

# Emergence of Clinical *Salmonella enterica* Serovar Typhimurium Isolates with Concurrent Resistance to Ciprofloxacin, Ceftriaxone, and Azithromycin

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**Salmonella** infection is an important public health issue for which the needs of antimicrobial treatment are increasing. A total of 546 human clinical *S. enterica* serovar Typhimurium isolates were recovered from patients in hospitals in China during the period of 2005 to ~2011. Twenty percent of the isolates exhibited resistance to ciprofloxacin, and 4% were resistant to ceftriaxone. Importantly, for the first time, 12 (2%) *S. Typhimurium* isolates resistant to both ciprofloxacin and ceftriaxone were recovered; among these 12 isolates, two were also resistant to azithromycin, and one was resistant to all other drugs tested. The combined effects of various transferrable extended-spectrum  $\beta$ -lactamase determinants and a novel efflux-based ciprofloxacin resistance mechanism encoded by the mobile efflux gene *oqxAB* were responsible for the emergence of these extremely (highly) drug-resistant (XDR) *S. Typhimurium* isolates. The dissemination of resistance genes, such as those encoding ESBLs and the OqxAB pump, among *Salmonella* organisms will speed up the selection of XDR *Salmonella*, posing a huge threat to public health and *Salmonella* infection control.

Food-borne salmonellosis is an important public health problem worldwide and the leading cause of food-borne illnesses in many countries, including the United States and China. Although antimicrobial treatment is not necessary for most cases of salmonellosis, it can be lifesaving in invasive infections (1). Ceftriaxone and ciprofloxacin are the key drugs of choice for treatment of invasive *Salmonella* infections (2). Recently, azithromycin was also approved by the FDA to be an additional agent for treatment of *Salmonella* infections (3). Resistance to ceftriaxone or other extended-spectrum beta-lactams is usually due to intracellular production of extended-spectrum  $\beta$ -lactamases (ESBLs). In *Salmonella*, the most commonly found ESBL types in Asia are in the CTX-M group (4), which are usually located on transmissible plasmids that tend to disseminate among members of the *Enterobacteriaceae* (5). The most commonly reported CTX-M enzymes in *Salmonella* strains are CTX-M-9, CTX-M-14, and CTX-M-15 (5). In other regions, such as the United States and Canada, the AmpC  $\beta$ -lactamase CMY-2 is the major contributor to ceftriaxone resistance in *Salmonella* (6). On the other hand, ciprofloxacin resistance is mainly attributed to double mutations in *gyrA* and a single mutation in *parC* in *Salmonella* (7). Efflux pumps and the presence of plasmid-mediated quinolone resistance (PMQR) determinants may also contribute to ciprofloxacin resistance. Qnr genes such as *qnrA*, *qnrB*, *qnrD*, and *qnrS* have been reported (8, 9). Recent studies also detected *oqxAB*, a plasmid-borne gene which encoded an RND efflux pump, in *Salmonella* isolated from food, animals, and humans (10–12). Although resistance to fluoroquinolone or extended-spectrum cephalosporins was increasingly reported in *S. enterica* serovar Typhimurium, concurrent resistance to ciprofloxacin and ceftriaxone (i.e., extremely drug resistant [XDR]) has been reported in other serovars, including *S. Choleraesuis*, *S. Kentucky*, *S. Senftenberg*, and *S. Oranienburg*, but not in *S. Typhimurium* (13–16). This work illustrated the

underlying molecular mechanisms responsible for the production of extremely drug-resistant phenotypes in *S. Typhimurium*.

## MATERIALS AND METHODS

**Bacterial isolates and serotyping.** Human clinical *S. Typhimurium* isolates were collected from the State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention (ICDC), Chinese Center for Disease Control and Prevention, Beijing, China. These isolates originated from hospitals in eight participating cities and provinces in mainland China, including Guangdong, Guangxi, Henan, Fujian, Sichuan, Beijing, Shanghai, and Chongqing. All isolates were serotyped according to the Kauffmann-White scheme.

