C2-1874 Molecular Epidemiology of Nosocomial CTX-M-Producing *Klebsiella pneumoniae* and *Escherichia coli* Isolates from 21 Russian Hospitals

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REVISED ABSTRACT

Background: CTX-M-group of extended-spectrum β-lactamases (ESBLs) represents a rapidly emerging problem in many countries. High prevalence of nosocomial CTX-M-producing *E.coli* and *K.pneumoniae* strains in Russian hospitals has been shown previously but molecular epidemiology of such strains has never been resolved. This study describes molecular typing of nosocomial CTX-M-producing *E.coli* and *K.pneumoniae* strains from Russia. **Methods:** PCR with primers specific for all the known CTX-M-coding genes was used to identify 28 *E.coli* and 87 *K.pneumoniae* strains producing CTX-M ESBLs from 21 Russian hospitals located in the areas of Western, South, Central Russia, Ural, Siberia and Far East during 1997-98. All the isolates underwent molecular typing by ERIC-PCR. Amplified 544bp bla_{CTX-M} internal fragment was sequenced for 3 *E.coli* and 3 *K.pneumoniae* strains representing clonal outbreaks. **Results:** Seventeen distinct ERIC-PCR patterns were obtained for *E.coli* and 49 for *K.pneumoniae*, respectively. Isolates from the same hospital mostly belonged to 1 or 2 major clones, but we also observed a number of strains with unique patterns. Two cases of clonal relatedness between the strains from geographically distinct centers were found. The bla_{CTX-M} internal fragment sequences from all the *K.pneumoniae* strains were identical to that of $bla_{CTX-M-3}$, whereas sequences from two *E.coli* strains differed by only one point mutation corresponding to UOE-1-coding gene. These genes were readily transferred to a recipient *E.coli* strain in conjugation experiments. One *E.coli* strain representing a group of 8 clonally related isolates from a single hospital carried the gene for CTX-M-5 which was not transferred by conjugation. **Conclusions:** Both horizontal and vertical gene transfers are responsible for broad dissemination of CTX-M-3 and closely related β-lactamases in nosocomial *E.coli* and *K.pneumoniae* in Russia. The occasional outbreak of CTX-M-5 β-lactamase producers in the single hospital was due to their clonal spread.

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 enzymes were found to be widespread or even predominant ESBL species in some countries, including Argentina (M.F.Galas,1999), Japan (N.Shibata, 2001) and Poland (M.Gniadkowski, 2002). Still, there are only few studies assessing the distribution and epidemiology of CTX-M-producers at the country-wide scale.

Recently we reported a high prevalence and broad geographic distribution of nosocomial CTX-M-producing *E.coli* and *K.pneumoniae* isolates in Russia (M.Edelstein, 2002). CTX-M-encoding genes were found in over one-third of the isolates expressing ESBL phenotype. The present study describes the molecular epidemiology of these isolates.

METHODS

Bacterial isolates. Consecutive nonduplicate nosocomial isolates of *E.coli* (n=346) and *K.pneumoniae* (n=346) were collected during 1997-1998 in acute and intensive care units of 21 hospitals located in 12 Russian cities: Ekaterinburg, Kazan, Krasnodar, Krasnojarsk, Moscow, Novosibirsk, Omsk, Smolensk, St.-Petersburg, Stavropol, Thomsk and Vladivostok (see Figure 1). All the strains were reidentified at the laboratory of the Institute of Antimicrobial Chemotherapy using API20E system (bioMérieux, France) and stored at -70°C until analysis.

Detection and subtyping of bla_{CTX-M} genes by PCR-RFLP. A pair of primers (CTX-M/F': 5'-TTTGCGATGTGCAGTACCAGTAA-3' and CTX-M/R': 5'-CGATATCGTTGGTGGTGCCATA-3') matching the conserved sequences at positions 205 to 227 and 748 to 727 with respect to the CTX-M translational starting point was used to amplify a specific 544-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, 2mM $MgCl_2$, 200μM of each dNTP, 0.5μM of each primer, 1 TaqBead Hot Start Polymerase (Promega, USA) and 5μl of template DNA extracted using the Lyse-N-Go PCR reagent (PIERCE, USA). 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. The PCR products were digested with Pst I and Pvu II restriction enzymes (Amersham Pharmacia Biotech, USA) and the digests were analyzed by agarose gel electrophoresis.

Sequencing of PCR products. Amplified 544-bp internal fragments of bla_{CTX-M} genes were directly sequenced using the primers CTX-M/F' and CTX-M/R' on the CEQ-2000 automated sequencer (Beckman-Coulter, USA). This procedure was done at the Eurogene company (Moscow, Russia).

