Japanese Journal of Infectious Diseases

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Received: July 18, 2017. Accepted: December 25, 2017. Published online: April 27, 2018. DOI: 10.7883/yoken.JJID.2017.321
Report of relapse typhoid fever cases from Kolkata, India: Recrudescence or Reinfection?

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Running Title: Typhoid fever Relapse cases from Kolkata, India

Keywords: Salmonella Typhi, recrudescence, reinfection, antimicrobial resistance, molecular subtyping

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SUMMARY:
Three relapse cases out of 107 hospital attending typhoid cases within a period of 2 years (2014–2016) from Apollo Geneagles hospital, Kolkata, India were reported. Of three cases, two were treated with ceftriaxone for 7 days and one was for 14 days in the first episode of typhoid fever. Six *Salmonella Typhi* (*S. Typhi*) isolates, obtained from three patients in both the episodes, were subjected to antimicrobial susceptibility test (AST), detection of QRDR mutation and molecular subtyping by PFGE, MLVA, MLST, CRISPR and H58 haplotyping. Pairs of the *S. Typhi* strains isolated from each of the two patients during 1st and 2nd episodes were similar with respect to the antimicrobial resistance (AMR) profiles, QRDR mutations and molecular subtypes, whereas *S. Typhi* strain pairs isolated from third patient during the two episodes were different in their AMR profiles, QRDR mutations and MLVA profiles. From the observation, it may be concluded that in spite of treatment of typhoid cases with ceftriaxone 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 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*). It is a global public health problem, with 20.6 million cases and 22,3000 deaths, majority occurring in Asia (1). The most common clinical manifestation seen in typhoid patients are 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 second attack, the relapse is defined as recrudescence. If the two strains are different, then the second attack is classified as reinfection (3). Relapse of typhoid fever occurs in 5–10% of cases. Most of the relapse cases occur after 2–3 weeks of resolution of initial fever usually following antibiotic treatment. The clinical severity of relapse episode is milder than the initial episode (4,5). Antimicrobial therapy contributes the mainstay for management of acute or relapse cases of typhoid fever; mortality being as high as 30% if remains untreated, which falls to <1% with appropriate antimicrobial therapy. Emergence of multidrug resistant (MDR) *S. Typhi* (resistant to ampicillin, chloramphenicol and cotrimoxazole) in 1970s and 1980s has led to the use of fluoroquinolones (FQs) for treatment. Again, indiscriminate use of FQs gave rise to rapid increase in 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, 7, 8). Recently, resistance to third generation cephalosporin and azithromycin in S. Typhi has been reported (9, 10). Several mechanisms of fluoroquinolone 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, 12, 13). Global emergence of drug resistant S. Typhi isolates has been shown to be mediated by the dissemination of specific lineage H58 across Asian and African countries (11). Most common typing methods used for epidemiological investigation in S. Typhi isolates are pulse field gel electrophoresis (PFGE), multi locus variable number of tandem repeats (VNTR) analysis, multilocus sequence typing (MLST) (13, 14, 15). Most recently clustered regularly interspaced short palindromic repeats (CRISPR) typing has been used as subtyping tool in several serovars of Salmonella (16). Reports on typhoid relapse cases were found from countries like Malaysia, Pakistan, Vietnam, Taiwan, Persia and Denmark (3, 17, 18, 19, 20, 21). The relapse cases of typhoid fever were not reported from India. Hence, the article reports three typhoid relapse cases and confirmation of reinfection or recrudescence of typhoid fever based on molecular subtyping of 3 pairs of S. Typhi strains isolated from hospital attending typhoid patients during first and second episodes of their illness.

