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Epidemic and Endemic Spread of *Klebsiella pneumoniae* Producing SHV-5 Beta-Lactamase in Dubrava University Hospital, Zagreb, Croatia

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Summary

Plasmid-encoded resistance to broad-spectrum cephalosporins and aztreonam is becoming a widespread phenomenon in clinical medicine. These antibiotics are inactivated by an array of different extended-spectrum β-lactamases (ESBLs) which have evolved by point mutations of parental TEM or SHV β-lactamases. In a previous study conducted during 1994-1995, SHV-2, SHV-2a and SHV-5 β-lactamases were found among *Klebsiella pneumoniae* isolates in Dubrava University Hospital. High prevalence of ESBLs among *K. pneumoniae* strains in this hospital (20%) required further investigation. In this investigation, β-lactamases from 42 *K. pneumoniae* strains collected in 1997 and 15 in 2004 from Dubrava University Hospital, were characterized in order to study the evolution of plasmid-encoded resistance to extended-spectrum cephalosporins and aztreonam in that hospital over a prolonged study period. Susceptibility to antibiotics was determined by disk-diffusion and broth microdilution method. β-lactamases were characterized by isoelectric focusing, determination of hydrolysis of β-lactam substrates, polymerase chain reaction and sequencing of bla_{SHV} genes. All *K. pneumoniae* strains and their *Escherichia coli* transconjugants produced β-lactamase with an isoelectric point of 8.2. Based on sequencing of bla_{SHV} genes enzymes of all transconjugants were identified as SHV-5 β-lactamase which conferred on the producing isolates high level of ceftazidime and aztreonam resistance. In this study, an outbreak of nosocomial infections caused by SHV-5 producing *K. pneumoniae* was described in 1997 which evolved to endemic spread of SHV-5 producing *K. pneumoniae* due to multiple plasmid transfer in the Dubrava University Hospital. The strains from 1997 and 2004 were not clonally related. Hospital hygiene measures should be applied in order to control the spread of epidemic strains through the hospital wards and the consumption of the broad-spectrum cephalosporins needs to be restricted to reduce the selection pressure which enables the proliferation of ESBL producers in hospital.

Key words: SHV-5 β-lactamase, ceftazidime, aztreonam, plasmids, *Klebsiella pneumoniae*

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INTRODUCTION

Plasmid-encoded resistance to broad-spectrum cephalosporins and aztreonam is becoming a widespread phenomenon in clinical medicine. These antibiotics are inactivated by an array of different extended-spectrum β-lactamases (ESBLs), which have evolved by point mutations of parental TEM or SHV β-lactamases [1-2]. In a previous study conducted during 1994-1995, SHV-2 was a more prevalent type of β-lactamase in *K. pneumoniae* isolates from Dubrava Hospital than SHV-5 which was also detected in some isolates [3-4]. A high prevalence of ESBLs in Dubrava Hospital (20%) in 1994-1995 required further investigation. In this study, β-lactamases from 42 *K. pneumoniae* strains collected in Dubrava Hospital in 1997 and 15 in 2004 were characterized in order to study the evolution of plasmid-encoded resistance to expanded-spectrum cephalosporins and aztreonam in that hospital over a prolonged time period. The aim of this study was to investigate the temporal perspective of resistance due to ESBLs in Dubrava University Hospital. The antibiotic susceptibility patterns, β-lactamase types, plasmid profiles and PFGE (pulsed field gel electrophoresis) types of the strains from two study periods (1997 and 2004) were compared. These results were later analyzed with regard of what was obtained in the earlier study (1994-1995), in order to show the temporary aspect of the resistance evolution due to ESBLs in the above mentioned hospital.

MATERIALS AND METHODS

Strains

Forty-two ceftazidime-resistant consecutive clinical isolates of *K. pneumoniae* (one from each patient) were collected in 1997 from January until May and from November until December from various clinical specimens (urine, bronchoalveolar aspirate, wounds, etc.) and from all wards in the Dubrava University Hospital in Zagreb which is one of the largest university hospitals in Croatia with 610 hospital beds receiving patients from all regions in Croatia. Another 15 isolates were collected in August 2004. The strains were identified by conventional biochemical tests.

