Clonal Spread of Cefotaxime-Resistant (CTX-R) Salmonella typhimurium in 006 / 51 Belarus: Epidemiology and Molecular Analysis of Resistance Mechanisms

M. EDELSTEIN¹, M. PIMKIN¹, I. EDELSTEIN¹, T. DMITRACHENKO², V. SEMENOV², L. STRATCHOUNSKI¹ ¹Institute of Antimicrobial Chemotherapy, Smolensk, Russia: ²Medical University, Vitebsk, Belarus

±7-0812-55 72 22 61 13 27

P.O.Box 5, 214019

REVISED ABSTRACT

In the present study we explored the genetic relatedness and resistance mechanisms to β-lactams of 15 cefotaxime-resistant (CTX-R) S.typhimurium isolated in 7 Belarussian hospitals during 1994 - 2000. Five previously characterized CTX-M-4 β-lactamase-producing isolates from St.-Petersburg (Russia) with similar resistance phenotype as well as 3 unrelated susceptible strains were also included for comparison. Susceptibility testing using Etests revealed a common antibiogram in CTX-R strains: susceptibility to celoxitin, resistance to celfriaxone, aztreonam, amoxicilin/clavulanate, piperacilin/tazobactam and decreased susceptibility to effazidime (Tz). An effo fold reduction of MICs of TZ in the presence of calvulanate indicated a production of ESBL Using molecular typing by ERIC-PCR and RAPPO without primers highly discriminative for S. Japhimunium identical fingerprints were obtained for all CTX-R isolates including those from St.-Petersburg, whereas susceptible strains were readily distinguished. The determinants of resistance were transferred by conjugation to E.col AB1456 (RIP). Two types of transconjugants (TRCs) were selected on agar containing rifampin with either cefotaxime or ampicillin. The TRCs of type 1 expressed a β-la The second of t

INTRODUCTION

Multiple drug resistance in salmonellae have emerged as important problem in many countries of the world. Development of resistance to oxyiminoβ-lactams is especially alarming because these drugs have been successfully used for empirical treatment of severe salmonellosis forms over the long time. Isolates of S.typhimurium resistant to cefotaxime were first identified in Argentina in 1990. The resistance was attributed to the production of plasmid-mediated ESBL designated CTX-M-2 (A.Bauernfeind et al., 1992). Subsequently there have been several reports of CTX-M-type β-lactamaseproducing S.typhimurium strains isolated in East and South European countries, including Latvia (P.Bradford et al., 1998), Greece (L.Tzouvelekis et al., 1998), Russia (M.Gazouli et al., 1998) and Hungary (P.Tassios et al., 1999). The latter report have pointed out the spread of single S.typhimurium clone resistant to expanded-spectrum cephalosporins in three European countries. In the present study we describe a long-time countrywide outbreak of salmonellosis in Belarus caused by CTX-R S.typhimurium clone and demonstrate its genetic relatedness to the CTX-M-4 β-lactamase-producing strains previously isolated in St.-Petersburg (Russia).

METHODS

Bacterial strains: This study was performed with 15 non-duplicate S.typhimurium isolates obtained from hospitalized children either during local nosocomial outbreaks or from sporadic cases of salmonellosis in 7 Belarussian hospitals located in Vitebsk Rechitsa Minsk Gomel and Volcovisk in 1994-2000. Five previously characterized CTX-M-4 8-lactamase-producing isolates with similar resistance phenotype isolated in St.-Petersburg Russia (M.Gazouli et al., 1998) as well as 3 unrelated susceptible strains were also included for comparison.

Susceptibility testing: MICs of ampicillin, amoxicillin/clavulanic acid (2:1), piperacillin, piperacillin/tazobactam (tazobactam fixed at 4mg/L), cefotaxime, ceftriaxone, ceftazidime, ceftaz Mueller-Hinton agar (Becton Dickinson, USA). Susceptibility to non-β-lactam agents: tetracycline, chloramphenicol, gentamicin, tobramycin, trimethoprim/sulfamethoxazole, and ciprofloxacin was determined by disk-diffusion method. The results of susceptibility testing were interpreted according to the current NCCLS standards. E.coli strains ATCC® 25922 and ATCC® 35218 were used for quality control.

