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Characterization of Extended-Spectrum β-Lactamases in Enterobacteriaceae Causing Nosocomial Infections in a Zagreb University Hospital

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Summary

The bacteria producing extended-spectrum β-lactamases (ESBLs) are increasingly reported. Production of ESBLs by Gram-negative bacteria is the major mechanism of resistance to oxymino-cephalosporins and aztreonam. The aim of the present study was to characterize ESBLs produced by *Enterobacteriaceae*, collected during 2003-2005 in a University Hospital in Zagreb, and to determine the risk factors associated with nosocomial infections due to them. 76 isolates of *Enterobacteriaceae* were included in the study. Antibiotic susceptibility testing was performed by disk-diffusion and broth microdilution method according to CLSI. β-lactamases were characterized by PCR and sequencing of bla_{ESBL} genes. Plasmids were extracted by alkaline lysis method and digested with *Eco*RI enzyme. Most of the strains displayed CAZ phenotype meaning a higher level of resistance to ceftazidime compared to cefotaxime and ceftriaxone. 50 strains produced SHV-ESBL, 28 TEM and 8 CTX-M β-lactamase. Sequencing of bla_{SHV} genes from representative strains revealed SHV-5 β-lactamase in 6 strains whereas sequencing of bla_{CTX-M} genes identified CTX-M-3 β-lactamase in 3 and CTX-M-15 in 5 strains. Strains were assigned to groups from A to F according to plasmid fingerprinting. The spread of SHV-5-producing strains throughout the hospital units could be due to selective pressure of ceftazidime which is widely prescribed in our hospital thus favoring survival of strains possessing a mutation at the Ambler position 240 responsible for ceftazidime and aztreonam resistance.

Key words: **Extended-spectrum β-lactamases, ceftazidime, plasmids, SHV-5 β-lactamase, CTX-M-3 β-lactamase, CTX-M-15 β-lactamase.**

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 from parental TEM-1, TEM-2 and SHV-1 β-lactamases by point mutations that alter the configuration of the active enzyme site and expand their spectrum of activity [1-2]. ESBL-producing Enterobacteriaceae are often associated with outbreaks of nosocomial infections all over the world which are difficult to control [1-8]. In most cases TEM and SHV-ESBLs are associated with nosocomial outbreaks [2-9].

In recent decades a new family of ESBLs with predominant activity against cefotaxime (CTX-M β-lactamases) has emerged in hospital and community settings [10]. In contrast to TEM or SHV-ESBLs, CTX-M β-lactamases are native ESBLs and are derived from the chro-

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mosomal β-lactamases of the genus *Kluyvera* [11].

Recently an increase of ESBL-producing Enterobacteriacea causing nosocomial infections in Sisters of Mercy University Hospital (Zagreb) was observed. The aim of the present study was to characterize ESBLs produced by *Enterobacteriaceae*, associated with nosocomial infections, collected during 2003-2005 in a University Hospital in Zagreb and to determine the risk factors associated with the nosocomial infections they caused.

MATERIAL AND METHODS

A total of 76 isolates of *Enterobacteriaceae*, collected from 2003 - 2005 in the Sisters of Mercy Hospital, were positive for ESBL by double-disk synergy test (DDST). DDST was performed on all enterobacterial isolates during the study period. The collection comprised 37 *Klebsiella pneumoniae*, 26 *Escherichia coli*, 5 *Klebsiella oxytoca*, 3 *Enterobacter cloacae* and 5 *Proteus mirabilis* isolates. The strains were causative agents of 5 different types of nosocomial infections, defined in accordance with the Centers for Disease Control and Prevention criteria [12].

Minimum inhibitory concentrations (MICs) of a wide range of antibiotics were determined by a twofold microdilution technique according to CLSI [13]. Susceptibility to co-trimoxazole, tetracycline, chloramphenicol and norfloxacin was determined by disk-diffusion test.

ESBL production was determined by double-disk synergy test [14] and confirmed by at least 8-fold reduction (three dilutions) of ceftazidime (MIC) by clavulanate.

Enterobacteriaceae were investigated for the transferability of their resistance determinants. Conjugation experiments were set up employing *E. coli* A15 R^- strain free of plasmids and resistant to rifampicin [15]. Transconjugants were selected on the combined plates containing ceftazidime (1 mg/L) and rifampicin (256 mg/L). The frequency of conjugation was expressed relatively to the number of donor cells.

