High Prevalence of CTX-M Group of Extended Spectrum Beta-Lactamases in *Enterobacteriaceae* strains isolated from the North-East of Romania

Prevalența crescută de beta-lactamaze de tip CTX-M cu spectru extins, la tulpini de *Enterobacteriaceae* izolate în Nord-Vestul României

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Abstract

The antibiotic resistance mediated by extended-spectrum beta-lactamases (ESBL) is a phenomenon that creates serious therapeutic problems around the world. The aim of this study was to detect the presence of CTX-M ESBLs enzymes in Enterobacteriaceae isolates from the North-East of Romania and the antimicrobial resistance profile of the CTX-M-producing strains. ***Material and methods.*** *One hundred and twelve Escherichia coli and Klebsiella pneumoniae strains isolated from patients admitted to "Sf. Spiridon" Teaching Hospital from Iasi, between January-October 2009 were included. ESBL production was determined using a double-disc synergy test. The presence of CTX-M-type beta-lactamase genes was initially screened by PCR-using universal primers for the conserved region of bla $_{CTX-M}$ genes. The PCR positive isolates were further tested using individuals pairs of primers to identify to which CTX-M group the gene belonged.* ***Results.*** *A total of 41 strains were positive for CTX-M genes: 39 strains (29 E. coli and 10 K. pneumoniae) carried CTX-M group 1 related genes, one strain of E. coli with CTX-M group 2 gene and another strain of E. coli with CTX-M group 9 gene. These enterobacteria strains demonstrated a high level of resistance to cephalosporins, with cefotaxime minimum inhibitory concentration (MIC) ≥ 4-fold greater than ceftazidime MIC.* ***Conclusions.*** *The study revealed that CTX-M-1 group is the dominant ESBL-type in both E. coli and K. pneumoniae tested strains. In addition, this is the first report of E. coli producing CTX-M -9 related ESBL in Romania.*

Key words: CTX-M, extended-spectrum beta-lactamases, enterobacteria

Rezumat

Rezistența la antibiotice prin producerea de beta-lactamaze cu spectru extins (BLSE) este un fenomen care creează serioase probleme terapeutice în întreaga lume. Scopul acestui studiu a fost de a detecta prezența BLSE tip CTX-M la tulpini de Enterobacteriaceae izolate în Nord-Estul României şi stabilirea rezistenței la antibiotice a izolatelor de enterobacterii producătoare de CTX-M. ***Material şi metode.*** *Am testat 112 tulpini de Escherichia coli şi Klebsiella pneumoniae izolate in perioada ianuarie-octombrie 2009, de la pacienți internați în Spitalul*

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Universitar "Sf. Spiridon" din Iaşi. Am testat producerea de BLSE prin testul dublului disc. Prezența genelor codante pentru beta-lactamaze de tip CTX-M a fost inițial investigată prin PCR cu primeri universali pentru regiunea conservată a genelor bla$_{CTX-M}$. Izolatele pozitive au fost ulterior investigate cu perechi de primeri specifici, pentru a identifica apartenența de grup a enzimelor. ***Rezultate.*** *Un număr de 41 izolate au fost pozitive pentru gene care codifică beta-lactamaze tip CTX-M: la 39 tulpini (29 E. coli şi 10 K. pneumoniae) am detectat BLSE din grupul CTX-M-1, iar la alte două izolate de E. coli, BLSE din grupul CTX-M-2, respectiv CTX-M-9. Aceste tulpini de enterobacterii au demonstrat un nivel ridicat de rezistență la cefalosporine, cu valori ale concentrației minime inhibitorii (CMI) la cefotaxim ≥ 4 ori decât valorile CMI la ceftazidim.* ***Concluzii.*** *Studiul demonstrează că grupul CTX-M-1 este dominant în cadrul BLSE produse atât de tulpinile de E. coli cât şi de K. pneumoniae testate. De asemenea, este prima raportare din România a unei tulpini de E. coli producătoare de BLSE din grupul CTX-M-9.*