Bacterial strains typing by PCR-based methods: The genetic relatedness of Styphimurium clinical isolates was studied by two independent methods: 1) ERIC-PCR with primers ERIC1R (5'-atgtaagctcctgggggttcac-3') and ERIC2 (5'-aagtaagtgactggggtgagcg-3'); 2) RAPD typing with primer OPB-17 (5'-aggaacgag-3') described by A.Lin et al. (1996). Template DNA was extracted from 3-4 colonies of each strain grown overnight on MacConkey agar using the InstaGene matrix (BioRad, USA), The ERIC-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-HCI (pH 9.0), 50mM KCI, 1.5mM MgCl₂, 200µM of each dNTP and 1.5U of Tag-polymerase after addition of primers (50 pmoles each), 10ul of template DNA and water to a final volume of 25ul. 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 RAPD mixes were also prepared with Ready-To-Go PCR Beads and contained 50pmoles of primer OPB-17 and 2ul of template DNA. Thermal cycling was carried out as described for ERIC-PCR, except that annealing temperature was set to 35°C. PCR products were analysed by electrophoresis in 1.3% agarose gel and ethidium bromide staining.

Isoelectric focusing: Crude sonic extracts containing β-lactamases were examined on a PhastSystem apparatus on preformed polyacrilamide gels covering the pH ranges 5-8 and 3-9 (Amersham Pharmacia Biotech, USA) and stained with nitrocefin. β-Lactamases with known pls (TEM-1 (pl 5.4), TEM-2 (pl 5.6), TEM-3 (pl 6.3), SHV-1 (pl 7.6), and SHV-5 (pl 8.2)) were used as standards.

PCR detection of β-lactamase genes: A pair of primers (5'-ataaaattcttqaaqacqaaa-3' and 5'-qacaqttaccaatqcttaatca-3') described by C.Mabilat and S.Goussard (1993) was used to amplify specific 1080-bp fragment of bla_{TEM} gene. The PCR mixes contained: 12.5mM Tris-HCl (pH 8.3), 62.5mM KCl, 2mM MgCl₂, 200µM of each dNTP, 0.25µM of each primer, 1.25U AmpliTaq DNA polymerase (Perkin-Elmer, USA) and 20µl of template DNA prepared with InstaGene matrix in total volume of 50µl. The PCR was carried out in a PTC-200 thermocycler (MJ Research, USA) as follows: 1 min 50 sec initial denaturation at 94°C followed by 35 cycles of 10 sec denaturation at 94°C, 10 sec annealing at 54°C, and 45 sec elongation at 72°C with final elongation step extended to 3 min.

PCR detection of blacTX-M genes was performed using the primes (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. The PCR mixes contained in 50µl volumes: 50mM KCl, 10mM Tris-HCl (pH 9), 0.1% TritonX-100, 1. 5mM MqCl₂, 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. To verify that the amplified sequences correspond to either the bla_{CTX-M-1} or bla_{CTX-M-2}-related genes, PCR products purified by ethanol/sodium acetate precipitation were subjected to restriction enzyme digests with Pstl and Pvull. The PCR products and restriction fragments were separated in 3.5% AmpliSize agarose (BioRad, USA) gel and stained with ethidium bromide.

A PCR with primers (OXA-1/F: 5'-atgaaaaacacaatacaatacaaca3' and OXA-1/R: 5'-tttcctgtaagtgcggacac-3') was used to detect 755-bp internal fragment of bla_{CXA-1}-related genes. The composition of PCR mixes and amplification conditions were the same as described for bla_{CTX-M} genes except the concentration of primers - 0.5µM (each) and annealing temperature - 48°C.

