β-Lactamase activity was determined spectrophotometrically in 50 mM sodium phosphate buffer, pH 7.0, at 30 °C, with a double-beam spectrophotometer (Hitachi model 150–20). The concentration of each antibiotic substrate was 100 μM. The wavelength of maximal absorption for each antibiotic was first determined. Then the absorption of the non-hydrolyzed antibiotic in the hydrolysis mixture was measured, and the hydrolysis activity of each enzyme was determined in relation to the activity it had in 100 mM ampicillin (100%). The hydrolysis of β-lactam antibiotics was attempted also with the microbiological method described by Bauernfeind et al. [18], according to which polyacrylamide gel, after the isoelectric focusing, is overlaid with a first layer of agar containing the β-lactam antibiotic (ceftazidime, aztreonam or cefotaxime) and with a second layer of tryptic soy agar mixed with an *E. coli* susceptible strain. After overnight incubation, the positive result of hydrolysis was shown by the

growth of *E. coli* colonies around the band of the enzyme.

Plasmid DNA preparation

A rapid procedure according to Kado and Liu was performed [19]. Large-scale preparation was performed with alkaline lysis according to Maniatis et al. [20]. As reference strain for plasmid DNA isolation, *E. coli* strain MXR, containing plasmid pULB113 (68 kb), was used [9].

Plasmid DNA restriction analysis

*Eco*RI (Gibco-BRL, Gaithersburg, MD, USA), *Hind* III (BioLabs, Beverly, MA) and restriction endonucleases were used for plasmid DNA restriction analysis according to the manufacturer's instructions. The digests were electrophorized on 0.8% agarose gels, with *Hind*III digests of bacteriophage λDNA and 1-kb DNA ladder as molecular size markers.

PCR amplification and sequencing of SHV gene

An 865-bp sequence of the SHV gene was PCR amplified using the oligonucleotide primers 5′-ATG CGT TAT ATT CGC CTG TG-3′ and 5′-GTT AGC GTT GCC AGT GCT CG-3′. Chromosomal DNA was prepared by using the Qiamp tissue kit (QIAGEN GmbH) as described by the manufacturer. The PCR reaction mixture was initially incubated at 94 °C for 5 min, followed by 30 cycles of denaturation (60 s at 94 °C), annealing (60 s at 56 °C) and extension (180 s at 72 °C). The extension was completed after 7 min of incubation at 72 °C. Reactions were carried out in a thermal Cycler 480 (Perkin Elmer Cetus, Emeryville, CA, USA). The nucleotide sequences of PCR-generated amplimers were determined using the Taq DyedeoxyTerminator Cycle sequence kit and analyzed in an automatic DNA Sequencer ABI (Applied Biosystems, Foster City, CA, USA; 373A).

Results

The ten *K. pneumoniae* isolates were resistant to ampicillin, ureidopenicillins, first-, second- and third-generation cephalosporins (with the exception of cefoxitin, for which two strains were resistant), aztreonam, co-trimoxazole, tetracyclines and all aminoglycosides. Three of them were also resistant to quinolones, and all strains remained susceptible to imipenem (Table 1), meropenem ($\leq$0.25) and combinations with β-lactamase inhibitors. The MICs of ceftazidime (128 to >512) and aztreonam (256 to >512) were higher than the MICs of cefotaxime (32–128) and ceftriaxone (32–128) (Table 1). The impaired activities of ceftazidime, ceftriaxone, cefotaxime and aztreonam were significantly restored when they were combined with clavulanic acid or sulbactam in the DDST. These strains were isolated from urinary infections, pus or sputum from different patients (one per patient), different wards and departments, or even different hospitals. The ten *K. pneumoniae* strains were distinct according to bacteriocin typing (Table 1), and nine of them were distinct according to REP-PCR (Figure 1) too. Strains 568 and 584 had different bacteriocin types but shared the same amplification pattern in REP-PCR.

Ceftazidime resistance was transferred from all clinical isolates to *E. coli* recipient RC85, with a frequency ranging from 10^{-5} to 10^{-6}. The *E. coli* transconjugants had similar MICs to those observed for parent *K. pneumoniae* isolates for the extended-spectrum cephalosporins and aztreonam, with the exception of cefoxitin (Table 1). Synergism with clavulanic acid or sulbactam was similar to that observed with the clinical isolates. Aminoglycoside resistance was co-transferred with ceftazidime resistance to all ten isolates, as well as tetracycline and co-trimoxazole resistance, while chloramphenicol resistance was co-transferred in two (584, 568) of five (223, 584, 11, 568, 1290) resistant strains. Ciprofloxacin resistance was not transferable.