Susceptibility tests

Disk-diffusion test: Susceptibility to cefoxitin, tetracycline, chloramphenicol and sulfamethoxazole/trimethoprim (cotrimoxazole) was determined by disk-diffusion method. The test was performed and interpreted by standard procedures of the National Committee for Clinical Laboratory Standards [5].

Minimum inhibitory concentrations (MICs): MICs of amoxicillin, amoxycillin/clavulanate, piperacillin/tazobactam, cephalexin, cefuroxime, ceftazidime, ceftazidime/clavulante, cefotaxime, ceftriaxone, ceftibuten, cefepime, cefpirome, aztreonam, imipenem, meropenem, gentamicin and ciprofloxacin were determined by a twofold microdilution technique using microtiter plates and Mueller-Hinton broth inoculated with 5 x 10^5 CFU/ml [6]. Clavulanic acid was added to amoxycillin and ceftazidime and tazobactam to piperacillin in the fixed concentration of 4 mg/L.

Transfer of resistance determinants

Klebsiella pneumoniae isolates were investigated for the transferability of their resistance determinants. Conjugation experiments were set up employing *Escherichia coli* A15 R^- strain free of plasmids and resistant to rifampicin as recipient [7]. The strain was kindly provided from Prof. Adolf Bauernfeind, Max von Pettenkofer Institute, Munich, Germany. Transconjugants were selected on the combined plates containing ceftazidime (2 mg/L) and rifampicin (256 mg/L). The frequency of tranconjugation was expressed relative to the number of donor cells.

Characterization of β-lactamases

ESBLs were detected in *K. pneumoniae* isolates by double-disk synergy test. A central disk of amoxicillin/clavulanate was surrounded by disks of cefotaxime, ceftriaxone, ceftazidime and aztreonam at the distance of 2.5 cm on Mueller-Hinton agar plate previously inoculated with the test organism [8]. Distortion of the inhibition zones around cephalosporin and aztreonam disks towards central disk was considered as a positive result.

For the preparation of β-lactamases, cells were harvested from overnight Brain-Heart Infusion broth cultures (300 ml) by centrifugation, washed and resuspended in glycine buffer and β-lactamases were released by sonication. Cell debris was removed by centrifugation. Supernatant was used as crude enzyme.

Isoelectric focusing was performed on polyacrylamide gels (acrylamide 7%, bis-acrylamide 0.2%) containing ampholines with a pH range of 3.5 to 10. The β-lactamases were detected by staining the gel with nitrocefin (100 mg/ml), following IEF [9]. β-lactamases of known pI (isoelectric point) were used as standards: TEM-1, TEM-2, SHV-1, SHV-2, SHV-3, SHV-4 and SHV-5.

The crude β-lactamase extracts were used to evaluate their ability to hydrolyze β-lactam antibiotics (cefoxitin, ceftriaxone, cefotaxime, ceftazidime, aztreonam and imipenem) by microbiological method. Antimicrobial disks were impregnated with crude extract from different isolates. The inhibition zones produced by them, and by non-impregnated disks against *E. coli* ATCC 25922 were compared. A reduction of the inhibition zone with the impreg-

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nated disk was considered to be evidence for β-lactamase activity against the corresponding antimicrobial agent [10].

Template DNA for polymerase chain reaction (PCR) was extracted by the alkaline lysis method. Bla_{SHV} genes were amplified as described previously [11]. The primers 5' CGCCGGGTTATTCTTATTTGTCGC-3' and 5' TCTTTCCGATGCCGCCGCCAGTCA-3' were used for the synthesis of the 1016 bp amplicon. The PCR conditions employed were as follows: 94° for 5 min, the 30 cycles consisting of 95°C for 30 s, 68°C for 30 s, and 72°C for 50s each. The samples were run in 1% agarose gel, stained with ethidium bromide and the amplicons were visualized under UV light.

