ORIGINAL ARTICLE

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, cefazoline, 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 $5x10^{-4}$. Five *P. stuarti* 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*

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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 bla_{TEM} and bla_{SHV} 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. stuarti* 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 cefalosporine 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, ceftazidime, cefotaxime, 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 tranconjugation 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 bla_{ESBL} genes was determined by polymerase chain reaction (PCR) using primers targeting bla_{TEM}, bla_{SHV}, bla_{CTX-M} and bla_{PER} 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 CTT ATT TGT CGC-3') and MN-2 (5' TCT TTC CGA TGC CGC CGC CAG TCA-3') (25) 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') (26) for detection of TEM 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') (27) 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 (28) 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 (29). 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 (27). Primers IS*Ecp1*L1 (CAGCTTTTATGACTCG) and ALA-5 (CCTAAATTCCACGTGTGT) were applied to amplify the IS*Ecp1* insertion sequence (30). PCR mapping was performed with forward primer for IS*Ecp1* and reverse primer for bla_{CTX-M} (MA-3).

In order to determine the genetic context of bla_{CTX-M} genes PCR mapping was performed using primers for IS26 and IS*Ecp1* combined with forward and reverse primers for bla_{CTX-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 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). Reference strains producing CTX-M-15 and CTX-M-2 β-lactamases were provided by Neil Woodford from Health Protection Agency, London,UK.

Characterization of plasmids

Plasmids were extracted with Qiagen Plasmid Mini kit (QIAGEN, 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 β-lactamases

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

Five *P. stuarti* 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 lysosime (*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 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 room temperature for 2 h in PMSF (phenylmethanesulfonyl-fluoride, Aldrich) and then were washed 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 *Xba*I 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 colleagues for bacterial strain typing (32) 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 0.50% and position tolerance of 3.00% were applied during the comparison of PFGE fingerprinting patterns.

RESULTS

Detection of ESBL

All strains were positive for ESBL production by DDST. Combined disk method confirmed the production of ESBLs.

Antibiotic susceptibility testing

P. stuartii strains were uniformly resistant to amoxicillin alone and combined with clavulanate, cefazoline, cefuroxime, ceftazidime, cefotaxime, ceftriaxone, gentamicin and ciprofloxacin (Table 1). They were intermediately susceptible to aztreonam. No resistance to carbapenems, cefepime, cefoxitin, amikacin and piperacillin/tazobactam was observed (Table 1). *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 cefotaxime resistance to *E. coli* recipient strain with frequency ranging from 10^{-5} 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 ß-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 $bla_{\text{CTX-M}}$ genes revealed CTX-M-15 β-lactamase. IS*Ecp1* insertion sequence was found upstream of $bla_{\text{CTX-M-15}}$ gene. Sequen-

Table 1. Minimum inhibitory concentrations of ESBL-positive *Providencia* spp. strains

Strain number	Date of isolation	Specimen	Species	Hospital unit	AMX	AMC	PIP	CZ	CXM	CAZ	CTX	CTX/cl	CRO	FEP	FOX	AMT	TZP	IMI	MEM	GM	CIP
68	24.02.2010.	urine	*P. stuartii*	Medical	≥256	64	≥128	≥256	64	≥256	64	<0.06	64	4	8	16	2	2	<0.06	16	16
69	17.09.2010.	urine	*P. stuartii*	Infectology	≥256	64	≥128	≥256	64	≥256	64	<0.06	128	2	8	8	8	0.5	<0.06	16	8
72	06.09.2010.	urine	*P. stuartii*	Neurogusrgery	≥256	128	≥128	≥256	256	≥256	64	<0.06	64	4	4	16	8	2	<0.06	32	8
73	13.06.2011.	urine	*P. stuartii*	Medical	≥256	64	≥128	≥256	≥256	≥256	128	<0.06	64	4	4	16	8	2	<0.06	32	16
74	29.10.2011.	urine	*P. stuartii*	Infectology	≥256	64	≥128	≥256	≥256	≥256	32	<0.06	32	2	8	32	8	0.5	<0.06	16	2
75	14.05.2010.	urine	*P. rettgeri*	Neurology	≥256	4	64	≥256	32	32	2	<0.06	2	1	2	16	4	2	<0.06	4	4

Abbreviations: AMX-amoxycillin; AMC-amoxycillin/clavulanic acid; PIP-piperacillin; CXM-cefuroxime; CAZ-ceftazidime; CTX-cefotaxime, CTX/cl-cefotaxime/clavulanic acid; CRO-ceftriaxone; FEP-cefepime; FOX-cefoxitin; AMT-aztreonam; TZP-piperacillin/tazobactam; IMI-imipenem; MEM-meropenem; GM-gentamicin; CIP-ciprofloxacin

cing of bla_{TEM} genes identified TEM-1. *P. rettgeri* had only TEM amplicon.

