ORIGINAL ARTICLE

Emergence of CTX-M group 1 extended-spectrum ß-lactamase–producing *Klebsiella pneumoniae* strains in the community

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ABSTRACT

Aim Molecular characterization of ESBL-producing *K. pneumoniae* strains isolated from urine of outpatients in Zagreb region during the last five years.

Methods During the five-year study period a total of 2, 651 *K. pneumoniae* strains were isolated from urine of nonhospitalized patients with significant bacteriuria. ESBL production was detected by double-disk diffusion technique and by >3-dilution reduction in the minimal inhibitory concentration of ceftazidime in the presence of clavulanate. A total of 441 ESBL-producing *K. pneumoniae* strains (15.5%) were collected and 17 strains were characterised.

Double-disk synergy test was used to detect ESBLs. Minimum inhibitory concentrations (MICs) were determined by broth microdilution method according to CLSI. The transferability of cefotaxime resistance was tested by conjugation (broth mating method). PCR was used to detect alleles encoding ESBL enzymes. The genotypes of the strains were compared by pulsed-field gel electrophoresis (PFGE) of *Xba* I-digested genomic DNA.

Results A significant difference in frequencies of ESBL isolates was observed. In the first year of study only 4.9% of isolated strains were ESBL producers, while in the second year 17.3% ESBL strains were detected (p<0.01), and the frequency remains stabile within following years. Out of 17 characterized strains all strains yielded an amplicon with primers specific for SHV β-lactamases and CTX-M β-lactamases.. Based on sequencing of bla_{CTX-M} genes enzymes of nine strains were identified as CTX-M 15 β –lactamase and three as CTX-M-14. Isolates were not clonally related.

Conclusion The study demonstrated community-associated emergence of CTX-M 1 β-lactamase–producing *K. pneumoniae* strains.

Key words: community-acquired urinary tract infections, cefotaxime, extended-spectrum β-lactamase (ESBL, CTX-M 15 β-lactamase, CTX-M-14 β-lactamase

INTRODUCTION

Since plasmid-mediated extended-spectrum β-lactamases (ESBLs) were first detected in *Klebsiella oxytoca* isolate in 1983 in Germany, they have been increasingly reported worldwide (1). Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria (1-2). ESBLs are predominantly derivatives of plasmid-mediated TEM or SHV β-lactamases and arise through mutations that alter the configuration of the active site, thereby expanding the hydrolytic spectrum of the enzyme (3). The CTX-M family of β-lactamases groups evolutionary related ESBLs with a much higher level of activity against cefotaxime than ceftazidime; and their similarity to some species-specific β-lactamases, like those of *Klebsiella oxytoca* and *Citrobacter diversus*, has been known for years (4-5). The recent finding of 99% homology between the CTX-M-2 enzyme and the β-lactamase of *Kluyvera ascorbata* has indicated the origins of at least a fraction of the CTX-M-variants (6). In contrast to TEM and SHV- ESBLs which rely on point mutations in bla_{TEM} and bla_{SHV} genes to expand their substrate profiles, CTX-M β-lactamases have an intrinsic extended-spectrum profile. Whereas only three enzymes of this family (CTX-M-1, MEN-1, CTX-M-2, and Toho-1) were described between 1990 and 1995, in recent years the list has been increasing very quickly (6). In some countries CTX-M β-lactamases are the most prevalent types of ESBLs, for instance in Russia (7), Greece (8), Spain (9), Switzerland (10), Japan (11), Taiwan (12), China (13) and Argentina (14).

Recently an increase in the prevalence of ESBL producing *K. pneumoniae* was observed in Zagreb Institute of Public Health. Very little data regarding the molecular epidemiology of community acquired ESBLs in Croatia are available. The majority of strains showed unusual resistance phenotype with no inhibition zones around cefotaxime disks, consistent with the production of CTX-M β-lactamases. The aim of this study was to determine the prevalence and molecular epidemiology of CTX-M β-lactamases in community population in Zagreb region.

MATERIAL AND METHODS

Bacterial strains

Fourteen isolates from 2005 and 4 from 2007 were selected for further study. Isolates were collected from urine of non-hospitalised patients with significant bacteriuria.

