Spread of CTX-M-15 positive Providencia spp. causing urinary tract infections at the University Hospital Split in Croatia

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ABSTRACT

Aim During 2010-2011, six Providencia spp (five Providencia stuartii and one Providencia rettgeri) urine isolates with unusual resistance phenotype were collected from various hospital units at the University Hospital Split in Croatia. The aim of the study was to analyze the mechanisms of resistance to expanded-spectrum cephalosporins.

Methods The antimicrobial susceptibility to a wide range of antibiotics was determined by broth microdilution method according to CLSI guidelines. A double-disk-synergy test (DDST) was performed to detect ESBLs. The transferability of cefotaxime resistance was determined by conjugation. The presence of genes encoding ESBLs was determined by PCR while genotyping of the isolates was performed by PFGE.

Results All strains were positive for ESBL production by DDST. They were uniformly resistant to amoxicillin alone and combined with clavulanate, cefazolin, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, gentamicin and ciprofloxacin. P. stuartii strains transferred cefotaxime resistance to E. coli recipient strain with frequency ranging from $10^{-5}$ to $5 \times 10^{-4}$. Five P. stuartii strains were positive for TEM and CTX-M β-lactamases while P. rettgeri was positive only for TEM β-lactamases. Five CTX-M producing isolates were shown to be clonally related.

Conclusions Continuous surveillance in tracking CTX-M-15-producing P. stuartii in the hospitals is necessary to prevent their spread to other hospitals and community. Global spread of ESBL positive Providencia spp all over the world is of great clinical concern.

Key words: extended-spectrum β-lactamase, CTX-M-15 β-lactamase, cefotaxime, Providentia stuartii
INTRODUCTION

β-lactam agents such as penicillins, cephalosporins, monobactams and carbapenems are antibiotics of choice to treat a variety of infections. The introduction into therapy was rapidly followed by the reports of resistance. Microorganisms producing extended-spectrum β-lactamases (ESBLs) were identified in early 1980-ies, shortly after the introduction of oxyimino-cephalosporins (1). Production of ESBLs is the major mechanism of resistance to oxymino-cephalosporins and aztreonam in Gram-negative bacteria (2-3).

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). However, some CTX-M β-lactamases such as CTX-M-15 and CTX-M-28 can efficiently hydrolyze also ceftazidime. In contrast to TEM and SHV ESBLs which rely on point mutations in blaTEM and blaSHV genes to expand their substrate profiles, CTX-Ms have an intrinsic extended-spectrum profile. Whereas only three enzymes of this family (CTX-M-1 or 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 Switzerland (6), Russia (7), Greece (8), Spain (9), Japan (10), Taiwan (11), China (12) and Argentina (13).

Since the first isolation of ESBL-producing Klebsiella pneumoniae in Croatia (14) a growing variety of Enterobacteriaceae and ESBL enzymes have been detected (15-16). The first isolates found to produce CTX-M β-lactamases in Croatia were E. coli from the University Hospital in Split (17). Very soon after the first report CTX-M producing E. coli and K. pneumoniae were reported from other centers in Croatia (18-20).

Six strains of Providencia spp (five strains of P. stuartii and one P. rettgeri) with unusual resistance phenotype were isolated from urine samples from different hospital units of the University Hospital Split. The aim of the study was to analyze the mechanisms of resistance to expanded-spectrum cephalosporins.

MATERIAL AND METHODS

Bacterial strains

During 2010-2011, six Providentia spp isolates with unusual resistance phenotype (five strains of P. stuartii and one P. rettgeri) were collected from urine samples with significant bacteriuria from various hospital units of the University Hospital Split in Croatia. The strains were identified by conventional biochemical testing.

Detection of ESBL

ESBL production was determined by double-disk-synergy test (DDST) and confirmed by CLSI combined disk test (22) and at least three-fold reduction in cefotaxime minimal inhibitory concentration (MIC) by clavulanate (22).

Double- disk-synergy test (DDST)

For DDST, an overnight broth culture of test strain was diluted in saline, adjusted to McFarland standard suspension 0.5 and inoculated onto Mueller-Hinton 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 cefepime (30 µg). Plates were incubated overnight at 37ºC. Distortion of the inhibition zones around cephalosporine disks toward co-amoxiclav disk was indicative of ESBL production. ESBL production was confirmed by CLSI combined disk method (22).

