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High prevalence of nosocomial *Escherichia coli* and *Klebsiella pneumoniae* producing CTX-M-type extended spectrum β-lactamases in Russian hospitals

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REVISED ABSTRACT

BACKGROUND AND OBJECTIVES: ESBLs of a CTX-M-type represent rapidly emerging group of β-lactamases. On the basis of sequence similarity all enzymes of this group can be distributed into four subtypes epitomized by CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9. The aim of our study was to investigate the prevalence of different subtypes of CTX-M ESBLs among nosocomial *E.coli* and *K.pneumoniae* strains in Russian hospitals. **METHODS:** Consecutive nosocomial isolates of *E.coli* (n=494) and *K.pneumoniae* (n=410) were collected in 28 Russian hospitals during 1997-1998. ESBL production was detected by double-disc synergy test. All ESBL-producing strains were screened for CTX-M β-lactamases by PCR with primers specific to the conserved regions of the coding genes. A PCR was followed by RFLP analysis with *Pst*I and *Pvu*II restriction enzymes permitting the differentiation of the CTX-M subtypes. The *E.coli* strains producing the known CTX-M β-lactamases were used for quality control. **RESULTS:** The ESBL phenotype was observed in 78 (15.8%) and 248 (60.8%) *E.coli* and *K.pneumoniae* isolates, respectively. Among the ESBL producers 28 (35.9%) *E.coli* and 85 (34.3%) *K.pneumoniae* were found to possess genes for CTX-M β-lactamases. The majority of these strains (92.9%) produced the CTX-M-1-related ESBLs. The CTX-M-2-related enzymes were detected in 8 *E.coli* strains from a single hospital only. Neither CTX-M-8- nor CTX-M-9-related enzymes were found in this study. The CTX-M-producing strains were detected in 21 of 28 surveyed hospitals and predominated over the strains expressing all other types of ESBLs in the medical centers located in the areas of Ural and Siberia. In two hospitals the relative frequency of CTX-M-type ESBLs ranged up to 93 and 100%, respectively. **CONCLUSIONS:** We conclude that the CTX-M-type β-lactamases represent a significant and rapidly spreading group of ESBLs in Russia.

INTRODUCTION AND PURPOSE

During the past decade ESBLs of CTX-M-type emerged in many countries of the world. A rapid increase in the proportion of CTX-M variants to the classical TEM- and SHV-derived ESBLs was observed in Spanish (T.M.Coque, 2001), Canadian (M.P.Muller, 2001), Chinese (A.Chanawong, 2001) hospitals. Furthermore, CTX-M β-lactamases, mainly CTX-M-2 and CTX-M-3, became predominant ESBL types in Argentina (M.F.Galas,1999), Japan (N.Shibata, 2001) and Poland (M.Gniadkowski, 2002).
Currently the CTX-M family includes more than 20 β-lactamases which may be grouped on the basis of sequence similarity into 4 distinct subtypes epitomized by CTX-M-1, CTX-M-2, CTX-M-8 and CTX-M-9. Within each subtype there is a high degree of bla_{CTX-M} gene sequence identity (>95%), although members of different subtypes share only 70-77% homology at the level of gene sequences.
The classical phenotype of resistance conferred by CTX-M β-lactamases (significantly higher level of resistance to cefotaxime than ceftazidime) is not universal among all CTX-M producers, since many factors including production of additional β-lactamases (M.Gniadkowski, 2002), or mutations altering the substrate specificity of CTX-M enzymes (P.Nordman, 2001) can mask their presence. Therefore, phenotypic detection of CTX-M enzymes is difficult. Isoelectric focusing is also inadequate since the same pI can correspond to β-lactamases of different families. PCR has been used widely to detect bla_{CTX-M} genes, however detection of all the known variants required multiple reactions with primers specific for different genes.
We have developed a PCR-RFLP method for detection of all the known types of CTX-M ESBLs and applied this method to study the prevalence of CTX-M enzymes among nosocomial *E.coli* and *K.pneumoniae* strains in Russian hospitals.

METHODS

Bacterial isolates. Consecutive nonduplicate nosocomial isolates of *E.coli* (n=494) and *K.pneumoniae* (n=410) were collected during 1997-1998 in acute and intensive care units of 28 hospitals located in 12 Russian cities: Ekaterinburg, Kazan, Krasnodar, Krasnojarsk, Moscow, Novosibirsk, Omsk, Riazan, Smolensk, St.-Petersburg, Stavropol, Thomsk, Ufa and Vladivostok. All the strains were reidentified in the laboratory of the Institute of Antimicrobial Chemotherapy using API20E system (bioMérieux, France) and stored at -70°C until analysis.

