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MAJOR ARTICLE

Emergence of Resistance Mutations in *Salmonella enterica* Serovar Typhi Against Fluoroquinolones

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Background. Little is known about the evolutionary process and emergence time of resistance mutations to fluoroquinolone in *Salmonella enterica* serovar Typhi.

Methods. We analyzed *S.* Typhi isolates collected from returned travelers between 2001 and 2016. Based on ciprofloxacin susceptibility, isolates were categorized as highly resistant (minimum inhibitory concentration [MIC] ≥ 4 μg/mL [CIP^{HR}]), resistant (MIC = 1–2 μg/mL [CIP^{R}]), intermediate susceptible (MIC = 0.12–0.5 μg/mL [CIP^{I}]), and susceptible (MIC ≤ 0.06 μg/mL [CIP^{S}]).

Results. A total of 107 isolates (33 CIP^{HR}, 14 CIP^{R}, 30 CIP^{I}, and 30 CIP^{S}) were analyzed by whole-genome sequencing; 2461 single nucleotide polymorphisms (SNPs) were identified. CIP^{S} had no mutations in the *gyrA* or *parC* genes, while each CIP^{I} had 1 of 3 single mutations in *gyrA* (encoding Ser83Phe [63.3%], Ser83Tyr [33.3%], or Asp87Asn [3.3%]). CIP^{HR} had the same 3 mutations: 2 SNPs in *gyrA* (encoding Ser83Phe and Asp87Asn) and a third in *parC* (encoding Ser80Ile). CIP^{HR} shared a common ancestor with CIP^{R} and CIP^{I} isolates harboring a single mutation in *gyrA* encoding Ser83Phe, suggesting that CIP^{HR} emerged 16 to 23 years ago.

Conclusions. Three SNPs—2 in *gyrA* and 1 in *parC*—are present in *S.* Typhi strains highly resistant to fluoroquinolone, which were found to have evolved in 1993–2000, approximately 10 years after the beginning of the ciprofloxacin era. Highly resistant strains with survival advantages arose from strains harboring a single mutation in *gyrA* encoding Ser83Phe. Judicious use of fluoroquinolones is warranted to prevent acceleration of such resistance mechanisms in the future.

Keywords. evolution; fluoroquinolone resistance; molecular; *Salmonella enterica* serovar Typhi.

Salmonella enterica subspecies *enterica* serovar Typhi (*S.* Typhi), a human-specific pathogen, causes typhoid fever, a systemic infection. An estimated 11.9 to 20.6 million new infections and >200 000 typhoid fever–related deaths occur annually worldwide [1, 2]. *S.* Typhi can proliferate in macrophages/dendritic cells by suppressing the phagolysosomal reaction, thereby avoiding phagocytosis [3, 4]. Fluoroquinolones are widely accepted as the optimal treatment for typhoid fever, as they have excellent tissue penetration and bactericidal activity [5]. However, the H58 haplotype (genotype 4.3.1) [6] of *S.* Typhi—which is associated with multidrug resistance and decreased ciprofloxacin susceptibility [7, 8]—has spread from South Asia to Africa [8, 9], and fluoroquinolone-resistant strains have continued to proliferate in endemic areas, particularly in South Asia, posing a serious global health threat [10, 11].

Several mechanisms of fluoroquinolone resistance have been reported in *S.* Typhi, including efflux pumps, reduced outer membrane permeability, plasmid-mediated acquisition, and genetic mutations; the major mechanism is thought to be chromosomal mutations in genes encoding DNA gyrase (ie, *gyrA* and *gyrB*) and topoisomerase IV (ie, *parC* and *parE*) [8, 12, 13]. However, the evolutionary process and emergence time of fluoroquinolone resistance mutations in *S.* Typhi have not been fully investigated. This was addressed in the present study by analyzing mutations in DNA gyrase– and topoisomerase IV–encoding genes in *S.* Typhi isolates.