**Study subjects and case details:** Three patients with suspected cases of typhoid fever were admitted to Apollo Gleneagles Hospital situated in eastern part of Kolkata at different time period from February 2014 to January 2016. The details of patients are shown in Table 1. Diagnosis of typhoid fever was confirmed in all the three patients by isolation of S. Typhi from the blood culture. The duration of initial episodes of fever was 4 to18 days for all three patients, who were treated with 3rd generation cephalosporin (ceftriaxone, CRO) after hospital admission. The patients were discharged after the fever subsided. Within a month after discharge, the first patient (S1) was re-admitted to the hospital due to the second episode of fever with the provisional diagnosis of typhoid fever. The second patient (S2) also visited the outpatient department (OPD) of the hospital within a month after discharge from the hospital with diagnosis of suspected typhoid fever. The third patient (S3) came back to the hospital after 2 years after discharge with the history of high grade fever (39°C) for seven days, diarrhea for 5 days and cough and cold for 4 days. Further laboratory test results revealed leucopenia, increased inflammatory markers (CRF), increased liver enzymes (SGPT), triglycerides and fibrinogen. During the 2nd episode of fever the second patient (S2)
was treated with meropenem and doxycycline for 5 days followed by amoxicillin-clavulanate for 5 days and the third patient (S3) was treated again with CRO (1gm, ter die sumendum (TDS, three times a day)) for 13 days followed by cefixime (200mg) for 5 days. The information on treatment of first patient during second episode could not be obtained.

MATERIALS AND METHODS:

Microbiological culture of the blood samples: *Salmonella enteric serovar* Typhi (S. Typhi) blood culture isolate during each of the episode of the study patients were collected from Microbiology Department of Apollo Gleangle Hospital located in Eastern part of Kolkata, India. All S. Typhi strains were retested and confirmed by standard microbiological procedure and by serotyping using slide and tube agglutination test with *Salmonella* O, H polyvalent sera, serovar specific factor sera and Vi antisera (Denka Seiken Co Ltd., Tokyo, Japan).

Antimicrobial Susceptibility testing: The isolates were tested for their antimicrobial susceptibility using the Kirby–Bauer disk-diffusion method against a panel antimicrobials as follows: ampicillin (10 µg), chloramphenicol (30 µg), tetracycline (30 µg), cotrimoxazole (25 µg), nalidixic acid (30 µg), ciprofloxacin (5 µg), ofloxacin (5 µg), cefotaxime (30 µg), ceftazidime (30 µg), ceftriaxone (30 µg), amoxicillin-clavulanate (30 µg) and azithromycin (15 µg) (BD BBLTM Maryland, USA). Minimum inhibitory concentrations (MICs) of fluoroquinolones were determined by E-tests (AB Biodisk, Solna, Sweden). The results were interpreted according to Clinical and Laboratory Standards Institute guidelines (22) and *Escherichia coli* ATCC 25922 was used as control.

Determination of AMR genes and haplotypes of S. Typhi strains by PCR: To determine the mechanism of fluoroquinolone resistance, chromosomal mutations at QRDRs of DNA gyrase and topoisomerase IV were detected by PCR with published primers followed by sequencing (23). The PMQR genes like *qnr* (*qnrA, qnrB, qnrD, qnrS*), *aac(6′)-Ib-cr* and *qepA*, were determined by PCR following methods described earlier (24). The most common Haplotype H58 in AMR S. Typhi strains was determined by PCR using published specific primers (25). Amplification bands of 1100 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 Gel Red nucleic acid gel stain (Biotium, Inc, Hayward,CA). The amplicons were purified by QIAquick PCR purification kit (Qiagen,
Hilden, Germany) for direct sequencing using a 3730 DNA analyzer (Applied Biosystems, Foster City, CA), and analyzed by Basic Local alignment Search Tool (BLAST) database search program of the National Center for Biotechnology Information (NCBI) to determine any mutation at nucleotide level.