Extraction and analysis of plasmid DNA: Plasmid DNA was extracted from three S typhimurium isolates as well as from E coli transconjugants and transformants using the Prep-A-Gene DNA Miniprep Kit (BioRad, USA) as recommended by manufacturer and subjected to restriction enzyme digests with Avall, Pstl or Pvull (Amersham Pharmacia Biotech, USA). Native plasmids and restriction products were analysed by electrophoresis in 1.3% agarose gel and ethidium bromide staining.

Transfer of resistance: All resistant S.typhimurium isolates were mated in broth with E.coli AB1456 (Rif⁸). The transconjugants were selected on agar containing rifampin (100µg/ml) with either cefotaxime (10µg/ml) or ampicillin (100µg/ml). In addition, the plasmid DNA was transformed into competent cells of E.coli TOP10 (Promega, USA) and transformants were selected on agar containing cefotaxime (10µg/ml)

RESULTS AND DISCUSSION

Susceptibility. The resistance phenotypes and other characteristics of S.typhimurium isolates from St.-Petersburg and Belarus are shown in Table 1. All isolates were highly resistant to penicillins, cefotaxime, ceftriaxone and aztreonam, but susceptible to cefoxitin. MICs of ceftazidime were generally below the resistance level, however a synergy between all oxyimino-β-lactams and β-lactamase-inhibitors (especially tazobactam) suggested an ESBL-production. All salmonella from St.-Petersburg and 9 Belarussian isolates demonstrated high-level resistance to penicillininhibitor combinations, the remaining 6 isolates were fully susceptible to piperacillin/tazobactam and had the MICs of amoxicillin/clavulanate close to the resistance breakpoints (8-32 mg/L). In addition, 17 isolates were resistant to tetracycline and chloramphenicol, 15 - to gentamicin and tobramycin, and 9 - to trimethoprim/sulfamethoxazole

ERIC-PCR and RAPD typing. The isolation of multiple *Styphimurium* with similar and characteristic phenotype of β-lactam resistance may indicate dissemination of either a single clone or a specific ESBL-species among different strains. Molecular typing by ERIC-PCR and RAPD with primers highly discriminative for S.typhimurium (A.Lin et al., 1996;) showed that all CTX-R isolates from St.-Petersburg and Belarus were genetically related whereas control susceptible strains were clearly distinguishable (Fig. 1).

β-lactamase characterization. Isoelectric focusing revealed the production of β-lactamase with a pl of approximately 8.4 in all S.typhimurium isolates. The resistance phenotype and pl of the enzymes were indicative of a CTX-M-type ESBL. In support of this assumption, the specific internal fragments of bla_{CTX-M} genes were amplified by PCR from all isolates. Digestion of PCR-products with PstI and PvulI restriction endonucleases, which allow to distinguish the groups of blaction and blaction and blaction and blaction and blaction and blaction are leaded genes, demonstrated that all isolates carried the genes of the latter group (Fig. 2). In addition, 14 isolates resistant to piperacillin/tazobactam produced a second β-lactamase with pl 7.5, which correlated with OXA-4, and 2 isolates expressed a third enzyme with pl 5.4 (presumably TEM-1), which apparently did not affect the resistance phenotype. The presence of TEM- and OXA-1-type β-lactamases was confirmed by PCR with bla_{TEM} and bla_{OXA-1} specific