The ten *K. pneumoniae* isolates had similar β-lactamase profiles as revealed by isoelectric focusing (Figure 2a). There was one band with pI of 8.2 common for all strains which was also observed in their *E. coli* transconjugants. The hydrolytic properties of this β-lactamase (pI 8.2) were similar to those of SHV-5. Ceftazidimase activity was demonstrated by both methods used, with ceftazidime, cefotaxime, ceftriaxone and aztreonam hydrolyzed as by the SHV-5 enzyme, and hydrolysis inhibited by clavulanic acid and sulbactam (Figure 2b). DNA sequences of SHV genes from the transconjugant strains were identical to that of the SHV-5 β-lactamase gene published by Billot-Klein et al. [21].

Table 1 Main characteristics and susceptibility patterns to β-lactam antibiotics (MIC in mg/L) of 10 *K. pneumoniae* strains and their transconjugants

			Antibiotics						
Strain	**Species**	**Bacteriocin typing**	**Ceftazidime**	**Ceftriaxone**	**Cefotaxime**	**Aztreonam**	**Cefoxitin**	**Imipenem**	**MEM**
41[a]	*K. pneumoniae*	3, 5	>512	32	64	>512	16	1	≤0.25
41-t[b]	*E. coli* RC85		>512	64	128	>512	4	≤0.25	≤0.25
223[a]	*K. pneumoniae*	1, 3, 5, 6, 7	256	>128	64	512	>128	0.5	0.25
223-t[b]	*E. coli* RC85		64	8	16	256	4	≤0.25	≤0.25
584[a]	*K. pneumoniae*	3	>512	64	64	>512	16	2	≤0.25
584-t[b]	*E. coli* RC85		512	64	64	512	8	≤0.25	≤0.25
660[a]	*K. pneumoniae*	1, 7	256	32	128	>512	4	1	0.25
660-t[b]	*E. coli* RC85		512	64	256	256	8	≤0.25	≤0.25
1459[a]	*K. pneumoniae*	1, 2, 3, 7	128	64	32	256	8	0.5	0.25
1459-t[b]	*E. coli* RC85		64	4	32	256	4	≤0.25	≤0.25
2294[a]	*K. pneumoniae*	1, 3, 4, 5, 6, 7	256	32	32	512	8	0.5	0.25
2294-t[b]	*E. coli* RC85		128	16	16	64	4	≤0.25	≤0.25
2573[a]	*K. pneumoniae*	1, 2, 3, 5, 6, 7	256	32	32	512	2	0.5	≤0.25
2573-t[b]	*E. coli* RC85		256	16	32	256	4	≤0.25	≤0.25
11[a]	*K. pneumoniae*	1, 3	>512	32	32	>512	4	1	≤0.25
11-t[b]	*E. coli* RC85		256	32	64	256	4	≤0.25	≤0.25
568[a]	*K. pneumoniae*	0	>512	64	128	>512	8	0.5	0.25
568-t[b]	*E. coli* RC85		>512	128	128	>512	4	≤0.25	≤0.25
1290[a]	*Klebsiella pneumonia*	1, 3, 6	>512	64	64	>512	64	2	≤0.25
1290-t[b]	*E. coli* RC85		>512	32	64	>512	4	≤0.25	≤0.25
RC85	*E. coli*		1	0.25	0.07	0.5	2	≤0.25	≤0.25

[a]*K. pneumoniae* parent strain. [b]*E. coli* transconjugant strain.

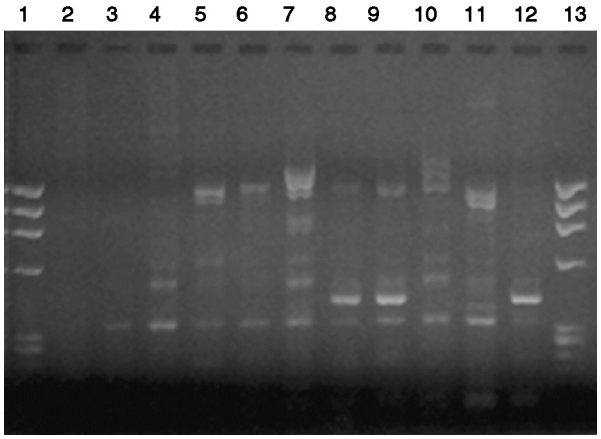

Figure 1 Repetitive extragenic palindromic (REP) PCR. Lanes 1 and 13: ϕX174 DNA/*Hae*III digest. Lane 2: PCR control. Lanes 3–12: Amplified DNA segments of strains 11, 41, 223, 568, 584, 660, 1290, 1459, 2294 and 2573, respectively.