**Antimicrobial susceptibility testing.** Confirmed *S. Typhimurium* isolates were subjected to antimicrobial susceptibility testing using the agar dilution method, and the results were interpreted according to the CLSI guidelines (17). Nineteen antimicrobials were tested: ampicillin, amoxicillin-clavulanic acid, ceftazidime, ceftiofur, cefotaxime, ceftriaxone, meropenem, chloramphenicol, gentamicin, kanamycin, streptomycin, nalidixic acid, ciprofloxacin, sulfamethoxazole, tetracycline, trimethoprim, erythromycin, amikacin, and azithromycin. Olaquinox was also tested. *Escherichia coli* strains ATCC 25922 and 35218, *Enterococcus faecalis* strain ATCC 29212, *Staphylococcus aureus* strain ATCC 29213, and *Pseudomonas aeruginosa* ATCC 27853 were used as quality controls.

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**PMQR genes and target gene mutation screening.** The quinolone resistance-determining regions (QRDRs) of *gyrA* and *parC* were amplified by PCR as previously described (18), followed by determination of their nucleotide sequences and comparison to the wild-type *Salmonella* Typhimurium LT2 strain to identify target gene mutations in the test isolates. The presence of the PMQR genes *qnrA*, *qnrB*, *qnrC*, *qnrD*, *qnrS*, *qepA*, *oqxAB*, and *aac(6')-Ib-cr* was determined by PCR using primers described previously and sequencing (18, 19). The prevalence of ESBLs was determined by PCR targeting known  $\beta$ -lactamases as previously described (10). The full lengths of  $\beta$ -lactamase genes were amplified by specific primers for CTX-M group 1 (forward [F], 5'-ATGGTTAAAAA TCACTGCG; reverse [R], 5' TTACAAACCGTCGGTGAC), CTX-M group 2 (F, 5'-ATGATGACTCAGAGCATTCGC; R, 5'-TCAGAAACCG TGGGTTACGAT), CTX-M group 9 (F, 5'-ATTCAGAGCTCATGGTGA CAAAGAGAGTGC; R, 5'-TAGTAGGATCCTTACAGCCCTTCGGCGA TG), and CMY (F, 5'-ATGATGAAAAATCGTTATGCT; R, 5'-ATTGC AGCTTTTCAAGAAT), followed by nucleotide sequencing to determine their type specificity.

**Conjugation experiments.** A conjugative experiment was carried out as previously described (20) using sodium azide-resistant *E. coli* strain J53 as the recipient. Briefly, overnight cultures of donor and recipient strains were mixed and collected on a filter, which was subjected to overnight incubation on a blood agar plate. The mixture was then spread on double selective blood agar plates containing ceftriaxone (2  $\mu$ g/ml) and sodium azide (100  $\mu$ g/ml) to select drug-resistant transconjugants. Plasmid replication typing was performed as previously described (21).

**Molecular typing.** The clonal relationship between representative *Salmonella* isolates was examined by pulsed-field gel electrophoresis (PFGE) according to the PulseNet PFGE protocol for *Salmonella* (22). S1-PFGE was conducted to determine the size of large plasmids. Briefly, agarose-embedded DNA was digested with S1 nuclease (New England BioLabs) at 37°C for 1 h. The restriction fragments were separated by electrophoresis in 0.5 mM Tris-borate-EDTA buffer at 14°C for 18 h using a CHEF Mapper electrophoresis system (Bio-Rad, Hercules, CA) with pulse times of 2.16 to 63.8 s. A phage Lambda PFGE ladder (New England BioLabs) was used as the DNA size marker. The gels were stained with GelRed, and DNA bands were visualized with UV transillumination (Bio-Rad). Multilocus sequence typing was performed using primer sets as suggested at [www.mlst.net](http://www.mlst.net).

