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Original Article

Report of Relapse Typhoid Fever Cases from Kolkata, India: Recrudescence or Reinfection?

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SUMMARY: Three relapse cases were reported out of 107 hospital-attending typhoid cases within a period of 2 years (2014–2016) from Apollo Gleneagles Hospital, Kolkata, India. During the first episode of typhoid fever, 2 of the 3 cases were treated with ceftriaxone (CRO) for 7 days, and 1 was treated for 14 days. Six *Salmonella* Typhi (*S.* Typhi) isolates, obtained from the 3 patients during both typhoid episodes, were subjected to antimicrobial susceptibility testing, detection of quinolone resistance-determining region (QRDR) mutation and molecular subtyping by pulsed-field gel electrophoresis (PFGE), multiple-locus variable number tandem repeat analysis (MLVA), multilocus sequence typing (MLST), clustered regularly interspaced short palindromic repeats (CRISPR), and H58 haplotyping. Pairs of the *S.* Typhi strains isolated from two of the patients during the 1st and 2nd episodes were similar with respect to the antimicrobial resistance (AMR) profiles, QRDR mutations, and molecular subtypes; whereas, the *S.* Typhi strain pair isolated from the 3rd patient were different in their AMR profiles, QRDR mutations, and MLVA profiles. From these observations, it may be concluded that in spite of treating typhoid cases with CRO for 7–14 days, relapse of typhoid fever might occur. The article also showed the advantage of MLVA typing over PFGE, MLST, and CRISPR typing for the discrimination of strains isolated from the same patient in case of relapse of typhoid fever.

INTRODUCTION

Typhoid fever is a systemic disease caused by *Salmonella enterica* subspecies *enterica* serotype Typhi (*S.* Typhi). This disease is a global public health concern, with 20.6 million cases and 223,000 deaths, the majority of which occur in Asia (1). The most common clinical manifestation of the disease is prolonged fever with headache, followed by abdominal pain and diarrhea. A relapse of typhoid fever may be due to recrudescence or reinfection (2). If the initial strain of *S.* Typhi is identical to the strain that causes 2nd attack, the relapse is defined as a recrudescence. If the 2 strains are different, the 2nd attack is classified as a reinfection (3). Relapse of typhoid fever occurs in 5–10% of cases. Most of the relapse cases occur 2–3 weeks after resolution of the initial fever, usually following antibiotic treatment. The clinical severity of a relapse episode is milder than that of the initial episode (4,5). Antimicrobial therapy contributes the mainstay for management of initial or relapse cases of typhoid fever; in general, mortality can be as high as 30% if left untreated, but falls to < 1% with appropriate antimicrobial therapy. The emergence of multidrug-resistant (MDR) *S.* Typhi (resistant to ampicillin [AMP], chloramphenicol [CHL], and sulfamethoxazole/trimethoprim [SXT]) in the 1970s and 1980s has led to the use of fluoroquinolones (FQs) for treatment. However, the indiscriminate use of FQs gave rise to a rapid increase in resistant organisms, namely, the decreased ciprofloxacin susceptible (DCS; MIC, 0.12–0.5 μg/ml) and ciprofloxacin-resistant (MIC ≥ 1 μg/ml) isolates in South and South-East Asia during the last decades (6–8). Recently, resistance to third-generation cephalosporins and azithromycin in *S.* Typhi has been reported (9,10). Several mechanisms of FQ resistance have been reported in *S.* Typhi, including efflux pumps, reduced outer membrane permeability, plasmid-mediated acquisition, and genetic mutations; however, the major mechanism is thought to be chromosomal mutations in genes encoding DNA gyrase and topoisomerase IV (11–13). The global emergence of drug resistant *S.* Typhi isolates has been shown to be mediated by the dissemination of the specific lineage H58 across Asian and African countries (11). The most common typing methods used for epidemiological investigation in *S.* Typhi isolates are pulsed-field gel electrophoresis (PFGE), multilocus variable number of tandem repeats (VNTR) analysis (MLVA), and multilocus sequence typing (MLST) (13–15). Recently, clustered regularly interspaced short palindromic repeats (CRISPR) typing has been used as a subtyping tool in several serovars of *Salmonella* (16). Reports on typhoid relapse cases have been found from countries such as Malaysia, Pakistan, Vietnam, Taiwan, France, and Denmark (3,17–21). To date, relapse cases of typhoid fever have not been reported from India. Hence, this article reports 3 typhoid relapse cases and the confirmation of reinfection or recrudescence of typhoid fever based on molecular subtyping of 3 pairs of *S.* Typhi strains isolated from hospital-attending patients with typhoid during the 1st and 2nd episodes of their illness.