DNA sequencing was performed using an ABI PRISM 377 Genetic Analyser (Applied Biosystems) only on the transconjugants originating from 1997. Using the primers pairs listed above, all amplicons of the bla_{SHV} genes spanned the entire open reading frame. All amplicons were sequenced on both strands along their entire length. The software Bioedit was used for sequence alignments and analysis [12].

Plasmid preparation

Plasmid DNA was extracted by the alkaline lysis procedure and subjected to electrophoresis in 0.8% agarose gel in TBE buffer [13]. After staining with ethidium bromide, the DNA was visualized by ultraviolet light. Transconjugant plasmid DNA from strains with different resistant markers was analyzed by restriction digestion with EcoRI.

Molecular typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA

Isolation of chromosomal DNA was performed as described by Kaufman *et al* [14]. The macrorestriction was performed with *Xba*I enzyme for 3 h at 37°C. Restriction fragments of DNA were separated by PFGE with a CHEF-DRII apparatus (Bio-Rad Laboratories) through 1% pulsed-field certified agarose (*Bio-Rad*) at a field strength of 6 V/cm for 20 h at 11°C**;** with pulses from 5 to 50 s in 0.5 TBE buffer with thiourea (50mM, *Sigma*). A lambda ladder (Roche) was used as the molecular size marker. After electrophoresis, gels were stained with ethidium bromide, rinsed, and photographed under UV light.The PFGE patterns were compared following the criteria of Tenover and coleagues for bacterial strain typing [15] and analyzed by computer software (*GelComparII*). The patterns obtained were compared by clustering methods (unweighted pair-group method with arithmetic averages) using the *Dice* coefficient. An optimization of 0.5% and position tolerance of 3,% were applied during the comparison of PFGE fingerprinting patterns.

RESULTS

Susceptibility testing

All except two strains from 1997 and two from 2004 displayed higher level of resistance to ceftazidime and aztreonam compared to cefotaxime and ceftriaxone (CAZ phenotype). Two strains from 1997 and two from 2004 showed equal levels of resistance to ceftazidime and cefotaxime and could not be assigned to either phenotype. MIC distribution is shown in *Table 1*. The isolates from two study periods have shown similar resistance patterns. Most isolates from both study periods were resistant to gentamicin. No resistance to ciprofloxacin, imipenem and meropenem was observed among our strains.

Transferability of resistance determinants

Thirty-three of 42 *K. pneumoniae* strains from 1997 and five from fifteen strains from 2004 trasferred ceftazidime resistance to *E. coli* recipient. Properties of transconjugant strains are shown in *Table 2*.

Characterization of β-lactamases

The production of ESBLs was detected by ≥8 fold reduction in ceftazidime MIC in the presence of clavulanate and by double-disk synergy test in all strains from both study periods.

Isoelectric focusing revealed a β-lactamase with the pI of 8.2 in the both *K. pneumoniae* donor strains and their transconjugants from both collections as shown in *Table 2*. The donor strains possessed an additional enzyme with the pI of 7.6 which corresponds to that of the chromosomaly encoded SHV-1 typical for *K. pneumoniae* strains.

Crude β-lactamases from transconjugant strains from both study periods antagonized the activities of disks containing ceftazidime, cefotaxime, ceftriaxone, and aztreonam (30 μg). Enzymes did not affect the inhibition zones around cefoxitin and imipenem disks.

All *K. pneumoniae* strains and their *E. coli* transconjugants from 1997 and 2004 yielded an amplicon of 1016 bp with primers specific for genes encoding SHV β-lactamases as shown in *Table 2*.

Sequencing of bla_{SHV} genes obtained from strains collected in 1997 revealed two mutations: at the Ambler amino-acid positions 238 (GGC→AGC, glycine→serine) typical for all SHV ESBLs, and at the Ambler amino-acid positions 240 (GAG→AAG, glutamic acid→ lysine) typical for SHV-5 β-lactamase (*Table 2*). Mutations typical for other SHV- ESBLs with the pI of 8.2 were not found. Based on sequencing of bla_{SHV} genes, enzymes from all the tranconjugants were identified as SHV-5 β-lactamase. The sequencing of bla_{SHV} genes from transconjugants obtained in 2004 is not yet performed.