## RESULTS AND DISCUSSION

A total of 546 human clinical *S. Typhimurium* isolates, accounting for approximately 40% (1,380) of all *S. Typhimurium* isolates and 9% (5,873) of all *Salmonella* isolates collected from the State Key Laboratory for Infectious Disease Prevention and Control, National Institute for Communicable Disease Control and Prevention (ICDC) of China, during 2005 to ~2011 were included in this study. A substantial proportion of these isolates displayed resistance (MIC,  $\geq 4$   $\mu$ g/ml) (20%) or intermediate resistance (MIC, 2  $\mu$ g/ml) (16%) to ciprofloxacin. Around 4% of the isolates were resistant to extended-spectrum cephalosporins (Table 1). Profiles of resistance to other antimicrobials are shown in Table 1. All *Salmonella* isolates were susceptible to meropenem.

The vast majority of 109 (20%) isolates which were resistant to ciprofloxacin also exhibited high-level resistance to other classes of antimicrobials, including sulfamethoxazole (100%), chloramphenicol (97%), ampicillin (94%), tetracycline (94%), kanamycin (92%), trimethoprim (92%), amoxicillin-clavulanic acid (87%), gentamicin (82%), streptomycin (67%), and extended-spectrum cephalosporins (9%) (Table 1). The annual distribution of ciprofloxacin-resistant *Salmonella* isolates from 2005 to ~2011 was 11%, 37%, 20%, 12%, 9%, and 33%, respectively. Among the 22 ceftriaxone-resistant *S. Typhimurium* isolates, the rates of resis-

TABLE 1 Rates of *S. Typhimurium* resistance to 18 different antimicrobial agents

	% Resistance <sup>a</sup>			
	Overall (n = 546)	CIP (MIC, $\geq 4$ $\mu$ g/ml) (n = 109)	CTZ (MIC, $\geq 4$ $\mu$ g/ml) (n = 22)	CIP, CTZ (MIC, $\geq 4$ $\mu$ g/ml) (n = 12)
Antimicrobial				
Ampicillin	47	94	100	100
Amoxicillin-clavulanic acid <sup>b</sup>	6 (20)	87 (6)	100	100
Ceftazidime	2	9	50	50
Ceftiofur	4	9	100	100
Cefotaxime	4	9	100	100
Ceftriaxone	4	9	100	100
Meropenem	0	0	0	0
Chloramphenicol	43	97	59	100
Gentamicin	35	82	82	92
Kanamycin	44	92	92	100
Streptomycin	28	67	64	92
Nalidixic acid	63	100	91	100
Ciprofloxacin <sup>b</sup>	20 (16)	100	55	100
Sulfamethoxazole	55	100	86	100
Tetracycline	49	94	86	92
Trimethoprim	42	92	73	100
Erythromycin	93 <sup>c</sup>	97 <sup>c</sup>	100 <sup>c</sup>	100
Amikacin	ND	ND	ND	17
Azithromycin	ND	ND	ND	17 <sup>d</sup>

<sup>a</sup> CIP, ciprofloxacin; CTZ, ceftriaxone; ND, not determined. CIP (MIC,  $\geq 4$   $\mu$ g/ml), *Salmonella* isolates with ciprofloxacin MIC of  $\geq 4$   $\mu$ g/ml; CTZ (MIC,  $\geq 4$   $\mu$ g/ml), *Salmonella* isolates with ceftriaxone MIC of  $\geq 4$   $\mu$ g/ml; CIP, CTZ (MIC,  $\geq 4$   $\mu$ g/ml), *Salmonella* isolates with both ciprofloxacin and ceftriaxone MICs of  $\geq 4$   $\mu$ g/ml.

<sup>b</sup> Values in parentheses indicate percentages of isolates with intermediate resistance.

<sup>c</sup> Percentage of isolates with a MIC of  $\geq 64$   $\mu$ g/ml.