Cuvinte cheie*: CTX-M, beta-lactamaze cu spectru extins, enterobacterii*

Introduction

The antibiotic resistance mediated by extended-spectrum beta-lactamases (ESBL) is a phenomenon that creates serious therapeutic problems. The CTX-M-type enzymes are a group of class A ESBLs that exhibit an overall preference for cefotaxime and ceftriaxone and they are rapidly spreading among *Enterobacteriaceae* worldwide (1). The CTX-M family is composed of more than 50 enzymes and can be subclassified into six major phylogenetic groups, including: CTX-M-1, CTX-M-2, CTX-M-8, CTX-M-9, CTX-M-25 group and CTX-M-45 (2). For some years, CTX-M ESBLs have been predominantly found in three geographic areas: South America, the Far East and Eastern Europe (3).

The objective of this study was to evaluate the presence of CTX-M ESBLs in *Enterobacteriaceae* isolates from the North-East of Romania and the antibiotic resistance profile of the CTX-M-producing strains.

Material and methods

Bacterial strains

A total of 112 *Enterobacteriaceae* isolates, collected between January-October 2009 from patients admitted to "Sf. Spiridon" Teaching Hospital from Iasi, were selected for this study according to their resistance profile to third generation cephalosporins. The strains were isolated from pus, blood, catheter tips, urine, and faeces. Identification was performed by conventional methods and with the mini API system (bioMérieux, Marcy-l'Étoile, France).

Antimicrobial susceptibility testing

The minimum inhibitory concentrations (MICs) for ceftazidime, cefotaxime, cefepime, cefpirome, aztreonam, imipenem and meropenem were determined by agar dilution method. Resistance rates were reported using the Clinical Laboratory Standard Institute (CLSI) breakpoints for the fully susceptible category; moderately susceptible isolates were classified as resistant (4).

Screening for and confirmation of ESBLs

ESBL production was detected by the double-disc synergy test using amoxicillin-clavulanic acid, cefotaxime and ceftazidime and by the phenotypic confirmatory test using cefotaxime (30µg) and ceftazidime (30 µg) discs alone and in combination with 10µg of clavulanic acid (Oxoid, Basingstoke, UK), according to CLSI guidelines. *Escherichia coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC 700603 were used as negative and positive controls, respectively.

Beta-lactamase expression

The expression of β-lactamases was detected by isoelectric focusing (IEF). Bacteria growing exponentially at 37^{0}C in Luria-Bertani medium were harvested and cell-free lysates were obtained by sonication. Isoelectric focusing was performed on a 5% polyacryamide gel containing ampholynes with a pH range of 3 to 10, in a LKB Multiphor 2117 apparatus. Enzymes were focused at a constant power of 1W for 60 min and detected by overlaying the gel with 1 mM nitrocefin solution.

Table 1. Specific primer pairs used for amplification

Primer		Sequence 5' → 3'	Amplicon size	Reference
CTX-MU_1		ATG TGC AGY ACC AGT AAR GT	593 bp	1
CTX-MU_2		GGG TRA ART ARG TSA CCA GA		
CTX-M-1 group	Forward	AAA AAT CAC TGC GCC AGT TC	415 bp	5
	Reverse	AGC TTA TTC ATC GCC ACG TT		
CTX-M-2 group	Forward	CGA CGC TAC CCC TGC TAT T	552 bp	5
	Reverse	CCA GCG TCA GAT TTT TCA GG		
CTX-M-8 group	Forward	TCG CGT TAA GCG GAT GAT GC	666 bp	5
	Reverse	AAC CCA CGA TGT GGG TAG C		
CTX-M-9 group	Forward	CAA AGA GAG TGC AAC GGA TG	205 bp	5
	Reverse	ATT GAA AAG CGT TCA TCA CC		
CTX-M-25 group	Forward	GCA CGA TGA CAT TCG GG	327 bp	5
	Reverse	AAC CCA CGA TGT GGG TAG C		