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TABLE 1 - *Comparative minimum inhibitory concentrations (MICs) for Klebsiella pneumoniae from 1997 and 2004. Concentrations necessary to inhibit 50 and 90% of the isolates and the percentage of resistant strains according to the National Committee for Clinical Laboratory Standards (NCCLS) are given.*

Antibiotic and the NCCLS breakpoint	Strain collection-1997 (n=42) MIC range	MIC_{50}	MIC_{90}	% of resistant strains	Strain collection-2004 (n=15) MIC range	MIC_{50}	MIC_{90}	% of resistant strains
amoxycillin (≥32)	>4096->4096	>4096	>4096	42/42 (100%)	>4096->4096	>4096	>4096	42/42 (100%)
amoxycillin/clavulanate (≥32/4	16/4->128/4	64/4	>128/4	42/42 (100%)	64/4-256/4	128/4	256/4	42/42 (100%)
cephalexin (≥32)[1]	32->1024	256	1024	42/42 (100%)	128->1024	256	>1024	15/15 (100%)
cefuroxime (≥32)	8->1024	64	>1024	35/42 (83%)	64->1024	128	>1024	15/15 (100%)
ceftazidime (≥32)	64->1024	512	>1024	42/42 (100%)	128->1024	256	>1024	15/15 (100%)
ceftazidime/clavulanate (32/4)	0.12/4-4/4	1/4	4/4	0/42 (0%)	0.06/4-2/4	1/4	2/4	0/15 (0%)
cefotaxime (≥64)	4->1024	32	128	11/42 (26%)	32->1024	128	256	13/15 (80%)
ceftriaxone (≥64)	8->1024	64	512	25/42 (60%)	32->1024	128	256	14/15 (93%)
ceftibuten (≥32)	2-64	16	64	19/42 (48.72)	2-64	16	64	19/42 (48.72)
aztreonam (≥32)	256->1024	>1024	>1024	42/42 (100%)	256->1024	512	>1024	15/15 (100%)
cefpirome (≥32)	0.5-64	8	32	8/42 (19%)	8-256	16	64	7/15 (46%)
cefepime (≥32)	2-64	16	64	11/42 (26%)	4-256	32	128	7/15 (46%)
imipenem (≥16)	0.06-0.25	0.25	0.25	0/42 (0%)	0.5-2	1	2	0/15 (0%)
meropenem (≥16)	0.0015-0.25	0.06	0.25	0/42 (0%)	0.06-0.25	0.12	0.25	0/15 (0%)
piperacillin/tazobactam (≥128/4)	4/4-32/4	8/4	32/4	0/42 (0%)	4-32	16	32	0/15 (0%)
gentamicin (≥8)	8->1024	64	256	42/42 (100%)	0.25-256	32	256	14/15 (93%)
ciprofloxacin (≥4)	0.03-0.5	0.12	0.25	0/42 (0%)	0.03-0.12	0.06	0.12	0/15 (0%)

[1] Breakpoint for cefaclor was applied.

Plasmids encoding β-lactamases

Most enzymes described in this investigation were encoded on large plasmids of 120 to >150 kb. Plasmid analysis after digestion with EcoRI showed that strains with different resistant markers harbored plasmids with different fingerprinting patterns (*Figure 1*). Plasmids from 1997 harbored resistance genes for tetracycline only in most strains whereas plasmids from 2004 contained resistance genes for gentamicin, chloramphenicol, tetracycline and cotrimoxazole as well.