Susceptibility testing. MICs of cefotaxime and ceftazidime were determined using Etests (AB Biodisk, Sweden) on Mueller-Hinton agar (Becton Dickinson, USA). *E.coli* strain ATCC® 25922 was used for quality control.

Phenotypic ESBL detection. ESBL production was detected by double disk synergy test (DDST) using disks with cefotaxime (30μg) and ceftazidime (30μg) placed 20mm and 30mm (center to center) from a disk with amoxicillin/clavulanic acid (20/10μg).

Detection and subtyping of bla_{CTX-M} genes by PCR-RFLP. A pair of primers (CTX-M/F: 5'-tttgcgatgtgcagtaccag-3' and CTX-M/R: 5'-gatatcgttggtggtgccat-3') matching the conserved sequences at positions 205 to 224 and 747 to 728 with respect to the CTX-M translational starting point was designed to amplify a specific 543-bp fragment of all the known bla_{CTX-M} genes. The PCR mixes contained in 50μl volumes: 50mM KCl, 10mM Tris-HCl (pH 9), 0.1% TritonX-100, 1. 5mM $MgCl_2$, 200μM of each dNTP, 0.4μM of each primer, 1 TaqBead Hot Start Polymerase (Promega, USA) and 5μl of template DNA prepared with Lyse-N-Go PCR reagent (PIERCE, USA) as recommended by manufacturer. The amplification was carried out in a PTC-200 thermocycler (MJ Research, USA) under the following conditions: 2 min initial denaturation at 94°C followed by 35 cycles of 20 sec denaturation at 94°C, 30 sec annealing at 51°C, and 30 sec elongation at 72°C with a final elongation step extended to 3 min. A computer analysis was used to identify restriction endonucleases capable of distinguishing the subtypes of bla_{CTX-M} genes. These restriction endonucleases and their predicted patterns are shown in Table 1. The amplified DNA (10μl) was directly subjected to digestion with 9U *Pst* I and 4U *Pvu* II enzymes (Amersham Pharmacia Biotech, USA) in One-Phor-All Plus Buffer (10mM Tris acetate (pH 7.5), 10mM magnesium acetate, 50mM potassium acetate) for 3 h at 37°C . The PCR products and restriction fragments were analysed by electropforesis in 3.5% AmpliSize agarose (BioRad, USA) and ethidium bromide staining. Bacterial strains producing the known β-lactamases: *E.coli* AB1456 (CTX-M-4), *Citrobacter freundii* 2525 (CTX-M-3), *E.coli* C600 (TEM-1), *E.coli* J53 (SHV-1), *Kluyvera ascorbata* T861 (KluA, species-specific), *Klebsiella oxytoca* (OXY, species-specific) were used for quality control and assessment of specificity of PCR-RFLP.

Transfer of resistance. Nine CTX-M β-lactamase producing isolates were mated in broth with *E.coli* AB1456 (F', Rif^R). The transconjugants were selected on agar containing rifampin (100μg/ml) and cefotaxime (2μg/ml).

RESULTS AND DISCUSSION

Validation of the PCR-RFLP method for detection and discrimination of bla_{CTX-M} genes. Using the described primers and conditions of PCR a single DNA fragment of expected size (543 bp) was amplified from the control strains producing the known CTX-M β-lactamases of different genetic groups, and from the *K.ascorbata* type strain T861 carrying the chromosomal gene for species-specific β-lactamase KluA which shares >99% identity with CTX-M-2. The specificity of PCR was confirmed by negative amplification results with bacteria possessing other class A plasmid mediated β-lactamases and *K.oxytoca* bla_{OXY} gene which is partially homologous to bla_{CTX-M}. Further digestion of PCR products with selected restriction endonucleases made it possible to distinguish between the subtypes of bla_{CTX-M} genes as shown on Fig. 1.

Table 1. Restriction patterns of the PCR products showing differentiation between the CTX-M subtypes

CTX-M Subtype[a]	Number of restriction sites		Expected length of *Pst* I - *Pvu* II digestion products
	Pst I	*Pvu* II	
CTX-M-1-related (CTX-M-3, -10, -11, -12, -22, UOE-1)	0	2	267, 156, 120 bp
CTX-M-2-related (CTX-M-4, -5, -6, -7, -20, Toho-1, KluA)	1	0	355, 188 bp
CTX-M-8	0	0	543 bp
CTX-M-9-related (CTX-M-13, -14, -15, -16, -18, -19, -21)	1	1	426, 72, 45 bp

a - grouping is based on ≥95% sequence identity of the coding genes

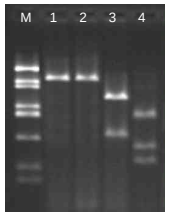

Figure 1. bla_{CTX-M}-gene amplification products and their RFLP patterns obtained with *Pst* I-*Pvu* II restriction endonucleases.