MATERIALS AND METHODS

Bacterial Strains

This study was conducted at the Department of Bacteriology I at the National Institute of Infectious Diseases (NIID) in Tokyo, Japan. *S.* Typhi was identified at regional public health centers, and all autochthonous and imported *S.* Typhi isolates in Japan have been stored at the NIID. We analyzed pooled isolates collected between January 2001 and February 2016. Based on susceptibility to ciprofloxacin (in accordance with Clinical Laboratory Standard Institute M100-S21 and M100-S26), isolates were classified as highly resistant (minimum inhibitory concentration [MIC] of ciprofloxacin ≥ 4 μg/mL), resistant (MIC = 1–2 μg/mL), intermediate susceptible (MIC = 0.12–0.5 μg/mL), or fully susceptible (wild-type; MIC ≤ 0.06 μg/mL)

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(Figure 1). All highly resistant (n = 33) and resistant (n = 14) isolates originating from returned travelers were analyzed. As controls, we analyzed randomly selected intermediate susceptible (n = 30) and all fully susceptible (n = 30) isolates from South Asia. We applied the following exclusion criteria to identify intermediate susceptible strains: isolates from fellow travelers, participants in the same tour, chronic carriers, and more than 1 episode (eg, recurrence). Two isolates from each year were randomly selected using the RAND function of Microsoft Excel software (Redmond, WA). We defined South Asia as the following countries: Afghanistan, Bangladesh, Bhutan, India, Nepal, Maldives, Pakistan, and Sri Lanka. The MIC of ciprofloxacin was determined by Etests (bioMérieux, Marcy-l'Étoile, France) from 2001 to 2006 and by the microdilution technique using Dry Plate Eiken (Eiken Chemical, Tokyo, Japan) from 2007 to 2016 [14]. The requirement for ethical approval and written informed consent was waived due to the use of unidentifiable epidemiological and microbiological data.

Whole-Genome Sequencing

Genomic DNA was prepared using the DNeasy Blood and Tissue kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Genomic DNA libraries were prepared using the Nextera XT DNA sample prep kit (Illumina, San Diego, CA), and paired-end (300 × 2 bp) short reads for each library were sequenced on a MiSeq sequencer (Illumina). Sequence reads were assembled with the de novo genome assembly program CLC Genomics Workbench v. 8.5.1 (CLC Bio, Aarhus, Denmark) to generate a multicontig draft genome for each sample. All contigs were compared with the reference genome for *S.* Typhi (strain Ty2; AE014613.1) to detect variants of *glpA*, *gyrA*, *gyrB*, *parC*, and *parE* genes. Haplotype H58 (genotype 4.3.1) was defined by the single nucleotide polymorphism (SNP) *glpA*-C1047T [7, 15]. Nucleotide sequence data have been submitted to the DNA Data Bank of Japan Sequenced Read Archive under the accession numbers DRX064929–065021 [16].

SNP Detection for Phylogenetic and Temporal Analyses

To generate short-read mapping data for all strains compared with the reference chromosomal sequence of *S.* Typhi strain Ty2, bwasw [17] and samtools [18] were used with default parameters. SNPs were extracted with VarScan v. 2.3.4 [19] with default parameters; the minimum mapping quality was set to 50. Prophage and repetitive sequences as well as recombinogenetic regions identified by RecHMM [20] were removed from further analyses. Exact and inexact repeat regions with a length ≥50 were detected using the nucmer, repeat-match, and exact-tandems function of MUMmer v. 3.23 [21]. The remaining 2461 SNPs were concatenated to generate a pseudo-sequence for phylogenetic analysis; maximum likelihood phylogenetic analysis was performed using RAxML v. 8.2.0 [22] with 1000 bootstrap iterations.

To further assess the divergence dates of haplotype H58 (genotype 4.3.1), we performed a temporal analysis using Bayesian Evolutionary Analysis Sampling Trees (BEAST) [23] under previously described conditions [8]. Briefly, a Bayesian skyline model for population growth was used, with a general time-reversible gamma substitution model and an uncorrelated, log-normal relaxed clock. Markov chain Monte Carlo generations were conducted for 100 000 000 steps, with sampling at every 1000 steps and the first 10% of states discarded as burn-in. The effective sample sizes of all parameter were ≥200.