The aminoglycoside-modifying enzymes AAC (6′)-I and AAC(3)-I were produced by all *K. pneumoniae* clinical isolates and by their *E. coli* transconjugants, as demonstrated by the AGRP method and DNA probe analysis. APH(3′)-I was also observed in seven donor strains and their transconjugants (Table 2).

A plasmid of approximately 80 kb, with closely related *Eco*RI and *Hind*III restriction patterns, was isolated from all *E. coli* transconjugants, encoding the SHV-5-type β-lactamase, the aminoglycoside-modifying enzymes, and tetracycline and co-trimoxazole resistance. Slight differences in restriction patterns of plasmids also encoded for APH(3′)-I were observed (Figure 3).

Table 2 Determination of aminoglycoside-modifying enzymes produced by 10 *K. pneumoniae* clinical isolates and their transconjugants after hybridization with 10 different DNA probes[a]

Indicative number of strain	Enzymes produced
41	AAC(3)-I, AAC(6′)-I, APH(3′)-I
223	AAC(3)-I, AAC(6′)-I
584	AAC(3)-I, AAC(6′)-I, APH(3′)-I
660	AAC(3)-I, AAC(6′)-I, APH(3′)-I
1459	AAC(3)-I, AAC(6′)-I, APH(3′)-I
2294	AAC(3)-I, AAC(6′)-I
2573	AAC(3)-I, AAC(6′)-I
11	AAC(3)-I, AAC(6′)-I, APH(3′)-I
568	AAC(3)-I, AAC(6′)-I, APH(3′)-I
1290	AAC(3)-I, AAC(6′)-I, APH(3′)-I

[a]The correlation of the results of AGRP resistance patterns and DNA probe analysis was 100%.