Transfer of resistance. In broth mating experiments two different types of transconjugants (TRCs) were obtained from isolates resistant to cefotaxime and piperacillin/tazobactam. The TRCs of type 1 were selected on plates containing rifampin and ampicillin at a high frequency (10³-10⁴). The results for only two representative TRCs of type 1 (AB1465/SP891 and AB1456/6570-1) are shown in table 1. All clones of this type produced an OXA-1-related β-lactamase (most likely – OXA-4) providing resistance only to penicillins and decreasing susceptibility to penicillin-inhibitor combinations, but vary in the number of co-transferred non-β-lactam resistance markers. These data may suggest that different resistance determinants were located on multiple separate plasmids, and that the differences in antibiogram of genetically related S.typhimurium isolates could be attributed to the variation in their plasmid spectrum. The TRCs of type 2 were obtained on selective plates containing rifampin and cerotaxime at a very low frequency (-10°). In spite of the multiple attempts and prolonged incubation of mating mixtures only two isolates transferred the cerotaxime resistance to recipient strain. The respective TRCs (AB1456/6570-2 and AB1456/1358-2) produced a single CTX-M-β-lactamase conferring ESBL phenotype of the donor strains, but were susceptible to piperacillin/tazobactam and all non-β-lactam antibiotics. The laboratory clones (TOP10/SP891 and TOP10/1358) obtained by plasmid-DNA transformation and selected on cefotaxime-containing plates displayed the same resistance phenotype and produced a CTX-M-β-lactamase. Notably, one of these transformants additionally produced a TEM-1 β-lactamase

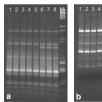
Analysis of plasmids carrying the blaction genes. Each of the CTX-M-β-lactamase producing transconjugants and transformants acquired a single plasmid. The length of CTX-M-β-lactamase encoding plasmids from different donor strains varied from 7.3 to 12 kb. When these plasmids were digested with restriction endonuclease Pstl or Pvull, the patterns obtained were rather similar differing by 3 bands at most (Fig. 3). All but one plasmid originating from Russian isolate (SP891) did not contain Avall restriction sites. The digestion patterns and the presence of bla TELLA, gene on this differing plasmid indicated a possible insertion of a TnA-type transposon. In addition to the identical ERIC-PCR and RAPD patterns, the similarity of CTX-M-β-lactamase-encoding plasmids further supported the clonal origin of the CTX-R isolates. These plasmids were probably non-self-transferable, but their transmission in vitro was facilitated by coexisting conjugative plasmids.

Possible relationship between CTX-R S.tvphimurium isolates from Belarus and other European countries. In this study we described a clonal spread of CTX-R S.tvphimurium in Belarus. To our knowledge, this study revealed and characterized the biggest number of CTX-M-β-lactamase producing S.typhimurium that have been isolated in one country over a long period of time. Moreover, we demonstrated a possible clonal relationship between isolates from Belarus and St.-Petersburg (Russia), Notably, the Russian isolates included in this study were previously compared with Hungarian and Greek strains and all of them were found to be highly related on the basis of PFGE typing (P. Tassios et al., 1999). Unfortunately, direct comparison of Belarussian clone and Latvian CTX-M-5-β-lactamase-producing isolates described by P.Bradford et al. (1998) was not possible in this study. However, a number of common features, including resistance spectrum and properties of CTX-M-β-lactamase encoding plasmids, suggests a possible relationship between CTX-R S.typhimurium isolates from nearby located Latvia and Belarus.

Table 1. Susceptibilities and other characteristics of S.tvphimurium isolates and E.coli transconjugants and transformants

