CONTINUOUS SPREAD OF ESBL-PRODUCING SALMONELLAE IN RUSSIAN HOSPITALS

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INTRODUCTION

Emergence of multi-drug resistant salmonellae is an important chemotherapeutic problem worldwide. Severe salmonellosis, especially in children, is often treated empirically with expanded-spectrum cephalosporins (ESCs). Therefore, the spread of ESC-resistant salmonellae is of particular concern in terms of possible therapeutic setback. Several plasmid mediated extended-spectrum β-lactamases (ESBLs), in particular, the enzymes of the CTX-M group, are known to confer ESC-resistance phenotype in *Salmonella* spp.

In our previous study we revealed the clonal origin of 34 ESC-resistant *Salmonella* serovar Typhimurium (ST) isolates from multiple nosocomial outbreaks of gastroenteritis in Russia and Belarus in 1994-2003. All the isolates were found to produce the CTX-M-5 ESBL encoded by small non-self-transferable plasmid [1].

In the years 2003-2004 an active surveillance program was initiated by the Institute of Antimicrobial Chemotherapy with the aim to trace the possible dissemination of ESC-resistant salmonellae in the hospitals throughout Russia. A total of eleven ESC-resistant nosocomial isolates of ST and one strain of *Salmonella* serovar Infantis (SInf) were collected in six hospitals, including five newly surveyed centers, of four geographically distant Russian cities (Table 1). In the present study we explored the mechanisms of ESC resistance in these strains and their potential relation to the previously characterized ST clone.

MATERIALS AND METHODS

Bacterial strains. The sources of ESC-resistant *Salmonella* strains are listed in Table 1. All strains were isolated from patients with nosocomial salmonellosis. Notably, two strains were isolated from extraintestinal sites. Serotyping was performed with respect to cell wall (O) and flagellar (H) antigens. Two additional strains (one ESC-resistant, representing the previously characterized epidemic clone, and one epidemiologically unrelated susceptible strain isolated in hospital H4 in 2003) were included for comparison.

Susceptibility testing. Susceptibilities to ampicillin, amoxicillin-clavulanate (2:1), piperacillin, piperacillin-tazobactam (4 mg/L fixed inhibitor conc.), ceftriaxone, cefotaxime, cefotaxime-clavulanate (4 mg/L fixed inhibitor conc.), ceftazidime, ceftazidime-clavulanic acid (4 mg/L fixed inhibitor conc.), cefoxitin, tetracycline, chloramphenicol, gentamicin, amikacin, co-trimoxazole and ciprofloxacin were determined using agar dilution method according to the NCCLS (2004) guidelines.

Molecular typing. Arbitrary primed PCR (AP-PCR) with primers ERIC1R and ERIC2 and PCR with a random primer, OPB-17, were used to type all the strains [1].

Molecular characterization of β-lactamases. All isolates were screened by PCR for the presence of the genes for TEM-, SHV-, OXA- and CTX-M-type β-lactamases as described previously [2, 3, 1]. The *bla*CTX-M-gene amplification products were directly sequenced on both strands using an ABI PRISM Big Dye Terminator V3.1 cycle sequencing kit and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems, Stafford, TX).

Detection of mobile elements upstream of the *bla*CTX-M genes. Two PCRs with ISEcp1-specific primers were used to identify the linkage of *bla*CTX-M genes with ISEcp1. One forward primer (F1: 5'-TGTCTGGTATAATAAGAATATCATC-3') matched the 3'-end sequence of tnpA and another (F2: 5'-AAACACACGTGGAATTTAGG-3') matched the right terminal repeat (RTR) sequence of ISEcp1. A common reverse primer (CTX-M/R': 5'-CGATATCGTTGGTGGTGCCATA-3') was located internally to *bla*CTX-M [4]. The same primers and the primer CTX-M-F' (5'-TTTGCGATGTGCAGTACCAGTAA-3') were used to sequence the PCR products.

Transfer of resistance to ESCs and analysis of CTX-M-coding plasmids. All ESC-resistant *Salmonella* isolates were mated in broth with *Escherichia coli* AB1456 (F-, RifR). Selection of transconjugants was attempted on plates containing cefotaxime (1 mg/L) and rifampin (100 mg/L). Plasmids were purified from five ST isolates (one from each hospital) using a Wizard Plus SV Minipreps Kit (Promega, Madison, WI) and used to transform *E. coli* TOP10 competent cells. Native CTX-M-coding plasmids isolated from the transformants and restriction fragments obtained after digestion of these plasmids with *PstI* and *PvuII* endonucleases (Promega) were compared by agarose gel electrophoresis.

Table 1. Sources of ESC-resistant *Salmonella* isolates.