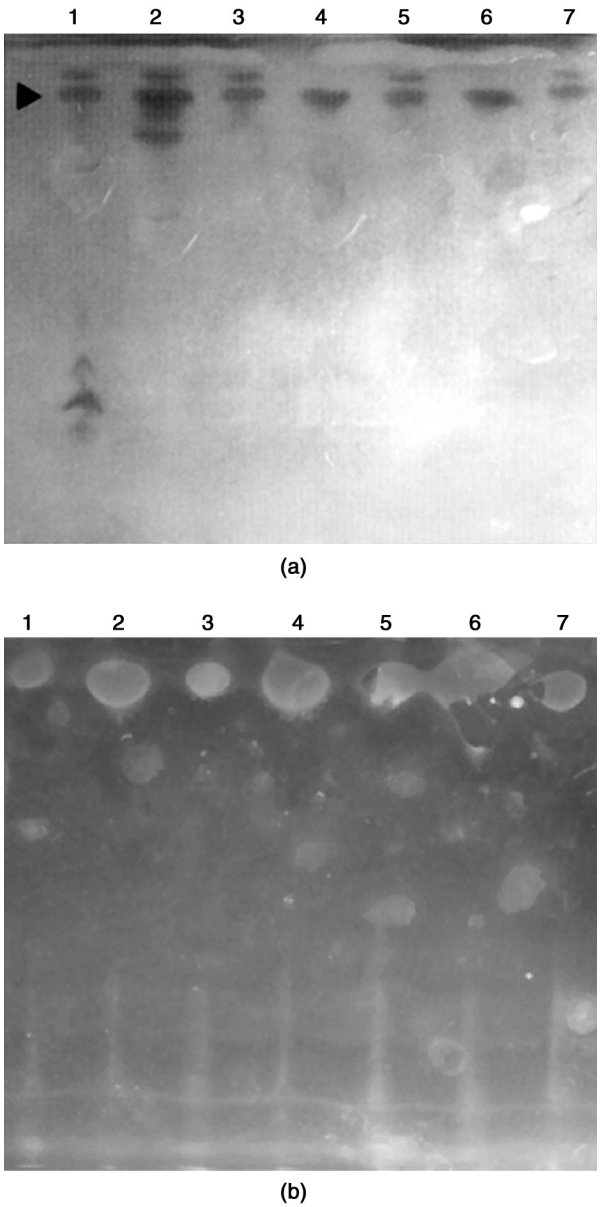

Figure 2 (a) Isoelectric focusing (pH range 3.5–9.5) of β-lactamases. Lane 1: *K. pneumoniae* 160 (SHV-5). Lane 2: *K. pneumoniae* clinical isolate 223. Lane 3: *E. coli* transconjugant 223-1. Lane 4: *K. pneumoniae* clinical isolate 1459. Lane 5: *E. coli* transconjugant 1459-1. Lane 6: *K. pneumoniae* clinical isolate 2294. Lane 7: *E. coli* transconjugant 2294-1. The arrow identifies a β-lactamase with a pI of 8.2. (b) Microbiological method of hydrolysis of ceftazidime, cefotaxime, ceftriaxone and aztreonam by β-lactamases. Lane 1: *K. pneumoniae* 160 (SHV-5). Lane 2: *K. pneumoniae* clinical isolate 223. Lane 3: *E. coli* transconjugant 223-1. Lane 4: *K. pneumoniae* clinical isolate 1459. Lane 5: *E. coli* transconjugant 1459-1. Lane 6: *K. pneumoniae* clinical isolate 2294. Lane 7: *E. coli* transconjugant 2294-1. The arrow identifies the hydrolysis of ceftazidime, cefotaxime, ceftriaxone or aztreonam by the β-lactamase with pI 8.2.

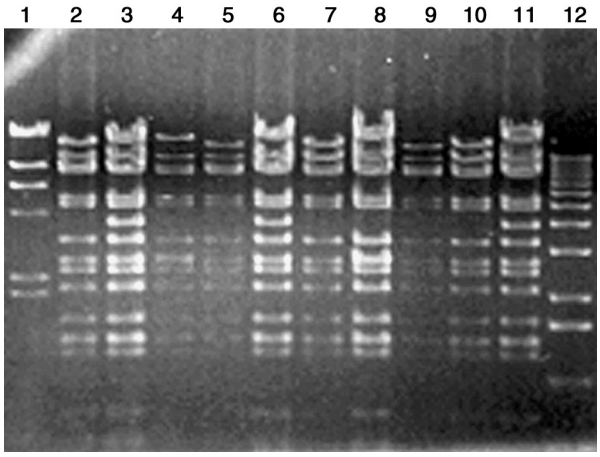

Figure 3 *Eco*RI restriction patterns of the transferred plasmid isolated from *E. coli* transconjugants. Lane 1: λDNA/*Hind*III digest. Lanes 2–11: *Eco*RI digests of plasmid isolated from strains 1290-1, 2294-1, 2573-1, 41-1, 223-1, 584-1, 1459-1, 11-1, 568-1, 1201-1. Lane 12: 1-kb DNA ladder.

DISCUSSION

Extended-spectrum cephalosporins are often used as primary agents for the treatment of severe, mostly nosocomial, infections. However, soon after the introduction of the newer extended-spectrum cephalosporins into clinical practice, the emergence of resistance with subsequent therapeutic failures was observed. Extended-spectrum enzymes were first recognized in Europe in 1983 [22], but have since been reported in many countries [7,8,22–27]. The prevalence of strains producing extended-spectrum β-lactamases is increasing worldwide, and *K. pneumoniae* is by far the most common species in which these enzymes have been recognized. Plasmids responsible for extended-spectrum β-lactamase production tend to be large (80 kb or more in size) and tend to carry resistance to several agents [7,28–30], an important limitation to be taken into consideration in the design of treatment alternatives. Curiously, none of these enzymes has been shown to be transposable [28], although TEM-1 and TEM-2 can be transposon encoded [30,31]. The usual transmissibility of the responsible plasmids, however, allows resistance to spread readily to other pathogens, so that extended-spectrum enzymes have been found not only in *E. coli*, *Klebsiella* spp., *Pseudomonas aeruginosa*, *Citrobacter freundii* and *Enterobacter cloacae*, but also in *Enterobacter aerogenes*, *Levinea malonatica*, *Morganella morganii*, *Salmonella* spp. and *Serratia marcescens* [29,32]. In many outbreaks, related plasmids from isolates at a single hospital encoded different extended-spectrum enzymes, as though sequential mutations were occurring in a common β-lactamase gene [33,34]. For these reasons, we examined ten multiresistant *K. pneumoniae* clinical isolates derived from different wards, departments or hospitals in Greece to determine their resistance mechanisms to third-generation cephalosporins, aztreonam and aminoglycosides. It should be pointed out that recent resistance rates of *K. pneumoniae* strains to ceftazidime ranged from 35% to 83% and to amikacin from 22% to 61% (WHONET Greece, 1999; A. Vatopoulos, personal communication). There is no doubt that, in order to solve the difficult therapeutic problems with infections caused by multiresistant bacteria and by *K. pneumoniae* in particular in Greek hospitals, it is vital to investigate their underlying resistance mechanisms to several antimicrobials, since empirical treatment of nosocomial infections is very often initiated with a combination of a broad-spectrum cephalosporin and an aminoglycoside.

