**BRIEF COMMUNICATION**

# First Report of CTX-M Extended-Spectrum Beta-Lactamase-Producing Isolates from Croatia

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Antimicrobial resistance in Gram-negative bacteria due to the production of extended-spectrum beta-lactamases (ESBLs) has become a worldwide problem [1]. CTX-M type enzymes are molecular class A of ESBLs. Most CTX-M enzymes provide a high level of resistance to the cefotaxime and ceftriaxone whereas minimum inhibitory concentrations (MICs) of ceftazidime often remain within the susceptible range [2]. The CTX-M family can be divided by amino acid sequence similarity into five major groups: CTX-M-1 group (CTX-M-1, -3, -10, -12, -15, -22, -23, -28, and FEC-1), CTX-M-2 group (CTX-M-2, -4, -4L, -5, -6, -7, -20, and Toho-1), CTX-M-8 (one plasmid-mediated member), CTX-M-9 group (CTX-M-9, -13, -14, -16, -17, -19, -21, -27, -24, and Toho-2), and CTX-M-25 group (CTX-M-25 and CTX-M-26) [2]. The first CTX-M ESBL-producing clinical isolate was detected in Germany in 1989 [3]. After 1995, the emergence of the CTX-M enzymes has been observed in many parts of the world, especially in South America, the Far East, and Europe [4-6]. These enzymes have been identified in *Enterobacteriaceae* from countries near Croatia such as Italy [7], Hungary [8] and Austria [9], as well as other Mediterranean countries (Greece, Turkey) [2]. Recently, CTX-M enzymes have also been found in Algeria [10].

Only SHV-2, SHV-5 and SHV-12 extended-spectrum β-lactamases have been detected in *K. pneumoniae* isolates from Croatia so far [11].

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ESBL-producing *Escherichia coli* strains with unusual resistance phenotypes, being more resistant to cefotaxime than to ceftazidime, and isolated during 2002-2003 at the Split University Hospital, were studied to determine the type of ESBL produced. Five ESBL-producing *E. coli* isolates were studied: 3 strains (N. 32, 36, and 86) were isolated from the urine or bronchial aspirates of pediatric patients; 2 (N. 16 and 100) were isolated from the urine of adult patients from Nephrology and Surgery. Production of an ESBL was detected using a double disk synergy test with a cefotaxime disk and confirmed by ESBL E test. MICs were determined by agar dilution test and interpreted using NCCLS breakpoints [12]. A phenotype consistent with production of CTX-M-type β-lactamase was defined by a 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. The isolates were more resistant to cefotaxime and ceftriaxone than to ceftazidime (*Table 1*). Four strains were resistant to gentamicin, and all were susceptible to imipenem. The two isolates from adults were resistant to ciprofloxacin while the three from children were susceptible to this antibiotic.

Isolates with a CTX-M phenotype were screened for $bla_{CTX-M}$ alleles by multiplex PCR with primers 5'SCS-ATG-TGC-AGY-ACC-AGT-AA-3' (MA-1) and 5'CCG-CRA-TAT-GRT-TGG-TGG-TG-3' (MA-2). Cycling conditions were: initial denaturation at 94°C for 3 min; 35 cycles of 94°C for 30 d, 55°C for 30 s, and 72°C for 45 s; and a final elongation at 72°C for 5 min [6]. All five isolates were confirmed to produce group 1 CTX-M enzyme. Four of the five tested isolates gave a 400bp PCR product apparently linking an IS26 element with $bla_{CTX-M}$ which was previously reported in the UK epidemic CTX-M-15-producing *E. coli* (strain A) [6].