Strains	Location	Hospital	Isolation date	Patient age, years	Specimen type	Diagnosis
ST-356	Voronezh	H1	Jan. 2004	36	Feces	Enteritis
ST-458	Voronezh	H1	May. 2004	30	Feces	Enteritis
ST-490	Voronezh	H1	Aug. 2004	31	Feces	Enteritis
ST-516	Voronezh	H1	Apr. 2004	17	Feces	Enteritis
ST-725	Voronezh	H2	Aug. 2004	69	Feces	Enteritis
ST-1196	Jartsevo	H3	Oct. 2003	6	Feces	Enteritis
ST-1197	Jartsevo	H3	Oct. 2003	6	Feces	Enteritis
ST-30	Jartsevo	H3	Jan. 2004	2	Feces	Enteritis
ST-930	Smolensk	H4	Jul. 2004	1	Feces	Enteritis
ST-977	Smolensk	H4	Aug. 2004	20	Feces	Carriage
ST-1732	Irkutsk	H5	Apr. 2003	5	Urine	UTI
SInf-349	Moscow	H6	Oct. 2003	57	Blood	Bacteraemia
ST-81 [a]	Jartsevo	H3	Jan. 2003	31	Feces	Enteritis

[a] Previously characterized epidemic clone

Table 2. Phenotypic and genotypic characteristics of Salmonella isolates.

Strains	MICs of β-lactams, mg/L [b]										Co-resistance markers [c]	AP-PCR pattern	Presence of ISEcp1 upstream of *bla*CTX-M	CTX-M-coding plasmid	β-lactamases detected
	AM	XL	PP	PTc	TX	CT	CTL	TZ	TZL	FX					
ST-356	≥256	16	≥256	2	≥256	≥256	0.25	8	0.25	4	Tet, Chl, Gm	A	Yes,- 20 bp	ND [d]	CTX-M-5
ST-458	≥256	16	≥256	2	≥256	≥256	0.5	16	0.5	4	Tet, Chl, Gm	A	Yes,- 20 bp	ND [d]	CTX-M-5
ST-490	≥256	32	≥256	≥256	≥256	≥256	0.5	16	0.5	4	Tet, Chl Gm, Sxt	A	Yes,- 20 bp	ND [d]	CTX-M-5, OXA-1-like
ST-516	≥256	16	≥256	2	≥256	≥256	0.5	16	0.5	4	Tet, Chl, Gm	A	Yes,- 20 bp	7.4 kb	CTX-M-5
ST-725	≥256	32	≥256	≥256	≥256	≥256	0.5	8	0.5	4	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	7.4 kb	CTX-M-5, OXA-1-like
ST-1196	≥256	32	≥256	128	≥256	≥256	0.25	4	0.25	4	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	ND [d]	CTX-M-5, OXA-1-like
ST-1197	≥256	32	≥256	128	≥256	≥256	0.25	4	0.25	2	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	ND [d]	CTX-M-5, OXA-1-like
ST-30	≥256	32	≥256	≥256	≥256	≥256	0.25	4	0.25	4	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	7.4 kb	CTX-M-5, OXA-1-like
ST-930	≥256	8	≥256	2	≥256	≥256	0.06	4	0.25	4	-	A	Yes,- 20 bp	ND [d]	CTX-M-5
ST-977	≥256	8	≥256	2	≥256	≥256	0.25	4	0.25	4	-	A	Yes,- 20 bp	7.4 kb	CTX-M-5
ST-1732	≥256	16	≥256	2	≥256	≥256	0.5	4	1	4	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	-	CTX-M-5
SInf-349	≥256	8	≥256	4	≥256	≥256	0.5	64	0.5	16	Chl, Gm	B	Yes, -48 bp	ND [d]	CTX-M-15
ST-81 [b]	≥256	≥256	≥256	≥256	≥256	≥256	0.5	8	1	4	Tet, Chl, Gm, Sxt	A	Yes,- 20 bp	7.4 kb	CTX-M-5, OXA-1-like

[b] AM, ampicillin; XL, amoxicillin-clavulanate; PP, piperacillin; PTc, piperacillin-tazobactam; TX, ceftriaxone; CT, cefotaxime; CTL, cefotaxime-clavulanate; TZ, ceftazidime; TZL, ceftazidime-clavulanate; FX, cefoxitin.

[c] Tet, tetracycline; Chl, chloramphenicol; Gm, gentamicin; Sxt, co-trimoxazole.

[d] ND - not determined.

RESULTS AND DISCUSSION

Antibiograms. The results of susceptibility testing are summarized in Table 2. All the isolates studied were highly resistant to penicillins, cefotaxime and ceftriaxone (MICs >256 mg/L). The Sinf strain was also resistant to ceftazidime (MIC 64 mg/L) while the ST isolates were more susceptible (4-16 mg/L). The described phenotype was typical for the earlier reported epidemic ST clone producing the CTX-M-5.

Resistance to piperacillin-tazobactam was detected in five ST isolates. Furthermore, ten of the twelve isolates were non-susceptible to gentamicin and chloramphenicol, nine - to tetracycline, and six - to co-trimoxazole. No resistance was observed to cefoxitin, amikacin and ciprofloxacin, although the MICs of the latter drug were markedly elevated in two strains (SInf-349 and ST-1732) as compared to the naturally susceptible strains (0.5-1 mg/L vs. 0.003-0.006 mg/L).

β-Lactamase content. Results of PCR and sequencing were indicative of the presence of CTX-M-5 ESBL in all the ST isolates. In addition, five isolates from different hospitals carried the OXA-1-like β-lactamase conferring resistance to piperacillin-tazobactam.