Nine of the ten strains examined were distinct, as confirmed by bacteriocin typing and REP-PCR, suggesting that one resistant clone has not been spread, but rather, multiresistant plasmids harboring the *bla* gene and genes encoding for aminoglycoside-modifying enzymes between different strains of enterobacteria have spread.

The enzyme isolated with a pI of 8.2, with hydrolytic activity against ceftazidime, cefotaxime, ceftriaxone and aztreonam, that is inhibited by clavulanic acid and sulbactam, suggests a close relationship with the SHV-5 β-lactamase. Sequencing of the bla_{SHV} genes showed that the strains produced SHV-5, and not one of the recently described β-lactamases that have the same pI, i.e. SHV-9, SHV-10 [5], or SHV-12 [35]. SHV-5 is one of the most frequently encountered ESBLs produced by *K. pneumoniae* strains worldwide [1]. It was first reported in Chile [8] as well as in France (CAZ-4 β-lactamase) [36], and has already been described in Greece [7]. Gutmann et al. [8] reported that SHV-5 was encoded by a plasmid of 150 kb that was self-transferable to other *K. pneumoniae* strains, but not to *E. coli*. It was not reported whether this plasmid carried resistance genes for non-β-lactam antibiotics. The CAZ-4 enzyme, isolated in Clermont-Ferrand Hospital in France [36,37], was also encoded by a 150-kb plasmid,

but was transferable to *E. coli* at a 1ow frequency ($\leq 10^{-6}$) and co-transferred resistance to amikacin, kanamycin, netilmicin, tobramycin, chloramphenicol and sulfonamides. Vatopoulos et al. [7] reported that the SHV-5-type enzyme isolated from Greek *K. pneumoniae* isolates was encoded by related 91-kb plasmids that were easily self-transferred to *E. coli*, and some of them also encoded resistance to aminoglycosides tobramycin, amikacin and netilmicin. Shannon et al. reported another SHV-5-type β-lactamase produced by a *K. pneumoniae* strain isolated from a patient from Greece, which was encoded by a 90–100-MDa (137–152-kb) plasmid, and which also encoded lactose fermentation, but not resistance to tobramycin, sulfamethoxazole or trimethoprim.

The aminoglycoside resistance mechanisms in *K. pneumoniae* clinical isolates comprise the production of the aminoglycoside-modifying enzymes AAC(6′)-I, mediating resistance to gentamicin, tobramycin, netilmicin and amikacin, and AAC (3)-I, mediating resistance to gentamicin and sisomycin, and were encoded in all isolates and their *E. coli* transconjugants. APH(3′)-I, mediating resistance to kanamycin and neomycin, was also produced by seven strains and their transconjugants. Studies on the aminoglycoside resistance mechanisms in aminoglycoside-resistant isolates undertaken between 1988 and 1993 in several countries showed a much greater incidence of complexity of those mechanisms than did earlier studies [38,39]. The resistance rates among aminoglycoside-resistant *Klebsiella* species to the clinically available aminoglycosides in these surveys were quite different from those observed in earlier surveys. As expected, in the earliest surveys, rates of resistance to gentamicin and tobramycin were quite high, and resistance rates to netilmicin, amikacin and isepamicin were quite low. Later surveys in Europe, after the latter aminoglycosides had been frequently used, showed higher netilmicin resistance. In Greek hospitals, where amikacin and netilmicin usage (40% and 50%, respectively) was very high [40], the incidence of AAC(6′)-I (tobramycin, netilmicin, amikacin, dibekacin, and kanamycin) as a single aminoglycoside resistance mechanism was 8.9%, but a single combination of AAC(6′)-I with AAC(3)-I (gentamicin) was very common (59.5%), especially in the 14 Greek hospitals and one Turkish hospital surveyed [41]. This combination occurred in many different types of Enterobacteriaceae in each of the individual Greek hospitals, where it seemed to be acting as a plasmid epidemic. It was almost always found together with APH(3′)-I (kanamycin and neomycin). Our results agree with those findings, and a combination of the previous three enzymes is established in the transferable plasmid examined. The restriction patterns of the plasmid isolated from each donor strain and its *E. coli* transconjugant were identical for the strains harboring the *aph(3)-I* gene, showing extended homology in their endonuclease restriction analysis. Isolates expressing no APH(3′)-I activity (three of ten) showed small differences in their restriction patterns compared to the previous ones.