The presence of bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{PER-1} genes was determined by polymerase chain reaction (PCR) using primers and conditions as described previously [16]. Primers used in this study were: MN-1 (5' CGC CGG GTT ATT CTT ATT TGT CGC-3') and MN-2 (5' TCT TTC CGA TGC CGC CGC CAG TCA-3')[16] OT-3 (5'-ATG-AGT-ATT-CAA-CAT-TTC-CG-3') and OT-4 (CCA-ATG-CTT-AAT-CAG-TGA-GG-3')[17], MA-1 (5'-SCS-ATG-TGC-AGY-ACC-AGT-AA-3') and MA-2 (5'-CGC-CRA-TAT-GRT-TGG-TGG-TG-3')[18] and PER-1-F (5'GGG- ACA -(A/G) TC- (G/C)(G/T)-ATG- AAT-GTC A and PER-1R: 5' gg (C/T) (G/C) GCT-TAG ATA-GTG-CTG-AT [19]. Primers IS26F (5'-GCG-GTA-AAT-CGT-GGA-GTG-AT-3) and IS26R (5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3') [18] were used to amplify IS26 insertion sequence in CTX-M producing isolates [18]. Strains positive for CTX-M β-lactamases were further tested with primers specific for CTX-M groups 1, 2 and 9 to amplify the whole coding sequence. The PCR products were visualized by agarose gel electrophoresis, after staining with ethidium bromide.

Amplicons from representative strains producing TEM, SHV and CTX-M β-lactamases were column purified (Quiagen, Quiaquick purification kit, Hilden, Germany) and subjected to DNA sequencing using an ABI PRISM 377 Genetic Analyzer (Applied Biosystems). After sequencing of the PCR products obtained, we used the BLAST program (multiple sequence alignment, pairwise comparison of sequences) to look for sequence homology with the other bla_{ESBL} genes. Bla_{SHV} genes, not subjected to sequencing were tested by PCR *Nhe* test to distinguish between bla_{SHV-1} and $bla_{SHV-ESBL}$ [16].

Plasmid DNA was extracted from 35 strains that yielded tranconjugants in the mating experiments by alkaline lysis method [20] and subjected to electrophoresis in 0.8% agarose gel. After staining with ethidium bromide, the DNA was visualized by ultraviolet light. The sizes of plasmids in the isolates were estimated from a standard curve of the logarithm of a molecular size of four plasmids with molecular weights of 148, 64, 36 and 7 kb from *E. coli* NTCC 50192 against the logarithm of relative mobility. Plasmids were digested with *EcoRI* endonuclease.

PFGE of *Xba*-digested genomic DNA was performed with a CHEF-DRIII system (Bio-rad) as described previously [21] on the strains producing CTX-M β-lactamases. The images were processed using the GelCompar software, and a dendrogram was computed after band intensity correlation using global alignement with 2% optimization and UPGMA (unweighted pair-group method using arithmetical averages) clustering [22].

Statistical analysis

The statistical analysis determined the significance of differences between the parameters of experimental and control groups using the Mann-Whitney U test and Hi Quadrat test. To determine the risk factors for development of nosocomial infection caused by *Enterobacteriaceae*-producing ESBLs, logistic regression was done. The experimental group contained 60 patients with nosocomial infection caused by *Enterobacteriaceae* harboring ESBLs whereas the control group consisted of 32 patients with hospital infection caused by non-ESBL-producing *Enterobacteriaceae* hospitalized in the same period as those in the experimental group.

RESULTS

Antibiotic susceptibilities

Very high resistance rates to fluoroquinolones (90% norfloxacin, 76% ciprofloxacin), cotrimoxazole

(90%) and gentamicin (84%) were observed in *K. pneumoniae* (*Table 1*). In contrast, most *E. coli* strains were susceptible to fluoroquinolones (70% to norfloxacin and 96% to ciprofloxacin). Resistance rates for aminoglycosides were high in all tested species. No resistance was observed to carbapenems (*Table 1*). Resistance phenotypes are shown in *Table 2*. Most isolates displayed CAZ phenotype. CTX phenotype was associated with CTX-M β-lactamases.

Conjugation

Resistance to third-generation cephalosporins was transferred by conjugation to *E. coli* recipient strain by 35 out of 76 strains (46%). The highest percentage of transferability of resistance to expanded-spectrum cephalosporins was noticed for *K. oxytoca* (80%) followed by *E. cloacae* (66%), *E. coli* (58%) and *K. pneumoniae* (38%). The frequency of conjugation ranged from 10^{-3} to 10^{-5}. Various resistance markers to non β-lactam antibiotics were co-transferred alongside cephalosporin resistance.