Detection of ESBL

ESBL production was determined by double-disk synergy test (DDST) and confirmed by at least threefold reduction in ceftazidime minimal inhibitory concentration (MIC) by clavulanate (15). For DDST, an overnight broth culture of test strain was diluted in saline, adjusted to Mc-Farland standard suspension 0.5 and inoculated onto Mueller-Hinton (MH) agar. Disk containing amoxycillin/clavulanate (20/10 µg) was placed in the middle of the plate and surrounded by disks containing ceftazidime, cefotaxime, ceftriaxone and aztreonam (30 µg). Plates were incubated overnight at 37ºC. Distortion of the inhibition zones around cefalosporine disks toward co-amoxiclav disk was indicative of ESBL production. ESBL production was confirmed by CLSI combined disk test and by 8 fold reduction in ceftazidime MIC in the presence of clavulanate.

Antibiotic susceptibility testing

Antibiotic susceptibilities to a wide range of antibiotic were determined by disk diffusion and broth microdilution method in MH broth and 96 well microtiter plates according to CLSI (16).

A phenotype consistent with production of a CTX-M-type β lactamases was defined by cefotaxime MIC≥8-fold higher than the ceftazidime MIC, with the MICs of both agents reduced significantly (again ≥8-fold) in the presence of 4 mg/L clavulanic acid.

Transfer of resistance determinants

K. pneumoniae isolates 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 (17). Overnight BHI (Brain-Heart Infusion) broth cultures of *K. pneumoniae* donor

strain and *E. coli* recipient strain were mixed in the ratio 1:2 in 5 ml BHI broth and incubated 18 h at 37 °C without shaking. 100 µl of the mating mixture will be seeded on the Mueller-Hinton plates containing cefotaxime (1 mg/L), rifampicin (256 mg/L) and combined plates containing cefotaxime + rifampicin. Transconjugants were selected on the combined plates containing cefotaxime (1 mg/L) and rifampicin (256 mg/L). The frequency of conjugation was expressed relatively to the number of donor cells.

Characterization of extended-spectrum ß-lactamases

The presence of bla_{ESBL} genes was determined by polymerase chain reaction (PCR) using primers targeting bla_{TEM}, bla_{SHV}, bla_{CTX-M} and conditions as described previously (18-20). Bacterial DNA was extracted by the boiling method. PCR mix (50 µl) contained: 21 µl of ultrapure water, 25 µl of master mix (Roche), 1 µl of each primer and 2 µl of template DNA. PCR was performed under the following conditions: 94° for 3 min, the 35 cycles consisting of 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s each, followed by a final extension at 72°C for 5 min. 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')(18) for detection of SHV β-lactamases, OT-3 (5'-ATG-AGT-ATT-CAA-CAT-TTC-CG-3') and OT-4 (CCA-ATG-CTT-AAT-CAG-TGA-GG-3') for detection of TEM (19) β-lactamases, MA-1 (5'-SCS-ATG-TGC-AGY-ACC-AGT-AA-3') and MA-2 (5'-CGC-CRA-TAT-GRT-TGG-TGG-TG-3') (20) for detection of CTX-M β-lactamases, 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 (21) for detection of PER β-lactamases. Strains were further tested by multiplex PCR with primers specific for CTX-M groups 1, 2, 8, 9 and 25 (22). Primers IS26F (5'-GCG-GTA-AAT-CGT-GGA-GTG-AT-3) and IS26R (5'-ATT-CGG-CAA-GTT-TTT-GCT-GT-3') were used to amplify 400 bp fragment spanning the link between IS26 insertion sequence and bla_{CTX-M} gene in CTX-M producing isolates (23). Primers IS*Ecp1*L1 (CAGCTTTTA-TGACTCG) and ALA-5 (CCTAAATTCCA-CGTGTGT) were applied to amplify the IS*EcpI* insertion sequence (23).

Reference strains producing CTX-M-15 and CTX-M-2 β-lactamases were provided by Neil Woodford from Health Protection Agency, London,UK. The PCR products were visualized by agarose gel electrophoresis, after staining with ethidium bromide. Amplicons were then column-purified (Quiagen DNA purification kit) and sequenced directly using the ABI PRISM 377 Genetic Analyser (Applied Biosystems). After sequencing the PCR products obtained, we used the BLAST program to look for sequence homology with the other bla_{ESBL} genes. More specific primers for each cluster of the CTX-M-family were then used to amplify the entire coding sequence of the bla_{CTX-M} gene. Reference strains producing CTX-M-15 and CTX-M-2 β-lactamases were provided by Neil Woodford (Health Protection Agency, London,UK). Reference strains producing TEM-1, TEM-2 and SHV-1 β-lactamase were kindly provided by Prof. A. Bauernfeind (Microer, Munich, Germany).