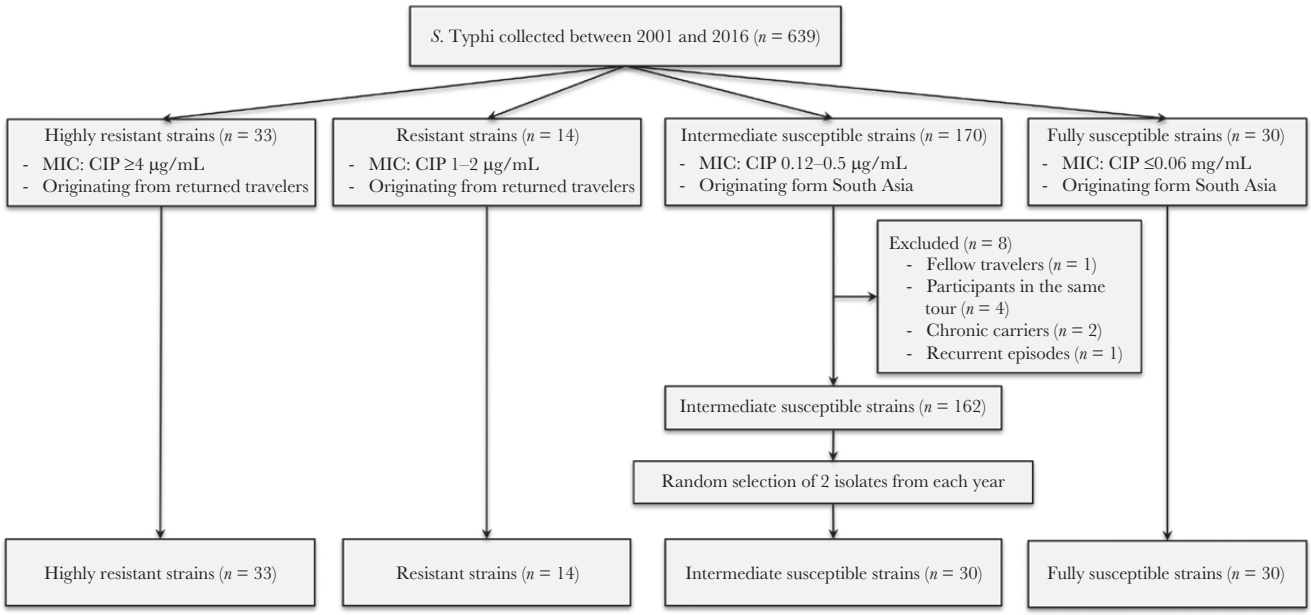

Figure 1. Flow diagram of ciprofloxacin-susceptible *S.* Typhi isolates. Abbreviations: CIP, ciprofloxacin; MIC, minimum inhibitory concentration.

Identification of Genetic Variations Fixed in the Highly Resistant Strains

To further explore genetic variations between highly resistant strains and other H58 *S.* Typhi strains, we used a kmer-based approach in which the genome sequence of each isolate was fragmented into unique, overlapping, 31-bp DNA motifs or kmers that were used to identify any genetic variations such as SNPs, indels, and the presence/absence of whole genes or gene regions [24, 25]. We extracted kmers present in all of the highly resistant strains and absent in all other H58 *S.* Typhi strains, and then identified nonsynonymous substitutions corresponding to kmers.

RESULTS

A total of 107 *S.* Typhi isolates originating from 107 subjects were analyzed, including 33 highly resistant, 14 resistant, 30 intermediate susceptible, and 30 fully susceptible strains (Figure 1). Of the 107 subjects, the median age was 25 years (range, 1–67 years); 17% were children (age 0 to <15 years), 83% were adults, and 63% were male (Table 1). There were 106/107 isolates (99%) originating from patients with acute illness (ie, typhoid fever) and 1 from a chronic carrier (160010TY). In total, 100/107 of isolates (93%) originated from South Asia: 55 (59%) from India, 24 (22%) from Nepal, and 15 (14%) from Bangladesh. Each fully susceptible strain was detected before 2008 (2001–2007), while each of the 33 highly resistant strains was detected after 2005 (2006–2016). Among the latter, 31/33 (94%) originated from South Asia (predominantly India [21/33] and Nepal [8/33]) and 2/33 (6%) from Southeast Asia (Myanmar); the distribution of ciprofloxacin susceptibility was as follows: MIC = 8 μg/mL (n = 5, 15%); MIC = 16 μg/mL (n = 23, 70%); and MIC ≥ 32 μg/mL (n = 5, 15%).