<sup>d</sup> Since there is no breakpoint for azithromycin, it is commonly accepted that azithromycin MIC of  $\geq 64$   $\mu$ g/ml in *Salmonella* is considered resistant to azithromycin.

tance to different antimicrobials were the following: kanamycin (92%), nalidixic acid (91%), sulfamethoxazole (86%), tetracycline (86%), gentamicin (82%), trimethoprim (72%), streptomycin (64%), and chloramphenicol (59%). Most importantly, 12 of the 22 ceftriaxone-resistant *S. Typhimurium* isolates also exhibited resistance to ciprofloxacin. Not surprisingly, these isolates exhibited extremely high rates and levels of resistance to all other antimicrobials. Three of them were susceptible to only one antimicrobial (Table 1). Among these 12 extremely drug-resistant (XDR) *S. Typhimurium* isolates, two were also resistant to azithromycin; thus, these two isolates were concurrently resistant to ciprofloxacin, ceftriaxone, and azithromycin (Table 1). Human clinical *Salmonella* isolates that were resistant to all three of these choices of treatment were only recently reported for *S. Kentucky* ST198-X1, a much less prevalent serotype (16). The emergence of *S. Typhimurium* strains resistant to these three antimicrobials warrants continuous monitoring of the trend of development of antimicrobial resistance in China.

Mechanisms of resistance to ciprofloxacin and ceftriaxone in the 12 XDR *S. Typhimurium* isolates were investigated. Double *gyrA* (S83F, D87N) mutations and a single *parC* (S80R) mutation were detectable in 8 out of 12 XDR *S. Typhimurium* isolates. The PMQR genes *aac(6')-Ib-cr* and *qeqA* were detected in two isolates (Table 2). In contrast to other reports, the remaining 4 isolates harbored only a single *gyrA* (D87N) mutation. However, *oqxAB* was detected in all four of these isolates. One of them also con-

TABLE 2 Summary of genotypes and phenotypes of *S. Typhimurium* isolates and their corresponding transconjugants

Isolate	MLST	MIC <sup>a</sup> (μg/ml)			Non-β-lactam resistance profile <sup>b</sup>	β-Lactamase	QRDR mutation(s) <sup>c</sup>			Plasmid replicon(s)	Plasmid size(s) (ca. kb)
		CIP	CTX	CEF			GyrA	ParC	PMQR gene(s)		
ST414	19	≥8	≥128	≥64	NAL-CHL-GEN-KAN-SUL-TRI-TET	CTX-M-28	S83F, D87N	S80R		FIB, FIIAs	ND
ST429	19	≥8	≥128	8	NAL-CHL-AMK-GEN-KAN-SUL-TRI-TET-AZR	CTX-M-14	S83F, D87N	S80R	<i>qepA</i>	FIB, FIIAs	70, 120, 170
TC-ST429		<0.025	≥128	4	AMK-GEN	CTX-M-14	ND	ND		FIB	70
ST494	34	≥8	≥128	2	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-104	S83F, D87N	S80R	<i>aac(6′)-Ib-cr</i>	HI2	ND
ST249	19	≥8	≥128	8	NAL-CHL-GEN-SUL-KAN-TRI-AZR	CTX-M-14	S83F, D87N	S80R		FIB, FIIAs	50, 97
ST512	19	≥8	≥128	4	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-14	S83F, D87N	S80R		FIIAs	100
ST588	34	4	≥128	4	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-14	D87N	WT	<i>oqxAB</i>	I1-Iγ	120, 200
TC-ST588		<0.025	≥128	4	GEN	CTX-M-14	ND	ND		I1-Iγ	120
ST297	19	4	≥128	4	NAL-GEN-TET-SUL-KAN-TRI	CTX-M-14	S83F, D87N	S80R		I1-Iγ, FIIAs	47, 90, 110, 200
TC-ST297		<0.025	64	4	GEN	CTX-M-14	ND	ND		I1-Iγ	110
ST277	19	≥8	≥128	64	NAL-CHL-KAN-SUL-TRI-TET	CTX-M-79	S83F, D87N	S80R		I1-Iγ, HI1	80, 220
TC-ST277		<0.025	64	32		CTX-M-79	ND	ND		I1-Iγ	80
ST241	34	≥8	≥128	4	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-14	S83F, D87N	S80R	<i>aac(6′)-Ib-cr, oqxAB</i>	FIB, FIIAs	90, 97
ST426	34	4	≥128	4	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-14	D87Y	WT	<i>oqxAB</i>	B/O, HI2	48.5, 242
ST566	19	4	64	64	NAL-CHL-GEN-SUL-KAN-TRI-TET	CMY-2	S83F	WT	<i>oqxAB</i>	Y, FIB	ND
SH111302	34	4	≥128	32	NAL-CHL-GEN-SUL-KAN-TRI-TET	CTX-M-27	D87Y	WT	<i>oqxAB</i>	HI2	ND