**Molecular typing of S. Typhi study strains:**

**Pulsed Field Gel Electrophoresis (PFGE):** PFGE was performed using the PulseNet one-day standardized laboratory protocol for *Salmonella* species from the Centers for Disease Control & Prevention (26). Overnight grown TSB culture of bacterial cells were suspended in cell suspension buffer (100mM Tris, 100mM EDTA, pH 8.0), and the O.D was adjusted to an absorbance of 0.8 to 1.0 at 610nm. Proteinase K was added to a final concentration of 0.5mg/ml, and 200µl of cell suspensions was added to 200µl of 1% Seakem agarose (Lonza). 200µl of the agarose mixture was pipetted into disposable plug molds (Bio-Rad, Hercules, Calif.). Solidified agarose plugs was transferred to a tube containing 5 ml of lysis buffer (50mM Tris, 50mM EDTA, 1% Sarkosyl (pH 8.0) and 25µl of proteinase K (20mg/ml) incubated in shaking water bath at 54°C for 2hrs. Plugs were washed two times with type I water and four times with TE buffer (10mM Tris, 1mM EDTA, pH 8.0) for 15 min each time in shaking water bath. Agarose-embedded DNA plugs were restricted with 50U of XbaI (New England Biolabs, Ipswich, MA) for 2 hrs at 37°C. 1% pulse field certified agarose gel (Bio-Rad) was prepared using 0.5X tris-buffered EDTA buffer (Sigma, St. Louis, Mo.) and the digested DNA plugs were inserted into the wells. The electrophoresis was performed using a CHEF DR-III (Bio-Rad) with switch times of 2.2 to 63.8sec at 6 V/cm for 19 hrs at 14°C. The Gel was stained using ethidium bromide (1µg/ml) and de-stained with two deionized water washes. Gel image was obtained using A GelDoc-1000 imager (Bio-Rad). Analysis of PFGE patterns was done using FPQuest software version 4.0 (Bio-Rad) and their similarities was scored by the method of Tenover et al (27). A *Salmonella* serovar Braenderup strain (H9812) was used as a reference standard. Dice similarity coefficients and unweighted pair group method with arithmetic means (UPGMA) algorithm was used to calculate similarity coefficients.

**Multi locus VNTR (Variable Number of Tandem Repeat) analysis:** 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 this tandem repeats in the genomic sequence of *serovar* Typhi strain
CT18 with the parameters reported earlier (14). PCR amplification was carried out in 25µl reaction volume containing 100ng of template DNA, 0.5μM of forward and reverse primers, 250μM deoxynucleoside triphosphates, 1U Taq DNA polymerase and 1X PCR buffer with 1.5mM MgCl\(_2\) (New England Biolabs, Ipswich, MA). After an initial denaturation at 94°C for 2 min, the PCR reaction was performed for 35 cycles at 94°C for 30 sec, 55°C for 30 sec and 72°C for 1 min, followed by an extension step of 72°C for 7 min on a Perkin-Elmer Gene Amp PCR system 9700 (ABI Biosystems). The PCR products was separated on a 1.5% agarose gel (Sigma) and visualized using a Gel Doc (BioRad). 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.

**Multi locus sequence typing (MLST):** Seven housekeeping genes of known function and chromosome position, *thrA* (aspartokinase + homogenize 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).

**Clustered regularly interspaced short palindromic repeat (CRISPR):** CRISPR typing was based on detection of 32-bp sequences (spacers) within CRISPR1 array regions (30). PCR amplification followed by DNA sequencing was carried out in similar manner as stated earlier. 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 three typhoid patients, considering one isolate from each of the two fever episodes. It was observed that the antimicrobial resistance (AMR) profiles of *S.* Typhi strains isolated from S1 (Strains: ST1a and ST1b) and S2 (strains: ST2a, ST2b) patients during their 1\(^{st}\) and 2\(^{nd}\) episodes of fever were similar (Table2). ST1a and ST1b strains were nalidixic acid resistant (Na\(^R\)) with decreased susceptible to ciprofloxacin (DCS). Similarly, ST2a and ST2b strains showed resistance to both nalidixic acid and
fluoroquinolones (Ciprofloxacin, ofloxacin, levofloxacin). However, ST3a and ST3b strains did not show similar AMR profiles; ST3a was resistant to nalidixic acid and ciprofloxacin (Na\textsuperscript{R}Ci\textsuperscript{R}) and ST3b showed Na\textsuperscript{R} and DCS (Table 2).

While determining the mutation in QRDR of the study isolates (Table 2), the pair of strains (ST1a, ST1b) isolated from S1 patients showed a single point mutation in gyrA gene (S83Y); strains (ST2a, ST2b) from S2 patients showed double point mutations in gyrA (S83F and D87V) and single point mutation in parC gene (S80I). S. Typhi strain (ST3a) from S3 patients had double point mutation in gyrA (S83F, D87N) and single mutation in parC (E84G) whereas ST3b strain had single mutation in each of gyrA (S83F) and parC (E84G) gene. To explore, the plasmid mediated mechanism for quinolone resistance (PMQR), three genes qnr, aac(6\textsuperscript{-})-Ib-cr and qepA were found negative in all six S. Typhi study strains.