Table 1. Baseline Characteristics of 107 Subjects With *Salmonella enterica* Serovar Typhi Infection

Characteristics	Subjects (n = 107)[a]
Age, median (range), y	25 (1–67)
Male, No. (%)	67 (63)
Travel destination,[b] No. (%)	
South Asia	100 (93)
India	59 (55)
Nepal	24 (22)
Bangladesh	15 (14)
Pakistan	8 (7)
Sri Lanka	2 (2)
Afghanistan	1 (1)
Bhutan	1 (1)
Southeast Asia	7 (7)
Myanmar	4 (4)
Cambodia	2 (2)
Vietnam	1 (1)
Malaysia	1 (1)

[a]Including 106 subjects with typhoid fever and 1 chronic carrier.

[b]Including duplicate counts.

The haplotype H58 (genotype 4.3.1) was detected in 78/107 isolates (73%), with the proportion increasing over time; the haplotype H58 (genotype 4.3.1) was detected in 17/41 isolates (41%) from 2001 to 2005, in 19/21 (90%) from 2006 to 2010, and in 42/45 (93%) from 2011 to 2016 (Supplemental Figure S1). All resistant and highly resistant, 20% of fully susceptible and 83% of intermediate susceptible strains were H58 (genotype 4.3.1). Overall, 2461 SNPs (overall average, 152 SNPs) were identified among the 107 *S.* Typhi isolates. The phylogenetic analysis revealed that the following paired isolates were probably clonal (Figure 2): fellow travelers (030067TY and 030082TY: 3 SNPs), travelers to the same destination in the same month and year (030006TY and 030011TY: 3 SNPs; or 040034TY and 040054TY: 1 SNP), and an infected patient (160011TY) from a chronic carrier (160010TY: 0 SNPs). Furthermore, haplotype H58 (genotype 4.3.1) and highly resistant strains clustered together in the tree.

There were no mutations in *gyrA*, *parC*, and *parE* genes encoding DNA gyrase and topoisomerase IV in fully susceptible strains (Figure 2); of these strains, only 1 isolate (020022TY) had a mutation in the *gyrB* gene encoding Ser464Tyr. In intermediate susceptible strains, each isolate had 1 of 3 single mutations in *gyrA* encoding Ser83Phe (63.3%, 19/30), Ser83Tyr (33.3%, 10/30), or Asp87Asn (3.3%, 1/30) (Supplemental Table S2). However, each resistant strain harbored the same single mutation in *gyrA* encoding Ser83Phe, and 29% (4/14) and 21% (3/14) of strains had a second SNP in *parC* encoding Glu84Gly or in *parE* encoding Asp420Asn, respectively. Notably, all 33 highly resistant strains had the same 3 genetic mutations: 2 SNPs in *gyrA* encoding Ser83Phe and Asp87Asn, and a third in *parC* encoding Ser80Ile.

We analyzed the divergence time of H58 (genotype 4.3.1) *S.* Typhi (n = 78) using BEAST and found that H58 (genotype 4.3.1) isolates originated 27 years ago (95% heist posterior density [HPD], 20–40 years] from a common ancestor that evolved into an intermediate susceptible or resistant strain with a single SNP in *gyrA* (Figure 3). Each strain with this mutation had different coding changes, namely Ser83Phe or Ser83Tyr. However, the 6 isolates (040070TY, 080014TY, 120046TY, 120048TY, 130052TY, and 150012TY) that were the closest neighbors of highly resistant strains had the same single SNP in *gyrA* encoding Ser83Phe, and 5/6 isolates (83%) had a second SNP in *parC* encoding Glu84Gly. The neighboring 6 strains shared a recent common ancestor with a subset of highly resistant strains harboring the 3 SNPs (2 in *gyrA* and 1 in *parC*). The analyses also suggested that highly resistant strains emerged between 16 (95% HPD, 11–24) and 23 (95% HPD, 18–30) years ago.

