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Long-Term Dissemination of CTX-M-5-Producing Hypermutable *Salmonella enterica* Serovar Typhimurium Sequence Type 328 Strains in Russia, Belarus, and Kazakhstan

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In this paper, we present evidence of long-term circulation of cefotaxime-resistant clonally related *Salmonella enterica* serovar Typhimurium strains over a broad geographic area. The genetic relatedness of 88 isolates collected from multiple outbreaks and sporadic cases of nosocomial salmonellosis in various parts of Russia, Belarus, and Kazakhstan from 1996 to 2009 was established by multilocus tandem-repeat analysis (MLVA) and multilocus sequence typing (MLST). The isolates belong to sequence type 328 (ST328) and produce CTX-M-5 β-lactamase, whose gene is carried by highly related non-self-conjugative but mobilizable plasmids. Resistance to nalidixic acid and low-level resistance to ciprofloxacin is present in 37 (42%) of the isolates and in all cases is determined by various single point mutations in the *gyrA* gene quinolone resistance-determining region (QRDR). Isolates of the described clonal group exhibit a hypermutable phenotype that probably facilitates independent acquisition of quinolone resistance mutations.

Salmonella enterica subspecies I (*enterica*) is recognized as the most common cause of acute gastroenteritis (1). During the past 10 years, the incidence of salmonellosis in Russia remained stably high at 30 to 35.5 cases per 100,000 of population (2). The reported incidence of salmonellosis in the neighboring countries of Kazakhstan and Belarus varies between 13.7 and 55 cases per 100,000 of population, respectively (3). Although salmonellosis is typically a food-borne disease, direct person-to-person transmission may also occur, especially in hospitals where the close proximity of patients to one another, contact by health care workers, and use of shared sanitary facilities favor the spread of the pathogen (4). Nosocomial salmonellosis poses a particular threat to neonates, elderly patients, and individuals with weakened immune systems who are at higher risk of severe complications requiring antibiotic treatment (5, 6).

Resistance of *S. enterica* to oxyimino-cephalosporins and fluoroquinolones, which are used as primary treatments for acute salmonellosis, is an emerging problem worldwide (7, 8). Quinolone resistance in *Salmonella* is commonly caused by mutations in the quinolone resistance-determining regions (QRDRs) of the DNA-gyrase subunit A (GyrA), primarily at amino acid positions 83 and 87, or topoisomerase IV subunit A (ParC) (9–11). Other mechanisms of low-level fluoroquinolone resistance, such as impaired permeability, efflux (9), and production of Qnr proteins (12, 13) or the ciprofloxacin-modifying enzyme *aac(6′)Ib-cr* (14), can complement mutations of the target gene and increase MICs. Resistance to oxyimino-cephalosporins in salmonellae is associated with production of acquired class C cephalosporinases (AmpC) (15) or class A extended-spectrum β-lactamases (ESBLs), among which CTX-M-type enzymes are currently most common (7, 16).

CTX-M-5, which was first described in 1998 in an *S. enterica* serovar Typhimurium from Latvia (17), is most often associated with this species. There were several reports of nosocomial outbreaks and sporadic isolations of CTX-M-5 ESBL-producing *S.* Typhimurium in European countries (Latvia [1991 to 1998] [17], Greece [1996 to 1998] [18], Hungary [1996 to 1998] [19], Russia [1996 to 1997] [19, 20], and Belarus [2007] [21]), as well as in the United States (2000, 2003, and 2007) (22–24). Previously, we reported on the isolation of clonally related CTX-M-5-producing strains of *S.* Typhimurium in Russia and Belarus from 1994 to 2003 (25). Since then, we have been continuously receiving cefotaxime-resistant *S.* Typhimurium isolates from outbreaks and sporadic cases of nosocomial salmonellosis in various regions of Russia, Belarus, and Kazakhstan. This study was undertaken to reveal the mechanisms of resistance to β-lactams and quinolones and the genetic relatedness of cefotaxime-resistant *S.* Typhimurium isolates circulating in Russia and neighbor countries.

MATERIALS AND METHODS

***Salmonella* Typhimurium clinical isolates.** Eighty-eight nonduplicate (one per patient) cefotaxime-resistant *S.* Typhimurium isolates were obtained from stool samples of hospitalized patients with acute gastroenteritis in 10 regions of Russia, Belarus, and Kazakhstan from 1996 to 2009 (Table 1). It is notable that all but 3 cases of infection were considered nosocomial based on the criteria of onset of symptoms ≥48 h after hospitalization. Fifteen of 88 isolates were isolated from children younger than 3 years of age. In addition, three epidemiologically unrelated *S.* Typhimurium strains were included in the study as controls for clonality assessment, two cefotaxime-

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TABLE 1 Sources of cefotaxime-resistant *S.* Typhimurium isolates

Country	City	No. of hospitals	No. of isolates	Source[a]	Isolation dates
Russia	St. Petersburg	2	6	HAI outbreaks and 1 CAI	1996–1997, 2003
	Moscow	1	1	HAI outbreak	1998
	Irkutsk	1	1	Sporadic HAI	2003
	Voronezh	2	5	HAI outbreaks	2004
	Yaroslavl	1	2	HAI outbreak	2004
	Smolensk	3	26	HAI outbreaks	2004, 2006, 2009
	Tomsk	1	3	HAI outbreak	2007–2008
Belarus	Gomel	3	22	HAI outbreaks and 2 CAI	1997, 2006–2007
	Vitebsk	3	13	HAI outbreaks	1999–2000
Kazakhstan	Karaganda	1	9	HAI outbreak	2006–2007

[a] HAI, hospital-acquired infection; CAI, community-acquired infection.

susceptible *S.* Typhimurium isolates from the Smolensk and Gomel regions (which were collected at the same hospitals as the resistant strains but were from patients with community-acquired infection), and one CTX-M-2-producing strain (CAS-5) from Argentina (26).