ERIC-PCR fingerprinting. Molecular typing of all the CTX-M-positive strains was performed with primer ERIC1 as described elsewhere with some minor modifications. PCR mixes were set up in Ready-To-Go PCR Bead format (Amersham Pharmacia Biotech, USA) providing the following composition of reaction mixture: 10mM Tris-HCl (pH 9.0), 50mM KCl, 1.5mM $MgCl_2$, 200μM of each dNTP and 1.5U of Taq-polymerase after addition of ERIC1 primer (50 pmoles), 2μl of template DNA and water to the final volume of 25μl. The amplification was carried out in a PTC-200 thermocycler (MJ Research, USA) under the following conditions: 2 min 30 sec initial denaturation at 94°C followed by 35 cycles of 30 sec denaturation at 94°C, 1 min annealing at 47°C, and 1 min elongation at 72°C with a final elongation step extended to 4 min. The PCR products were electrophoresed in 1.3% agarose gel and stained with ethidium bromide. The gels were documented using a PhotoDoc-IT Link Gel Documentation System (UVP, USA). Cluster analysis of genomic fingerprints was done using the GelCompar software v.4.1 (Applied Maths BVBA, Belgium) by the unweighted pair group method using arithmetic averages (UPGMA).

RESULTS AND DISCUSSION

Table 1. Prevalence of CTX-M-producing strains in the surveyed hospitals and multiplicity of their ERIC-PCR profiles.

Center	*E.coli*			*K.pneumoniae*			CTX-M cluster (type)[b]
	No. of isolates	No. of CTX-M-positive	No. of ERIC-PCR profiles[a]	No. of isolates	No. of CTX-M-positive	No. of ERIC-PCR profiles[a]	
Ekaterinburg-1	1	0		14	6	5	1
Ekaterinburg-2	6	0		9	6	3	1 (CTX-M3)
Kazan	19	8	1	21	0		2 (CTX-M-5)
Krasnodar	10	1	1	4	1	1	1
Krasnojarsk-1	13	2	2	19	2	2	1
Krasnojarsk-2	13	1	1	10	1	1	1
Moscow-1	14	3	3	10	2	2	1
Moscow-2	13	1	1	18	0		1
Moscow-3	14	5	2	26	7	4	1 (UOE-1)
Moscow-4	46	0		7	1	1	1
Moscow-5	40	0		30	1	1	1
Moscow-6	21	1	1	3	0		1
Moscow-7	27	0		11	1	1	1
Moscow-8	19	0		15	1	1	1
Novosibirsk	5	1	1	34	20	2	1 (CTX-M3)
Omsk	29	0		8	1	1	1
Smolensk	10	0		31	5	4	1
St.-Petersburg	6	2	1	14	2	1	1 (CTX-M3)
Stavropol	13	1	1	34	17	14	1
Tomsk	19	2	2	20	12	6	1
Vladivostok	8	0		8	1	1	1
All centers	346	28	17	346	87	48	

[a] Numbers of distinct ERIC-PCR profiles among CTX-M producers.
[b] CTX-M clusters (as determined by by PCR-RFLP analysis):
1 – CTX-M-1-related enzymes; 2 - CTX-M-2-related enzymes.
Types of enzymes are indicated according to the sequence data.

Prevalence and distribution of different CTX-M ESBLs. A total of 115 CTX-M-producing *E.coli* and *K.pneumoniae* isolates were identified by PCR screening. These isolates represented 21 hospitals from 12 Russian cities (Table 1). As demonstrated by restriction analysis of PCR products, the vast majority (92.9%) of CTX-M β-lactamases found in this study belonged to the CTX-M-1 cluster. Direct sequencing of the amplified internal fragments of $bla_{CTX-M-1}$-related genes from selected strains representing major clonal outbreaks (see below) has shown that in three *K.pneumoniae* isolates (NOV37, EK-Ro171 and SP477.1) from Novosibirsk, Ekaterinburg and St.-Petersburg these sequences corresponded to $bla_{CTX-M-3}$ (GenBank # Y10278). Previously, a country-wide spread of CTX-M-3 β-lactamase-producing organisms was reported in Poland, where this ESBL was found in 6 species of the family *Enterobacteriaceae*, including *K.pneumoniae* (M.Gniadkowski, 2002). In addition, similar sequences were obtained from two *E.coli* strains (BH6884, BH3223/2) isolated in Moscow. Both sequences differed from $bla_{CTX-M-3}$ by a single point mutation leading to the Asp240Gly substitution in the deduced amino acid sequence. This mutation is known to distinguish the UOE-1 enzyme (GenBank # AY013478) which was first described in *E.coli* isolate from Japan (T.Muratani, 2000) and more recently was identified in a single isolate of the same species in Bulgaria (I.Schneider, 2002).