Isolation of chromosomal DNA was performed as described by Kaufman *et al.*[13]. 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 (75 mM NaCl; 25 mM EDTA, Sigma), bacteria were resuspended in 500 µl SE buffer with 10 µl lysosime (Boehringer Mannheim GmbH). Next, 500 µl of this bacterial suspension were 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°C in 2 ml of ESP buffer (1% N-lauril sarcosine; 0.5 M $EDTANa_2$, 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 washed three times for 30 min at



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TABLE 1 - *MICs of the five CTX-M β-lactamase-producing Escherichia coli isolates.*

| Isolate | MIC (μg/ml)[a] | | | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | AN | GN | AMP | AMC | CZ | CXM | CTX | CTX/CA | CRO | FOX | CTB | CAZ | CAZ/CA | FEP | IPM | PIP | TZP | CIP |
| 16 | 64 | 32 | 1024 | 4 | 512 | >1024 | 64 | 0.125 | 1024 | 4 | 0.064 | 1 | 0.125 | 128 | 0.125 | 256 | 2 | >32 |
| 32 | 16 | 128 | >1024 | 8 | 1024 | 1024 | 1024 | <=0.06 | 1024 | 16 | 0.5 | 4 | 0.5 | 2 | 0.25 | >64 | 16 | <=0.125 |
| 36 | 2 | 1 | >1024 | 8 | >1024 | >1024 | 512 | <=0.06 | 1024 | 8 | 0.5 | 4 | 0.25 | 256 | 0.25 | >64 | 4 | <=0.125 |
| 86 | 4 | >32 | 1024 | 8 | >1024 | >1024 | 1024 | 0.125 | 1024 | 16 | 1 | 8 | 0.5 | 512 | 0.25 | >64 | >64 | <=0125 |
| 100 | >64 | >32 | >1024 | 8 | 128 | >1024 | 128 | <=0.06 | 1024 | 8 | 0.25 | 4 | 0.5 | 32 | 0.5 | >64 | 4 | 8 |

[a] AN, amikacin; GN, gentamicin; AMP, ampicillin; AMC, amoxicillin/clavulanic acid; CZ, cefazolin; CXM, cefuroxime; CTX, cefotaxime; CTX/CA, cefotaxime/clavulanic acid; CRO, ceftriaxone; FOX, cefoxitin; CTB, ceftibuten; CAZ, ceftazidime; CAZ/CA, ceftazidime/clavulanic acid; FEP, cefepime; IPM, imipenem; PIP, piperacillin; TZP, tazobactam/piperacillin; CIP, ciprofloxacin.

4°C with TE buffer (10 mM Tris-Hcl, pH 8, 0.1 mM EDTA, Sigma) before macrorestriction with 10 U/1 μl *Xba*I for 3 h at 37°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°C**;** with pulses from 5 to 50 s in 0.5 TBE buffer with thiurea (50 mM, 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 banding patterns obtained by PFGE of genomic DNA digests were compared following the criteria of Tenover for bacterial strain typing [14] and analyzed with GelComparII and BioNumerics computer software. The patterns obtained were compared by clustering methods (unweighted pairgroup method with arithmetic averages) using the Dice coefficient. An optimization of 0.50% and position tolerance of 3.00% was applied during the comparison of PFGE fingerprinting patterns.

Two pediatric isolates were clonally related, but the other 3 isolates had distinct PFGE patterns (*Figure 1*); all isolates were distinct from UK strain A (*Figure 2*).

The total spectrum of multiple drug resistance was transferred by conjugation (broth mating method) [15] from three isolates to a rifampin-resistant recipient strain; resistance to aminoglycosides was cotransferred with the ESBL from strain 16, resistance to tetracycline was cotransferred from strain 36 and resistance to aminoglycosides, tetracycline and co-trimoxazole from strain 100. Transconjugants were selected on Mueller-Hinton medium containing rifampin (256 μg/ml) and ceftazidime (2 mg/L).

In the present study, we report the first group 1 CTX-M ESBLs found in Croatia. Origins of our clinical isolates (urinary tract) are in agreement with previous reports [2]. The emergence of CTX-M enzymes highlights the importance of using either ceftazidime and cefotaxime, or cefpodoxime screens to detect ESBL production[16]. Delayed recognition of infections caused by CTX-M producers may lead to inappropriate treatment and to high mortality [17]. Carbapenems could be recommended as antibiotics of choice for the treatment of infections caused by our CTX-M producing *E. coli* isolates.

Fluoroquinolones could be considered as an option for adults if *in vitro* tests show susceptibility. Susceptibility to ciprofloxacin of the isolates obtained from children could be due to non-prescribing of this antibiotic in this age group.