The Sinf strain produced the CTX-M-15 ESBL that is known to confer increased resistance to ceftazidime as a result of D240G substitution relative to CTX-M-3 [5].

Neither *bla*TEM nor *bla*SHV genes were detected by PCR in any of the isolates studied.

Genetic context of *bla*CTX-M genes and transferability of resistance. An ISEcp1 insertion sequence was detected 20 bp upstream of the *bla*CTX-M-5 in all the ST isolates and 48 bp upstream of the *bla*CTX-M-15 in the Sinf strain. Sequences directly preceding the *bla*CTX-M-5 were all identical and differed from the published sequence (GeneBank accession # AF286192) of plasmid pCLL3477 from Latvian ST strain by only three nucleotide changes. Sequence of the *bla*CTX-M-15 gene and its upstream vicinity in the Sinf strain completely matched that of the Indian *E. coli* strain (GenBank accession # AY044436).

In mating experiments, none of the analyzed strains transferred cefotaxime resistance to the recipient *E. coli*.

The CTX-M-5-encoding plasmids were isolated from four ST isolates (one per hospital) and introduced to the *E. coli* TOP10 by transformation. The resulting transformants resembled the original strains in ESC-resistance phenotype but were susceptible to β-lactam-inhibitor combinations and all non-β-lactam agents (data not shown). Therefore the transferred CTX-M-5-coding plasmids contained no additional resistance determinants. Three isolates had the same restriction patterns of *bla*CTX-M-5-carrying plasmids as earlier reported strain (ST-81) belonging to the epidemic clone and one isolate (ST-725) contained the plasmid differing at only one PstI restriction site. Several attempts failed to isolate CTX-M-5-coding plasmid from the strain ST-1732. It was therefore suggested that the *bla*CTX-M-5 gene was probably transferred by ISEcp1 to the chromosome of this strain.

Genetic and epidemiological relatedness among ESC-resistant strains. The AP-PCR profiles of all the ESC-resistant ST isolates and the previously characterized strain ST-81 were identical and differed from that of the epidemiologically unrelated susceptible strain ST-1878 (Figure 2). Therefore, the resistant isolates probably belong to the earlier reported clone broadly disseminated in Russia.

Unfortunately, available epidemiological data are insufficient to trace the spread of ESC-resistant ST clone between geographically distant cities. Nevertheless, both recent and previously published data [1] indicate that infected patients may have transferred this clone locally between hospitals. For example, in the hospital of Jartsevo (H3) a large outbreak of salmonellosis caused by ESC-resistant ST occurred in the end of 2002. This outbreak was terminated in January 2003 by applying strict disinfection measures and no new cases of the disease were registered for almost nine months. However, between October 2003 and July 2004, ESC-resistant ST was isolated from four hospitalized patients one of whom, a one-year-old girl, was transferred from H3 to the Smolensk City Hospital (H4).

The same strain was later cultured from stool specimen of an asymptomatic person who nursed that girl in the H4.

The origins of the CTX-M-15-producing strain of Sinf isolated in the hospital H6 in Moscow remain unknown. However, it may be speculated that the *bla*CTX-M-15 gene was acquired by Sinf from another nosocomial strain of the Enterobacteriaceae family, since the production of CTX-M-15 was earlier established among strains of *E. coli*, *K. pneumoniae* and *S. marcescens* isolated in the same hospital (unpublished data).

CONCLUSION

- The identity of AP-PCR patterns and the mechanisms of resistance to ESCs support the clonal origin of the *S.* Typhimurium isolates. Thus, our study provides the evidence of the continuous spread of the CTX-M-5-producing *S.* Typhimurium clone in several hospitals throughout Russia.
- This study also reports for the first time the production of CTX-M-15 in *S.* Infantis.

Figure 1. Geographic location of the hospitals where ESC-resistant salmonellae were isolated before ( ) and after 2003 ( ).

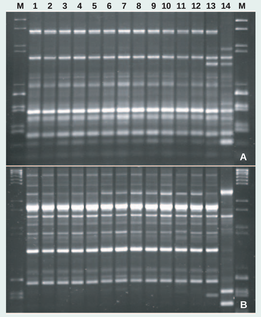

Figure 2. AP-PCR profiles of *Salmonella* isolates amplified with primers ERIC1R and ERIC2 (A) and OPB-17 (B). Lanes 1 to 12, ESC-resistant ST isolates; Lane 13, unrelated ESC-susceptible ST strain; Lane 14, SInf-349; Lane M, MW marker α-*Bst*EII.

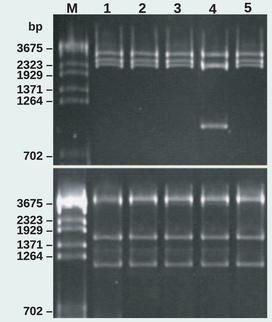

Figure 3. Restriction profiles of CTX-M-coding plasmids digested with *Pst*I (A) and *Pvu*II (B). Lanes 1 to 5, ST-977, ST-30, ST-516, ST-725, ST-81; Lane M, MW marker α-*Bst*EII.

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