Antibiotic-susceptibility testing and phenotypic ESBL detection. MICs were determined by an agar dilution method on Mueller-Hinton agar (Becton, Dickinson, Sparks, MD) for the antibiotics ampicillin, amoxicillin-clavulanic acid (2:1), aztreonam, cefotaxime, cefotaxime-clavulanic acid (with clavulanate at a fixed concentration of 4 mg/liter), ceftazidime, ceftazidime-clavulanic acid (with clavulanate at a fixed concentration of 4 mg/liter), cefepime, piperacillin, piperacillin-tazobactam (with tazobactam at a fixed concentration of 4 mg/liter), imipenem, meropenem, ertapenem, doripenem, gentamicin, trimethoprim-sulfamethoxazole (1:19), nalidixic acid, and ciprofloxacin. Susceptibilities of isolates to tetracycline and chloramphenicol were determined by the disk diffusion method with commercial disks (Becton, Dickinson, Cockeysville, MD). Susceptibility testing results were interpreted according to current CLSI standards (27). ESBL production was inferred by the double-disk synergy test (28) and by estimating the reduction of MICs of cefotaxime and ceftazidime in the presence of clavulanic acid (4 mg/liter). *Escherichia coli* ATCC 25922, *E. coli* ATCC 35218, and *K. pneumoniae* ATCC 700603 were used for quality control of susceptibility testing and ESBL detection.

Characterization of β-lactamase genes and their genetic context. Genes of β-lactamases of OXA-1, TEM, SHV, and CTX-M groups were detected by PCR as described previously (25, 29, 30). Associations of $bla_{\text{CTX-M}}$ genes with known mobile elements (IS*Ecp1* or IS*CR1*) were revealed by PCR mapping and sequencing (25). DNA sequencing was performed on both strands using a Big Dye Terminator v. 3.1 cycle sequencing kit and an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA).

Mutation detection in *gyrA* QRDR. Amplification and direct sequencing of the internal fragment of the *gyrA* gene were carried out as described previously (1), except that the sequence of the reverse primer STGYRA12 (11) was extended at the 3′ end (5′-CGTTGATGACTTCCG TCAGGT-3′).

Transfer and analysis of plasmids mediating cefotaxime resistance. Plasmids were extracted from isolates with the plasmid midi kit (Qiagen, Hilden, Germany) and transferred by transformation into the competent *E. coli* EPI300 strain (Epicentre Biotechnologies, Madison, WI). Transformants were selected on agar plates containing cefotaxime (1 mg/liter). CTX-M-coding plasmids extracted from transformants were analyzed by restriction fragment length polymorphism (RFLP) with PstI (Promega, Madison, WI) and PvuII (Amersham, Piscataway, NJ) endonucleases (25).

Conjugational transfer of plasmids from isolates into the recipient strain *E. coli* AB1456 (rifampin-resistant [Rif^r]) (31) was carried out in Mueller-Hinton broth, with selection of transconjugants on agar plates containing both rifampin (100 mg/liter) and cefotaxime (1 mg/liter).

Complete nucleotide sequences of CTX-M-5-coding plasmids (pCTX-M-5-like plasmids) representing each RFLP type were determined by the automated Sanger method using a Big Dye Terminator v. 3.1 cycle sequencing kit and an ABI Prism 3730 genetic analyzer (Applied Biosystems, Foster City, CA). Sequences were read directly from plasmid DNA purified from transformants using the starting oligonucleotide primers for the $bla_{\text{CTX-M-5}}$ gene and consecutive primers designed according to gradually accumulated sequence data. Gene prediction was performed for the complete plasmid sequence with Artemis v. 8 (Sanger Institute, Hinxton, United Kingdom). Pairwise alignment was performed by a BLASTN and BLASTP homology search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) and using CLC Main Workbench v. 5.7.1 software (CLC bio, Aarhus, Denmark).

For the detection of pCTX-M-5-like plasmids in *S.* Typhimurium isolates, two primers (pCTXM5-F [5′-GACACAGAGAAGTTGATAGGCG A-3′] and pCTXM5-R [5′-TTCCAAATAGATCCACGGTGAC-3′]) were designed to target a specific hypothetical open reading frame (ORF) within a common part of the plasmid backbone in all CTX-M-5-coding plasmids (corresponding to the nucleotide positions 3393 to 4226 in GenBank accession number JX017306).

Hypermutability testing. In order to assess the hypermutable phenotype of clinical *S.* Typhimurium isolates, a disc-diffusion-based method with a nalidixic acid disc (30 μg) was performed as described in Galán et al. (32). A suspension of tested culture in saline (a McFarland standard of 2) was applied with a swab on a surface of Mueller-Hinton agar. After incubation at 35°C for 24 h, the presence of scattered mutant colonies inside the nalidixic acid zone of inhibition was estimated.

The rate of spontaneous nalidixic acid-resistant mutants among originally susceptible isolates was determined by a standard method. Overnight bacterial cultures in serial 10-fold dilutions were seeded on agar with nalidixic acid (32 mg/liter) and without antibiotic. The spontaneous mutant rate was calculated as a ratio of the number of colonies on plates with antibiotic to the number of colonies on plates without antibiotic. *E. coli* GM2995 (*mutD5*) (33) and *E. coli* ATCC 25922 were used as mutator and normomutable controls, respectively.