Antibiotic susceptibility testing

Antibiotic susceptibilities to a wide range of antibiotics were determined by disk-diffusion and broth microdilution method. Minimum inhibitory concentrations (MICs) of amoxycillin, ceftazidime, ceftriaxone, cefepime, aztreonam, piperacillin/tazobactam, imipenem, meropenem gentamicin, and ciprofloxacin were determined in microtiter plates and Mueller-Hinton broth according to CLSI.
guidelines (22-23). E. coli ATCC 25922 and K. pneumoniae 700603 were used for quality control. Antibiotic susceptibility to chloramphenicol, tetracycline, sulphamethoxazole, trimethoprim, amikacin was determined by disk-diffusion test.

Transfer of resistance determinants

Providencia spp 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 (24). Overnight BHI (Brain-Heart Infusion) broth cultures of Providencia spp 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. Transconjugants were selected on the combined plates containing cefotaxime (1 mg/L) and rifampicin (256 mg/L). The frequency of transconjugation was expressed relatively to the number of donor cells. E. coli A15 R strain was kindly provided by Prof. A. Bauernfeind (Microer, Munich).

Characterization of extended-spectrum ß-lactamases

The presence of blaESBL genes was determined by polymerase chain reaction (PCR) using primers targeting blaTEM, blaSHV, blaCTX-M, and blaPER genes, and conditions as described previously (25-28). Bacterial DNA was extracted by boiling method. PCR mix (50 µl) contained: 22 µl of ultrapure water, 25 µl of master mix (Roche), 1 µl of each primer and 3 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 CTG TTG TGT CGC-3’) and MN-2 (5’ TCT TTC CGA TGC CGC CAG TCA-3’) (25) for detection of SHV ß-lactamases, OT-3 (5’-ATG-AGT-ATT-CAT-TTC-CG-3’) and OT-4 (CCG-ATG-CTT-ATG-CTA-3’) (26) for detection of TEM ß-lactamases, MA-1 (5’-SCS-ATC-TGC-ATC-3’) (27) and MA-2 (5’-CGC-CRA-TAT-GRT-TGG-TGG-3’) (27) for detection of CTX-M ß-lactamases, and PER-1-F (5’GGG-ACA-(A/G) TC- (G/C)(G/T)-ATG-ATT-GCTCA and PER-1R: 5’ gg (C/T) (G/C) GCT-GATAG-ATA-GTG-CTG-AT (28) for detection of PER ß-lactamases. Strains were further tested by multiplex PCR with primers specific for CTX-M groups 1, 2, 8, 9 and 25 (29). Primers IS26F (5’-GGG-GTA-AAT-CTG-CAA-GTA-GTG-AT-3) and IS26R (5’-TTC-AGG-CAG-ATT-ATT-ATG-GTC-3’) were used to amplify 400 bp fragment spanning the link between IS26 insertion sequence and blaCTX-M gene in CTX-M producing isolates (27). Primers ISEcpIL1 (CAGCCTTATTAGACTCG) and ALA-5 (CCCTATTACGCTGTTG) were applied to amplify the ISEcpIL insertion sequence (30). PCR mapping was performed with forward primer for ISEcpIL and reverse primer for blaCTX-M (MA-3).

In order to determine the genetic context of blaCTX-M genes PCR mapping was performed using primers for IS26 and ISEcpIL combined with forward and reverse primers for blaCTX-M genes (30). 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 ABI PRISM 377 Genetic Analyzer (Applied Biosystems). After sequencing the PCR products obtained, we used BLAST program to look for sequence homology with the other blaESBL genes. More specific primers for each cluster of the CTX-M-family were then used to amplify the entire coding sequence of the blaCTX-M gene. Reference strains producing CTX-M-15 and CTX-M-2 ß-lactamases were provided by Neil Woodford (Health Protection Agency, London,UK). Reference strains producing TEM-1, TEM-2 and SHV-1 ß-lactamase were kindly provided by Prof. A. Bauernfeind (Microer, Munich, Germany). Reference strains producing CTX-M-15 and CTX-M-2 ß-lactamases were provided by Neil Woodford from Health Protection Agency, London,UK.