Lane M, pUC18-*Hae* III;

Lanes 1 and 2, undigested 543-bp PCR-products;

Lanes 3 and 4, RFLP patterns corresponding to *E.coli* AB1456 (CTX-M-4) and C. *freundii* 2525 (CTX-M-3), respectively.

Prevalence of ESBL production and proportion of isolates possessing CTX-M-type ESBLs. The percentage of ESBL-producing *E.coli* and *K.pneumoniae* isolates varied from 8.1 to 90% in different hospitals, although the overall frequency of ESBL producers observed in this study was alarmingly high: 15.8% in *E.coli* and 60.8% in *K.pneumoniae* (Table 2). The rate of ESBL production did not depend on the geographic location of the hospital. For example, the frequency of ESBL-positive strains ranged between 10 and 90% in eight Moscow hospitals, and between 26.1 and 56.3% in two Krasnojarsk hospitals thus reflecting a specific situation in each medical center.
All the ESBL-producing strains were examined by PCR for the presence of bla_{CTX-M} genes and positive amplification results were observed for 28 (35.9%) *E.coli* and 85 (34.3%) *K.pneumoniae* expressing ESBL phenotype. These results for the first time demonstrate the high proportion of CTX-M enzymes among different ESBL species produced by nosocomial strains in Russian hospitals. CTX-M β-lactamases were detected in 21 (75%) surveyed hospitals from geographically distant areas of Russia and in 5 (17.9%) hospitals were the predominant ESBL types. The highest rates of CTX-M β-lactamase-producing *E.coli* and *K.pneumoniae* was observed in the hospitals of Ekaterinburg, Thomsk, Krasnodar and Stavropol which represent the areas of Ural, Siberia and South Russia.
Distribution of different CTX-M subtypes. As demonstrated by PCR-RFLP analysis, the vast majority (92.9%) of CTX-M β-lactamases belonged to the group of CTX-M-1-related enzymes. The CTX-M-2-related enzymes were detected in 8 *E.coli* isolates from a single hospital only.

Table 2. Percentage of ESBL-producing *E.coli* and *K.pneumoniae* and proportion of CTX-M-positive isolates in different hospitals

Center	*E.coli*			*K.pneumoniae*			Both species		
	n[a]	% ESBL+[b]	% CTX-M+[c]	n[a]	% ESBL+[b]	% CTX-M+[c]	n[a]	% ESBL+[b]	% CTX-M+[c]
Ekaterinburg-1	1	100.0	0.0	14	85.7	50.0	15	86.7	46.2
Ekaterinburg-2	6	0.0	-	9	66.7	100.0	15	40.0	**100.0**
Kazan	19	42.1	100.0	21	50.0	0.0	40	46.2	44.4
Krasnodar	10	20.0	50.0	4	50.0	50.0	14	28.6	50.0
Krasnojarsk-1	13	23.1	66.7	19	78.9	13.3	32	56.3	22.2
Krasnojarsk-2	13	7.7	100.0	10	50.0	20.0	23	26.1	33.3
Moscow-1	14	35.7	60.0	10	90.0	22.2	24	58.3	35.7
Moscow-2	13	84.6	9.1	18	88.9	0.0	31	87.1	3.7
Moscow-3	14	85.7	41.7	26	92.3	25.0	40	90.0	30.6
Moscow-4	46	10.9	0.0	7	57.1	25.0	53	17.0	11.1
Moscow-5	40	2.5	0.0	30	20.0	16.7	70	10.0	14.3
Moscow-6	21	9.5	50.0	3	66.7	0.0	24	16.7	25.0
Moscow-7	27	18.5	0.0	11	36.4	25.0	38	23.7	11.1
Moscow-8	19	5.3	0.0	15	53.3	12.5	34	26.5	11.1
Novosibirsk	5	20.0	100.0	34	88.2	66.7	39	79.5	67.7
Omsk	29	0.0	-	8	37.5	33.3	37	8.1	33.3
Smolensk	10	10.0	0.0	31	77.4	16.7	41	61.0	16.0
St.-Petersburg	6	50.0	66.7	14	92.9	15.4	20	80.0	25.0
Stavropol	13	7.7	100.0	34	67.6	73.9	47	51.1	75.0
Tomsk	19	10.5	100.0	20	65.0	92.3	39	38.5	**93.3**
Vladivostok	8	12.5	0.0	8	25.0	50.0	16	18.8	33.3
Other hospitals	148	8.1	0.0	64	27.0	0.0	212	13.7	0.0
All centers	**494**	**15.8**	**35.9**	**410**	**60.8**	**34.3**	**904**	**36.1**	**34.7**

a - number of isolates; **b** - percentage of ESBL-producing isolates;
c - percentage of isolates carrying bla_{CTX-M} genes among ESBL producers