MLVA typing. Multilocus variable-number tandem-repeat analysis (MLVA) was performed according to Lindstedt et al. (2004) (34) with fluorescent detection by 5 variable-number tandem-repeat (VNTR) loci (STTR3, STTR5, STTR6, STTR9, and STTR10pl). PCR products were analyzed with an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) and Peak Scanner software v. 1.0 (Applied Biosystems, Foster City, CA). Cluster analysis was performed using BioNumerics Software v. 6.1 (Applied Maths, Kortrijk, Belgium) with application of the minimum spanning tree (MST) algorithm for categorical variables of STTR allele sizes.

TABLE 2 Antimicrobial susceptibilities of cefotaxime-resistant *S.* Typhimurium isolates

Antibiotic	MIC (mg/L)			Susceptibility categories (no. [%])[a]			
	Range	50%	90%	S	SDD	I	R
Ampicillin	≥256	≥256	≥256	0 (0)	—	0 (0)	88 (100)
Amoxicillin-clavulanic acid	8 to 64	32	32	9 (10.2)	—	13 (14.8)	66 (75)
Piperacillin	≥256	≥256	≥256	0 (0)	—	0 (0)	88 (100)
Piperacillin-tazobactam	2 to ≥256	≥256	≥256	22 (25)	—	4 (4.5)	62 (70.5)
Cefotaxime	128 to ≥256	≥256	≥256	0 (0)	—	1 (1.1)	87 (98.9)
Cefotaxime-clavulanic acid	0.06 to 1	0.25	0.5	—	—	—	—
Ceftazidime	1 to ≥256	8	16	6 (6.8)	—	43 (48.9)	39 (44.3)
Ceftazidime-clavulanic acid	0.25 to 1	0.5	0.5	—	—	—	—
Cefepime	8 to ≥256	128	≥256	0 (0)	5 (5.7)	—	83 (94.3)
Aztreonam	8 to ≥256	64	64	3 (3.4)	—	1 (1.1)	84 (95.5)
Imipenem	0.06 to 0.5	0.25	0.25	88 (100)	—	0 (0)	0 (0)
Doripenem	0.06 to 1	0.06	0.5	88 (100)	—	0 (0)	0 (0)
Meropenem	0.06 to 0.125	0.06	0.125	88 (100)	—	0 (0)	0 (0)
Ertapenem	0.06 to 0.5	0.06	0.06	88 (100)	—	0 (0)	0 (0)
Gentamicin	1 to ≥256	16	32	12 (13.6)	—	4 (4.5)	72 (81.8)
Trimethoprim-sulfamethoxazole	0.125 to ≥256	≥256	≥256	29 (33)	—	0 (0)	59 (67)
Nalidixic acid	4 to ≥512	8	≥512	50 (56.8)	—	0 (0)	38 (43.2)
Ciprofloxacin	0.03 to 0.5	0.03	0.5	51 (58)	—	37 (42)	0 (0)
Chloramphenicol	—[b]	—	—	10 (11.4)	—	0 (0)	78 (88.6)
Tetracycline	—	—	—	8 (9.1)	—	0 (0)	80 (90.9)

[a] S, susceptible; SDD, susceptible-dose dependent; I, intermediate; R, resistant.
[b] —, not applicable.

MLST typing. Isolates were typed by seven housekeeping genes (*aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*) according to the standard scheme for *Salmonella enterica* (http://mlst.warwick.ac.uk/mlst/dbs/Senterica) (35) Allele sequences were obtained using an ABI Prism 310 genetic analyzer (Applied Biosystems, Foster City, CA) and identified using the MLST plug-in of BioNumerics software.

Nucleotide sequence accession numbers. The complete sequences of plasmids pCTXM5-637, pCTXM5-891, pCTXM5-1358, and pCTXM5-1845 have been deposited into GenBank under the accession numbers JX017306, JX017307, JX017308, and JX017309, respectively.

RESULTS

Antimicrobial susceptibility. The results of susceptibility testing of 88 cefotaxime-resistant *S.* Typhimurium isolates are presented in Table 2. All isolates exhibited resistance or reduced susceptibility to oxyimino-cephalosporins (MIC range, $MIC_{90\%}$) (cefotaxime [128 to ≥256, ≥256 mg/liter], cefepime [8 to ≥256, ≥256 mg/liter], ceftazidime [1 to ≥256, 16 mg/liter]) and to aztreonam (8 to ≥256, 64 mg/liter). Significant (≥8-fold) reductions of MICs of oxyimino-cephalosporins in the presence of clavulanic acid and positive results of double-disk synergy tests, indicating ESBL production, were observed for all isolates.

Seventy-nine (89.8%) and 66 (75%) isolates were nonsusceptible to amoxicillin-clavulanic acid and piperacillin-tazobactam, respectively. All isolates remained susceptible to carbapenems.

Resistance to one or more non-β-lactam drugs was detected in 85 (96.6%) isolates. Resistance to nalidixic acid was established in 38 (43.2%) isolates, of which all but one were also nonsusceptible to ciprofloxacin. The latter isolate had a borderline ciprofloxacin MIC (0.06 mg/liter) and thus was formally referred to as fluoroquinolone susceptible.