We identified genetic variations that were completely differentiated between the highly resistant strains (n = 33) and other H58 *S.* Typhi strains (n = 45). All genetic variations were SNPs and, with the exception of Asp87Asn in GyrA and Ser80Ile in ParC, there were 10 nonsynonymous substitutions in 9 genes

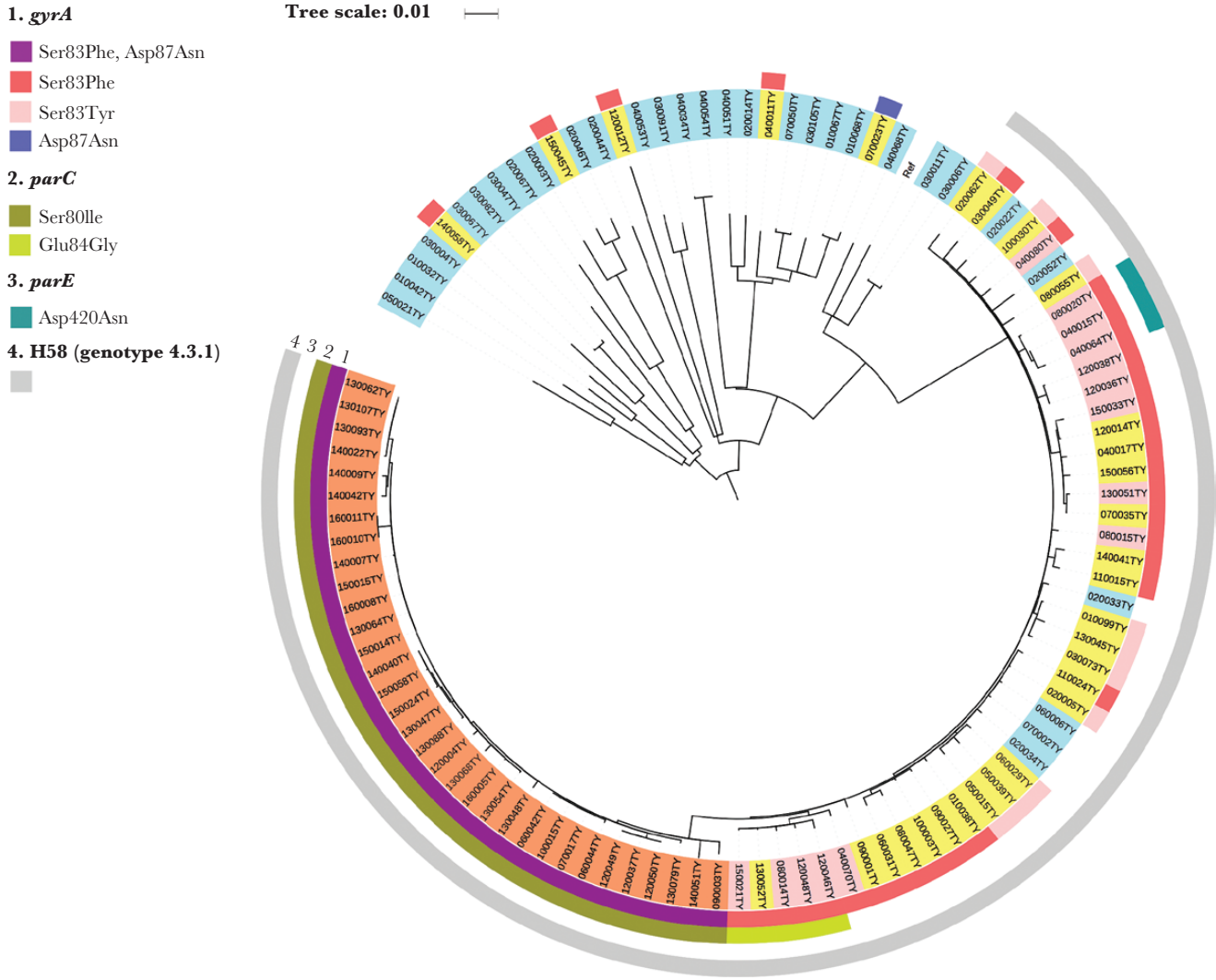

Figure 2. Phylogenetic distribution of genetic mutations in *gyrA*, *parC*, and *parE* genes in 107 *S.* Typhi isolates. Highlighted strains are susceptible to ciprofloxacin (orange, highly resistant; pink, resistant; yellow, intermediate susceptible; sky blue, fully susceptible). The nonhighlighted strain (Ty2; AE014613.1) is a reference genome for *S.* Typhi. Abbreviations: Asn, asparagine; Asp, aspartic acid; Glu, glutamic acid; Gly, glycine; Ile, isoleucine; Phe, phenylalanine; Ser, serine; Tyr, tyrosine.