PCR experiments

The presence of *bla_{CTX-M}* was detected by PCR. The isolates were initially screened with universal primers, CTX-MU_1 and CTX-MU_2, targeting the conserved region of the encoding genes (1). PCR positive isolates were further tested in a multiplex PCR using individual pairs of primers specific for CTX-M groups 1, 2, 8, 9 and 25, as previously described (5). The primers used in this study are listed in *Table 1*. All PCR reactions were carried out in 50 µl final volumes made up of 200 mM dNTPs, 1,5 mM $MgCl_2$, 1 U Taq Polymerase (Promega Corp., Madison WI, USA), 50 pmol of each primer, on a Mastercycler Personal instrument (Eppendorf). The template DNA was obtained by 15 minutes boiling of a dense bacterial suspension in 500 µl distilled water, followed by centrifugation at 13000 rpm, 5 minutes. The PCR products were separated by electrophoresis in 1% agarose gels, 60 min at 100V in 0.5 x TBE buffer. DNA was stained with ethidium bromide and the gels were visualized under UV light. We used positive controls for groups CTX-M-1, CTX-M-2 and CTX-M-9.

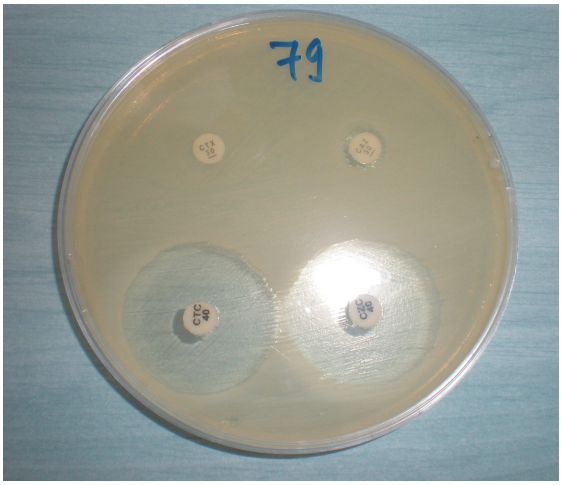

Figure 1. Phenotypic detection of ESBL production for *Escherichia coli* strain number 79: there is an increase of ≥ 5 mm of the inhibition zone around the discs with cefotaxime and ceftazidime in combination with clavulanic acid (CTC and CZC), compared with the cefotaxime (CTX) and ceftazidime (CAZ) discs alone

Results

Screening for and confirmation of ESBLs

The double-disc synergy test was positive for 55 isolates from 112 tested strains. In the phenotypic confirmatory test, all these 55 strains were confirmed as ESBL producers: an increase of ≥ 5 mm of the cefotaxime or ceftazidime inhibition zone in the presence of clavulanic acid was considered positive test (*Figure 1*).

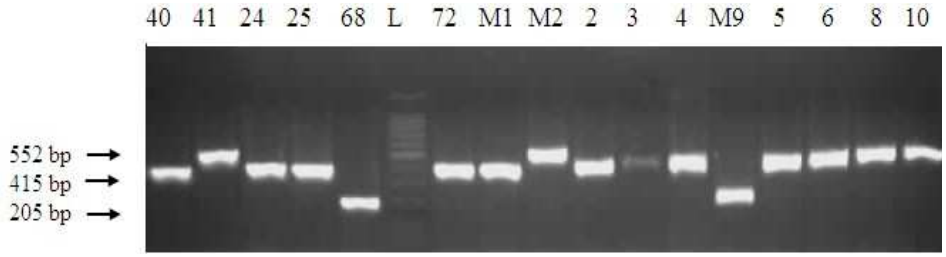

Figure 2. PCR products obtained with specific primers for CTX-M groups. L = 100bp DNA ladder (Promega Corp., Madison WI, USA); M1, M2, M9 = positive controls for groups CTX-M-1, CTX-M-2 and CTX-M-9; 40 = *K. pneumoniae* strain harboring group1 CTX-M alleles; 24, 25, 72, 2, 3, 4, 5, 6, 8, 10 = *E. coli* strains harboring group1 CTX-M alleles; 41= *E. coli* strain harboring a group 2 CTX-M gene; 68 = *E. coli* strain harboring a group 9 CTX-M gene.