Molecular characterization of β-lactamase-encoding genes. The ESBL gene $bla_{CTX\text{-}M\text{-}5}$ was detected by PCR and sequencing in all cefotaxime-resistant isolates and in all cases was found 19-bp downstream of the IS*Ecp1* mobile element. In contrast, the $bla_{CTX\text{-}M\text{-}2}$ gene of the Argentine *S.* Typhimurium index strain CAS-5 is associated with the IS*CR1* element.

Four (4.5%) isolates had the $bla_{TEM\text{-}1}$ penicillinase gene, and 66 (75%) isolates carried $bla_{OXA\text{-}1}$-like genes. No genes of SHV β-lactamases were detected. The presence of OXA-1-like β-lactamases resistant to inhibition by clavulanic acid and tazobactam (36) correlated with resistance of isolates to piperacillin-tazobactam, as expected.

Analysis of CTX-M-5-coding plasmids. In plasmid transfer experiments, cefotaxime-resistant transformants were readily obtained from the majority of isolates; however, transconjugants nonsusceptible to cefotaxime were obtained only from 10 isolates with very low frequency (10^{-7} to 10^{-8}). All cefotaxime-resistant recipients had a single low-molecular-weight plasmid (7 to 12 kb) carrying the $bla_{CTX\text{-}M\text{-}5}$ gene and were resistant to penicillins and oxyimino-cephalosporins but susceptible to piperacillin-tazobactam and all non-β-lactam antibiotics.

RFLP analysis of CTX-M-5-coding plasmids of 26 isolates from different hospitals distinguished 4 related plasmid types (representative restriction profiles are shown in Fig. 1). The predominant restriction profile A1 (prototype plasmid pCTXM5-637) was observed for 20 isolates; 11 of these were isolated from Belarus, 8 from Russia, and 1 from Kazakhstan. Plasmids with highly similar profiles (A2 [prototype plasmid pCTXM5-1358] and A3 [prototype plasmid pCTXM5-1845]) differing from A1 by a single restriction site of the PstI endonuclease were found in 4 isolates from Belarus and 1 isolate from Russia. Plasmid profile A4 (plasmid pCTXM5-891) was found in a single isolate from Russia and differed from A1 by the presence of additional bands in PstI and PvuII restriction patterns.

Complete DNA sequences were determined for the four prototypic plasmids corresponding to the different restriction types found in this collection. All four plasmids shared an identical backbone, showing 98% nucleotide identity with the 4,995-bp

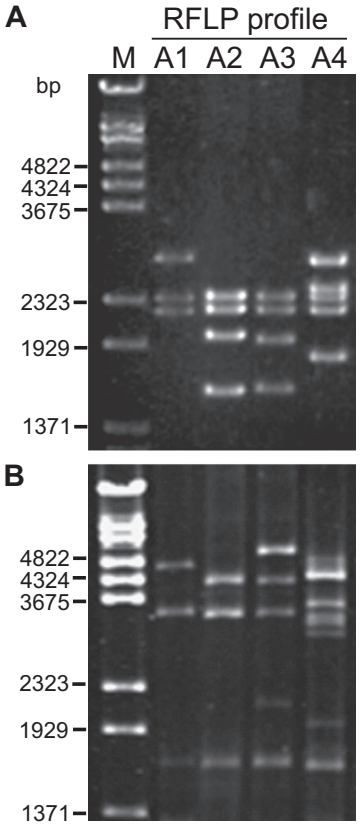

FIG 1 Representative RFLP profiles of CTX-M-5-coding plasmids obtained with PstI (A) and PvuII (B) endonucleases. Lane M, molecular weight marker (λ DNA-/*Bst*EII digest + pUC18/HaeIII).

pIGRW12 cryptic plasmid, isolated in 2006 in Poland from a clinical strain of *Escherichia coli* (accession number EF088686) and 97 to 95% nucleotide identity with the small pS51B (accession number AB583678) and pEC01 (accession number AB117929) plasmids identified in *Enterobacter cloacae* in Japan, which may represent the plasmid scaffold before the acquisition of the resistance determinants. The scaffold contained *mobA*, *mobB*, *mobC*, *mobD*, RNAI, RNAII, and the plasmid replication initiation protein gene, promoting mobilization in *trans*, replication, and control of copy number of the plasmid, respectively. The integration of the IS*Ecp1*::$bla_{CTX\text{-}M\text{-}5}$ resistance determinant occurred in the region between *orfX* and the *mobD* gene in the plasmid scaffolds of RFLP type A1 plasmids (pCTXM5-637). The acquisition of the IS*1* insertion sequence was observed in the A2 (pCTXM5-1358) and A3 (pCTXM5-1845) plasmid variants, while plasmid A4 (pCTXM5-891) showed the acquisition of the transposase (*tnpA*) and resolvase (*tnpR*) genes of the Tn*3* transposon carrying the $bla_{TEM\text{-}1}$ gene (Fig. 2). Finally, an open reading frame, designated *orfX*, was identified in the plasmid backbone, encoding a conserved hypothetical protein of unknown function. This *orfX* was selected to devise a couple of primers to search by PCR for the pCTXM5-like plasmids in all the strains of our collection.

PCR typing detected the presence of pCTXM5-like backbones in 80 (90.9%) of the cefotaxime-resistant isolates, but not in the Argentine strain CAS-5 or in the cefotaxime-susceptible isolates. The remaining eight CTX-M-5-producing isolates (5 from Russia ([Tomsk, Irkutsk, and S.-Petersburg], 2 from Kazakhstan [Karaganda], and 1 from Belarus [Vitebsk]) negative by this PCR were also unsuccessful in transferring $bla_{CTX\text{-}M\text{-}5}$ by conjugation or transformation, suggesting a possible transposition of the IS*Ecp1*::$bla_{CTX\text{-}M\text{-}5}$ resistance determinant to a chromosomal locus in these isolates.