**Molecular subtypes of 3 pairs of S. Typhi strains:** PFGE analysis of each pairs of S. Typhi strains obtained from each of the three typhoid patients during their 1\textsuperscript{st} and 2\textsuperscript{nd} episodes of fever revealed distinct banding patterns (P1, P2, P3) in each pair of strains (Fig 1). Pairs of the S. Typhi isolates from patient 1 (S1) and patient 3 (S3) belonged to H58 haplotype and MLST type 1 (ST1). Pair of S. Typhi isolates from patient 2 (S2) belonged to non-H58 and MLST type 2 (ST2). Overall in MLVA types of S. Typhi study strains, 2 to 4 alleles were observed, and the no. of repeats ranged from 5 to 35 considering the six VNTR loci. Similar MLVA profiles were observed in the pairs of S. Typhi strains from patient 1 (S1) and patient 2 (S2). But, MLVA profiles were different in the pair of S. Typhi strains from patient 3 and were differed by VNTR loci (TR2, TR4699 and Sal20). All of the 6 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 Geneagles hospital. Of 107 cases of typhoid fever visiting the hospital only 3 cases of relapses were observed, who were admitted to this hospital. Relapse cases of typhoid were common (5–10\%) in other countries like Vietnam especially when third generation cephalosporin was used for treatment (18). This case report may not contribute in the estimated occurrence of typhoid relapse in a particular region. The reasons may be that recurrent attacks of typhoid yield negative blood cultures in most of the 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 earlier (18). In the present study the period of recurrence for the first and second patients (S1 and S2) was 4 weeks and a period of 2 years for the third patient (S3). In other studies, the period of recurrence was mostly observed as 2 to 3 weeks and the relapse episode was reported milder and less severe than original attack (4,5), but worse report was also documented during the second episode in a study from Malaysia (3). In this study, similar laboratory reports were found in both the episodes for two study patients (S1 and S2) and the third patient (S3) developed leucopenia in the 2nd episode. Among the admitted study patients, S1 was treated with 3rd generation cephalosporin (ceftriaxone, CRO, 1gm bd) for 14 days and S2 and S3 were treated with same antibiotic for 7 days in their first episode. A study from Pakistan documented 14% of children receiving CRO for 7 days for typhoid fever treatment and had a confirmed bacteriological relapse within 4 weeks of stopping therapy (32). Another study from Egypt showed that five day course of ceftriaxone (CRO) was associated with lower relapse rate (95% cure rate) (33). Single and/or double point mutations in QRDRs of DNA gyrase and topoisomerase IV genes were found in the study isolates, which was reported earlier from Kolkata (34). The pair of S. Typhi isolates from S2 patient showed a novel point mutation in gyrA (D87V) which was reported by another study from Nepal (35). The data on molecular subtyping of the isolates by PFGE, MLVA, MLST, H58 haplotyping showed that each pair of S. Typhi isolates from relapse patients (S1 and S2) were identical and responsible for recrudescence of the disease among those patients. For the third patient (S3) MLVA typing of the pair of S. Typhi isolates showed variations between the two isolates (ST3a and ST3b) by 3 VNTR loci. It was suggested by the earlier authors that the isolates with single/double locus variants have been isolated from same outbreak (14, 36). Thus, the third case differed not only in their AMR profile and QRDR mutation but also in their MLVA profile by three VNTR loci between the 1st and 2nd episode of typhoid fever hence the third case may be designated as reinfection of typhoid fever. 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 this article, the study isolates showed ST1 type in two pairs of strains (ST1a, ST1b, ST3a and ST3b) from S1 and S3 patients and ST2 type in one pair of strains (ST2a and ST2b) from S2 patient. A study on global MLST analysis of S. Typhi isolates confirms the predominance of this two S. Typhi types (ST1 and ST2) in the endemic regions including India (38, 15). The association of H58 S. Typhi isolates with multidrug resistance and reduced susceptibility to
FQs was well informed (11). This study also showed the occurrence of H58 haplotype in the drug resistant study strains.

