HIGH FREQUENCY OF ASSOCIATION BETWEEN CTX-M-β-LACTAMASE-CODING GENES AND THE ISEcp1 INSERTION ELEMENT

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REVISED ABSTRACT

Objectives: CTX-M beta-lactamases are becoming an increasingly alarming problem throughout the world due to their fast spread. This is often addressed to the possible transfer of CTX-M-coding genes with mobile genetic elements. Indeed, several studies have shown an association of bla_{CTX-M} genes with the ISEcp1 insertion sequence or the putative recombinase gene *orf513*. However, such studies have been confined to relatively small numbers of strains. We have studied the association of bla_{CTX-M} genes with ISEcp1 using a large collection of CTX-M-producing enterobacterial strains obtained from 21 Russian hospitals.

Methods: The strains studied were 28 *Escherichia coli* (EC), 87 *Klebsiella pneumoniae* (KP) and 36 *Proteus mirabilis* (PM). They comprised 17, 48 and 22 distinct genetic types, respectively, as determined by ERIC-PCR and RAPD typing and produced CTX-M-1-cluster enzymes (94.7%), i.e. CTX-M-3 and CTX-M-15, and a CTX-M-2-cluster enzyme (5.3%) - CTX-M-5. Two PCRs with ISEcp1-specific primers were used to identify the linkage of bla_{CTX-M} genes with ISEcp1. One forward primer (F1) matched the 3'-end sequence of *tnpA* and another (F2) matched the right terminal repeat (RTR) sequence of ISEcp1. A common reverse primer (R) was located internally to bla_{CTX-M}. Previously characterized EC and *Citrobacter freundii* strains harboring bla_{CTX-M} genes associated with either ISEcp1 or *orf513* were included as positive and negative controls, respectively.

Results: Positive PCR results with primers F2-R were observed for 28 (100%) EC, 86 (98.9%) KP and 35 (97.2%) PM isolates. Amplification with primers F1-R additionally confirmed the association of bla_{CTX-M} genes with ISEcp1 in 25 (89.3%) EC, 85 (97.7%) KP and 34 (94.4%) PM isolates. According to the length of PCR products, ISEcp1 was located approximately 50 bp upstream of the CTX-M ORFs in all EC, KP and PM isolates expressing CTX-M-1-cluster enzymes and approximately 20 bp upstream of $bla_{CTX-M-5}$ in 8 EC isolates. An insertion of the IS26 sequence between tnpA gene and IRR sequence of ISEcp1 was detected in a single KP isolate of a unique genetic type. Two isolates (PM and KP) which failed to produce PCR products with either of the primer pairs also represented unique genetic types.

Conclusion: : We conclude that CTX-M-coding genes of different genetic subtypes have a strong association with the ISEcp1 insertion element in nosocomial *Enterobacteriaceae* from Russia. This in part may explain their notoriously rapid spread.

INTRODUCTION AND PURPOSE

During the past decade ESBLs of CTX-M-type emerged in many countries of the world. 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. Genetic fingerprinting of a large collection of CTX-M-producing enterobacteria collected on a countrywide scale revealed a great genetic diversity of CTX-M producers. Hence, well in agreement with other reports, our data suggested the predominant role of horizontal gene transfer in CTX-M distribution (Pimkin, 2002).

The extremely rapid spread of CTX-M is believed to be facilitated by mobile genetic elements with which CTX-M ESBL coding genes have been found associated. Indeed, several studies have shown bla_{CTX-M} genes to reside in close proximity to the ISEcp1 insertion sequence (CTX-M-1, CTX-M-9 groups) (Karim, 2001; Poirel, 2003) or the putative recombinase gene *orf513* (CTX-M-2 group) (Eckert, 2004; Melano, 2003). However, it remains largely unraveled whether this is ubiquitous in all CTX-M-producing bacteria. In the present study we have assessed the frequency of association of CTX-M β-lactamases with the ISEcp1 insertion sequence on a large collection of previously characterized CTX-M-producing *Enterobacteriaceae* strains from 21 Russian hospitals.

MATERIALS AND METHODS

Bacterial Isolates: A previously characterized in our institute collection of CTX-M-producing strains (Edelstein, 2002; Pimkin 2002) was used in this study. The isolates were 28 *Escherichia coli* (EC), 87 *Klebsiella pneumoniae* (KP) and 36 *Proteus mirabilis* (PM), 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 (see Fig. 1). The production of CTX-M was confirmed in our previous study in a PCR with a pair of universal primers matching CTX-M conserved sequences. As determined by PCR-RFLP analysis with *Pvu* II and *Pst* I restriction endonucleases (Edelstein, 2002), the vast majority (94.7%) of strains produced CTX-M-1 subtype ESBLs. Subsequent direct sequencing of PCR products from several isolates allowed to identify these β-lactamases as CTX-M-3-like and CTX-M-15-like. These strains comprised 16 (EC), 48 (KP) and 22 (PM) distinct genetic types, as determined by ERIC-PCR and RAPD typing, thus showing considerable genetic diversity. (Pimkin, 2002). CTX-M-2-related enzymes (CTX-M-5, as shown by sequencing) were possessed by 8 clonally-related *E.coli* isolates (5.3%) from a single hospital of Kazan.