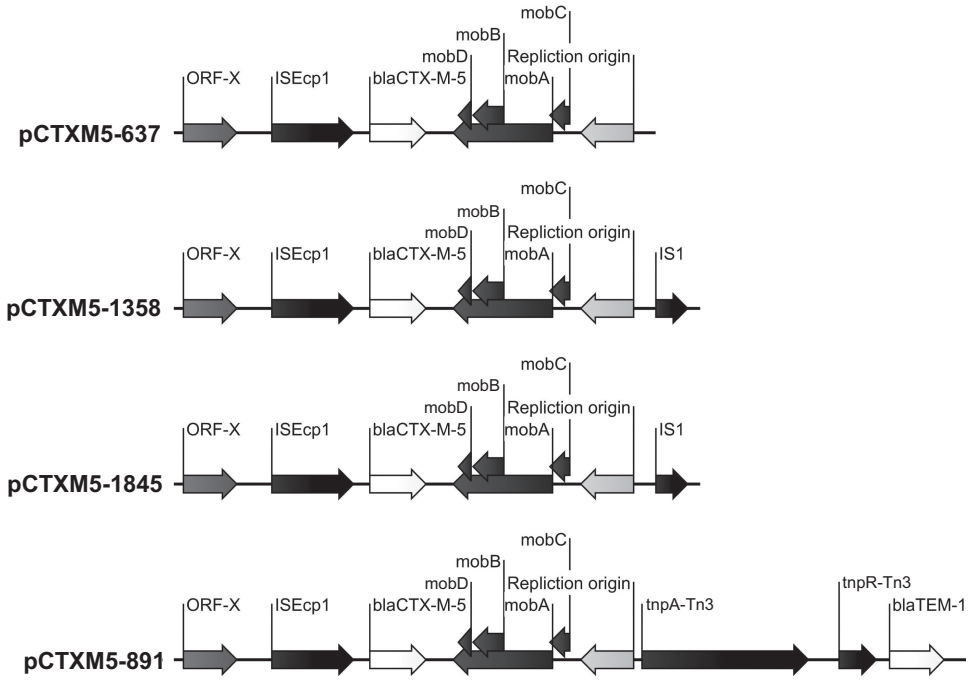

FIG 2 Sequence alignment of pCTXM5 plasmids. Arrows represent putative coding regions and key structural elements: conserved hypothetical protein (*orfX*), mobilization proteins (*mobA* to *D*), replication origin, β-lactamase genes ($bla_{CTX\text{-}M\text{-}5}$ and $bla_{TEM\text{-}1}$), insertion sequences (IS*Ecp1* and IS*1*), and Tn*3* transposon transposase (*tnpA*) and resolvase (*tnpR*) genes.

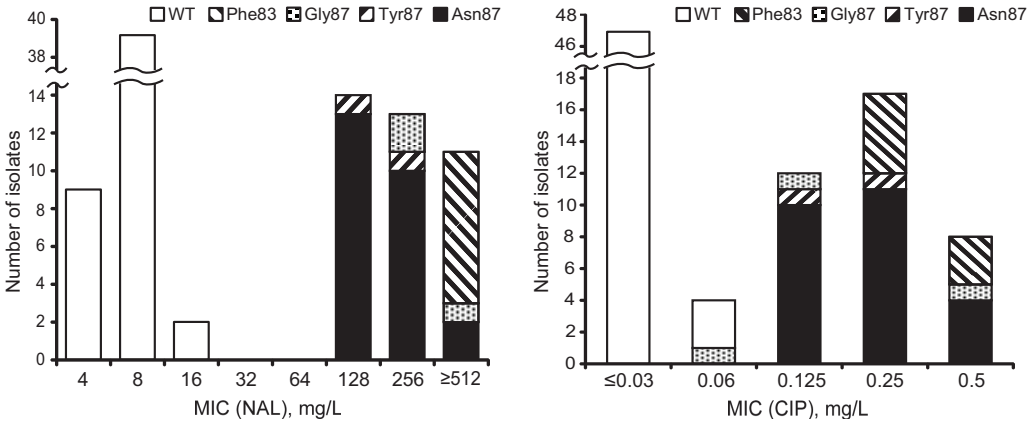

FIG 3 Distribution of studied *S.* Typhimurium isolates by nalidixic acid (NAL) and ciprofloxacin (CIP) MICs and type of mutation in GyrA QRDR. WT, wild type.

Molecular characterization of quinolone resistance determinants. Using PCR and sequencing, all quinolone nonsusceptible *S.* Typhimurium isolates ($n = 38$) were found to carry various single point mutations in the *gyrA* QRDR corresponding to amino acid substitutions (Asn-87 [$n = 25$], Gly-87 [$n = 3$], Tyr-87 [$n = 2$], and Phe-83 [$n = 8$]), while no such mutations were found in quinolone-susceptible isolates. The correlation between mutation type and MICs of nalidixic acid and ciprofloxacin is shown in Fig. 3.