Characterization of β-lactamases

Thirty-three *K. pneumoniae* strains and their respective transconjugants yielded a 1016 bp product with primers specific for SHV β-lactamases. Sequencing of three representative bla_{SHV} genes (32, 47, 50) revealed the presence of SHV-5 β-lactamase (*Table 2*). At the position 238 glycin was substituted for serin (GGC→AGC) whereas at the position 240 glutamic acid was replaced by lysine (GAG→AAG). Six *K. pneumoniae* strains were found to produce CTX-M β-lactamases (two CTX-M-3 and four CTX-M-15). Two *K. pneumoniae* strains and their transconjugants gave only a product of 858 bp with primers specific for TEM β-lactamases (*Table 2*) whereas five strains possessed bla_{TEM} gene combined with bla_{SHV} gene.

E. coli isolates were mostly associated with TEM-ESBLs (13), eleven possessed SHV-ESBLs, but only two strains were found to possess CTX-M β-lactamases (one corresponded to CTX-M-3 and the other to CTX-M-15). IS26 was found upstream of $bla_{CTX-M-3}$ genes. The bla_{SHV} genes of two representative *E. coli* strains were sequenced and corresponded to bla_{SHV-5} gene (*Table 2*). Two *E. cloacae* and four *K. oxytoca* strains were SHV-ESBL producers. The bla_{SHV} gene of one representative *K. oxytoca* strain has been sequenced and was identical with bla_{SHV-5} gene. Five *P. mirabilis* strains were identified as TEM-52 producers. PER-1 β-lactamase was not found.

Bla_{SHV} genes, not subjected to sequencing, were identified as $bla_{SHV-ESBL}$ by PCR *Nhe* test.

Characterization of plasmids encoding ESBLs

According to restriction profiles, plasmids from transconjugant strains were assigned to groups A to F. All tested strains harbored large multiresistant plasmids ranging in size from 110 to 150 kb.

TABLE 1 - *MICs of various antibiotics for ESBL-producing E. coli and K. pneumoniae strains.*

Antibiotic and resistance breakpoint (mg/L)		MIC range	% of resistant strains
E. coli			
Co-amoxiclav	≥32/4	8/4-512/4	16/26 (61%)
Piperacillin/ tazobactam	≥128/4	2/4-64/4	0/26 (0%)
Cefuroxime	≥ 32	32-512	26/26 (100%)
Ceftriaxone	≥ 64	2-512	8/26 (31%)
Cefotaxime	≥ 64	1-512	8/26 (31%)
Ceftazidime	≥ 32	1-512	18/26 (69%)
Caz/clavulanate	≥32/4	0.06/4-4/4	0/26(0%)
Cefepime	≥ 32	0.5-32	3/26 (11%)
Cefoxitin	≥ 32	0.5-8	0/26 (0%)
Aztreonam	≥ 32	32-512	26/26 (100%)
Gentamicin	≥ 16	0.5-256	20/26 (77%)
Ciprofloxacin	≥ 4	0.005-64	1/26 (4%)
Imipenem	≥ 16	0.125-0.5	0/26 (0%)
Meropenem	≥ 16	0.01-0.125	0/26 (0%)
K. pneumoniae			
Co-Amoxiclav	≥32/4	16/4-256/4	31/37 (84%)
Piperacillin/ tazobactam	≥128/4	8/4-512/4	14/37 (38%)
Cefuroxime	≥32	32-512	37/37 (100%)
Ceftriaxone	≥64	4-512	26/37 (70%)
Cefotaxime	≥64	2-512	21/37 (57%)
Ceftazidime	≥32	8-512	32/37 (86%)
Caz/clavulanate	≥32/4	0.125/4-2/4	0/37 (0%)
Cefepime	≥32	0.5-512	10/37 (27%)
Cefoxitin	≥32	2-128	1/37 (2.7%)
Aztreonam	≥32	32-512	37/37 (100%)
Gentamicin	≥16	0.25-512	31/37 (84%)
Ciprofloxacin	≥4	0.01-256	28/37 (76%)
Imipenem	≥16	0.125-1	0/37 (0%)
Meropenem	≥16	0.03-0.5	0/37 (0%)

Genotyping of the isolates by PFGE

All CTX-M-producing *E. coli* and four *K. pneumoniae* isolates showed distinct PFGE patterns and were not clonally related. Two *K. pneumoniae* isolates (36 and 28) showed identical PFGE patterns (*Figure 1*).