	Strains		Etest MICs, mg/L										non-β-lactams					PCR for bla genes			pl of
Ottains		AM	XL	PP	PTc	CT	TX	TZ	TZL	FX	ΑT	ESBL	Tet	Chl	Ag	Sxt	Cip	TEM	OXA-1	CTX-M	β-lactamases
S.typhimunium clinical isolates	SP829*	256	256	256	256	256	256	4	0.8	2	64	+	R	R	S	S	S		+	+	7.5; ~8.4
	SP832*	256	256	256	256	256	256	6	0.8	2	64	+	R	R	S	S	S	-	+	+	7.5; ~8.4
	SP893*	256	256	256	256	256	256	4	0.8	2	64	+	R	R	S	S	S	-	+	+	7.5; ~8.4
	SP838*	256	256	256	256	256	256	8	1	2	64	+	R	R	R	S	S	+	+	+	5.4; 7.5; ~8.4
	SP891*	256	256	256	256	256	256	12	1.5	2	64	+	R	R	R	S	S	+	+	+	5.4; 7.5; ~8.4
	6570	256	96	256	128	256	256	8	1	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	3078	256	192	256	128	256	256	4	1	2	64	+	R	R	-1	R	S	-	+	+	7.5; ~8.4
	13205	256	64	256	256	256	256	8	0.4	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	13953	256	96	256	256	256	256	12	0.8	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	13526	256	96	256	256	256	256	12	0.5	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	14242	256	96	256	256	256	256	16	1	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	16753	256	96	256	256	256	256	16	1	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	26	256	96	256	128	256	256	12	1	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	16	256	96	256	256	256	256	12	0.8	2	64	+	R	R	R	R	S	-	+	+	7.5; ~8.4
	1358	256	32	256	2	256	256	12	0.8	2	64	+	R	R	R	S	S	-	-	+	~8.4
	1603	256	16	256	2	256	256	16	1	2	64	+	R	R	R	S	S	-	-	+	~8.4
	700	256	12	256	3	256	256	12	0.5	2	64	+	R	R	R	S	S	-	-	+	~8.4
	27	256	32	256	2	256	256	12	0.8	2	64	+	S	S	R	S	S	-	-	+	~8.4
	147	256	12	256	2	256	256	4	0.8	2	64	+	S	S	S	S	S	-	-	+	~8.4
	9837	256	8	256	3	256	256	3	0.8	2	64	+	S	S	S	S	S		-	+	~8.4
	TOP10/SP891	256	128	256	6	256	256	16	1.5	8	64	+	S	S	S	S	S	+	-	+	5.4; ~8.4
9	TOP10/1358	256	48	256	2	256	256	12	1.5	8	64	+	S	S	S	S	S	-	-	+	~8.4
iloo.	AB1456/6570-2	256	64	256	2	256	256	16	1	4	64	+	S	S	S	S	S	-	-	+	~8.4
Ш	AB1456/SP891	256	192	256	64	0.5	0.1	0.5	0.5	4	0.1	-	R	R	S	S	S	-	+	-	7.5
	AB1456/6570-1	256	256	256	64	0.8	0.1	0.5	0.5	4	0.1		R	R	R	R	S		+	-	7.5

 Strains from St.-Petersburg (SP).
Susceptibility is colour-coded: resistant (R) – red. intermediate (I) – vellow, susceptible (s) – white. AM = ampicillin XI = amovicillin/clavulanic acid PP = piperacillin PTc = piperacillin/tazobactam CT = cefotayime TX = ceftrayone



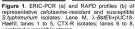




Figure 2. Pstl-Pvull double digests of bla_{CTX-M}-gene amplification products. Lane M, pUC18-Haelli; lanes 1 to 15, CTX-R Belarussian isolates; lane 16, C.freundii (CTX-M-3): lane 17, S.typhimurium (CTX-M-4): lane 18. undigested 543-bp PCR-product

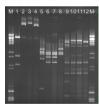


Figure 3. Digestion patterns CTX-M-β-lactamase-encoding

λ-BstEII+pUC18-HaeIII Lanes M. lanes 1 to 4, digestion with Avall; lanes 5 to 8, digestion with Pst; lanes 9 to 12, digestion with Pvull; lanes 1, 5, 9, E.coli TOP10/SP891; lanes 2, 6, 10, E.coli TOP10/1358: lanes 3 7 11 F coli AR1465/1358-2

CONCLUSIONS

Based on all assays performed, we conclude that the CTX-R isolates of S.typhimurium probably represented a single clone broadly disseminated in Belarus and perhaps in some other countries of East and South Europe. The resistance of this clone to extended-spectrum cephalosporins and penicillin-inhibitor combinations was attributed to simultaneous production of a CTX-M-type ESBL

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