PCR *Nhe* test was performed to distinguish between SHV-1 and SHV-ESBL. The PCR products of $bla_{SHV-ESBL}$ genes are cleaved in three fragments of 1017, 770 i 247 bp as described previously (18) in contrast to bla_{SHV-1} which is not digested by *NheI* restriction endonuclease. 10 µl of the PCR product was mixed with 1 µl of Nhe enzyme, 2 µl of buffer and 10 µl pf water. The samples were incubated for 2 h at 37 °C in water bath. The fragments were detected after electrophoresis in 1% agarose gel, after staining with ethidium bromide.

Molecular typing of the isolates by pulsed-field gel electrophoresis

Isolates were compared by PFGE of *X*ba I-digested genomic DNA as described previously. Isolation of chromosomal DNA was performed as described by Kaufman et al (24). For each isolate 1,0 ml (optical suspension density 0,6-0,7 at 540 nm) of an overnight culture grown in BHI broth was pelleted by centrifugation at 10 000 rpm for 2 min. After being washed in 1 ml SE buffer (75mM NaCl; 25mM EDTA, *Sigma*), bacteria were resuspended in 500µl SE buffer with 10 µl lysosime (*Boehringer Mannheim GmbH*) Next, 500 µl of this bacterial suspension was mixed with 500 µl 2,0% low- melting-temperature aga-

Table 1. Distribution of ESBL–producing *Klebsiella pneumoniae* strains

Year	*Klebsiella pneumoniae*		
	No of ESBL isolates (%)	No of non ESBL isolates (%)	Total No of isolates (%)
2003	27 (1,02)	520 (19,60)	547 (20,62)
2004	96 (3,62)	460 (17,34)	554 (20,96)
2005	94 (3,54)	215 (8,10)	309 (11,64)
2006	109 (4,11)	493 (18,58)	602 (22,69)
2007	114 (4,29)	525 (19,80)	639 (24,09)
Total	440 (16,58)	2213 (83,42)	2651 (100,00)

rose (InCert agarose; FMC Bioproducts) and left to solidify. Solid agarose plugs were then incubated for 24h at 56^0 C in 2ml of ESP buffer (1% N-lauril sarcosine; 0,5 M EDTANa2, pH 9,5; 500 µg/ml proteinase K, Sigma). After 24h, the plugs were incubated at the room temperature for 2 h in PMSF (phenylmethanesulfonyl-fluoride, Aldrich) and then were bedded three times for 30 min at 4^0 C with TE buffer (10mM Tris-Hcl,pH 8, 0,1 mM EDTA, Sigma) before macrorestriction with 10U / 1 µl XbaI for 3 h at 37^0 C. Restriction fragments of DNA were separated by PFGE with a CHEF-DRIII apparatus (Bio-Rad Laboratories) through 1% pulsed-field certified agarose (Bio-Rad) at a field strength of 6 V/cm for 20 h at 11^0 C; with pulses from 5 to 50 -s in 0,5 TBE buffer with thiurea (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 (25) and analysed 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 0,50% and position tolerance of 3,00% were applied during the comparison of PFGE fingerprinting patterns.

RESULTS

A dramatic increase in the prevalence of ESBL positive *K. pneumoniae* was observed between 2003 (4.9%) and 2007 (17.8%) as shown in Table 1. Very high prevalence of ESBLs persisted from 2004 till 2007.

ESBLs were detected in all tested strains by DDST and confirmed by >3 fold decrease of ceftazidime MICs by clavulanate.

All strains were uniformly resistant to amoxycillin and narrow and expanded-spectrum cephalosporins (Table 2). Isolates belonging to group 1 CTX-M β-lactamases were resistant to gentamicin, netilmicin and ciprofloxacin. Three strains producing CTX-M-2 group showed susceptibility to ceftazidime, gentamicin, netilmicin and ciprofloxacin.

Strains isolated in 2002-2003 were susceptible to piperacillin/tazobactam and susceptible or intermediate susceptible to amoxycillin/clavulanate

Table 2. Minimum inhibitory concentrations of *Klebsiella pneumoniae* isolates from 2002-2003 and 2007*