TABLE 2 - *Resistance phenotypes of ESBL positive Enterobacteriaceae. Abbreviations: CAZ-ceftazidime, CTX-cefotaxime, CRO-ceftriaxone, AZT-aztreonam, Te-tetracycline, Sxt-sulfamethoxazole/trimethoprim, C-chloramphenicol, Gm-gentamicin.*

				MIC (mg/L)				
Species	**Hospital unit**	**Resistance phenotype**	**Type of ESBL**	**CAZ**	**CTX**	**CRO**	**AZT**	**Associated resistance markers**
K. pneumoniae								
1	Internal	CAZ	SHV	256	16	16	256	Te, Sxt, C, Gm
2	Internal	CAZ	SHV	>512	128	128	>512	Te, Sxt, C, Gm
5	Internal	CAZ	TEM, SHV	64	16	16	256	Te, Sxt, C, Gm
7	Pediatric	CAZ	SHV	512	16	32	256	Te
11	Neurology	CAZ	SHV	128	32	32	64	Te, Sxt, C, Gm
12	Surgery	CAZ	SHV	>512	64	64	>512	Te, Sxt, C, Gm
13	Neurology	CAZ	SHV	128	32	64	64	Te, Sxt, C, Gm
17	Internal	CAZ	SHV	64	8	16	128	Te, Sxt, C, Gm
18	Internal	CTX	CTX-M-15, SHV	64	512	512	128	Te, Sxt, C
21	Surgery	CAZ	SHV	>512	32	64	>512	Te, Sxt, C, Gm
23	Neurology	CAZ	SHV	512	64	64	256	Te, Sxt, C, Gm
25	Surgery	CAZ	SHV	>512	128	64	512	Te, Sxt, C, Gm
27	Pediatric	CAZ	SHV	512	64	64	512	Te, Ge
28	ICU	CTX	CTX-M-3, SHV	8	256	512	32	Te, Sxt, C, Gm
30	Neurology	CAZ	SHV	128	32	32	256	Te, Sxt, C, Gm
32	Neurology	CAZ	SHV-5	>512	64	128	>512	Te, Ge
33	ICU	CAZ	SHV	256	32	32	256	Te, Sxt, C, Gm
34	ICU	CAZ	SHV	128	16	32	64	Te, Sxt, C, Gm
36	Surgery	CTX	CTX-M-15	8	256	512	32	Te, Sxt, C, Gm
38	Urology	CAZ	SHV	512	32	64	>512	Te, Sxt, C, Gm
41	ICU	CAZ	TEM	128	32	32	128	Te
42	Internal	CAZ	SHV	512	64	64	512	Te, Sxt, C, Gm
44	ICU	CTX	CTX-M-15,SHV	32	256	512	32	Te, Sxt, C, Gm
46	Internal	CAZ	SHV	512	64	64	256	Te, Ge
47	Neurology	CAZ	SHV-5, TEM-1	256	64	64	256	Te, Sxt, C, Gm
48	Internal	CAZ	TEM	256	32	64	>512	Te, Sxt, C, Gm
50	ICU	CAZ	SHV-5	128	32	32	64	Te, Sxt, C, Gm
52	Internal	CAZ	SHV, TEM	512	64	64	256	Te, Sxt, C, Gm
56	ICU	CAZ	SHV,TEM	128	32	64	512	Te, Sxt, C
60	Neurology	CTX	CTX-M-15	16	128	512	64	Te, Sxt, C, Gm
62	Surgery	CAZ	SHV	64	8	16	64	Te, Gm
65	ICU	CAZ	SHV	256	16	32	512	Te, Sxt, C, Gm
70	Surgery	CTX	CTX-M-3,SHV	8	128	512	64	Te, Sxt, C, Gm
86	Urology	CAZ	SHV	16	2	2	64	Te, Sxt, Gm
87	Internal	CTX	SHV, TEM	64	512	512	64	Sxt, C, Gm
92	Surgery	CAZ	SHV	128	16	32	256	Te, Sxt, C, Gm
93	Neurosurgery	CTX	SHV	64	512	512	128	Sxt, C, Gm
E. coli								
3	Internal	CAZ	TEM	128	4	2	128	Te, Sxt, C, Gm
6	Pediatric	CAZ	SHV	16	2	2	16	Te, Gm
8	Pediatric	CTX	TEM	64	512	512	32	Te, Sxt, Gm