Hypermutable phenotype detection. In the simple test for hypermutability with the nalidixic acid disc, all quinolone-susceptible *S.* Typhimurium isolates along with the control mutator strain *E. coli* GM2995 (*mutD5*) revealed an appearance of multiple mutant colonies inside the inhibition zone. No such colonies were observed in the normomutable *E. coli* ATCC 25922 strain (see Fig. S1 in the supplemental material). By plating serial dilutions of exponential-phase cultures, we found the frequency of spontaneous nalidixic acid-resistant mutants in the studied isolates to be about 1×10^{-5}, which is four orders of magnitude higher than in normomutable *S.* Typhimurium strains (37, 38). Therefore, the studied isolates were regarded as strong mutators. It is worth mentioning that sequencing of *gyrA* QRDRs from randomly picked mutants obtained *in vitro* revealed the presence of mutations identical to those described in the quinolone-resistant clinical isolates (data not shown).

Genetic relationship between isolates. MLVA typing classified all 88 cefotaxime-resistant isolates from Russia, Belarus, and Kazakhstan into 17 types which differ only at one or two VNTR loci and cluster together into a single clonal group (Fig. 4). All these isolates as well as the Argentine strain were PCR negative for the STTR10pl locus located on the pSLT virulence plasmid of *S.* Typhimurium (39) and thus were probably lacking this plasmid. The Argentine CTX-M-2-producing strain (MLVA type T) differs from the most closely related Russian CTX-M-5-producing strain (MLVA type F) at 2 VNTR loci. In contrast, the cefotaxime-susceptible *S.* Typhimurium isolates (MLVA types R and S) diverge by 3 VNTR loci from the closest members of the described clonal group.

MLST typing of the five cefotaxime-resistant *S.* Typhimurium isolates belonging to different MLVA types and collected from

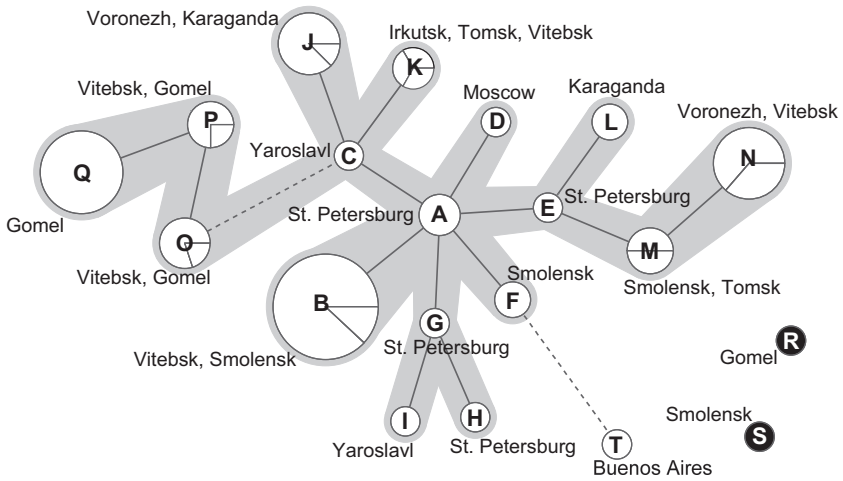

FIG 4 Minimum spanning tree of MLVA profiles of *S.* Typhimurium isolates. For each MLVA type there is a matching circle marked with a letter (A through T). Geographical locations where isolates were collected are shown along with corresponding MLVA types. Circle size is proportional to the number of isolates belonging to an MLVA type. Length and type of connecting lines reflect genetic distance (number of differing VNTR loci) between the connected types. Short solid lines connect two MLVA types differing by a single MLVA locus (single-locus variants) and long dotted lines connect double-locus variants. MLVA types divergent by two or fewer loci are regarded as a clonal group (marked by gray background color). White circles represent cefotaxime-resistant isolates and black circles represent cefotaxime-susceptible isolates.

different regions (MLVA type A [Saint-Petersburg, Russia], type D [Moscow, Russia], type K [Irkutsk, Russia], type L [Karaganda, Kazakhstan], and type Q [Gomel, Belarus]) revealed the identity of their allele profiles (*aroC-dnaN-hemD-hisD-purE-sucA-thrA* [116-7-12-9-5-9-2] corresponding to the sequence type 328 [ST328]). The respective isolates of ST328 (SE004/Sp-891, SE020/Mo-20, SE081/Ir-1732, SE079/Ka-5120, and SE066/Go-469) were submitted to the *S. enterica* MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica). The Argentine strain CAS-5 differed at the *aroC* allele and was identified as ST19 (allele profile, 10-7-12-9-5-9-2).

DISCUSSION

In recent years, there has been an increasing number of reports of clonal dissemination of cephalosporin- and quinolone-resistant *S.* Typhimurium strains in community and hospital settings in different countries worldwide (8, 40, 41). As *Salmonella* infections in humans are commonly assumed to originate from animal sources, many studies have highlighted the risk of transmission of cefotaxime-resistant *Salmonella* from animals to humans via the food chain (42–46). Production of CMY-type cephalosporinases (47–49) or CTX-M-type ESBLs, mainly CTX-M-2 (45), CTX-M-3 (50), CTX-M-15 (51), CTX-M-9 (46), and CTX-M-14 (52), has been identified as the most frequent mechanism of resistance to cefotaxime in *Salmonella* isolated from animal and human cases where potential food-borne transmission has been suggested. Furthermore, the genes for these enzymes have been found on similar or highly related plasmids in animal and human isolates, suggesting the common source of resistance (44, 47–49). Interestingly, however, to our knowledge, CTX-M-5 has been detected only in human *Salmonella* isolates (17, 18, 20–24, 53).