<sup>a</sup> CIP, ciprofloxacin; CTX, cefotaxime; CEF, ceftazidime.

<sup>b</sup> NAL, nalidixic acid; CHL, chloramphenicol; AMK, amikacin; GEN, gentamicin; KAN, kanamycin; SUL, sulfamethoxazole; TRI, trimethoprim; TET, tetracycline; AZR, azithromycin.

<sup>c</sup> WT, wild type; ND, not determined.

tained *aac(6′)-Ib-cr* (Table 2). A single *gyrA* mutation, together with *oqxAB*-mediated efflux, was shown to be a novel mechanism of ciprofloxacin resistance in *S. Typhimurium*. Further works will be needed to evaluate the relative roles of *OqxAB* and single-target-gene mutation in the development of fluoroquinolone resistance in *S. Typhimurium*. Recently, our laboratory confirmed that clonal expansion of an *S. Typhimurium* ST34 clone carrying multiple resistance determinants, in particular *oqxAB* and *aac(6′)-Ib-cr*, occurred in both China and Hong Kong (12). Therefore, findings of this work further highlight the role of the mobile *OqxAB* pump in the emergence of XDR phenotypes in *Salmonella* and the subsequent clonal expansion of such XDR isolates.

The ESBL screening and characterization for these *S. Typhimurium* isolates showed that different variants of the CTX-M family of β-lactamases were detectable in XDR *S. Typhimurium* and that they most likely contributed to the ceftriaxone resistance phenotypes of the host isolates. *bla*<sub>CTX-M-14</sub> was detectable in seven isolates, and *bla*<sub>CTX-M-27</sub>, *bla*<sub>CTX-M-28</sub>, *bla*<sub>CTX-M-79</sub>, *bla*<sub>CTX-M-104</sub>, and *bla*<sub>CMY-2</sub> could be detected in each of the other five *S. Typhimurium* isolates (Table 2). Detection of *bla*<sub>CTX-M-27</sub> and *bla*<sub>CTX-M-28</sub> was reported for *S. Livingstone* and *S. Isangi*, respectively, in 2005 (23, 24). This work constitutes the first report of *bla*<sub>CTX-M-79</sub> and *bla*<sub>CTX-M-104</sub> in *Salmonella*, and *bla*<sub>CTX-M-27</sub> and *bla*<sub>CTX-M-28</sub> were also found in *S. Typhimurium* for the first time. Both CTX-M-104 and CTX-M-79 have been identified in *E. coli* (25, 26). CTX-M-104 differs from CTX-M-14 at position 275, with N<sup>275</sup> in CTX-M-104 and S<sup>275</sup> in CTX-M-14. CTX-M-79 differs from CTX-M-55 at position 289, with N<sup>289</sup> in CTX-M-79 and D<sup>289</sup> in CTX-M-55. Conjugation experiments were conducted for all XDR *Salmonella* isolates listed in Table 2 and showed that four *S. Typhimurium* clones, ST429, ST588, ST297, and ST277, were able to transfer their ESBL determinants to *E. coli* J53. The gentamicin resistance determinants of ST588 and ST297 and gentamicin and amikacin resistance determinants of ST429 were also transferrable (Table 2). The resistance determinant for azithromycin was not transferable.