TABLE 2 - *Continued*

Species	Hospital unit	Resistance phenotype	Type of ESBL	MIC (mg/L) CAZ	CTX	CRO	AZT	Associated resistance markers
9	Internal	CAZ	TEM	32	4	1	32	Te, C, Gm
14	Internal	CAZ	SHV-5	16	1	2	32	Te
16	Pediatric	CAZ	TEM	16	4	2	16	Te, Gm
20	Gynecology	CAZ	SHV	64	2	4	16	Te, Gm
24	Internal	CTX	CTX-M-15	1	64	64	4	Te, Sxt
39	Pediatric	CAZ	SHV	32	8	8	128	Te
40	Pediatric	CAZ	SHV-5	256	32	32	256	Te, Gm
43	Internal	CAZ	TEM	64	8	8	64	Te, Gm
49	Neurology	CTX	CTX-M-3,TEM-1	8	128	64	8	Te, Gm
54	Pediatric	CAZ	SHV	512	16	32	256	Te, Gm
57	Pediatric	CTX	TEM	32	128	128	64	Te, Gm
63	Pediatric	CAZ	TEM	32	4	4	32	Te, Gm
64	Internal	CAZ	TEM	512	128	128	512	Gm
67	Pediatric	CAZ	SHV	64	16	16	512	Te, Gm
75	Pediatric	CAZ	SHV	128	16	8	256	Te, Gm
76	Urology	CAZ	TEM	16	2	2	32	Te, C, Gm
77	Neurology	CAZ	SHV	64	4	4	32	Gm
78	Neurology	CAZ	TEM	512	64	64	512	Te, Gm
79	Pediatric	CAZ	TEM	32	2	2	32	Sxt, Gm
80	Pediatric	CAZ	SHV	>512	64	64	>512	Te, Gm
81	Pediatric	CAZ	SHV	128	32	32	256	Te
82	Internal	CAZ	TEM	16	4	4	32	Te, Gm
98	Internal	CTX	TEM	4	256	256	16	-
K. oxytoca								
4	Pediatric	CAZ	SHV	64	8	4	128	Te, Sxt
10	Pediatric	CAZ	SHV	64	8	8	64	Te, Sxt, Gm
15	Pediatric	CAZ	SHV	32	4	8	32	Te, Sxt
66	Pediatric	CAZ	SHV-5	256	32	32	64	Te, Sxt, Gm
102	Urology	CAZ	TEM	128	32	16	256	Gm
E. cloacae								
26	Pediatric	CAZ	SHV	256	16	16	256	Te, Sxt, C, Gm
68	Pediatric	CAZ	SHV	512	32	64	256	Te
101	Internal	CAZ	TEM	256	64	32	256	Te
P. mirabilis								
19	ICU	CAZ	TEM-52	128	32	64	256	Te, Sxt, C, Gm
29	Pediatric	CAZ	TEM-52	512	128	128	256	Te, Sxt, C
58	Internal	CAZ	TEM-52	512	64	64	256	Te, Sxt, C, Gm
72	Internal	CTX	TEM-52	32	128	256	32	Te, Sxt, C
99	Surgery	CAZ	TEM-52	512	64	32	512	Te, Sxt, C