Co-trimoxazole resistance in the *K. pneumoniae* clinical isolates examined here is plasmid encoded, and was co-transferred in *E. coli* transconjugants with third-generation cephalosporin and aminoglycoside resistance.

The presence of various multiresistant self-transferable plasmids in *K. pneumoniae* strains isolated in Greek hospitals has been reported in previous studies [7], along with the production of the SHV-5-type β-lactamase. This study reports, for the first time, the production of aminoglycoside-modifying enzymes encoded by genes harbored in the same self-transferable plasmid as that harbors the β-lactamase SHV-5.

In conclusion, the results of the present study indicate clearly that self-transferable plasmids, which encoding a variety of resistance genes, seem to have become established in *K. pneumoniae* in Greece. Genes that encode (1) the extended-spectrum SHV-5 β-lactamase causing unusually high resistance to ceftazidime and aztreonam; (2) aminoglycoside-modifying enzymes such as AAC(6′)-I, AAC(3)-I and APH(3)-I causing resistance to all clinical available aminoglycosides, and (3) co-trimoxazole and tetracycline resistance, are all located in one plasmid. Owing to the high in vitro frequency of transfer of the R-factor carrying the previous genes, its dissemination in vivo must be anticipated. Accordingly, the excessive consumption of third-generation cephalosporins in Greek hospitals, either as monotherapy or as combination therapy, should be restricted. The latter indication is based on the results of several studies which proved that reduction in overuse or misuse of ceftazidime was followed by a remarkable reduction in resistance rates of *K. pneumoniae* strains to cephalosporins [42,43]. As the use of aminoglycosides, at least in Greece, is limited

because of the fear of nephrotoxicity, they are not, therefore, considered to perpetuate resistance to advanced cephalosporins.

ACKNOWLEDGMENTS

We thank Professor A. Bauernfeind (Max von Pettenkofer-Institute, Munich, Germany) for performing the bacteriocin typing of the *K. pneumoniae* clinical isolates.