a. 2002-2003

Strain	AMX	CN	CXM	CAZ	CTX	FEP	FOX	AMC	CAZ/cl	TZP	IMI	GM	NET	AMI	CIP
19	>128	>128	>128	2	>128	>128	8	16	0.12	2	0.25	1	0.5	8	1
22	>128	>128	>128	2	>128	>128	8	8	0.12	2	0.25	1	0.5	4	0.12
34	>128	>128	>128	2	>128	>128	8	4	0.06	4	0.5	2	4	128	0.25
48	>128	>128	>128	>128	>138	>128	8	16	0.5	64	0.5	64	32	8	128
49	>128	128	32	>128	32	8	8	8	1	16	0.25	0.5	0.5	1	0.12
50	>128	>128	>128	>128	>128	>128	8	8	0.5	16	0.25	32	32	64	0.03
51	>128	>128	>128	>128	>128	>128	8	16	1	16	0.25	64	16	2	64
53	>128	>128	>128	>128	>128	>128	8	16	1	16	0.5	64	32	8	64
57	>128	>128	>128	>128	>128	>128	4	16	1	16	0.5	128	64	32	>128
61	>128	>128	>128	>128	>128	>128	8	16	2	16	0.5	128	32	8	128
65	>128	>128	>128	>128	>128	>128	8	16	1	16	0.25	64	32	4	128
67	>128	>128	>128	64	>128	>128	8	16	2	16	0.25	128	64	8	64
69	>128	>128	>128	64	>128	>128	8	16	2	16	0.25	128	64	4	128

b. 2007

| Strain	AMX	CN	CXM	CAZ	CTX	FEP	FOX	AMC	CAZ/cl	TZP	IMI	GM	NET	AMI	CIP
22598	>128	>128	>256	>256	>256	>128	32	128	4	>128	0.5	128	32	4	128
23857	>128	>128	>256	>256	>256	>128	32	64	0.5	128	0.25	8	4	4	256
4986	>128	>128	>256	128	>256	>128	16	64	0.25	64	1	0.5	0.5	1	32
7590	>128	>128	>256	256	>256	>128	32	128	2	128	0.25	128	64	4	256

*AMX, amoxycillin; AMC, amoxycillin/clavulanic acid; CAZ/cl-ceftazidime/clavulanic acid; CXM, cefuroxime;CN,cephalexin; CAZ, ceftazidime; CAZ/cl, ceftazidime/clavulanic acid; CTX,cefotaxime; CRO,ceftriaxone; FEP,cefepime; FOX, cefoxitin; AMT, aztreonam; TZP, piperacillin/tazobactam; MEM,meropenem; GM, gentamicin; NET, netilmicin; AMI,amikacin; CIP, ciprofloxacin

contrary to the strains collected in 2007 which showed resistance to β-lactam combinations with inhibitors. Resistance to imipenem was not observed as shown in Table 2. Interestingly the strains from 2007 showed reduced susceptibility or resistance to cefoxitin unlike those from 2002-2003.

Six out of 13 isolates collected in 2002-2003 transferred resistance to *E. coli* recipient. Resistance to gentamicin and netilmicin was cotransferred alongside with cefotaxime resistance from four strains. Tranconjugants showed similar resistance phenotype to expanded-spectrum cephalosporins as their respective donors. Only one isolate from 2007 transferred cefotaxime resistance to recipient strain with the frequency of 10^{-4}. Gentamicin resistance was cotransferred.

All isolates yielded an amplicon of 545 bp with concensus MA primers (Figure 1). Multiplex PCR was positive for group 1 of CTX-M β-lactamases in all except three strains. Three strains (19,22,34) collected in 2005 yielded an amplicon with group 2 primers. Sequencing of selected amplicons belonging to group 1 revealed the presence of $bla_{CTX-15/28}$ with coding regions containing identical nucleotide sequences. The obtained DNA sequence of the PCR products exhibited 98% similarity with $bla_{CTX-M-15}$ from the gene bank. Three strains were positive for group 2 CTX-M β-lactamases and were identified by sequencing of *bla* genes as CTX-M-14. PCR-*Nhe* test was negative indicating the presence of naturally occurring SHV-1 β-lactamase. TEM β-lactamases were detected in 8 strains (19, 22,
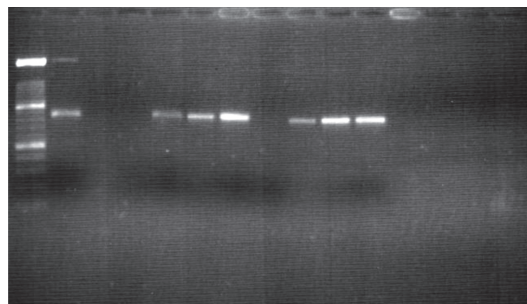
Lane
1. *E. coli*-CTX-M-15-positive control
2. *E. coli* ATCC 25922-negative control
3. *K. pneumoniae* 48
4. *K. pneumoniae* 51
5. *K. pneumoniae* 61
6. empty lane
7. *K. pneumoniae* 65
8. *K. pneumoniae* 67
9. *K. pneumoniae* 69

Figure 1. Multiplex PCR for detection of CTX-M groups

34, 57, 61, 65, 67 and 69). Clinical isolates of *K. pneumoniae* yielded an amplicon of 1016 bp with primers specific for SHV β-lactamases.