IEF experiment

A broad range of isolectric points (pI) was observed (pI=5.5-9). Some of strains produced two or multiple β-lactamases. The IEF is only a presumptive test to identify the type of β-lactamases.

PCR experiments

After the PCR screening with CTX-M universal primers, from the 55 strains, 41 (31 *E. coli* and 10 *K. pneumoniae*) were positive for *bla* $_{CTX-M}$. The rest of 14 isolates were CTX-M negative, but express ESBL phenotype. The nature of the other ESBLs was not investigated in this work. Alleles encoding group 1 CTX-M enzymes were found in 39 strains, comprising 29 *E. coli* and 10 *K. pneumoniae*. The remaining isolates included one *E. coli* that produced a group 2 CTX-M enzyme and one *E. coli* that produced a group 9 enzyme (*Figure 2*). No producers of group 8 or 25 CTX-M enzymes were detected.

Antimicrobial susceptibility tests

The cefotaxime MICs for all CTX-M positive strains were found to be higher than those to ceftazidime in the agar dilution method. The resistance phenotype of these isolates was suggestive for the production of CTX-M-type ESBL. The cumulative data for *in vitro* activity of the antibiotics tested against the CTX-M-1 group ESBL-producing isolates are summarized in *Table 2* and *Table 3*. All the *K. pneumoniae* strains were resistant to cefotaxime, ceftazidime, cefpirome, cefepime and aztreonam, with MICs values ranging from 4 mg/L to 512 mg/L. For *E. coli* strains, resistance rates for ceftazidime, cefotaxime, cefepime and cefpirome were very high: 86.2 %, 100%, 96.5 % and 100%, with the MIC90 values ≥ 128 mg/L for each of the tested cephalosporins. The MICs for cefotaxime ranged from 64 mg/l to 512 mg/L. No isolates resistant to imipenem or meropenem were revealed (*Table 2*). Both imipenem and meropenem inhibited 90% of strains at MIC values ≤ 0.03 mg/L.

For *E. coli* producing CXT-M-2-like ESBL the MIC value for cefotaxime was 256 mg/L, while for *E. coli* positive for CXT-M-9 related enzyme the MIC value was 64 mg/L. The MIC for ceftazidime for these two strains was 4 mg/L.

Discussions

The dimension and complexity of the survey of CTX-M-type ESBLs production at international level are serious reasons for the development at a national level of such researches, having the main purpose that of establishing the *status* of the enterobacterial resistance to the antimicrobial agents in our country at the present moment.

We have examined by PCR 55 ESBL positive isolates of *E. coli* and *K. pneumoniae* collected between January-October 2009 in the North-East of Romania. According to the specialized literature, there are a few data regard-

Table 2. *In vitro* activities of antimicrobial agents against CTX-M-1 group ESBL producing strains (n=39 strains), as MIC 50, MIC 90 and resistance percent

Species	Antimicrobial agent	MIC 50 (mg/L)	MIC 90 (mg/L)	Range (mg/L)	Resistance %
E. coli (n=29)	Cefotaxime	128	512	64-512	100
	Ceftazidime	64	128	4-256	86.2
	Cefepime	64	128	8-256	96.5
	Cefpirome	128	256	64-256	100
	Aztreonam	128	256	4-512	96.5
	Imipenem	0.03	0.03	0.03-0.125	0
	Meropenem	0.03	0.03	0.03-0.03	0
Klebsiella pneumoniae (n=10)	Cefotaxime	256	512	128-512	100
	Ceftazidime	64	256	16-256	100
	Cefepime	64	128	16-128	100
	Cefpirome	128	256	64-256	100
	Aztreonam	256	512	4-512	90
	Imipenem	0.03	0.03	0.03-0.03	0
	Meropenem	0.03	0.03	0.03-0.03	0

ing CTX-M-type ESBLs producing in Romania. Increased use of new generations of beta-lactam antibiotics in Central Europe and Eastern Europe has created, in the recent years, an increased hazard of efficient selection of *Enterobacteriaceae* ESBL producing strains (6). In Latvia there was characterized a CTX-M-2 variant, designated CTX-M-5. In Russia a CTX-M-4 producing *Salmonella enterica* was reported. The CTX-M-3 enzymes were identified in Poland and Greece (7). In Bulgaria and in Poland a CTX-M-15 variant was reported (8, 9). In Spain, rates of CTX-M enzymes production were found 52.3% and 12.5% among ESBL-producing isolates of *E. coli* and *K. pneumoniae*, respectively, with a predominance of group 9 (CTX-M-9 and CTX-M-14) and group 1 (CTX-M-10) (10).