Typing of Klebsiella pneumoniae strains

Five different PFGE types were found in collection from 1997. Thirty-eight strains belonged to type 1 and displayed more than 95% of molecular relatedness whereas 4 other strains showed different PFGE patterns (*Figure 2*). Type 1 comprised 10 different subtypes. Fifteen strains from 2004 were assigned into 5 different PFGE types (*Figure 2*). The strains from two study periods (1997 and 2004) were not clonally related.

DISCUSSION

In this study, an outbreak of nosocomial infections caused by SHV-5 producing *K. pneumoniae* was described between February and May 1997 which evolved into an endemic spread of the enzyme due to multiple plasmid transfer by the end of the year and has continued until 2004. The previous study from 1994-1995 found that SHV-2 β-lactamase, which is a cefotaximase according to the substrate profile, was more prevalent in Dubrava Hospital than SHV-5, but in 1997 it was completely replaced by SHV-5 which confers a high level ceftazidime and aztreonam resistance which persisted in that hospital for over a decade. The sequencing of β-lactamases from 2004 has not yet been performed but according to the isoelectric point, hydrolysis of β-lactam substrates and PCR, we concluded that enzymes from that period are most likely to be SHV-5 β-lactamase or its derivative. The persistence of SHV-5 β-lactamase for such a long period could be due to the antibiotic policy of the hospital, since emergence of mutation at the position 240, which is typical for SHV-5 and some other SHV- ESBLs is linked to selective pressure from the use of ceftazidime. Ceftazidime is the most widely prescribed expanded-spectrum cephalosporin in Croatian hospitals and apparently exerts selection pressure which enables proliferation and persistence of SHV-5-producing *Klebsiellae* in Dubrava University Hospital. SHV-5 β-lactamase with similar properties was previously described in Germany [16], Austria [17], Poland [18], Italy [19], France [20], United Kingdom [21], Greece [22], and Hungary [23] so it can be concluded that this type of β-lactamase is widespread in Europe. Apart from

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TABLE 2 - *Phenotypic and genetic characteristics of ESBLs produced by E. coli tranconjugants from 1997 and 2004.*

Strain N.	Isoelectric point	Resistance phenotype	Frequency of transconjugation	PCR[1]-SHV	Cotransferred R markers[2]	Codon for aminoacid at the aminoacid -position[3]		
						35 L→Q	238 G→S	240 E→K
Standard-SHV-1				+		CTA	GGC	GAG
Standard-SHV-5				+		CTA	AGC	AAG
Strains-1997								
4203	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
5023	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
5129	8.2	CAZ	10^{-7}	+	T	CTA	AGC	AAG
5166	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
5034	8.2	CAZ	10^{-5}	+	T, Gm	CTA	AGC	AAG
5603	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
5972	8.2	CAZ	10^{-7}	+	T	CTA	AGC	AAG
5859	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
5827	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
542/2	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
6359	8.2	CAZ	10^{-7}	+	T	CTA	AGC	AAG
469	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
6216	8.2	CAZ	10^{-5}	+	T, Gm	CTA	AGC	AAG
6274	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
6275	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
6306	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
6823	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
549	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
6504	8.2	CAZ	10^{-7}	+	T	CTA	AGC	AAG
470	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
7467	8.2	CAZ	10^{-8}	+	none	CTA	AGC	AAG
7557	8.2	CAZ	10^{-7}	+	T	CTA	AGC	AAG
7700	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
112	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
161	8.2	CAZ	10^{-6}	+	T,Gm	CTA	AGC	AAG
7788	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
272	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
303	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
569	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
887	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
892	8.2	CAZ	10^{-8}	+	T	CTA	AGC	AAG
21786	8.2	CAZ	10^{-5}	+	T	CTA	AGC	AAG
Strains 2004								
23217	8.2	CAZ	10^{-5}	+	C,Smx, T, Gm	ND[4]	ND	ND
24137	8.2	CAZ	10^{-5}	+	C, Smx, T,Gm	ND	ND	ND
24215	8.2+5.4	CAZ	10^{-4}	+	C, Smx, Gm	ND	ND	ND
24299	8.2	CAZ	10^{-5}	+	C, Smx, T,Gm	ND	ND	ND
24879	8.2	CAZ	10^{-6}	+	C, Smx, T,Gm	ND	ND	ND