In contrast to many other reports, our earlier study (25) and this study were focused on cefotaxime-resistant *S.* Typhimurium from human cases that predominantly acquired their etiologic agents in hospital settings via person-to-person transmission. Previously, we reported on several outbreaks of nosocomial salmonellosis which occurred in Russia and Belarus between 1994 and 2003 caused by a single clone of CTX-M-5-producing *S.* Typhimurium. Clonal relatedness of cefotaxime-resistant strains isolated before 2003 was established by pulsed-field gel electrophoresis (PFGE) and arbitrarily primed (AP) PCR (25). In an unrelated study, carried out as part of the SENTRY Antimicrobial Surveillance Program, clonality of 15 CTX-M-5-producing *S.* Typhimurium isolates collected in Smolensk, Russia, was also established using PFGE and ribotyping (54). Other research has shown genetic relatedness between 31 cefotaxime-resistant *S.* Typhimurium isolates from the Gomel region, Belarus, using PFGE and MLVA typing (21).

Here, cefotaxime-resistant *S.* Typhimurium isolates from our previous study and those collected later on (until 2009) in a broader geographic area spanning three countries, Russia, Belarus, and Kazakhstan, were typed by MLVA and MLST. Both these methods revealed the genetic relatedness of the studied isolates.

MLVA typing demonstrated that all cefotaxime-resistant nosocomial isolates represent a single clonal group and clearly differ from cefotaxime-susceptible community-acquired isolates. The seven MLVA types included cefotaxime-resistant isolates which were derived from at least two different geographic locations. This fact illustrates the remarkable ability of the strains of the studied clonal group to spread over a vast territory. Interestingly enough, the earliest three isolates from this study collected in 1996 in Saint Petersburg belonged to MLVA-type A, which according to minimum spanning tree analysis of MLVA profiles may represent a "progenitor" strain whose descendants spread over the three countries.

Genetic relatedness of cefotaxime-resistant *S.* Typhimurium isolates was also confirmed by MLST, which assigned them to the same sequence type, ST328. By querying the MLST database (http://mlst.warwick.ac.uk/mlst/dbs/Senterica), we identified another 10 records of ST328 isolates, all of which were isolated from humans in Asia (Taiwan and the Philippines) and Europe (United Kingdom) in 2001 to 2007. As part of the study of integron-mediated resistance in a global collection of *S. enterica* isolates, Kraulland et al. have reported on clonal dissemination of ST328 *S.* Typhimurium isolates with the ACSSuT phenotype (resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline) in Taiwan and the Philippines (55). However, it is not known whether any of the ST328 isolates from Asia or the United Kingdom exhibited resistance to oxyimino-cephalosporins or quinolones. The ST328 is a single-locus variant of the most globally spread sequence type of *S.* Typhimurium, ST19. Since the CTX-M-2-producing strain from Argentina (CAS-5) was typed as ST19, distant relatedness between our isolates and the CAS-5 indicated by MLVA was also supported by MLST.

While CTX-M-5 ESBL, a close variant of CTX-M-2, was detected in all cefotaxime resistant isolates from Russia, Belarus, and Kazakhstan, our analysis identified different genetic structures at the origin of acquisition of $bla_{\text{CTX-M-5}}$ and $bla_{\text{CTX-M-2}}$ genes. In the Argentine strain CAS-5 (ST19), the $bla_{\text{CTX-M-2}}$ gene is associated with the IS*CR1* element and is carried by a large self-conjugative plasmid, whereas in all isolates of the ST328 clonal group, $bla_{\text{CTX-M-5}}$ is associated with IS*Ecp1* and in most cases located on small (7.4- to 12.4-kb) closely related plasmids. A similar association, with only a 3-nucleotide difference in the noncoding region between $bla_{\text{CTX-M-5}}$ and IS*Ecp1*, harbored within a small (~10-kb) plasmid, was found in an *S.* Typhimurium strain from Latvia (GenBank number AF286192) (17), which might serve as circumstantial evidence of its common origin with the isolates described in our study.

The presence of CTX-M-5-coding plasmids, designated pCTXM5, which shared distinctive restriction profiles and a highly conserved genetic backbone, was specific to the isolates of the ST328 clonal group. We suggest that these plasmids may have evolved from pIGRW12 or similar plasmids found in different species of *Enterobacteriaceae* by acquisition of the IS*Ecp1*::$bla_{\text{CTX-M-5}}$ resistance determinant. Among our isolates, a predominant variant of pCTXM5 (pCTXM5-637) can be considered a prototype for 3 other less prevalent variants that diverged through the insertion of mobile elements (IS*1*, in the case of pCTXM5-1358 and pCTXM5-1845, and Tn*3*, in the case of pCTXM5-891). The absence of the *tra* operon and the presence of the mobilization genes (*mobA* to *-D*) in the backbone of pCTXM5 plasmids indicate that they are not self-transmissible but can be mobilized in *trans* by other plasmids, which is consistent with the fact that cefotaxime resistance was transferred in mating experiments with low frequency (10^{-7} to 10^{-8}) only from a small number of our isolates. Eight isolates of the ST328 clonal group were $bla_{\text{CTX-M-5}}$ positive but pCTXM5 negative by PCR targeting of the conserved segment of the plasmid backbone. The absence of pCTXM5 plasmids in these isolates can be explained by IS*Ecp1*-mediated transposition