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MATERIALS AND METHODS

Study subjects and case details: Three patients with suspected cases of typhoid fever were admitted to the Apollo Gleneagles Hospital, situated in the eastern part of Kolkata, India, at different times from February 2014 to January 2016. The details of the patients are shown in Table 1. A diagnosis of typhoid fever was confirmed in the 3 patients by the isolation of *S.* Typhi from blood culture. The duration of the initial episodes of fever ranged from 4 to 18 days for the patients, who were treated with third-generation cephalosporin (ceftriaxone [CRO]) after hospital admission. The patients were discharged after the fever subsided. Within a month of being discharged, the 1st patient (S1) was re-admitted to the hospital owing to a 2nd episode of fever, with a provisional diagnosis of typhoid fever. The second patient (S2) also visited the Outpatient Department of the hospital within a month of being discharged with a diagnosis of suspected typhoid fever. The third patient (S3) came back to the hospital after 2 years of being discharged with a history of high-grade fever (39°C) for 7 days, diarrhea for 5 days, and cough and cold for 4 days. Further laboratory test results revealed leukopenia and increased inflammatory markers, liver enzymes (ex., alanine aminotransferase), triglycerides, and fibrinogen. During the 2nd episode of fever, the patient S2 was treated with meropenem (MEM) and doxycycline (DOX) for 5 days, followed by amoxicillin-clavulanate (AMC) for 5 days, and the patient S3 was treated again with CRO (1 g TDS; to be taken 3 times a day) for 13 days, followed by cefixime (CFM, 200 mg) for 5 days. Unfortunately, information pertaining to the treatment of the patient S1 during the 2nd episode could not be obtained.

Microbiological culture of the blood samples: *Salmonella enterica* serovar Typhi (*S.* Typhi) blood culture isolates from each disease episode of the study patients were collected from the Microbiology Department of Apollo Gleneagles Hospital. All *S.* Typhi strains were retested and confirmed by standard microbiological procedures and serotyping, using slide and tube agglutination tests with *Salmonella* O, H polyvalent sera, serovar specific factor sera, and *Salmonella* Vi antisera (Denka Seiken, Tokyo, Japan).

Antimicrobial susceptibility testing: The isolates were tested for their antimicrobial susceptibility, using the Kirby-Bauer disk-diffusion method against a panel of antimicrobials as follows: AMP (10 μg), CHL (30 μg), tetracycline (30 μg), SXT (25 μg), nalidixic acid (NAL, 30 μg), ciprofloxacin (CIP, 5 μg), ofloxacin (OFX, 5 μg), cefotaxime (30 μg), ceftazidime (30 μg), CRO (30 μg), AMC (30 μg), and azithromycin (15 μg; Becton Dickinson, Franklin Lakes, NJ, USA). MICs of fluoroquinolones were determined using Etests (AB Biodisk, Solna, Sweden). The results were interpreted according to Clinical and Laboratory Standards Institute (CLSI) guidelines (22) with *Escherichia coli* ATCC 25922 used as the control.