S1-PFGE showed that most of the isolates possessed several large-sized plasmids (Fig. 1 and Table 2). Transconjugants of *S. Typhimurium* harbored plasmids with sizes ranging from 70 kb to

120 kb. IncF- and I1-Iγ-type plasmids were the most commonly found replicon types, followed by FIIAs, F1B, HI2, Y, and B/O (Table 2). Among the transconjugants, 3 out of 4 harbored an I1-Iγ-type plasmid, and the remaining one carried the F1B type. The I1-Iγ-type plasmids were found to be dominant among *Salmonella* strains carrying *bla*<sub>CMY-2</sub> (6). This study reports the detection of different *bla*<sub>CTX-M</sub>-containing I1-Iγ-type plasmids for the first time and provides evidence that plasmids of this type are disseminating among *S. Typhimurium* strains in China.

The clonal relationships among the 12 XDR *S. Typhimurium* isolates were determined. These 12 isolates were found to display unrelated PFGE profiles which were not the dominant PFGE types in China (Fig. 2). XDR *Salmonella* isolates were commonly seen in Henan Province in 2007 and 2008 and became common in Shanghai in 2010 and 2011 (Fig. 2). Nevertheless, multilocus sequence typing showed that 7 of the *S. Typhimurium* isolates belonged to ST19, a common clone causing human infections worldwide (27–29). The remaining 5 isolates belonged to ST34, which is associ-

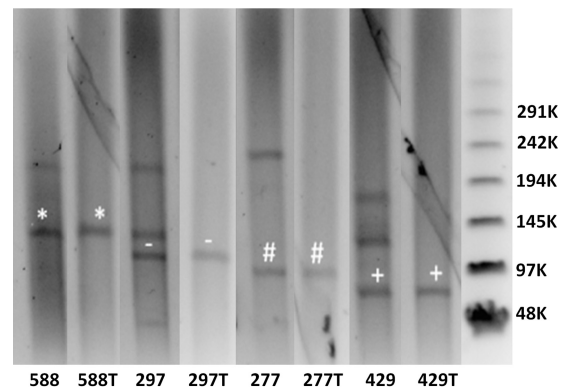


FIG 1 Plasmid profiles of representative *S. Typhimurium* isolates and transconjugants determined by S1-PFGE. Transmissible plasmids from *S. Typhimurium* and transconjugants (T) are highlighted. The same symbol in the parental isolate and transconjugant indicates the position of the conjugative plasmid. The marker is a phage lambda concatemer with different bands labeled.



FIG 2 PFGE patterns of clinical *S. Typhimurium* isolates that are resistant to both ciprofloxacin and ceftriaxone. The isolate identity (ID), year of isolation, origin, and location of isolation, as well as the ESBL, PMQR gene(s), and multilocus sequence types, are also indicated.

ated with a monophasic variant of *S. Typhimurium* in Europe with the ASSuTe R-type (27) (Table 2 and Fig. 2).

In conclusion, this study reported the emergence of *S. Typhimurium* human clinical isolates that were resistant to all classes of antimicrobials tested, including ceftriaxone, ciprofloxacin, and azithromycin, which are common choices of treatment. The selection of these XDR *S. Typhimurium* isolates may be due to the increasing transmission of different ESBL-encoding elements among ciprofloxacin-resistant *S. Typhimurium* organisms whose resistance phenotypes are in turn mediated by conventional and novel mechanisms, including a single *gyrA* mutation and a PMQR gene, *oqxAB*. Transmission of resistance genes such as those encoding ESBLs and the OqxAB pump in *Salmonella* will speed up the selection of these XDR *Salmonella* organisms, which poses a huge threat to human *Salmonella* infection control. Further works are required to trace the transmission routes of XDR *Salmonella* strains in clinical settings and develop appropriate intervention strategies.

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