(Table 2) and 4 synonymous substitutions. The results suggested that several of the genetic variations are advantageous for the survival and spread of highly resistant strains. AraC family transcriptional regulators are involved in carbon metabolism and responses to environmental stress [26]. The arabinose operon regulatory protein activates the transcription of the araBAD operon, which has been well studied in *Escherichia coli* as a regulatory system for cellular response to environmental changes [27]. The symporter YicJ is associated with bacterial fitness (resistance to heat shock) and increase in growth rate in *E. coli* [28, 29]. LysR family transcriptional regulators are involved in virulence regulation in *Salmonella* [30]. A type VI secretory protein is encoded in the middle of an T6SS island (from t2564 to t2621 in the reference genome Ty2), which is involved in various biological processes ranging from interbacterial interaction to pathogenesis [31]. The mannose-specific phosphoenolpyruvate-dependent phosphotransferase system (PTS) is related to growth rate and biomass yield in *Listeria* [32]. The polymyxin resistance protein ArnA is a key lipopolysaccharide modification enzyme in the lipid A modification pathway of Gram-negative bacteria that confers resistance to antimicrobial peptides of the innate immune system and antibiotics such as polymyxin [33].

DISCUSSION

In the present study, we evaluated the molecular evolutionary process and emergence time of fluoroquinolone resistance mutations in *S.* Typhi. We found that highly resistant *S.* Typhi had 3 genetic mutations—2 in *gyrA* (encoding Ser83Phe and Asp87Asn) and 1 in *parC* (encoding Ser80Ile)—and shared a recent ancestor with strains harboring a single mutation in *gyrA* encoding Ser83Phe. Moreover, we found that highly resistant