Determination of antimicrobial resistance (AMR) genes and haplotypes of *S.* Typhi strains using PCR: To determine the mechanism of flouroquinolone resistance, chromosomal mutations at the quinolone resistance-determining regions (QRDRs) of DNA gyrase and topoisomerase IV were detected using PCR with published primers, followed by sequencing (23). The plasmid-mediated quinolone resistance (PMQR) genes, such as *qnr* (*qnrA, qnrB, qnrD,* and *qnrS*), *aac(6')-Ib-cr*, and *qepA*, were determined using PCR, following methods described by Accou-Demartin et al (24). The most common haplotype H58 in AMR *S.* Typhi strains was determined using PCR with published specific primers (25). Amplification bands of 1,100 bp and 107 bp (deletion of 993 bp) represent the non-H58 and H58 haplotypes of *S.* Typhi strains, respectively. PCR amplicons were visualized on 1.5% agarose gels after staining with GelRed Nucleic Acid Gel Stain (Biotium, Fremont, CA, USA). The amplicons were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany) for direct sequencing using a 3730 DNA analyzer (Applied Biosystems, Foster City, CA, USA), and then analyzed using the Basic Local Alignment Search Tool (BLAST) database search program of the National Center for Biotechnology Information (NCBI) to determine any mutation at the nucleotide level.

Molecular typing of *S.* Typhi study strains: PFGE: PFGE was performed using the PulseNet one-day standardized laboratory protocol for *Salmonella* species from the Centers for Disease Control and Prevention (26). Overnight-grown tryptic soy broth (TSB) cultures of bacterial cells were suspended in cell suspension buffer (100 mM Tris, 100 mM EDTA [pH 8.0]), and the optical density was adjusted to an absorbance of 0.8 to 1.0 at

Table 1. Details of patients reported at tertiary care center as recurrence of typhoid fever cases

Patient ID	Age (yr)/ Sex	1st episode					2nd episode				
		Date of 1st admission/ Visit	Date of discharge	Duration of fever (day)	Antibiotics used	Blood culture (Str. ID)	Date of 2nd admission/ Visit	Date of discharge	Duration of fever (day)	Antibiotics used	Blood culture (Str. ID)
S1	31/M	Feb. 7, 2014	Feb. 22, 2014	6	CRO (1 g) for 14 days	*S.* Typhi (ST1a)	Mar. 19, 2014	Mar. 26, 2014	4	Not available	*S.* Typhi (ST1b)
S2	23/F	Jun. 4, 2015	Jun. 12, 2015	4	CRO (1 g) for 7 days	*S.* Typhi (ST2a)	Jul. 3, 2015	NA	3	MEM and DOX for 5 days followed by AMC for 5 days	*S.* Typhi (ST2b)
S3	11/M	Apr. 9, 2014	Apr. 16, 2014	18	CRO (1 g) for 7 days	*S.* Typhi (ST3a)	Jan. 4, 2016	Jan. 10, 2016	7	CRO (1g, TDS) for 13 days followed by CFM (200 mg) − 1 tab for 5 days	*S.* Typhi (ST3b)

M, male; F, female; CRO, ceftriaxone; NA, not applicable (treated as an outpatient); MEM, meropenem; DOX, doxycycline; AMC, amoxycillin/ clavulanate; TDS, to be taken 3 times a day; CFM, cefixime; tab, tablet.

610 nm. Proteinase K was added to a final concentration of 0.5 mg/ml, and 200 µl of cell suspension was added to 200 µl of 1% Seakem agarose (Lonza, Basel, Switzerland). Then, 200 µl of the agarose mixture was pipetted into disposable plug molds (Bio-Rad, Hercules, CA, USA). Solidified agarose plugs was transferred to a tube containing 5 ml of lysis buffer (50 mM Tris, 50 mM EDTA, 1% sarkosyl [pH 8.0], and 25 µl of proteinase K [20 mg/ml]) and incubated in a shaking water bath at 54°C for 2 h. Plugs were washed 2 times with type I water and 4 times with TE buffer (10 mM Tris, 1 mM EDTA [pH 8.0]) for 15 min each time in a shaking water bath. Agarose-embedded DNA plugs were restricted with 50 U of *Xba*I (New England Biolabs, Ipswich, MA, USA) for 2 h at 37°C. A 1% pulsed field certified agarose gel (Bio-Rad) was prepared using 0.5 × Tris-buffered EDTA (Sigma-Aldrich, St. Louis, MO, USA), and the digested DNA plugs were inserted into the wells. The electrophoresis process was performed using a CHEF DR-III (Bio-Rad) with switch times of 2.2 to 63.8 s at 6 V/cm for 19 h at 14°C. The gel was stained using ethidium bromide (1 µg/ml) and de-stained using 2 deionized water washes. A gel image was obtained using a GelDoc-1000 imager (Bio-Rad). PFGE patterns were analyzed using FPQuest software ver. 4.0 (Bio-Rad), and their similarities were scored using the method of Tenover et al (27). A *Salmonella* serovar Braenderup strain (H9812) was used as the reference standard. Dice similarity coefficients and the unweighted pair group method with arithmetic means (UPGMA) were used to calculate similarity coefficients.