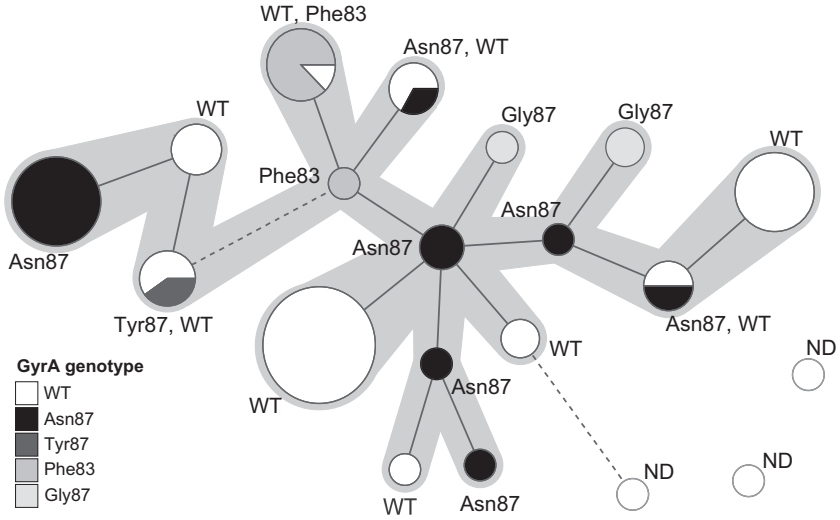

FIG 5 Distribution of GyrA mutations among isolates of various MLVA types within the ST328 clonal group. The color of circles and circle segments correspond to the GyrA genotype (white, wild type; black and various shades of gray, mutants). Circle size is proportional to the number of isolates belonging to an MLVA type. Lengths and types of connecting lines reflect genetic distance (number of differing VNTR loci) between the connected types. Short solid lines connect two MLVA types differing by a single MLVA locus (single-locus variants) and long dotted lines connect double-locus variants. MLVA types divergent by two or fewer loci are regarded as a clonal group (marked by gray background color).

of the $bla_{\text{CTX-M}}$ gene to the chromosome, described in several previous studies (56–59).

In addition to resistance to penicillins and cephalosporins conferred by CTX-M-5, many of the isolates studied are resistant to penicillin-inhibitor combinations owing to production of OXA-1-like penicillinases. As shown in our previous study, $bla_{\text{OXA-1}}$-like genes are located on large conjugative plasmids which also harbor resistance determinants to non-β-lactam antibiotics (tetracycline, chloramphenicol, gentamicin, trimethoprim, and sulfamethoxazole) (25).

The high frequencies of resistance to nalidixic acid (43.2%) and intermediate resistance to ciprofloxacin (42.0%) among the CTX-M-5-positive isolates of the ST328 clonal group are a particular cause of concern. Sequencing of the *gyrA* gene QRDR showed that in all cases resistance is caused by various single point mutations leading to amino acid substitutions at GyrA positions 83 or 87. These mutations are known to be associated with quinolone resistance in various species of *Enterobacteriaceae* (60), and their effect is well studied for *Salmonella enterica* (11, 61). Asn-87 and Phe-83 were most often detected in our isolates (28.4% and 9.1%, respectively). Consistently with previous reports (61), the latter mutation was associated with particularly higher levels of resistance to both nalidixic acid (median MIC of ≥512 mg/liter) and ciprofloxacin (median MIC of 0.25 mg/liter) (Fig. 3). The GyrA genotype precisely correlated with the phenotype of resistance to nalidixic acid. The wild-type isolates were all susceptible (MICs of ≤16 mg/liter), while mutants bearing substitutions at positions 83 or 87 were all resistant (MICs of ≥128 mg/liter). All but the one mutant isolate were also nonsusceptible to ciprofloxacin (MICs of ≥0.125); however, the latter isolate with Gly-87 had an MIC of 0.06 mg/liter and was therefore categorized as susceptible. In our opinion, this observation suggests that resistance to nalidixic acid is a more sensitive indicator of chromosome-mediated resistance to fluoroquinolones in *Salmonella*. Although nalidixic acid may not detect all mechanisms of fluoroquinolone resistance, its use as a surrogate marker is still recommended by CLSI, which states that "*Salmonella* that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with salmonellosis" (27).

The diversity of GyrA mutations found and analysis of their distribution among isolates of various MLVA types within the described clonal group (Fig. 5) clearly suggest that quinolone resistance has been acquired independently through the evolution of the ST328 genetic lineage. This is also consistent with our finding of a strong mutator phenotype of studied isolates, as evidenced by an increased (up to four orders of magnitude) rate of spontaneous resistance to nalidixic acid. Numerous studies have demonstrated that hypermutability plays an important role in the development of quinolone resistance in many species, including *S. enterica*, and is common among nosocomial strains (37, 62, 63). We accordingly suggest that hypermutability facilitates emergence of quinolone resistance in *S.* Typhimurium ST328 and thus contributes to its adaptation to the hospital environment. The exceptionally high resistance rates to antimicrobial agents of various classes among ST328 isolates may also reflect their persistence under antibiotic pressure exerted in nosocomial settings.

In conclusion, we provide evidence that cefotaxime-resistant isolates of *S.* Typhimurium collected from patients with nosocomial salmonellosis in three countries (Russia, Belarus, and Kazakhstan), over the period of more than 14 years, belong to one successful clone, ST328. Members of this clone harbor specific, low-molecular-weight plasmids (pCTXM5, carrying the IS*Ecp1*::$bla_{\text{CTX-M-5}}$ resistance determinant) and share a strong mutator phenotype which facilitates acquisition of quinolone resistance via mutations in the *gyrA* QRDR. Results obtained in our study may assist in identification of this multidrug-resistant high-risk clone and prevention of its further spread.

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