PCR detection of linkage between ISEcp1 and bla_{CTX-M}: Two PCRs with ISEcp1-specific primers were used to identify the linkage of bla_{CTX-M} genes with ISEcp1. One forward primer (F1: 5' - TGT CTG GTA TAA GAA TAT CAT C - 3 ') matched the 3'-end sequence of *tnpA* and another (F2: 5' - AAA CAC ACG TGG AAT TTA GG - 3') matched the right terminal repeat (RTR) sequence of ISEcp1. A common reverse primer (R: 5' - ACY TTA CTG GTR CTG CAC AT - 3') was located internally to bla_{CTX-M}

Fig. 1. Geographic location of the surveyed hospitals.

(Saladin, 2002). Previously characterized EC and *Citrobacter freundii* strains harboring bla_{CTX-M} genes associated with either ISEcp1 or *orf513* were included as positive and negative controls, respectively. The PCR mixes contained in 50 µl volumes: 50 mM KCl, 10 mM Tris-HCl (pH 9), 0.1% TritonX-100, 1.5 mM MgCl2, 200 µM of each dNTP, 0.4 µM of each primer, 1 TaqBead Hot Start Polymerase (Promega, USA) and 5 µl 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 95°C followed by 35 cycles of 20 sec denaturation at 95°C, 30 sec annealing at 43°C (for both primer pairs), and 30 sec elongation at 72°C with a final elongation step extended to 3 min. The PCR products were analysed by agarose gel electropforesis and ethidium bromide staining.

Transfer of resistance by conjugation: 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 (100 µg/ml) and cefotaxime (2 µg/ml).

Plasmid RFLP analysis: Plasmid DNA was extracted from 6 *E.coli* AB1456 recombinant strains obtained in conjugation experiments. A Qiagen MIDI Plasmid Extraction Kit (Qiagen, USA) was used. The pDNA was digested with either *Pst* I or *Pvu* II restriction endonucleases and digests were analysed by agarose gel electrophoresis.

DNA sequencing: DNA fragments amplified with F1 and R primers were directly sequenced on an ABI-310 automated genetic analyzer (Applied Biosystems, USA). A long PCR product derived from KP 237.7 isolate was cloned in pGEM-T plasmid vector (Promega, USA) in accordance with manufacturer recommendations. Sequencing of the insert was performed using vector-specific M13 primers.

RESULTS AND DISCUSSION

Positive PCR results with primers F2-R were observed for 28 (100%) EC, 86 (98.9%) KP and 35 (97.2%) PM isolates. A product of ca. 300 bp was derived from all positive CTX-M-1-subtype producers (see Fig. 2). It is worth noting that in our previous studies these isolates had shown considerable genetic diversity in PCR genetic fingerprinting experiments and readily transferred cefotaxime resistance determinants in conjugation experiments. Transconjugants were used to purify 6 CTX-M-coding plasmids originating from genetically non-related clinical isolates. No obvious similarities were revealed in plasmid RFLP analysis with either *Pst* I or *Pvu* II enzymes (Fig. 3). Along with the high frequency of CTX-M association with ISEcp1, these data, in our opinion, may evidence in favour of bla_{CTX-M}-gene transfer by this mobile element.

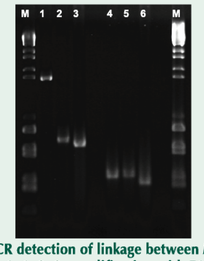

Fig. 2. PCR detection of linkage between bla_{CTX-M} and ISEcp1; 1, 2, 3, amplification with F1 and R primers; 4, 5, 6, amplification with F2 and R primers; 1, 4, KP 237.7 (CTX-M-1-subtype); 2, 5, EC 1236 (CTX-M-1 subtype); 3, 6, EC 1670 (CTX-M-5); M, λ-*Pst*E II + pUC18-*Hae* III molecular weight standard.

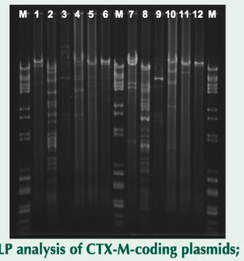

Fig. 3. RFLP analysis of CTX-M-coding plasmids; 1 – 6, *Pst* I restriction enzyme; 7 – 12, *Pvu* II restriction enzyme; 1, 7, EC 1160; 2, 8, KP 238; 3, 9, EC 1098; 4, 10, PM 1066; 5, 11, PM 1236, 6, 12, EC 395; M, λ-*Pst*E II + pUC18-*Hae* III molecular weight standard.

A shorter fragment of ca. 280 bp was amplified from 8 clonally related EC isolates from a single hospital of Kazan (see Fig. 2). These isolates had been found to produce the CTX-M-5 ESBL and were not capable of transferring cefotaxime resistance determinants in conjugation experiments. To our best knowledge this clone still remains the only EC in Europe reported to produce a CTX-M-2-subtype enzyme.