In the present study we have detected the CTX-M-1, CTX-M-2 and CTX-M-9 groups among enterobacteria producing ESBLs. It is the first detection of *E. coli* producing CTX-M-9 group ESBL in Romania. We observed that CTX-M-1 group was the most common ESBL type in North-Eastern Romania, like in Italy, were the rates of CTX-M production were found to be 54.8% and 12.3% among ESBL-producing isolates of *E. coli* and *K. pneumoniae*, respectively, with a predominance of group 1 enzymes (mostly CTX-M-1 and CTX-M-15 and less frequently, CTX-M-3) (2). Recently, in our country, the genotypic analysis by PCR and sequencing showed the presence of bla_{TEM} and group 1 CTX-M genes in 75% of *E. coli* strains isolated from patients admitted to the Neuropsichiatry Clinical Hospital of Craiova (11). In addition, in Romania it was detected the CTX-M-15, a variant of the CTX-M-3, which is included in the group 1 CTX-M (6, 7). The emergence of CTX-M-type ESBLs production reflects, most probably, the overuse of third generation cephalosporins in the North-East of Romania.

Table 3. Cumulative percent frequencies of MICs (mg/L) against CTX-M-1 group producers

CTX-M-1 group producers	Agent	Cumulative % of strains inhibited at a concentration (mg/L) of:							
		4	8	16	32	64	128	256	512
E. coli (n=29)	Cefotaxime					20.6	41.3	72.4	100
	Ceftazidime	6.9	13.7	27.5	27.5	55.1	82.7	100	
	Cefepime		3.4	13.8	34.4	55.1	89.6	100	
	Cefpirome					27.5	58.6	100	
	Aztreonam	3.4	3.4	3.4	10.3	17.2	44.8	82.7	100
K. pneumoniae (n=10)	Cefotaxime						30	70	100
	Ceftazidime			30	30	60	60	100	
	Cefepime			10	30	60	100		
	Cefpirome					10	60	100	
	Aztreonam	10	10	10	10	10	40	60	100

In our study, we have noticed for CTX-M producing strains high resistance rates for ceftazidime, cefotaxime, cefpirome and cefepime. Aztreonam was also a good substrate for beta-lactamase producers. The carbapenems were the only agents active against all strains, with very low MIC 50 and MIC 90 values (0.03 mg/L).

In summary, the overall prevalence of CTX-M ESBLs enzymes in *Enterobacteriaceae* isolates in the North-East of Romania was 41 of 55 ESBL producers (74.54%).

Conclusions

The present study demonstrated that CTX-M-1 group is dominant in both *E. coli* and *K. pneumoniae* tested strains, but we have also detected two strains producing CTX-M-2 and the CTX-M-9 related ESBLs. This is the first report of *E. coli* producing CTX-M -9 related ESBL in Romania. Further investigations are necessary to identify the type of these enzymes and the clonal relationship of the strains.

Acknowledgements

This study was carried out research grant no. 16843 from the "Gr. T. Popa" University of Medicine and Pharmacy Iasi, Romania.

We thank Ştefania Brînduşa Copăcianu, Head of Microbiology Department - Synevo Clinical Laboratory, Iasi, candidate for a doctor's degree "Gr. T. Popa" University of Medicine and Pharmacy Iasi, Romania and Aurora Ambăruş, Head of Microbiology Department" Sf. Spiridon" Hospital, Iasi, Romania, for providing the tested strains.

Conflict of interests: None to declare.

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