REFERENCES

1. Medeiros AA. Evolution and dissemination of beta-lactamases accelerated by generations of β-lactam antibiotics. *Clin Infect Dis* 1997; 24(suppl 1): S19–45.
2. Jacoby GA, Medeiros AA. More extended-spectrum β-lactamases. *Antimicrob Agents Chemother* 1991; 35: 1697–704.
3. Podbielski A, Schonling J, Melzer B, Warnatz K, Leusch H-G. Molecular characterization of a new plasmid-encoded SHV-type β-lactamase (SHV-2 variant) conferring high level cefotaxime resistance upon *Klebsiella pneumoniae*. *J Gen Microbiol* 1991; 137: 569–78.
4. Prinarakis EE, Tzelepi E, Gazouli M, Mentis AF, Tzouvelekis LS. Characterization of a novel SHV beta-lactamase variant that resembles the SHV-5 enzyme. *FEMS Microbiol Lett* 1996; 139: 229–34.
5. Prinarakis EE, Miriagou V, Tzelepi E, Gazouli M, Tzouvelekis LS. Emergence of an inhibitor-resistant β-lactamase (SHV-10) derived from SHV-5 variant. *Antimicrob Agents Chemother* 1997; 41: 838–40.
6. Giamarellou H. Surveillance of gram-negative resistance in Greece in 1994: The third Amphiaraion Study [abstract 0609]. In: *Program and Abstracts of the 19th International Congress of Chemotherapy*. Montreal: Pulses Group Inc., 1995: 282C.
7. Vatopoulos AC, Philippon A, Tzouvelekis LS, Komninou Z, Legakis NJ. Prevalence of a transferable SHV-5 type β-lactamase in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* in Greece. *J Antimicrob Chemother* 1990; 26: 635–48.
8. Gutmann L, Ferre B, Goldstein FW *et al.* SHV-5, a novel SHV-type β-lactamase that hydrolyzes broad-spectrum cephalosporins and monobactams. *Antimicrob Agents Chemother* 1989; 33: 951–6.
9. Van Gijsegem F, Toussaint A. Chromosome transfer and R-prime formation by an RP4::mini-Mu derivative in *Escherichia coli*, *Salmonella typhimurium*, *Klebsiella pneumoniae* and *Proteus mirabilis*. *Plasmid* 1982; 7: 30–44.
10. Bauernfeind A, Petermuller C, Schneider R. Bacteriocins as tools in analysis of nosocomial *Klebsiella pneumoniae* infections. *J Clin Microbiol* 1981; 33: 15–19.
11. Versalovic J, Koeuth T, Lupski JR. Distribution of repetitive DNA sequences in eubacteria and application to fingerprinting of bacteria genomes. *Nucleic Acids Res* 1991; 19: 6823–31.
12. Bauer AW, Kirby WMM, Sherris JC, Turck M. Antibiotics susceptibility testing by a standardized single disk method. *Am J Clin Pathol* 1966; 45: 493–6.
13. Jarlier V, Nicolas M-H, Fournier G, Philippon A. Extended broad-spectrum β-lactamases conferring transferable resistance to newer β-lactam agents in Enterobacteriaceae: hospital prevalence and susceptibility patterns. *Rev Infect Dis* 1988; 4: 867–78.
14. Legrand P, Fournier G, Bure A *et al.* Detection of extended broad-spectrum beta-lactamases in Enterobacteriaceae in four French hospitals. *Eur J Clin Microbiol Infect Dis* 1989; 8: 527–9.
15. National Committee for Clinical Laboratory Standards. *Performance standards for antimicrobial susceptibility testing, ninth informational supplement*. M100-S9 (M2-A6). Villanova, PA: NCCLS, 1999.
16. Miller GH, Sabatelli FJ, Hare RS, Waitz JA. Survey of aminoglycoside resistance patterns. *Dev Industrial Microbiol* 1980; 21: 91–104.
17. Miller GH, Hare RS, Shaw KJ, Sabatelli FJ. *Aminoglycoside resistance mechanisms booklet*. Manati: Schering-Plough Research, 1989.
18. Bauernfeind A, Grimm H, Schweighart S. A new plasmidic cefotaximase in a clinical isolate of *Escherichia coli*. *Infection* 1990; 18(5): 294–8.
19. Kado CI, Liu ST. Rapid procedure for detection and isolation of large and small plasmids. *J Bacteriol* 1981; 145: 1365–73.
20. Sambrook J, Fritsch EF, Maniatis T. *Molecular cloning: a laboratory manual*. Plainview: Cold Spring Harbor Laboratory Press, 1989.
21. Billot-Klein D, Gutmann L, Collatz E. Nucleotide sequence of the SHV-5 β-lactamase gene of the *Klebsiella pneumoniae* plasmid. *Antimicrob Agents Chemother* 1990; 34: 2439–41.
22. Knothe H, Shah P, Krcmery V, Antal M, Mitsuhashi S. Transferable resistance to cefotaxime, cefoxitin, cefamandole and cefuroxime in clinical isolates of *Klebsiella pneumoniae* and *Serratia marcescens*. *Infection* 1983; 11: 315–17.
23. Brun-Buisson C, Legrand P, Philippon A, Montravers F, Ansquer M, Duval J. Transferable enzymatic resistance to third generation cephalosporins during nosocomial outbreak of multiresistant *Klebsiella pneumoniae*. *Lancet* 1987; ii: 302–6.
24. Sirot D, Sirot J, Labia R *et al.* Transferable resistance to third generation cephalosporins in clinical isolates of *Klebsiella pneumoniae*: identification of CTX-1, a novel β-lactamase. *J Antimicrob Chemother* 1987; 20: 323–34.
25. Spencer RC, Wheat PF, Winstanley TG, Cox DM, Plested SJ. Novel β-lactamase in a clinical isolate of *Klebsiella pneumoniae* conferring unusual resistance