MLVA: Six previously described MLVA loci designated TR1, TR2, TR4699, Sal02, Sal16, and Sal20 were used for the genotyping of *S.* Typhi (28). A publicly available database <http://minisatellites.u-psud.fr> was used to identify these tandem repeats in the genomic sequence of serovar Typhi strain CT18, using the parameters reported previously (14). PCR amplification was carried out in a 25 µl reaction volume containing 100 ng template DNA, 0.5 µM forward and reverse primers, 250 µM dNTP, 1U Taq DNA polymerase, and 1 × PCR buffer with 1.5 mM $MgCl_2$ (New England Biolabs). After an initial denaturation at 94°C for 2 min, the PCR reaction was performed for 35 cycles at 94°C for 30 s, 55°C for 30 s, and 72°C for 1 min, followed by an extension step at 72°C for 7 min, on a Perkin-Elmer GeneAmp PCR system 9700 (Applied Biosystems). The PCR products was separated on a 1.5% agarose gel (Sigma-Aldrich) and visualized using a GelDoc. To confirm the identity of the amplicons and to assess the copy numbers of the repeats, the amplicons were purified using the QIAquick PCR Purification Kit (Qiagen) and subjected to unidirectional sequence analysis.

MLST: Seven housekeeping genes of known function and chromosome position, *thrA* (aspartokinase + homoserine dehydrogenase), *purE* (phosphoribosyl aminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase), *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase), and *dnaN* (DNA polymerase III beta subunit), were targeted by PCR amplification followed by DNA sequencing for the MLST scheme (29).

CRISPR: CRISPR typing was based on the detection of 32-bp sequences (spacers) within CRISPR1 array regions (30). PCR amplification followed by DNA sequencing was carried out in a similar manner, as stated above. Spacers were identified using the web-based CRISPR-Finder program and visualized as described previously (31).

RESULTS

AMR of 3 pairs of *S.* Typhi strains: A total of 6 *S.* Typhi strains were isolated from the duplicate blood samples of the 3 typhoid patients, considering 1 isolate from each of the 2 fever episodes. It was observed that the AMR profiles of *S.* Typhi strains isolated from the patient S1 (strains: ST1a, ST1b) and the patient S2 (strains: ST2a, ST2b) during their 1st and 2nd episodes of fever were similar (Table 2). ST1a and ST1b were NAL-resistant (NAL^R) with DCS. Similarly, ST2a and ST2b showed resistance to both NAL and fluoroquinolones (CIP, OFX). However, ST3a and ST3b did not show similar AMR profiles; ST3a was resistant to NAL and CIP (NAL^RCIP^R), and ST3b showed NAL^R and DCS.