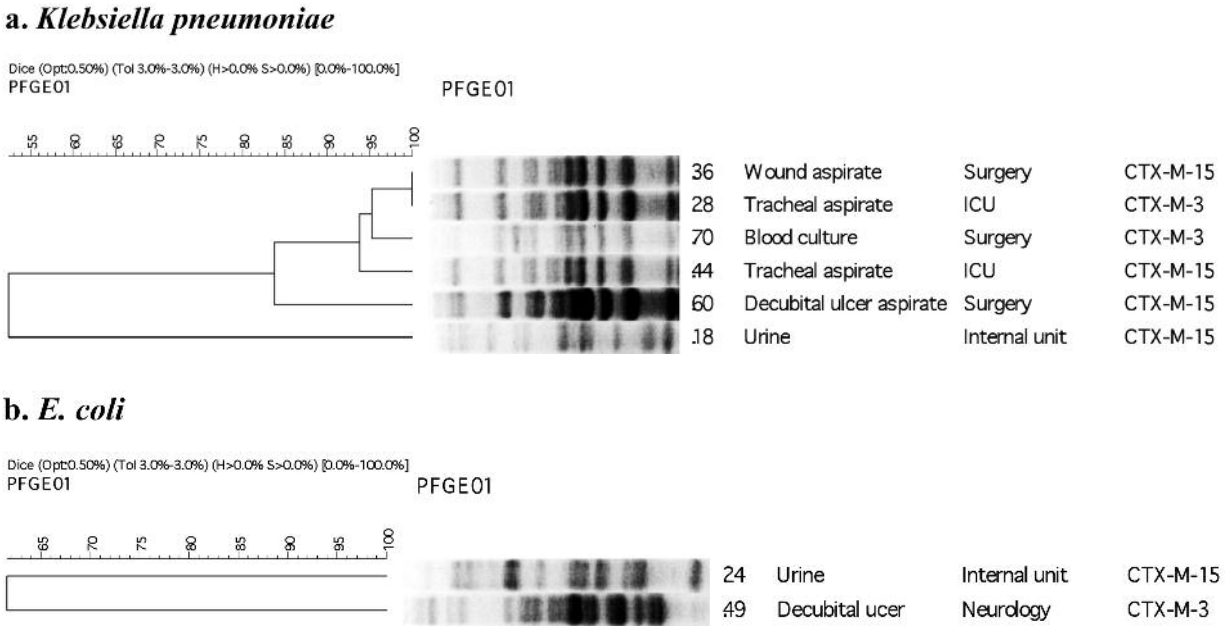

FIGURE 1 - Pulsed-field gel electrophoresis of genomic DNA of bacteria producing CTX-M β-lactamases.

TABLE 3 - *Risk-factors (logistic regression analysis) for acquisition of nosocomial infection caused by ESBL- producing Enterobacteriaceae, displayed in order of importance.*

	OR *	95% CI **		p	Range ***
		Lower	Upper		
No. of antibiotics in prior antibiotic exposure	5.588	2.727	11.451	0.0000	1
Prior antibiotic exposure	16.077	4.982	51.884	0.0000	2
Prior exposure to expanded-spectrum cephalosporins	8.077	1.719	37.955	0.0087	4
Prior exposure to aminoglycosides	4.351	1.329	14.243	0.0156	5
Use of invasive devices	1.767	1.093	2.855	0.0206	6
Central venous catheter	3.500	1.061	11.542	0.0398	7
Stay in ICU (yes/no)	2.500	1.022	6.116	0.0448	8

*Odds Ratio. ** CI – Confidence Interval. *** Risk factors displayed according to statistical significance with the most important risk factor being at the top of the table.

Statistical analysis

Table 3 shows risk factors (as results of logistic regression analysis) for acquisition of nosocomial infection caused by ESBL-producing *Enterobacteriaceae*. These are: prior antibiotic exposure, number of antibiotics administered, prior exposure to expanded-spectrum cephalosporins and amino- glycosides, use of invasive devices, central venous catheter and stay in ICU.

DISCUSSION

The prevalence of ESBL producers among *Enterobacteriaceae* in the Sisters of Mercy University Hospital is in agreement with other surveillance studies in Croatia [23-24]. Previous studies showed the highest prevalence of ESBL-positive Enterobacteriaceae in Italy (40%), Poland (31%), Russia (24%) Turkey (23%) [25-26] and South America [27].