Global dissemination of CTX-M-producing strains in recent years emphasizes the need for their epidemiological monitoring and prudent use of antimicrobial agents. Since ESBL detection procedures are not always sensitive, the occurrence of CTX-M enzymes suggests that it is important for our labora-

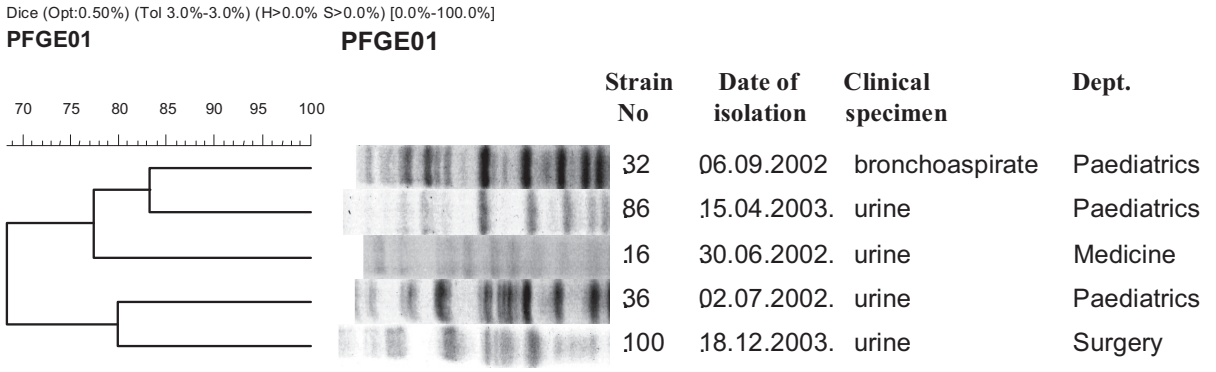


FIGURE 1 - Pulsed-field gel electrophoresis of genomic DNA of five group 1 CTX-M-producing *E. coli* isolates.

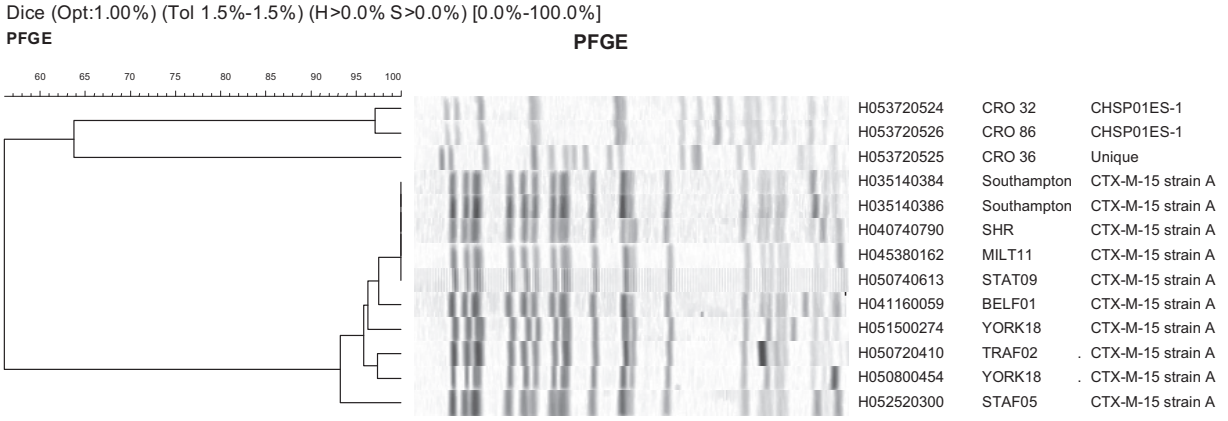


FIGURE 2 - Pulsed-field gel electrophoresis of genomic DNA of Croatian and UK group 1 CTX-M-producing *E. coli* isolates.

tories to perform double-disk synergy testing with cefpodoxime which is degraded by TEM, SHV and CTX-M ESBLs.

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