[1] + the strains yielded amplicon with primeres specific for SHV β-lactamases
[2] T-tetracycline,Gm- gentamicin, C-chloramphenicol, Smx- sulphamethoxazole
[3] Amino-acids according to the Ambler numbering scheme. L-leucine, Q-glutamine, E, -glutamic acid, K-lysine, G-glycine, S-serine.
[4] ND- not determined

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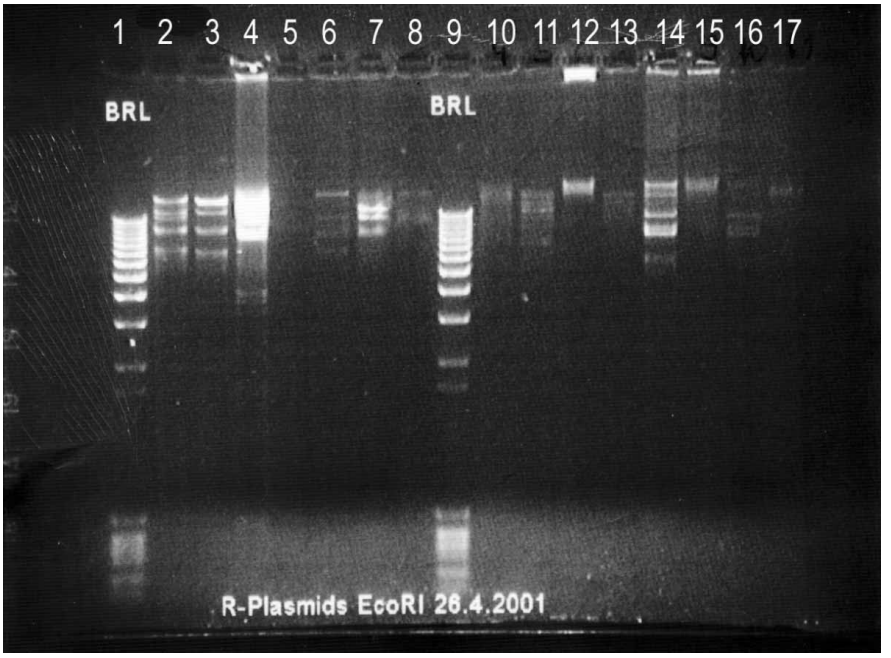

FIGURE 1 - Plasmid profiles of *E. coli* transconjugant strains (strains from 1997). Lane:
1. BRL size marker
2. *E. coli* A15 R+470
3. *E. coli* A15 R+6275
4. *E. coli* A15 R+303
5. *E. coli* A15 R+5972
6. *E. coli* A15 R+549
7. *E. coli* A15 R+272
8. *E. coli* A15 R+569
9. *E. coli* A15 R+542/2
10. BRL size marker
11. *E. coli* A15 R+7467
12. *E. coli* A15 R+7557
13. *E. coli* A15 R+21786
14. *E. coli* A15 R+892
15. *E. coli* A15 R+569

Europe, SHV-5 β-lactamase was found in Australia [24], Canada [25], Mexico [26] and Thailand [27].

The strains were in high percentage resistant to non-β-lactam antibiotics such as aminoglycosides, tetracyclines, chloramphenicol, and cotrimoxazole. Most transconjugant strains from 1997 harbored resistance genes only for tetracycline except of ESBL encoding genes whereas transconjugants from 2004 possessed the resistance genes for gentamicin, chloramphenicol, and cotrimoxazole as well. This finding is in concordance with the situation described in other countries where cotransfer is frequent [19-23] and may enhance the spread of ESBLs due to selective pressure exerted by other drugs, especially aminoglycosides which are used extensively in intensive care settings. Typically the genes for ESBLs reside on large plasmids, which also carry resistance genes for other classes of antimicrobials. In our previous study from 1994-1995, bla_{SHV-5} genes were encoded on large multiresistant plasmids of 120-150 kb [3]. The SHV-5 encoding plasmids from 1997 and 2004 were of the similar size. The plasmid location of ESBL genes strongly facilitated their spread among *K. pneumoniae* strains from different wards of the hospital. Persistence of SHV-5 β-lactamase for such a long period was not due to persistence of the same clones because the isolates from different study periods were shown not to be clonally related by PFGE. This supports the thesis that new clones of *K. pneumoniae* harboring SHV-5 β-lactamase replaced those with SHV-2 from the previous study period (1994-1995) and had not evolved from them by acquisition of a single point mutation in the SHV-2-encoding gene.