Characterization of plasmids

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

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

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

DISCUSSION

The study reported spread of CTX-M-15 producing *P. stuartii* causing urinary tract infections at the University Hospital in Split. CTX-M-15 β-lactamase was previously reported in *E. coli*, *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 *K. pneumoniae* and *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 *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 $bla_{CTX-M-3}$ and $bla_{CTX-M-15}$ genes have been found so far (33). IS*Ecp1* insertion sequence found upstream of the gene is able to mobilize and promote the expression of $bla_{CTX-M-15}$ gene acting as a significant factor in the rapid spread of CTX-M-15 enzyme in Croatia. Slight differences in cefotaxime and cefepime MICs could be attributable to variable expression of 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 *P. stuartii* in the Split University Hospital and horizontal transfer of re-

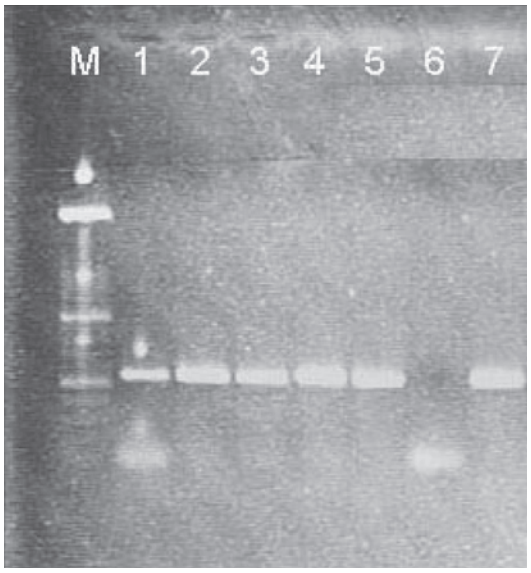

Figure. 1. Polymerase chain reaction with primers for CTX-M ß-lactamases
Lane 1. *Providencia stuarti* 68; 2. *Providencia stuarti* 69; 3. *Providencia stuarti* 72; 4. *Providencia stuarti* 73; 5. *Providencia stuarti* 74; 6. *E. coli* ATCC 25922 (negative control); 7. *E. coli* CTX-M-3 (positive control).

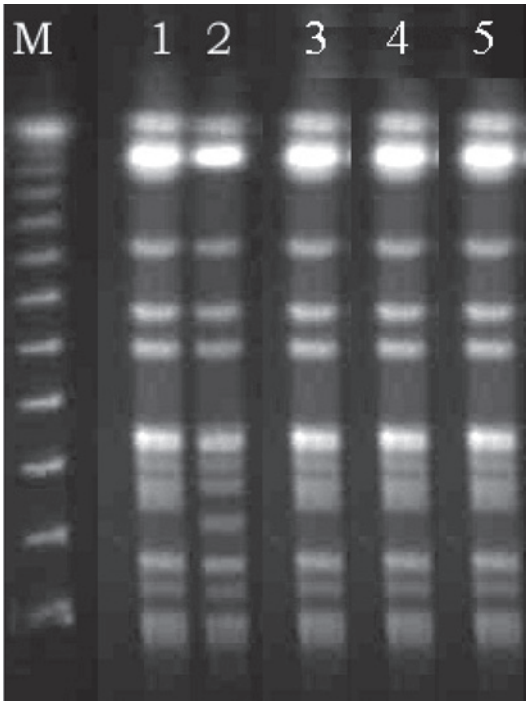

Figure 2. Pulsed-field electrophoresis of chromosomal DNA
Lane 1. *Providencia stuarti* 68; 2. *Providencia stuarti* 69; 3. *Providencia stuarti* 72; 4. *Providencia stuarti* 73; 5. *Providencia stuarti* 74.

lated plasmids containing bla_{CTX-M} genes which also contained resistance genes for aminoglycosides, tetracycline, choramphenicol, sulphonamides and trimethoprim. It is possible that also the consumption of non-β-lactam antibiotics like aminoglycosides exerts the selection pressure, which enables the spread of ESBL producing organisms in the University Hospital of Split. The fact that clonally related strains were collected during a prolonged study period raises concern that multiresistant strain persisted unnoticed in the hospital environment. ESBLs previously reported in *Providencia* spp. are most frequently of CTX-M-2 group-CTX-M-14 (43-48), TEM-116 (49), VEB-1(50-51), and PER-1 (52-53). CTX-M-15 producing *Enterobacteriaceae* are very often associated with urinary tract infections as reported previously (54-56).

Carbapenems and amikacin are antibotics of choice for the treatment of patients infected with ESBL positive *P. stuartii*. The strains were susceptible to cefepime and piperacillin/tazobactam but cephalosporins 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 producing *P.stuarti* in the hospital. Emergence of ESBL producing *P. stuarti* in our neighboring countries indicates regional dissemination of this important urinary tract pathogen (47,53).

Continuous surveillance in tracking CTX-M-15-producing *P. stuarti* 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.

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Klonsko širenje *Providencia* spp producenta CTX-M-15 ß-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 splitske 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.

Metode Osjetljivost na antibiotike širokog spektra djelovanja određena je metodom mikrodilucije prema CLSI smjernicama. Produkcija ESBLs dokazana je metodom dvostrukog diska (DDST). Konjugacijom je određen prijenos rezistencije na cefotaksim. Prisutnost gena koji kodiraju proizvodnju ESBL određen je PCR metodom, koristeći uvjete koji su opisani ranije. Genotipizacija sojeva izvedena je PFGE metodom.

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, ceftazidimom, cefotaksimom, ceftriaksonom, gentamicinom i ciprofloksacinom. *P. stuartii* je prenijela rezistenciju na *E. coli* s učestalosti u rasponu od 5 x 10^{-4} do 10^{-5}. Pet sojeva *P. stuarti* bilo je pozitivno na produkciju TEM i CTX-M β-laktamaze, dok je *P. rettgeri* bila pozitivna samo na TEM β-laktamazu. Sekvenciranje *bla*$_{CTX-M}$ gena je identificiralo CTX-M-15 β-laktamazu. Za pet izolata koji proizvode CTX-M β-laktamazu pokazalo se da su klonalno povezani.

Zaključak Neprekidnim se nadzorom želi spriječiti širenje *P. stuartii* pozitivne na CTX-M-15 β-laktamazu u bolnici i izvanbolničkom okruženju. Globalno širenje *P. stuartii* pozitivne na ESBL predstavlja veliki klinički problem u cijelome svijetu.

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