This article concluded that relapse typhoid cases did occur in patients having treated in tertiary care hospital in Kolkata after admission, by third generation of cephalosporins for a short duration (seven days in S2 and S3 and fourteen days in S1) and having discharged following remission of symptoms. Therefore prolong antimicrobial therapy in typhoid fever patients and discharge of the patients after bacteriological cure should be mademandatory in the hospital settings, which need to be followed by the attending physicians. The article also showed the advantage of MLVA and PFGE typing over CRISPR and MLST typing methods for discrimination of isolates obtained from the same patient in cases of typhoid relapse.

Acknowledgments
The work was supported by intramural fund of Indian council of medical research (ICMR) New Delhi, India (no. IM/SD-1/07-08/18). We would like to thank technical staff of NICED for his technical assistance during this study.

Conflict of interest
The authors declare that they have no conflict of interest in relation to this study.
REFERENCES:

1. Mogasale V, Maskery, B, Ochiai, RL et al. Burden of typhoid fever in low-income and middle-income countries: a systemic, literature-based update with risk-factor adjustment. Lancet Global Health. 2014;2:570-80.

2. Marmion DE, Naylor GR, Stewart IO. Second attacks of typhoid fever. J Hyg (Lond). 1953;51:260–67.

3. Deris ZZ, Noor SSM, Abdullah NH, Noor AR. Relapse typhoid fever in North–eastern state in Malaysia. Asian Pac J of Trop Med. 2010;3:48-50.

4. Parry CM, Hien TT, Dougan G, White NJ et al. Typhoid fever. N Engl J Med. 2002;347:1770-82.

5. Bhan MK, Bahl R, Bhatnagar S. Typhoid and paratyphoid fever. Lancet. 2005;366:749-62.

6. Chitnis S, Chitinis V, Hemvani N et al. Ciprofloxacin therapy for typhoid fever needs reconsideration. J Infect Chemother. 2006;12:402-4.

7. Menezes GA, Harish BN, Khan MA et al. Antimicrobial resistance trends in blood culture positive Salmonella Typhi isolates from Pondicherry, India, 2005-2009. Clin Microbiol Infect. 2012;18:239-45.

8. Humphries RM, Fang FC, Aarestrup FM, et al. In vitro susceptibility testing of fluoroquinolone activity against Salmonella: recent changes to CLSI standards. Clin Infect Dis. 2012;55:1107-13.

9. Kumarasamy K, Krishnan P. Report of a Salmonella enterica serovar Typhi isolate from India producing CMY-2 AmpC β-lactamase. J Antimicrob Chemother. 2012;67:775–6.

10. Hassing RJ, Goessens WHF, Pelt W et al. Salmonella subtypes with increased MICs in azithromycin in travelers returned to Netherlands. Emerg Infect Dis 2014;20:705-8.

11. Wong VK, Baker S, Pickard DJ et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. Nat Genet. 2015; 47:632-9.

12. Medalla F, Sjolund-karlsson M, Shin S et al. Ciprofloxacin-resistant Salmonella enterica Typhi, United States, 1999-2008. Emerg Infect Dis. 2011;17:1095-8.

13. Le AH, Fabre L, Roumagnac P et al. Clonal expansion and microevolution of quinolone-resistant Salmonella enterica serovars Typhi in Vietnam from 1996 to 2004. J Clin Microbiol. 2007;45:3485-92.
14. Octavia S, Lan R. Multiple-locus variable-number tandem-repeat analysis of *Salmonella enterica* serovar Typhi. J Clin Microbiol. 2009;47:2369-76.

15. Sharma P, Dahiya S, Balaji V et al. Typhoidal Salmonellae: Use of Multi-Locus Sequence Typing to Determine Population Structure. PLoS ONE. 2016;11:e0162530.

16. Shariat N, Sandt CH, DiMarzio MJ et al. CRISPR-MVLST subtyping of *Salmonella enterica* subsp. enterica serovars Typhimurium and Heidelberg and application in identifying outbreak isolates. BMC Microbiol. 2013;13:254.

17. Ahmad KA, Khan LH, Roshan B et al. Factors associated with typhoid relapse in the era of multiple drug resistant strains. J Infect Dev Ctries. 2011;5:727-31.

18. Wain J, Hien TT, Connerton P et al. Molecular typing of multiple-antibiotic-resistant *Salmonella enterica* serovar typhi from Vietnam: Application to acute and relapse cases of typhoid fever. J Clin Microbiol. 1999;37:2466-72.