IS26 insertion sequence was found upstream of the $bla_{CTX-M-15}$ gene. IS*EcpI* was not found.

The isolates showed distinct PFGE fingerprints. No clonal relatedness was observed among the isolates.

DISCUSSION

The results of this work provided insights into the molecular epidemiology of the community spread of ESBLs positive *Klebsiella pneumoniae*. The study found CTX-M-15 β-lactamase to be the most prevalent among isolates of *K. pneumoniae*. This type of CTX-M β-lactamase is the most prevalent around the world and was previously reported in our neighbouring countries such as Hungary (26), Austria (27), Slovenia (28) and Italy (29). It was for the first time described in New Delhi, India in 1999 (30). CTX-M-15 is also predominant in Switzerland (10), Bulgaria (31), and in France (32). Previous studies also found CTX-M-15 and CTX-M-3 β-lactamase among hospital isolates of *E. coli* from Croatia (33,34). CTX-M-15 differs from CTX-M-3 β-lactamase by a single amino-acid change (Asp-240-Gly). While these genes were found in all isolates, other rare types of ESBLs like WEB or IBC cannot be excluded without further testing, but were beyond this study. The spread of CTX-M-15 β-lactamase was not due to clonal dissemination of related isolates as expected for community isolates. Characterization of plasmids is necessary to be determined if dissemination of CTX-M-15 β-lactamase is due to the horizontal transfer of plasmids containing $bla_{CTX-M-15}$ genes.

Carbapenems are frequently the only therapeutic option available for treatment of severe infections caused by multiresistant ESBL producing *K. pneumoniae* isolates described in this work. Nevertheless, universal susceptibility to these last-line antimicrobials is no longer guaranteed. Resistance to ceftazidime observed in all strains is consistent with the production of CTX-M-15 β-lactamase which efficiently hydrolyze ceftazidime similarly as CTX-M-16 and CTX-M-28. These data suggest that phenotypic approach based on comparison of cefotaxime and cefta-

zidime MICs has a limited value in predicting the presence of CTX-M-ESBLs in clinical isolates. All strains were susceptible to combination of ceftazidime and clavulanate, but in general, β-lactam combinations with inhibitors are not recommended for the therapy of serious infections caused by ESBL producing bacteria due to pronounced inoculum effect and possibility of developing mutants hyperpoducing ESBL during the therapy (35). The strains displayed intermediate susceptibility or resistance to cefepime. The fluctuations in the level of cephalosporin resistance most probably reflects the variable levels of bla_{CTX-M} gene expression as previously reported for TEM and SHV-ESBLs (36,37). The strains with increased resistance could have the mutations in the promoter region, more gene copies or higher plasmid copy number. Associated aminoglycoside and fluoroquinolone resistance is frequent in ESBL producing bacteria. Some of the strains were resistant to cefoxitin but resistance was not transferable to *E. coli* recipient indicating chromosmal origin of the resistance gene. Concerning non β-lactam susceptibilities of CTX-M producers, all strains were resistant to ciprofloxacin. High rates of resistance to ciprofloxacin in CTX-M producers were also reported from Austria (27), Canada (38) and Italy (29). Coresistance to non β-lactam antibiotics is also frequent, either by the cotransfer of the resistance determinants in the same genetic elements (such as aminoglycosides resistance) or simply by the coselection of both resistance mechanism, as it occurs in fluoroquinolones.

Since plasmids encoding ESBLs also contain resistance genes for aminoglycosides it is possible that consumption of these antibiotics could also exert the selection pressure which favours the spread of plasmids with ESBL genes.

A number of risk factors have been identified as linked with the acquisition of community-acquired infections involving ESBL-positive isolates. The risk factors are a previous hospitalization or antibiotic therapy, within the past three months, old age (>60), male gander, confinement to bed with debilitation and urinary catheterization (39,40). The emergence of CTX-M enzymes in Croatia and elsewhere highlights the importance of using either both ceftazidime and cefotaxime, or cefpodoxime to detect possible ESBL production prior to performing cephalosporin-clavulanate synergy tests for confirmation (41).

This demonstrates the need to monitor both hospitalized and general practice patients for further emergence of transferable resistance to expanded-spectrum cephalosporins. Continuous surveillance in order to track CTX-M-producing *K. pneumoniae* in the community is necessary to prevent their influx into the hospitals. The appearance of CTX-M-15 β-lactamase in the community is a serious threat to public health.

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