Characterization of plasmids

Plasmids were extracted with Qiagen Plasmid Mini kit (QUIAGEN, Hamburg, Germany) according to manufacturer’s recommendations, run in 0.7% agarose gel, and stained with ethidium bromide. E. coli NTCC 50192 yielding four bands of known sizes of 148, 64, 36 and 7 kb was used as positive control. Plasmids were subjected to PCR with primers specific for TEM, SHV and CTX-M ß-lactamases.
Molecular typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA

Five *P. stuartii* isolates were subjected to PFGE of *Xba* I-digested genomic DNA as described previously. Isolation of chromosomal DNA was performed as described by Kaufman et al (31). 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 lysosyme (*Boehringer Mannheim GmbH*). Next, 500 μl of this bacterial suspension was mixed with 500 μl 2.0% low- melting-temperature agarose (InCert agarose; FMC Bioproducts) and left to solidify. Solid agarose plugs were then incubated for 24h at room temperature for 2 h in PMSF (phenylmethylsulfonyl-fluoride, Aldrich) and then were incubated at 540 °C; with pulses from 5 to 50-s and field strength of 6 V/cm for 20 h at 11°C; along with cefotaxime resistance. To gentamicin, chloramphenicol, tetracycline and sulphametoxazole/trimetoprim was cotransferred to cepotaxime resistance. *P. rettgeri* had similar resistance phenotype, but was susceptible to amoxycillin/ clavulanic acid, cefotaxime and ceftriaxone. Cefotaxime MICs were reduced by clavulanic acid for more than three dilutions confirming the production of ESBL.

Transfer of resistance determinants

Four strains (68, 69, 72, 73 and 74) transferred ceftotaxime resistance to *E. coli* recipient strain with frequency ranging from 10⁻¹ to 5 x10⁻⁴. Resistance to gentamicin, chloramphenicol, tetracycline and sulphametoxazole/trimetoprim was cotransferred alongside with cefotaxime resistance. *P. rettgeri* did not transfer cefotaxime resistance to *E. coli* recipient strain.

Characterization of extended-spectrum β-lactamases

Five strains were positive for TEM and CTX-M β-lactamases (68, 69, 72, 73 and 74). Multiplex PCR revealed group 1 of CTX-M β-lactamases (Figure 1). Sequencing of *bla* CTX-M genes revealed CTX-M-15 β-lactamase. *ISEcp1* insertion sequence was found upstream of *bla* CTX-M-15 gene. Sequen-
cing of \textit{bla}_{TEM} genes identified TEM-1. \textit{P. rettgeri} had only TEM amplicon.

\textbf{Characterization of plasmids}

Plasmid extracts were positive for TEM and CTX-M β-lactamases indicating plasmid origin of the \textit{bla} genes.

\textbf{Molecular typing by pulsed-field gel electrophoresis (PFGE) of bacterial DNA}

\textit{P. stuartii} strains were clonally related (68, 69, 72, 73, and 74) and showed identical PFGE patterns as shown in Figure 2.