19. Chiou CH, Wei HL, Mu JJ et al.. *Salmonella enterica* serovar Typhi Variants in Long-Term Carriers. J Clin Microbiol. 2013;51:669-72.

20. Caumes E, Ehya N, Nguyen J et al. Typhoid and Paratyphoid Fever: A 10-Year Retrospective Study of 41 Cases in a Parisian Hospital. J Travel Med. 2001;8:293-7.

21. Barrett FC, Knudsen JD and Johansen IS. Cases of typhoid fever in Copenhagen region: a retrospective study of presentation and relapse. BMC Res Notes. 2013;6:315.

22. Clinical and Laboratory Standards Institute. *Performance Standards for Antimicrobial Susceptibility Testing; Twenty sixth information supplement*. CLSI document M100-S26. Wayne, PA, 2016.

23. Parry CM, Thuy CT, Dongol S et al. Suitable disk antimicrobial susceptibility breakpoints defining *Salmonella enterica* serovars Typhi isolates with reduced susceptibility to fluoroquinolones. Antimicrob Agents Chemother. 2010;54:5201-8.

24. Accou-Demartin, M, Gaborieau V, Song Y et al. *Salmonella enterica* serotype Typhi with nonclassical quinolone resistance phenotype. Emerg Infect Dis. 2011;17:1091-4.

25. Murgia SR, Wain J, Gaind R et al. A novel broadly applicable PCR-RFLP method for rapid identification and subtyping of H58 *Salmonella* Typhi. J Microbiol Methods. 2016;127:219–23.

26. Ribot EM, Fitzgerald C, Kubota K et al. Rapid pulsed-field gel electrophoresis protocol for subtyping of *Campylobacter jejuni*. J Clin Microbiol. 2001;39:1889–94.
27. Tenover FC, Arbeit RD, Goering RV et al. Interpreting chromosomal DNA restriction patterns produced by pulsed-field gelelectrophoresis: criteria for bacterial strain typing. J Clin Microbiol. 1995; 33: 2233-9.
28. Kruy SL, van Cuyck H, Koeck JL. Multilocus variable-number tandem repeat analysis for Salmonella enterica subspecies. Eur J Clin Microbiol Infec Dis. 2011;4:465-73.
29. Kidgell C, Reichard U, Wain J et al. Salmonella Typhi, the causative agent of typhoid fever, is approximately 50,000 years old. Infect Genet Evol. 2002;2:39–45.
30. Fabre L, Zhang J, Guigon G et al. CRISPR typing and subtyping for improved laboratory surveillance of Salmonella infections. PLoS One. 2012;7:e36995.
31. Grissa I, Vergnaud G, Pourcel C. CRISPRFinder: a web tool to identify clustered regularly interspaced short palindromic repeats. Nucleic Acids Res. 2007;35:W52–7.
32. Bhutta ZA, Khan IA, Shadmani M et al. Failure of short-course ceftriaxone chemotherapy for multidrug-resistant typhoid fever in children: a randomized controlled trial in Pakistan. Antimicrob Agents Chemother. 2000;44:450-52.
33. Girgis NI, Sultan Y, Hammad O et al. Comparison of the efficacy, safety and cost of cefixime, ceftriaxone and aztreonam in the treatment of multidrug-resistant Salmonella typhi septicemia in children. Pediatr Infect Dis J. 1995;14:603-5.
34. Das S, Samajpati S, Ray U et al. Antimicrobial resistance and molecular subtypes of Salmonella enterica serovar Typhi isolates from Kolkata, India over a 15 years period 1998–2012. Int J Med Microbiol. 2016;307:28-36.
35. Koirala KD, Thanh DP, Thapa SD et al. Highly resistant Salmonella enterica serovar Typhi with a novel gyrA mutation raises questions about the long-term efficacy of older fluoroquinolones for treating typhoid fever. Antimicrob Agents Chemother. 2012;56:2761-2.
36. Das S, Samajpati S, Roy S et al. Molecular Subtyping of Salmonella enterica Serovar Typhi by Pulsed-Field Gel Electrophoresis and Multiple-Locus Variable-Number Tandem-Repeat Analysis in India: Their Association with Antimicrobial Resistance Profiles. Jpn J Infect Dis. 2017; 70:536-543.
Figure legends

