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 (6,7). 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.
Table 1. Details of patients reported at tertiary care center as recurrence of typhoid fever cases

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Age (yr)/Sex</th>
<th>Date of 1st admission/Visit</th>
<th>Date of discharge</th>
<th>Duration of fever (day)</th>
<th>Antibiotics used</th>
<th>Blood culture (Str. ID)</th>
<th>Date of 2nd admission/Visit</th>
<th>Date of discharge</th>
<th>Duration of fever (day)</th>
<th>Antibiotics used</th>
<th>Blood culture (Str. ID)</th>
</tr>
</thead>
<tbody>
<tr>
<td>S2 23/F</td>
<td>Jun. 4, 2015</td>
<td>Jun. 12, 2015</td>
<td>4</td>
<td>CRO (1 g)</td>
<td>S. Typhi</td>
<td>(ST2a)</td>
<td>Jul. 3, 2015</td>
<td>NA</td>
<td>3</td>
<td>MEM and DOX for 5 days</td>
<td>S. Typhi</td>
</tr>
<tr>
<td>S3 11/M</td>
<td>Apr. 9, 2014</td>
<td>Apr. 16, 2014</td>
<td>18</td>
<td>CRO (1 g)</td>
<td>S. Typhi</td>
<td>(ST3a)</td>
<td>Jan. 4, 2016</td>
<td>Jan. 10, 2016</td>
<td>7</td>
<td>CRO (1g, TDS) for 13 days followed by CFM (200 mg) - 1 tab for 5 days</td>
<td>S. Typhi</td>
</tr>
</tbody>
</table>

M, male; F, female; CRO, ceftriaxone; NA, not applicable (treated as an outpatient); MEM, meropenem; DOX, doxycycline; AMC, amoxicillin- clavulanate; TDS, to be taken 3 times a day; CFM, cefixime; tab, tablet.
Typhoid Fever Relapse Cases from Kolkata, India

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 were 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 XbaI (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 PFQuest 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 reference standard. Dice similarity coefficients and the unweighted pair group method with arithmetic means (UPGMA) were used to calculate similarity coefficients.

PCR amplification 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<sup>+</sup>) 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<sup>+</sup>CIP<sup>+</sup>), and ST3b showed NAL<sup>+</sup> and DCS.

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

<table>
<thead>
<tr>
<th>Patient ID</th>
<th>Strain ID</th>
<th>AMR profile</th>
<th>CIP MIC (μg/ml)</th>
<th>QRDR mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>ST1a</td>
<td>NAL, DCS</td>
<td>0.5</td>
<td>S83Y WT WT WT</td>
</tr>
<tr>
<td></td>
<td>ST1b</td>
<td>NAL, DCS</td>
<td>0.5</td>
<td>S83Y WT WT WT</td>
</tr>
<tr>
<td>S2</td>
<td>ST2a</td>
<td>NAL, CIP, OFX</td>
<td>&gt; 32</td>
<td>S83F, D87V WT S80I WT</td>
</tr>
<tr>
<td></td>
<td>ST2b</td>
<td>NAL, CIP, OFX</td>
<td>&gt; 32</td>
<td>S83F, D87V WT S80I WT</td>
</tr>
<tr>
<td>S3</td>
<td>ST3a</td>
<td>NAL, CIP</td>
<td>2</td>
<td>S83F, D87N WT E84G WT</td>
</tr>
<tr>
<td></td>
<td>ST3b</td>
<td>NAL, DCS</td>
<td>0.5</td>
<td>S83F WT E84G WT</td>
</tr>
</tbody>
</table>

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

This article reports 3 relapse cases of typhoid fever within a period of 2 years (2014–2016) from Apollo Glenegales 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 gyrA 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.

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 None to declare.
REFERENCES