Amplification with primers F1-R additionally confirmed the association of bla_{CTX-M} genes with ISEcp1 in 25 (89.3%) EC, 85 (97.7%) KP and 34 (94.4%) PM isolates. All EC, KP and PM isolates expressing CTX-M-1-cluster enzymes gave rise to a ca. 500 bp PCR product. Direct sequencing of two PCR products (EC 1236 and KP 238) confirmed their similarity with previously published sequences (see Fig. 4-5). ISEcp1 IRR was located 48 bp upstream of the CTX-M ORFs. Putative -35 and -10 promoter sequences were identified within the 3' end of ISEcp1 (see Tab. 1) (Karim, 2001).

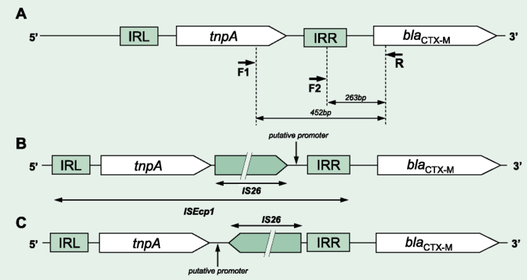

Fig. 4. Schematic maps of the sequences encoding ISEcp1 and bla_{CTX-M}. A, AY044436, EC 1236, KP 238; B, KP 237.7; C, AY462238

An insertion of the IS26 insertion sequence was detected between the *tnpA* gene and right terminal repeat of ISEcp1 in a single CTX-M-1-subtype-producing KP 237.7 isolate of a unique genetic type which yielded an extraordinary large fragment in the PCR with F1 and R primers. Interestingly, a similar sequence has been described by Woodford et al. in a CTX-M-15-producing *E.coli* strain from UK (GeneBank accession No. AY462238) but with slightly shifted IS26 insertion point and the IS26 sequence placed in the opposite direction (see Fig. 4-5).

A ca. 470 bp DNA fragment was obtained from the 8 CTX-M-5-producing EC isolates in the PCR with F1 and R primers. Direct sequencing of the PCR product from EC 1670 isolate revealed its absolute identity with *Salmonella typhimurium* pCLL3477 plasmid sequence (GeneBank accession No. AF286192), with CTX-M-5 ORF being located 20 bp downstream from ISEcp1 IRR (data not shown). It remains to be unraveled whether the entire CTX-M-5 encoding plasmids of these isolates are similar to pCLL3477 and/or plasmids of CTX-M-5-producing *Salmonella typhimurium* isolates that have caused multiple outbreaks of nosocomial salmonellosis in Russia (Edelstein, 2004; in print).

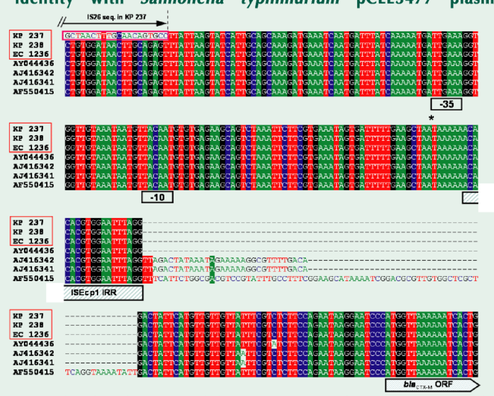

Fig. 5. ClustalW alignments of the sequences encoding 3' end region of ISEcp1 and 5' region of bla_{CTX-M}.*, the IS26 sequence insertion point in AY462238 (an *E.coli* with CTX-M-15 from UK; Woodford, 2003). AY044436, an *E.coli* with CTX-M-15 from India; Karim, 2003; AJ416342, a *Proteus mirabilis* with CTX-M-1 from France; Saladin, 2002; AJ416342, an *E.coli* with CTX-M-14 from France; Saladin, 2003; AF550415, a *Citrobacter freundii* with CTX-M-3 from Poland; Golebiewski. The IRR sequence, -35 and -10 putative promoter sequences are indicated in accordance with Karim, 2003)

Albeit rare, some strains failed to reveal the expected sort of association or even presented with none at all. Four isolates (EC 890 Burdenko Hosp., Moscow, EC 1057 Hosp.15, Moscow, EC 1096 Hosp.15, Moscow and PM 1050, Moscow) were negative in the PCR with F1 and R primer pair but yielded an expected size product when using F2 and R primers. Two isolates of unique genetic types (KP 827 Burdenko Hosp., Moscow and PM 96 Krasnodar) failed to produce PCR products with either of the primer pairs. Taking into account the sparseness of such findings, it may be speculated that they have originated from some rare recombination events.

CONCLUSIONS

CTX-M-β-lactamase-coding genes of different genetic subtypes have a strong association with ISEcp1 insertion elements in nosocomial strains of *Enterobacteriaceae* from Russian hospitals.

The ISEcp1 insertion sequence may play an important role in mobilizing CTX-M-coding genes and, hence, their broad dissemination among multiple strains and species.

The rare cases of no apparent association of bla_{CTX-M} with ISEcp1 may have evolved from some additional recombination events. Quite scarce, these findings may have very limited epidemiological significance.