\textbf{DISCUSSION}

The study reported spread of CTX-M-15 producing \textit{P. stuartii} causing urinary tract infections at the University Hospital in Split. CTX-M-15 β-lactamase was previously reported in \textit{E. coli}, \textit{Klebsiella pneumoniae} and other Enterobacteriaceae from Poland (33), but with time the variant has become the major CTX-M type in France (34), the UK (35), Portugal (36), Austria (37), India (38), Canada (39), Cameroon (40), Lebanon (41) and together with CTX-M-3, in Bulgaria (42). CTX-M-15 β-lactamase was also described in species other than \textit{K. pneumoniae} and \textit{E. coli} (41) which proves intergeneric spread of this enzyme. Previous studies on ESBLs at the University Hospital of Split revealed the presence of CTX-M-3 β-lactamase in \textit{E. coli}. CTX-M-3 and CTX-M-15 differ only by one amino acid from each other, aspartic acid versus glycine at position 240, respectively, and this difference is responsible for the ceftazidime-hydrolyzing activity of CTX-M-15, which contributed to elevated ceftazidime MICs of our strains. It is very likely that CTX-M-15 has evolved from CTX-M-3 by the D240G mutation, however, only in Poland the strictly related \textit{bla}_{CTX-M-15} and \textit{bla}_{CTX-M-3} genes have been found so far (33). IS\textit{Ecp1} insertion sequence found upstream of the gene is able to mobilize and promote the expression of \textit{bla}_{CTX-M-15} gene acting as a significant factor in the rapid spread of CTX-M-15 enzyme in Croatia. Slight differences incefotaxime and cefepime MICs could be attributable to variable expression of \textit{bla}_{CTX-M} genes. CTX-M β-lactamases are very often associated with urinary tract infections and this could be due to increased usage of oral expanded-spectrum cephalosporins such as ceftibuten and cefixime for the treatment of urinary tract infections. Other studies have shown the increase in the prevalence of CTX-M enzymes in Croatia. This observation may be related to the increased use of expanded-spectrum cephalosporins in Croatia, particularly ceftriaxone.

The present study revealed the clonal spread of CTX-M-15 producing \textit{P. stuartii} in the Split University Hospital and horizontal transfer of re-
lated plasmids containing bla_{CTX-M} genes which also contained resistance genes for aminoglyco-
side, tetracycline, chloramphenicol, sulphon-
amides and trimethoprim. It is possible that also
the consumption of non-β-lactam antibiotics like
aminoglycosides exerts the selection pressure,
the consumption of non-β-lactam antibiotics like
moxifloxacin and trimethoprim. It is possible that also
Carbapenems and amikacin are antibiotics of cho-

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halosporins and combinations with β-lactamase
inhibitors are generally not recommended for the
therapy of infections caused by ESBL producing
organisms according to CLSI. Infection control
measure limited the spread of CTX-M-15 produ-
ing P. stuartii in the hospital. Emergence of ESBL
producing P. stuartii in our neighboring countries
indicates regional dissemination of this important
urinary tract pathogen (47,53). Continuous surveil-
ance in tracking CTX-M-15-producing P. stuartii in
the hospitals is necessary to prevent their spread
to other hospitals and community. Global spread of ESBL positive Providencia
spp all over the world is of great clinical concern.

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TRANSPARENCY DECLARATIONS
Competing interests: none to declare.
Sekvenciranje produkciju TEM i CTX-M β-laktamaze, dok je
Univerzitet/Sveučilište "Vitez" Travnik, dimom, cefotaksimom, ceftriaksonom, gentamicinom i ciprofloksacinom.

- rezistenciju na amoksicilin sam i u kombinaciji s klavulanatom, cefazolinom, cefuroksimom, ceftazi


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CTX-M from India. J Chemother 2011; 23:131-4


Klonsko širenje Providencia spp producenta CTX-M-15 β-laktamaze kao urinarnog patogena u Kliničkoj bolnici Split

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SAŽETAK

Cilj Tijekom 2008-2009. godine prikupljeno je s različitih bolničkih odjela i uzoraka pacijenata iz sveučilišne splitiške bolnice u Hrvatskoj, šest sojeva Providencia spp. (pet Providencia stuartii i jedna Providencia rettgeri), s neobičnim fenotipom rezistencije. Cilj studije bio je analizirati mehanizme rezistencije na cefalosporine proširenog spektra.


Rezultati Svi su sojevi u DDST bili pozitivni na produkciju ESBL. Također su svi sojevi pokazivali rezistenciju na amoksicilin sâm i u kombinaciji s klavulanatom, cefazolinom, cefuroksimom, cefazidimom, ceftakosakim, ceftiranoksonom, gentamicinom i ciprofloksacinom. P. stuartii je prenijela rezistenciju na E. coli s učestalostima u rasponu od 5 x 10^4 do 10^5. Pet sojeva P. stuartii bilo je pozitivno na produkciju TEM i CTX-M β-laktamaze, dok je P. rettgeri bila pozitivna samo na TEM β-laktamazu. Sekvenciranje bla


Ključne riječi: CTX-M-15 beta-laktamaza, Providencia stuartii, Providencia rettgeri, ceftakosakim