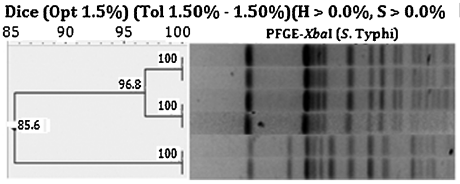
While determining the QRDR mutations in the study isolates (Table 2), ST1a and ST1b showed a single point mutation in *gyrA* (S83Y); ST2a and ST2b showed double point mutations in *gyrA* (S83F, D87V) and a single point mutation in *parC* (S80I). *S.* Typhi strain ST3a had double point mutations in *gyrA* (S83F, D87N) and a single mutation in *parC* (E84G), whereas ST3b had a single mutation in each of *gyrA* (S83F) and *parC* (E84G). To explore the mechanism of PMQR, 3 genes (*qnr*, *aac(6')-Ib-cr*, and *qepA*) were found to be negative in all 6 *S.* Typhi study strains.

Molecular subtypes of 3 pairs of *S.* Typhi strains: PFGE analysis of each pair of *S.* Typhi strains obtained from each of the 3 typhoid patients during their 1st and

Table 2. Details of AMR profile and QRDR mutation in the 6 *S.* Typhi isolates collected from the 3 patients

Patient ID	Strain ID	AMR profile	CIP MIC (µg/ml)	QRDR mutation			
				gyrA	*gyrB*	*parC*	*parE*
S1	ST1a	NAL, DCS	0.5	S83Y	WT	WT	WT
	ST1b	NAL, DCS	0.5	S83Y	WT	WT	WT
S2	ST2a	NAL, CIP, OFX	> 32	S83F, D87V	WT	S80I	WT
	ST2b	NAL, CIP, OFX	> 32	S83F, D87V	WT	S80I	WT
S3	ST3a	NAL, CIP	2	S83F, D87N	WT	E84G	WT
	ST3b	NAL, DCS	0.5	S83F	WT	E84G	WT

QRDR, quinolone resistance-determining region; CIP, ciprofloxacin; NAL, nalidixic acid; DCS, decreased ciprofloxacin susceptible; WT, wild type; OFX, ofloxacin.



Sample ID	Patient ID	R profile	PFGE type	MLVA profile	MLVA type	MLST type	H58 haplotype	CRISPR1 content
ST1a	S1	NAL/DCS	P1	05-31-10-11-11-23	M1	ST1	H58	1-2-3-4-5-6
ST1b	S1	NAL/DCS	P1	05-31-10-11-11-23	M1	ST1	H58	1-2-3-4-5-6
ST3b	S3	NAL/DCS	P2	05-06-11-11-11-11	M2	ST1	H58	1-2-3-4-5-6
ST3a	S3	NAL/CIP	P2	05-35-13-11-11-12	M3	ST1	H58	1-2-3-4-5-6
ST2a	S2	NAL/CIP/OFX	P3	10-23-06-07-22-09	M4	ST2	non-H58	1-2-3-4-5-6
ST2b	S2	NAL/CIP/OFX	P3	10-23-06-07-22-09	M4	ST2	non-H58	1-2-3-4-5-6

Fig. 1. Dendrogram showing the cluster analysis of 6 *S.* Typhi isolates from 3 relapse cases from Kolkata, India, by *Xba*I-PFGE. Band comparison was performed by using the Dice coefficient with 1.5% optimization (Opt) and 1.5% position tolerance (Tol). AMR (R) profile, PFGE type, MLVA profile, MLST type, H58 haplotype, and CRISPR1 content of the 6 strain is also shown in the figure. NAL, nalidixic acid; CIP, ciprofloxacin, OFX, ofloxacin.

2nd episodes of fever revealed distinct banding patterns (Fig. 1). The pairs of the *S.* Typhi isolates from S1 and S3 belonged to the H58 haplotype and MLST type 1 (ST1). In contrast, the pair of *S.* Typhi isolates from S2 belonged to the non-H58 haplotype and MLST type 2 (ST2). Overall, in the MLVA typing of the *S.* Typhi study strains, 2 to 4 alleles were observed, and the number of repeats ranged from 5 to 35 considering the 6 VNTR loci. Similar MLVA profiles were observed for the pairs of *S.* Typhi strains from S1 and S2. However, the MLVA profiles were different for the pair of *S.* Typhi strains from S3 and could be differentiated by their VNTR loci (TR2, TR4699, and Sal20). All study strains showed similar CRISPR1 content.