Notably, all these isolates were clonally related as determined by ERIC-PCR typing (data not shown) and did not transfer resistance to cefotaxime in conjugation experiments. This observation is in good agreement with previous publications describing the location of the $bla_{CTX-M-2}$-related genes on small nonself-transferable plasmids. It may also explain the relatively low proportion of CTX-M-2-related β-lactamases compared to CTX-M-1-related enzymes which are usually coded by conjugative plasmids. Neither CTX-M-8- nor CTX-M-9-related enzymes were found in this study.
Resistance phenotypes of ESBL-producing isolates. Figure 2 shows the frequency distribution of cefotaxime and ceftazidime MICs as well as their respective ratios in isolates producing CTX-M- and non-CTX-M-type ESBLs.

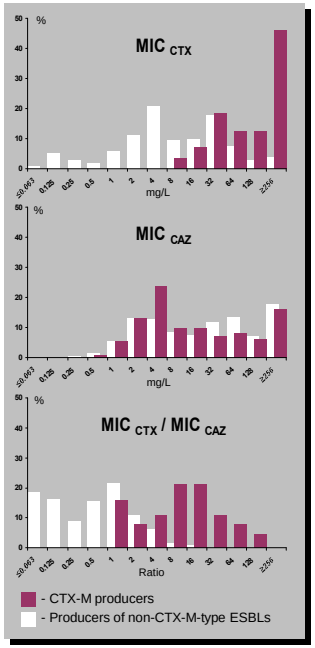

Figure 2. Frequency distribution of cefotaxime (CTX) MICs, ceftazidime (CAZ) MICs and their respective ratios in isolates producing CTX-M and non-CTX-M-type ESBLs.

Cefotaxime MICs were generally higher for CTX-M β-lactamase producers (mode 256 mg/L vs. 4 mg/L for non-CTX-M-type ESBL producers; p<0.0001, Wilcoxon test). Nevertheless, a high degree of diversity of the levels of resistance to cefotaxime was observed for CTX-M-positive isolates, as illustrated by broad MIC range (8 to 256 mg/L). More surprisingly, no statistically significant difference was observed in ceftazidime MICs between the groups of CTX-M- and non-CTX-M-type ESBL producers (p=0.1619, Wilcoxon test). For most of the CTX-M-positive isolates resistance phenotypes were consistent with the production of CTX-M ESBL with MICs of cefotaxime being higher by 2 to 9 twofold dilutions than those of ceftazidime. Nevertheless, equal or differing by 1 dilution MICs of cefotaxime and ceftazidime were observed in 27 (23.9%) CTX-M producers. All isolates equally resistant to cefotaxime and ceftazidime had the highest detectable MICs of 256 mg/L. Therefore, a limited range of MIC values determined by Etests, may partly explain the unusually low cefotaxime/ceftazidime ratio in CTX-M-producing strains. The high level of ceftazidime resistance in such strains is more likely explained by the presence of additional ceftazidime-hydrolyzing β-lactamases than an extended activity of the CTX-M enzyme. This was proved by conjugation experiments with 2 *E.coli* and 3 *K.pneumoniae* ceftazidime-resistant CTX-M-positive isolates, which transferred resistance to cefotaxime to the donor strain separately of ceftazidime resistance. On the other hand, there were 18 CTX-M-negative ESBL-producing isolates having 1 to 3 dilution higher MICs of cefotaxime than those of ceftazidime. All the above data suggest that phenotypic approach based on the comparison of cefotaxime and ceftazidime MICs is of limited value in predicting the presence of CTX-M-type ESBLs in clinical isolates.

CONCLUSIONS

- The proposed PCR-RFLP method is a useful tool for detecting and discriminating the CTX-M β-lactamases of different genetic groups.
- The CTX-M-type ESBL-producing isolates are globally disseminated in Russian hospitals. They comprise about one-third of all ESBL-producing *E.coli* and *K.pneumoniae* nosocomial strains.
- CTX-M-1-related β-lactamases predominate among different CTX-M subtypes in Russia.