The CTX-M-2-related enzymes were detected by PCR-RFLP analysis in 8 *E.coli* isolates from a single hospital of Kazan. None of these isolates transferred resistance to cefotaxime in conjugation experiments. Sequence of the internal bla_{CTX-M} gene fragment was determined for one isolate (KZ-Ma12) representing this group and was found to be identical to $bla_{CTX-M-5}$. The CTX-M-5 enzyme was first found in isolates of *Salmonella typhimurium* from Latvia (P.Bradford, 1998) and till now it was not identified in the other species of the family *Enterobacteriaceae*. This study therefore describes the first occurrence of the CTX-M-5-like enzyme in *E.coli*.

Molecular epidemiology of CTX-M-producing clinical isolates. ERIC-PCR typing revealed considerable diversity of genetic types of CTX-M-producers in Russia (Fig. 2). Seventeen distinct ERIC-PCR patterns were obtained for *E.coli* and 48 for *K.pneumoniae*, respectively. Nevertheless, several major clonal outbreaks caused by the strains of both species were recognized.

The largest outbreak occurred at the Novosibirsk hospital where 20 *K.pneumoniae* producing the CTX-M-3-like β-lactamase were isolated from patients of surgical and intensive care units during the two-year period. Out of these isolates 15 had identical ERIC-PCR patterns and the other 4 were grouped into a distinct cluster, suggesting a dissemination of two major clones in a single hospital. It is worth to mention that isolates which apparently belonged to the Novosibirsk epidemic clones were found in the hospitals of very distantly located cities: two strains were isolated in St.-Petersburg, and the other two – in Ekaterinburg. Alternative molecular typing methods (RAPD with different primers) confirmed the relatedness of these isolates (data not shown). However, the ways of their transmission remain unknown since the data about patient or personal transfer between these hospitals are lacking.

Figure 1. Geographic location of the surveyed hospitals.

Another outbreak of CTX-M-producing *K.pneumoniae* occurred at the Tomsk hospital. Eight of the 12 *K.pneumoniae* strains isolated in this hospital displayed very similar (differing by not more than 2 bands) profiles.

All the *E.coli* isolates from Kazan which produced the CTX-M-5-like enzyme were found to be clonally related too. Smaller groups of 2-4 isolates with identical or similar ERIC-PCR profiles were seen in many centers, but in almost all hospitals surveyed multiple clones of CTX-M-producing organisms were detected. Therefore, it is evident that in addition to the clonal spread of the strains plasmid transfer played important role in the global dissemination of CTX-M-coding genes among nosocomial strains in Russian hospitals. In support of this conclusion mating experiments with selected clinical isolates demonstrated that the genes for CTX-M-1-cluster enzymes were readily transferred by conjugative plasmids from all the isolates to the recipient strain.

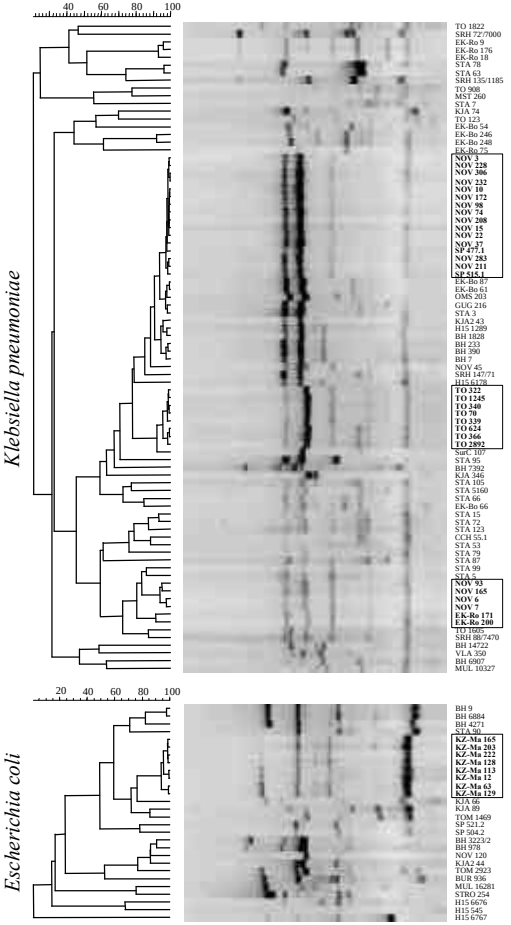

Figure 2. UPGMA clustering of ERIC-PCR profiles of CTX-M-producing strains. The isolates representing major clonal outbreaks are framed.

CONCLUSIONS

- β-Lactamases of the CTX-M-1 cluster, like CTX-M-3 and its single-mutation variant UOE-1, were the most prevalent CTX-M enzymes among nosocomial *E.coli* and *K.pneumoniae* strains isolated in Russian hospitals. Both horizontal and vertical gene transfers played important role in their broad dissemination within and between different hospitals.
- The dissemination of the CTX-M-5-like enzyme in the single hospital was attributed to clonal spread of the *E.coli* strain.
- The alarming situation with global dissemination of CTX-M-producing strains urges the need for their epidemiological monitoring and prudent use of III generation cephalosporins.