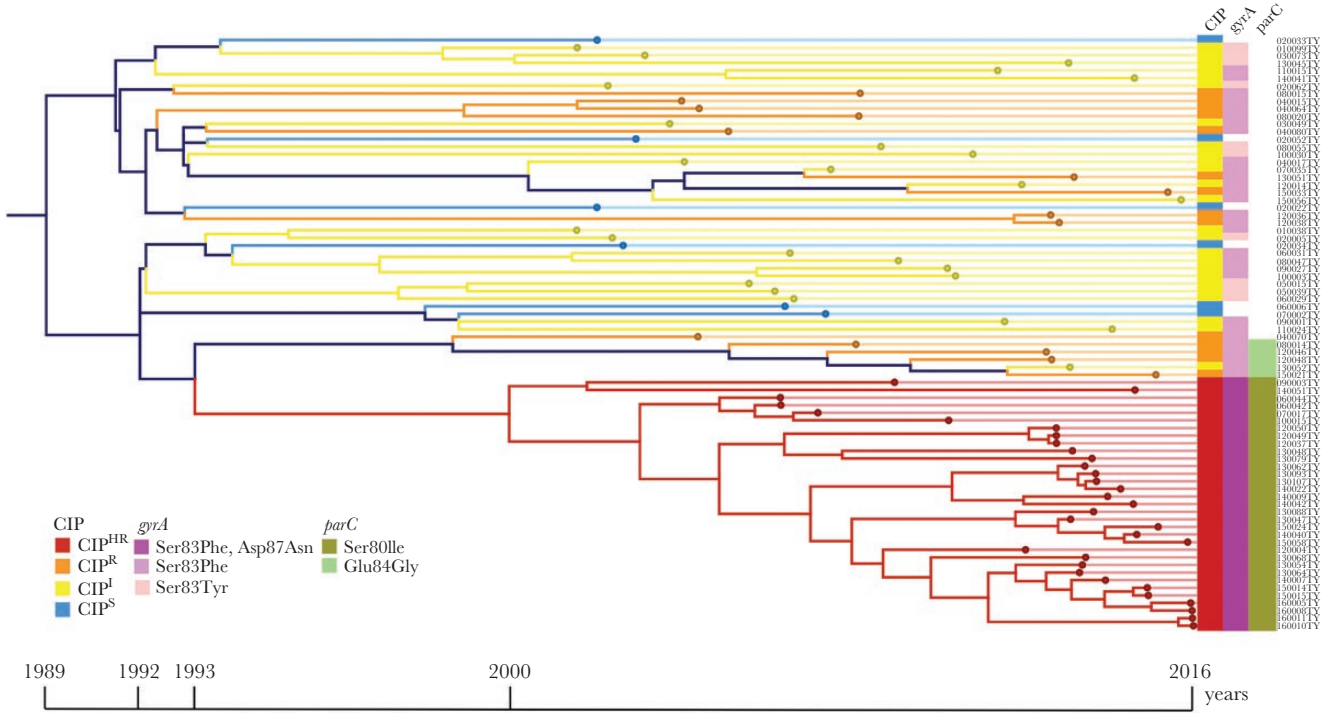

Figure 3. Maximum clade credibility tree of 78 H58 (genotype 4.3.1) *S.* Typhi isolates. The bottom scale represents years. Lines were colored based on susceptibilities to ciprofloxacin. H58 (genotype 4.3.1) isolates originated from the common ancestor 27 (95% HPD, 20–40) years ago. Strains that are highly resistant to ciprofloxacin were found to share a recent ancestor with those harboring a single mutation in the *gyrA* gene encoding Ser83Phe, and likely emerged between 16 (95% HPD, 11–24) and 23 (95% HPD, 18–30) years ago. Abbreviations: Asn, asparagine; Asp, aspartic acid; CIP, ciprofloxacin; CIP^{HR}, highly resistant; CIP^{I}, intermediate susceptible; CIP^{R}, resistant; CIP^{S}, fully susceptible; Glu, glutamic acid; Gly, glycine; HPD, heist posterior density; Ile, isoleucine; Phe, phenylalanine; Ser, serine; Tyr, tyrosine.

strains emerged around 1993–2000 and harbored genetic mutations that were potentially advantageous for bacterial survival and growth.

Our study has 3 important findings. First, this is the first study to evaluate the patterns of emergence of resistance mutations to fluoroquinolones in *S.* Typhi by whole-genome sequencing. The similar mutations in *gyrA* and *parC* identified here have been previously detected in 21 [8] and 12 [15] isolates, respectively, by whole-genome sequence analysis. However, the objectives of these earlier studies differed from our own; the multicenter investigation examined the epidemiological history of haplotype H58 (genotype 4.3.1) transmission, focusing mainly on multidrug-resistant *S.* Typhi [8], and the other study evaluated the relationship between ciprofloxacin-resistant strains and treatment failure [15]. In our study, all 33 highly resistant strains had the same 3 chromosomal mutations in the

Table 2. Nonsynonymous Substitutions Completely Differentiated Between the Highly Resistant Strains and the Others Among H58 *S.* Typhi

Locus Tag[a]	Description	Amino Acid Change[b]
T3083	AraC family transcriptional regulator	Gly123Ser
T108	Arabinose operon regulatory protein	Gly69Ser
T0155	Symporter YicJ	Ile39Thr
T4613	Purine-nucleoside phosphorylase	Ile58Phe
T0266	LysR family transcriptional	Arg20Trp
(Pseudogene in Ty2)	Regulator	Ala86Val
T2582	Type VI secretion protein	Pro248Ser
T1090	Uptake hydrogenase small	Thr17Ala
(Pseudogene in Ty2)	Subunit	
T0592	PTS system, mannose-specific IIAB component	Gly39Cys
T0564	Polymyxin resistance protein ArnA	Cys166Phe

Abbreviation: PTS, phosphotransferase system.

[a]In the reference complete genome of strain Ty2.

[b]The right amino acid is fixed in the highly resistant strains.

quinolone resistance-determining region. Previous studies have reported several distinct amino acid substitutions arising from the 3 mutations [8, 15, 34, 35]: namely, 2 in *gyrA* gene encoding Ser83Phe/Tyr/Leu and Asp87Asn/Tyr/Gly, and a third in *parC* encoding Ser80Ile/Glu84Lys. The most common of these were mutations encoding Ser83Phe and Asp87Asn in *gyrA* and 1 encoding Ser80Ile in *parC*, which is consistent with our results. Furthermore, our study design enabled us to identify mutation patterns according to fluoroquinolone susceptibility (Figure 2 and Table S2): Fully susceptible strains had no mutations in *gyrA*, *parC*, or *parE*; intermediate and resistant strains had a single mutation in *gyrA*, with a subset harboring a second mutation in *parC* or *parE*; and highly resistant strains had 3 mutations, 2 in *gyrA* and 1 in *parC*.