DISCUSSION

This article reports 3 relapse cases of typhoid fever within a period of 2 years (2014–2016) from Apollo Gleneagles Hospital. Of the 107 cases of typhoid fever visiting the hospital, only 3 relapses were observed. Relapse cases of typhoid were common (5–10%) in other countries such as Vietnam, especially when third-generation cephalosporin was used for treatment (18). This case report may not contribute to the estimated occurrence of typhoid relapse in a particular region. This could be because recurrent attacks of typhoid yield negative blood cultures in most patients. The patient may suffer from mild attacks during which samples were not collected, or they might attend different hospital for their second attack, as was reported by Wain et al (18). In the present study, the period of recurrence for the 1st and 2nd patients (S1 and S2) was 4 weeks and a period of 2 years for the 3rd patient (S3). In other studies, the period of recurrence was mostly observed as 2 to 3 weeks, with the relapse episode typically being milder than the original attack (4,5); however, a worse relapse was also documented in a study from Malaysia (3). In the current study, the laboratory reports were similar for both episodes of patients S1 and S2, whereas the patient S3 developed leukopenia in the 2nd episode. Among the admitted study patients, S1 was treated with third-generation cephalosporin (1g bid; twice a day) for 14 days, and S2 and S3 were treated with the same antibiotic for 7 days in their 1st episode.

A study from Pakistan documented that 14% of children with typhoid fever were treated with CRO for 7 days, and all of these children had a confirmed bacteriological relapse within 4 weeks of stopping therapy (32). Another study from Egypt showed that a 5-day course of CRO was associated with lower relapse rate (95% cure rate) (33). Single and/or double point mutations in the QRDRs of DNA gyrase and topoisomerase IV genes were found in the study isolates, which was also reported previously from Kolkata (34). The pair of *S.* Typhi isolates from S2 showed novel point mutations in *gyrA* (D87V), which was reported by another study from Nepal (35).

The data on molecular subtyping of the isolates by PFGE, MLVA, MLST, and H58 haplotyping showed that each pair of *S.* Typhi isolates from relapse patients (S1 and S2) were identical, and therefore, responsible for recrudescence. For S3, MLVA typing of the pair of *S.* Typhi isolates showed variations between the 2 isolates (ST3a and ST3b) in 3 VNTR loci. Previous researchers have suggested that isolates with single/double locus variants were isolated from the same outbreak (14,36). Thus, the 3rd case differed, not only in their AMR profile and QRDR mutation, but also in their MLVA profile, by 3 VNTR loci between the 1st and 2nd episode of typhoid fever, hence, the 3rd case may be designated a reinfection. However, in another study, it was shown that *S.* Typhi isolates with considerable genetic variations frequently at more than 2 VNTR loci could be excreted simultaneously from patients with long-term carrier status (19). In the current study, the isolates showed ST1 type in 2 pairs of strains (ST1a and ST1b; ST3a and ST3b) from S1 and S3 patients and ST2 type in 1 pair of strains (ST2a, ST2b) from S2. A study on the global MLST analysis of *S.* Typhi isolates confirms the predominance of the 2 *S.* Typhi types (ST1 and ST2) in endemic regions, including India (15,37). The association of H58 *S.* Typhi isolates with multidrug-resistance and reduced susceptibility to FQs was well known (11). This study also showed the occurrence of H58 haplotype with the drug-resistant strains.

This article concluded that relapse typhoid cases occurred in patients treated with third-generation cephalosporins for a short duration (7 days in S2 and S3; 14 days in S1) after admission into a tertiary care hospital in Kolkata and subsequent discharge following remission of symptoms. Therefore, prolonged antimicrobial therapy in typhoid fever patients and discharge of the patients only after bacteriological cure should be made mandatory in hospital settings, and need to be followed by attending physicians. The study also highlighted the advantage of MLVA and PFGE typing over CRISPR and MLST typing methods for the discrimination of isolates obtained from the same patient in cases of typhoid relapse.

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Conflict of interest None to declare.

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