Similarly to many other broad-spectrum β-lactamases, the SHV-5 enzyme did not affect resistance to β-lactams with a C6 or C7 alpha-methoxy constituent, although four clinical strains producing these enzymes had decreased susceptibility to cefoxitin. This might be explained by a defect in some of their outer membrane proteins, resulting in an impaired membrane permeability [20,22]. The fact that the resistance to cefoxitin was never transferable indicates that the decreased susceptibility of donors was not related to the mechanisms responsible for the resistance to the oxymino β-lactams. Fourth generation cephalosporins: cefepime and cefpirome were more active than third generation cephalosporins although they are hydrolyzed by some class A β-lactamases as well, including SHV-5 which was produced by our isolates.

On the therapeutic level, combinations of third-generation cephalosporins with a β-lactamase inhibitor should theoretically be useful for treating patients infected with our strains which produce the SHV-5 β-lactamase since all the enzymes were inhibited by clavulanate. However, the clinical efficacy of combinations of β-lactam antibiotics with a β-lactamase inhibitor is still controversial because of a pronounced inoculum effect [28]. Furthermore, hyperproduction of ESBLs and porin loss can lead to reduced activity of such combinations [29]. All our strains were susceptible to the combination of piperacillin with tazobactam but resistant to combinations of amoxycillin with clavulanic acid, contrary to the results obtained by other investigators who rarely reported resistance to amoxycillin/clavulanate for SHV-5 producers [23]. Ceftazidime combined with