Fig. 1. Dendrogram showing the cluster analysis of 6 S. Typhi isolates from 3 relapse cases from Kolkata, India, by XbaI-PFGE. Band comparison was performed by using the Dice coefficient with 1.5% optimization (Opt) and 1.5% position tolerance (Tol). R Profile, MLVA Profile, MLST Type, H58 haplotype and CRISPR1 content of the 6 strain is also shown in the figure.
Table 1. Details of patients reported at tertiary care center as recurrence of Typhoid fever cases

| Patient ID | Age (year)/Gender | 1st episode | 2nd episode |
|------------|-------------------|-------------|-------------|
|            | Date of admission/visit | Date of discharge | Duration of fever (days) | Date of admission/visit | Date of discharge | Duration of fever | Antibiotic used for treatment | Blood culture result (Str. ID) | Date of admission/visit | Date of discharge | Duration of fever | Antibiotic used for treatment | Blood culture positive |
| S1         | 31/M              | 07 Feb 2014  | 22 Feb 2014 | 6 | Ceftriaxone (1gm) for 14 days | S. Typhi (ST1a) | 19 March 2014 | 26 March 2014 | 4 | Not available | S. Typhi (ST1b) |
| S2         | 23/F              | 04 June 2015 | 12 June 2015 | 4 | Ceftriaxone (1gm) for 7 days | S. Typhi (ST2a) | 03 July 2015 | NA*           | 3 | Meropenem and doxycycline for 5 days followed by amoxy/clavulanate for 5 days | S. Typhi (ST2b) |
| S3         | 11/M              | 09 April 2014 | 16 April 2014 | 18 | Ceftriaxone (1gm) for 7 days | S. Typhi (ST3a) | 04 Jan 2016 | 10 Jan 2016 | 7 | Ceftriaxone 1 gm TDS, for 13 days followed by Cefixime (200mg) -1 tab for 5 days | S. Typhi (ST3b) |

* M, male; F, female

b TDS, ter die sumendum (three times a day); tab, tablet

*Not Applicable; treated as outpatient
Table 2. Details of R Profile and QRDR mutation in the 6 S. Typhi isolates collected from the 3 patients

| Patient ID | Strain ID | R Profile<sup>a</sup> | Cip MIC (µg/ml) | QRDR Mutation<sup>b</sup> | gyrA | gyrB | parC | parE |
|------------|-----------|------------------------|----------------|-----------------------------|------|------|------|------|
| S1         | ST1a      | Na<sup>R</sup>DCS       | 0.5            | S83Y, WT, WT, WT            |      |      |      |      |
|            | ST1b      | Na<sup>R</sup>DCS       | 0.5            | S83Y, WT, WT, WT            |      |      |      |      |
| S2         | ST2a      | Na<sup>R</sup>Ci<sup>R</sup>Of<sup>R</sup> | >32            | S83F, D87V, WT, S80I, WT    |      |      |      |      |
|            | ST2b      | Na<sup>R</sup>Ci<sup>R</sup>Of<sup>R</sup> | >32            | S83F, D87V, WT, S80I, WT    |      |      |      |      |
| S3         | ST3a      | Na<sup>R</sup>Ci<sup>R</sup> | 2              | S83F, D87N, WT, E84G, WT    |      |      |      |      |
|            | ST3b      | Na<sup>R</sup>DCS       | 0.5            | S83F, WT, E84G, WT          |      |      |      |      |

<sup>a</sup>Na, Nalidixic acid; DCS, Decreased ciprofloxacin susceptible; Ci, Ciprofloxacin; Of, Ofloxacin; Le, Lefloxacine

<sup>b</sup>WT, Wild type; S, Serene; Y, Tyrosine; F, Phenyalanine; D, Aspartate; N, Asparagine; V, Valine; I, Isoleucine; E, Glutamate; G, Glycine
Fig 1. Dendrogram showing the cluster analysis of 6 S. Typhi isolates from 3 relapse cases from Kolkata, India, by XbaI-PFGE. Band comparison was performed by using the Dice coefficient with 1.5% optimization (Opt) and 1.5% position tolerance (Tol). R Profile, MLVA Profile, MLST Type, H58 haplotype and CRISPR1 content of the 6 strain is also shown in the figure.