to β-lactam antibiotics. *J Antimicrob Chemother* 1987; 20: 919–27.
26. Vuye A, Verschraegen G, Claeys G. Plasmid mediated β-lactamases in clinical isolates of *Klebsiella pneumoniae* and *Escherichia coli* resistant to ceftazidime. *Antimicrob Agents Chemother* 1989; 33: 757–61.
27. Philippon A, Labia R, Jacoby G. Extended-spectrum β-lactamases. *Antimicrob Agents Chemother* 1989; 33: 1131–6.
28. Jacoby GA, Sutton L. Properties of plasmids responsible for extended-spectrum β-lactamase production. *Antimicrob Agents Chemother* 1991; 35: 164–9.
29. Philippon A, Ben Redjeb S, Fournier G, Ben Hassen A. Epidemiology of extended-spectrum β-lactamases. *Infection* 1989; 17: 347–54.
30. Smith CE, Tillman BS, Howell AW, Longfield RN, Jorgensen JH. Failure of ceftazidime–amikacin therapy for bacteremia and meningitis due to *Klebsiella pneumoniae* producing an extended-spectrum β-lactamase. *Antimicrob Agents Chemother* 1990; 34: 1290–3.
31. Saunders JR. Genetics and evolution of antibiotic resistance. *Br Med Bull* 1984; 40: 54–60.
32. Petit A, Gerbaund G, Sirot D, Courvalin P, Sirot J. Molecular epidemiology of TEM-3 (CTX-1) β-lactamase. *Antimicrob Agents Chemother* 1990; 34: 219–24.
33. Chanal CM, Sirot DL, Petit A *et al.* Multiplicity of TEM-derived β-lactamases from *Klebsiella pneumoniae* strains isolated at the same hospital and relationships between the responsible plasmids. *Antimicrob Agents Chemother* 1989; 33: 1915–20.
34. Rice LB, Willey SH, Papanicolaou GA *et al.* Outbreak of ceftazidime resistance caused by extended-spectrum β-lactamases at a Massachusetts chronic-care facility. *Antimicrob Agents Chemother* 1990; 34: 2193–9.
35. Nuesch-Inderbinen MT, Kayser FH, Hachler H. Survey and molecular genetics of SHV β-lactamases in Enterobacteriaceae in Switzerland: two novel enzymes, SHV-11 and SHV-12. *Antimicrob Agents Chemother* 1997; 41: 943–9.
36. Sirot D, Chanal C, Labia R, Meyran M, Sirot J, Cluzel R. Comparative study of five plasmid-mediated ceftazidimases isolated in *Klebsiella pneumoniae*. *J Antimicrob Chemother* 1989; 24: 509–21.
37. Shannon KP, King A, Phillips I, Nicolas MH, Philippon A. Importation of organisms producing broad-spectrum SHV-group β-lactamases into the United Kingdom. *J Antimicrob Chemother* 1990; 25: 343–51.
38. The Aminoglycoside Resistance Study Groups. The most frequently occurring aminoglycoside resistance mechanisms—combined results of surveys in eight regions of the world. *J Chemother* 1995; 7(suppl 2): 17–30.
39. Miller GH, Sabatelli FJ, Naples L, Hare RS, Shaw KJ, the Aminoglycoside Resistance Study Groups. The changing nature of aminoglycoside resistance mechanisms and the role of isepamicin—a new broad-spectrum aminoglycoside. *J Chemother* 1995; 7(suppl 2): 31–44.
40. Petrikkos G, Stamos G, Grammatikou M, Xirouchaki E, Miller G, Giamarellou H. Survey of the aminoglycoside resistance mechanisms in gram-negative bacteria [abstract 190]. In: *18th International Congress of Chemotherapy*, Stockholm: 1993: 190.
41. Miller GH, Sabatelli FJ, Hare RS *et al.* The most frequent aminoglycoside resistance mechanisms—changes with time and geographic area: a reflection of aminoglycoside usage patterns? *Clin Infect Dis* 1997; 24(suppl 1): S46–62.
42. Pena C, Pujol M, Ardanuy C *et al.* Epidemiology and successful control of a large outbreak due to *Klebsiella pneumoniae* producing extended-spectrum beta-lactamases. *Antimicrob Agents Chemother* 1998; 42(1): 53–8.
43. Rahal JJ, Urban C, Horn D *et al.* Class restriction of cephalosporin use to control total cephalosporin resistance in nosocomial *Klebsiella*. *JAMA* 1998; 280(14): 1233–7.