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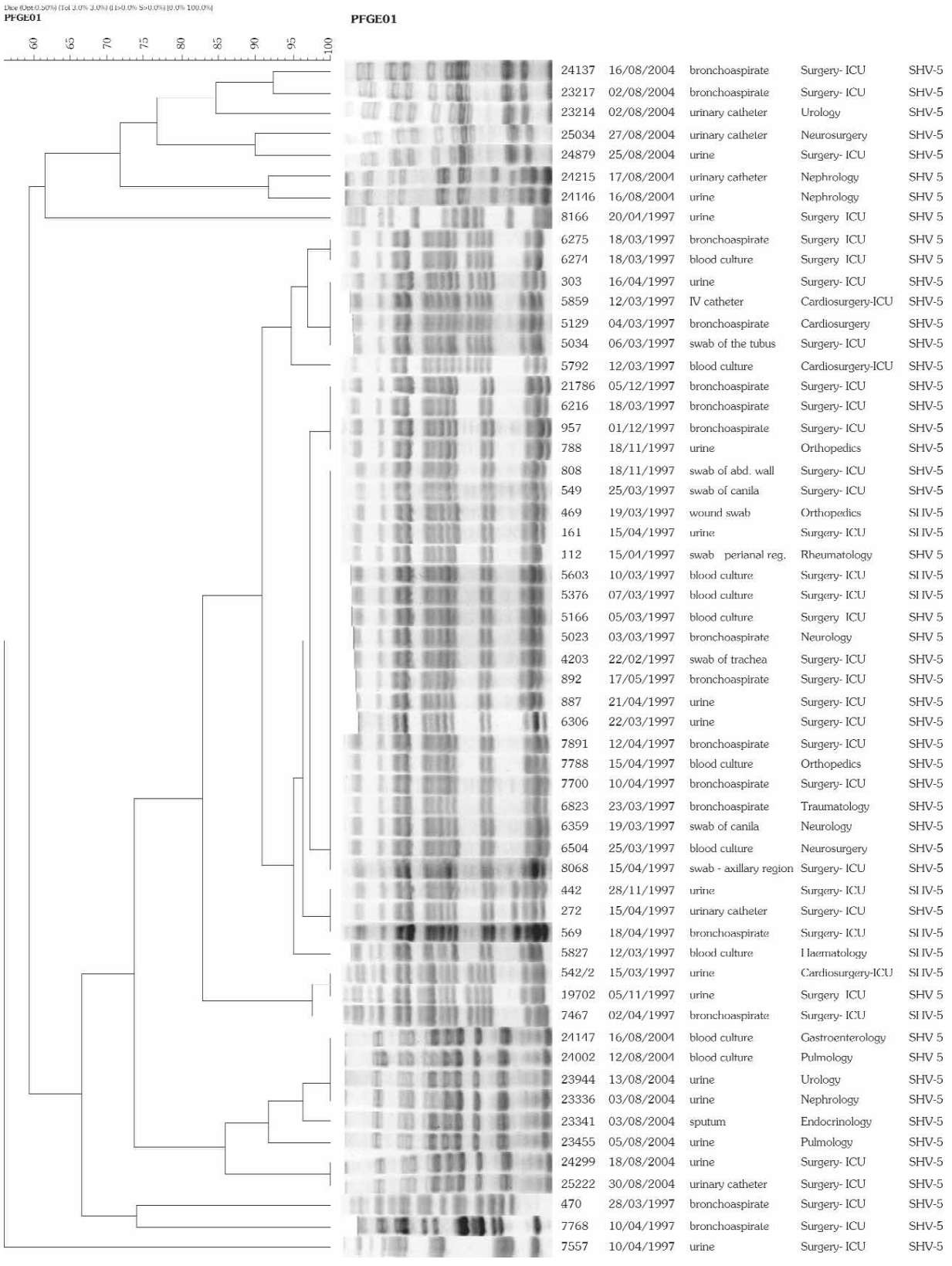

FIGURE 2 - Comparison of PFGE types of *K. pneumoniae* isolates from 1997 and 2004. Date of isolation, clinical specimen, hospital ward and type of β-lactamase are shown.

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clavulanate, which strongly inhibited all our strains, is not used at the clinical level although some combinations of expanded-spectrum cephalosporins with inhibitors such as cefoperazone/sulbactam are already marketed in some countries (France) [30].

Fluoroquinolone resistance among our isolates was not observed, and therefore these molecules could be considered as a therapeutic option although they can select quinolone-resistant *Acinetobacter baumannii* strains and enterococci which are difficult to treat pathogens in hospitals [30]. Carbapenems remain the antibiotics of choice for the treatment of infections caused by our SHV-ESBL-producing *K. pneumoniae* isolates. There was no resistance to imipenem or meropenem observed among our strains, but since the ESBLs conferring resistance to imipenem have already been described elsewhere [32-32] it is likely to expect that such strains would appear in Croatia in the future as well.

This study proved the epidemic and endemic presence of *K. pneumoniae* harboring SHV-5 β-lactamase in the Dubrava Hospital, which is not uncommon. Intensive care units were the most important reservoir of ESBL producers, as expected. Most isolates in both study periods originated from the respiratory and urinary tracts. Hospital hygiene measures should be applied to control the spread of epidemic strains throughout the hospital wards, and the consumption of the broad-spectrum cephalosporins needs to be restricted to reduce the selection pressure which enables the proliferation of ESBL producers in hospital. Ceftazidime, as a slowly penetrating antibiotic, has a higher potential of selecting mutations compared to cefotaxime or ceftriaxone and for that reason should be avoided [30]. We conclude that pathogens producing plasmid-mediated ESBLs, such as SHV-5-producing *K. pneumoniae*, may cause severe therapeutic and epidemiological problems as soon as they invade hospitals and spread among the patients and wards.

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