There are limited molecular studies in Croatia con-

cerning the type of ESBLs causing nosocomial infections. In this work we used a molecular approach to determine the epidemiology of ESBLs in the Sisters of Mercy University Hospital in Zagreb. The CAZ resistance phenotype was dominant in our hospital. It was associated with the predominance of SHV-5 β-lactamase which conferred a high level of ceftazidime and aztreonam resistance on the producing isolates. Some isolates also harbored an additional TEM-1 β-lactamase. SHV-5 β-lactamase is widespread in Middle and Eastern Europe and has been previously described in Austria [2], Germany [3], Hungary [28], Poland [29] and many other countries in the world [1]. The presence of additional TEM-1 β-lactamase in some isolates could be responsible for resistance to co-amoxiclav (amoxicillin/clavulanate). SHV-5 β-lactamase was reported to be the dominant ESBL type in *K. pneumoniae* [17]and *E. coli* in Croatia (unpublished results). The spread of SHV-5-producing *K. pneumoniae* strains throughout the hospital units was due to the horizontal transfer of plasmids containing bla_{SHV-5} genes, probably facilitated by selective pressure ceftazidime which is widely prescribed in our hospital thus favoring survival of the strains possessing mutation at the Ambler position 240 responsible for ceftazidime and aztreonam resistance. Ceftriaxone is the expanded-spectrum cephalosporin which is mostly used in our hospital, followed by ceftazidime whereas cefotaxime is not prescribed. It is a well known fact that slowly penetrating cephalosporins like ceftazidime have more potential for selecting mutations responsible for ESBL phenotype compared to quickly penetrating cephalosporins like cefotaxime and ceftriaxone. Infection control measures should be employed and the consumption of expanded-spectrum cephalosporins in the Sisters of Mercy Hospital should be restricted to reduce the spread of ESBL-producing enteric isolates throughout hospital units. Since plasmids encoding ESBLs also contain resistance genes for aminoglycosides, it is possible that consumption of these antibiotics could also exert the selective pressure which favored the spread of plasmids with ESBL genes.

Genotyping of SHV-5-producing bacterial isolates by PFGE would be necessary to determine if clonal dissemination of related strains occurred in our hospital. Only two *E. coli* and six *K. pneumoniae* strains were found to produce CTX-M β-lactamases. This is in contrast with a previous investigation which found CTX-M β-lactamase to be the most prevalent type in many countries such as Russia, Switzerland, Greece, Spain, Japan, Taiwan, China and Argentina [11]. Low prevalence of CTX-M β-lactamase could be due to not prescribing of cefotaxime in the Sisters of Mercy Hospital. CTX-M-3 β-lactamase was identified in three strains (one *E. coli* and two *K. pneumoniae*) by sequencing of bla_{CTX-M} genes. This type of ESBL is very frequent in Europe and was previously described in Poland, France, UK, Greece, and Russia [11]. It was also reported from another University Hospital in southern Croatia (unpublished results). CTX-M-15 β-lactamase found in five strains (one *E. coli* and four *K. pneumoniae*) was previously described in Poland, Austria, Bulgaria, Switzerland, Russia, Portugal, UK, Canada, Lebanon [11] and many other countries of the world [11]. In contrast to CTX-M-3 β-lactamase which was reported only in Europe and the Far East, CTX-M-15 β-lactamase is ubiquitous and found all over the world [11]. As expected, CTX-M-producing organisms were not clonally related because they were probably imported to the hospital from the community. Two *K. pneumoniae* strains showed identical PFGE patterns, but produced different types of CTX-M β-lactamases. Since CTX-M-3 and CTX-M-15 β-lactamases differ in only one amino acid (Asp-240→Gly) it is possible that the $bla_{CTX-M-15}$ gene of strain 36 evolved directly from $bla_{CTX-M-3}$ of strain 28 gene by point mutation in the bla_{CTX-M} gene. The resistance phenotype of CTX-M-producing organisms was consistent with the production of this type of ESBL. However, CTX-M-15 producing strains had elevated ceftazidime MICs as well which is in accordance with previous reports [11]. CTX-M-producing organisms were more susceptible to piperacillin/tazobactam than to amoxicillin/clavulanate (data not shown). This could be due to the fact that tazobactam inhibits CTX-M β-lactamases better than clavulanate. Variable β-lactam phenotypes conferred by CTX-M producing organisms most probably reflect fluctuations in the levels of bla_{CTX-M} gene expression. IS26 insertion sequence located upstream of the $bla_{CTX-M-3}$ gene most likely facilitated the mobilization and enhanced the expression of the genes.

TEM-52 β-lactamase found in our *P. mirabilis* strains was previously reported in *P. mirabilis* and *Salmonella enterica* [30]. In contrast to the data from the references, our isolates displayed elevated aztreonam MICs, probably due to other resistance mechanisms, unrelated to ESBLs.

The role of antibiotic restriction, infection control, and the development of new antimicrobials to address this problem are challenging issues for the future. The continued spread of ESBLs may eventually limit the utility of all β-lactam antibiotics, requiring the development of new classes of antimicrobials. This study highlights the need to establish an antimicrobial resistance surveillance network for Enterobacteriaceae to monitor the trends and new types of resistance mechanisms in hospitals. Also, the factors responsible for the selection and dissemination of the plasmids encoding ESBLs need to be identified, controlled and prevented.

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