Second, this is the first study to systematically investigate the emergence of the fluoroquinolone resistance mutations in *S.* Typhi in an evolutionary context. We found that *S.* Typhi evolved from fully susceptible strains without mutations in *gyrA* and *parC* via strains with a single mutation in *gyrA* encoding Ser83Phe, acquiring additional mutations in *gyrA* encoding Asp87Asn and in *parC* encoding Ser80Ile that become highly resistant. The phylodynamic analyses enabled us to identify the time at which highly resistant strains emerged. Previous studies concluded that haplotype H58 (genotype 4.3.1) existed more than 25 years ago (1985–1992) [8], and strains with decreased susceptibility to fluoroquinolones appeared in 1991 [7]. These earlier findings partly support our observation that the common ancestor of 78 clustered haplotype H58 (genotype 4.3.1) strains emerged between 1989 and 1992. In contrast, there has been little genetic information to date on the emergence time of highly fluoroquinolone-resistant strains. Strains with MIC to ciprofloxacin ≥2 μg/mL already existed in India between 1998 and 1999 [36], which is in agreement with our results that highly resistant strains emerged between 1993 and 2000. Ciprofloxacin was patented in 1981, and has been widely used since 1986 [37]. Our study provides new evidence that highly resistant *S.* Typhi evolved approximately 7 to 14 years after the beginning of the ciprofloxacin era.

Third, our study is first to investigate the genetic characteristics of highly fluoroquinolone-resistant *S.* Typhi. We found that several genetic mutations in highly resistant strains may confer benefits in terms of bacterial survival and spread, in addition to the resistance mutations in *gyrA* and *parC*. That is, the highly resistant strains may have advantages in their response to environmental stresses (related to AraC family transcriptional regulators, arabinose operon regulatory protein, and the YicJ symporter, as well as a higher growth rate (related to symporter YicJ and mannose-specific phosphoenolpyruvate-dependent PTS). Further studies are warranted to reveal these possibilities.

The present study has several limitations. First, the emergence times estimated based on Baysian phylogenetic inference are not supported by strong evidence, given the large 95% HPD. Nonetheless, our finding that highly resistant *S.* Typhi emerged between 1993 and 2000 is consistent with a previous epidemiological study reporting its appearance between 1998 and 1999 in India [36]. We therefore believe that our emergence times are reliable. Second, there may have been selection bias in our study as it was conducted at a single center (NIID in Japan). However, we have stored all autochthonous and imported *S.* Typhi strains collected throughout Japan since 1964 to the present; the strains used in our study have originated from various countries in endemic areas, including India (59%), Nepal (22%), and Bangladesh (14%) (Table 1). This suggests that our findings can be generalized. Finally, the emergence of antimicrobial-resistant isolates (not only *S.* Typhi but also various other microbials) is a serious global issue. Thus, unnecessary antimicrobial use, for example, the inappropriate use of over-the-counter antibiotics and the overuse of prophylactic macrolide or fluoroquinolone by travelers [38], should be avoided to prevent the increase in antimicrobial-resistant isolates.

CONCLUSIONS

The results of this study reveal the molecular evolutionary process by which fluoroquinolone resistance cultivated *S.* Typhi. Highly resistant *S.* Typhi evolved from fully susceptible strains via sequential acquisition of mutations in genes encoding DNA gyrase (*gyrA*) and topoisomerase IV (*parC*). We also found that highly resistant *S.* Typhi, which has certain survival advantages, emerged between 1993 and 2000, approximately 10 years after ciprofloxacin use became widespread. Our findings suggest that judicious use of fluoroquinolones is warranted in order to prevent the future emergence of novel resistance mutations.

Supplementary Data

Supplementary materials are available online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Acknowledgments

Author contributions. T.M. and M.O. conceived the original idea for the study. T.M. and M.M. collected the data and performed microbiological laboratory work. T.M., M.M., K.Y., and K.L. performed the analyses. T.M., M.M., and K.Y. drafted the original manuscript. All authors contributed to study design, data interpretation, and manuscript revision for intellectual content. All authors have read and approved the final version.

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