Ceftriaxone-resistant *Salmonella* Typhi carries an IncI1-ST31 plasmid encoding CTX-M-15

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**Abstract**

**Purpose.** Ceftriaxone is the drug of choice for typhoid fever and the emergence of resistant *Salmonella* Typhi raises major concerns for treatment. There are an increasing number of sporadic reports of ceftriaxone-resistant *S.* Typhi and limiting the risk of treatment failure in the patient and outbreaks in the community must be prioritized. This study describes the use of whole genome sequencing to guide outbreak identification and case management.

**Methodology.** An isolate of ceftriaxone-resistant *S.* Typhi from the blood of a child taken in 2000 at the Popular Diagnostic Center, Dhaka, Bangladesh was subjected to whole genome sequencing, using an Illumina NextSeq 500 and analysis using Geneious software.

**Results/Key findings.** Comparison with other ceftriaxone-resistant *S.* Typhi revealed an isolate from the Democratic Republic of the Congo in 2015 as the closest relative but no evidence of an outbreak. A plasmid belonging to incompatibility group I1 (IncI1-ST31) which included *bla*<sub>CTX-M-15</sub> (ceftaxone resistance) associated with *ISEcp-1* was identified. High similarity (90 %) was seen with pS115, an IncI1 plasmid from *S.* Enteritidis, and with pESBL-EA11, an IncI1 plasmid from *E. coli* (99 %) showing that *S.* Typhi has access to ceftriaxone resistance through the acquisition of common plasmids.

**Conclusions.** The transmission of ceftriaxone resistance from *E. coli* to *S.* Typhi is of concern because of clinical resistance to ceftriaxone, the mainstay of typhoid treatment. Whole genome sequencing, albeit several years after the isolation, demonstrated the success of containment but clinical trials with alternative agents are urgently required.

**INTRODUCTION**

Infection with *S.* Typhi, the causative agent of typhoid fever, is the predominant invasive bacterial disease in many developing countries [1–3]. Estimates for the burden of typhoid fever in low to middle income countries, 7–48 million [4], suffer from gaps in the data but it is clear that India, Bangladesh and Pakistan shoulder a major burden [5, 6]. Antibiotic treatment revolutionized the clinical management of typhoid fever, reducing mortality from around 30 % to less than 1 %, but antibiotic resistance has relentlessly followed the introduction of new drugs. Chloramphenicol, used through the 1950s and 1960s, was replaced in the 1970s by cotrimoxazole and amoxicillin after chloramphenicol-resistant strains of *S.* Typhi emerged [7]. In the 1990s multidrug-resistant (MDR) *S.* Typhi emerged with resistance to all three first line drugs. Of concern was that the pathogenicity of *S.* Typhi [8] was linked to plasmid-encoded resistance [9] and that one MDR strain (H58) [10], which expanded globally [11, 12], was associated with a single distinct plasmid type, IncHI1 PST6 (plasmid MLST type 6) [13]. The spread and persistence of the MDR phenotype led to the recommendation of third generation cephalosporins (ceftriaxone) or fluoroquinolones (ciprofloxacin) as first line therapy [14]. In many low to middle income countries, ciprofloxacin (or ofloxacin)

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**Abbreviations:** ESBL, extended spectrum beta-lactamase; Inc(I), incompatibility (group); MLST, multi-locus sequence typing; ST, sequence type; SPI, *Salmonella* pathogenicity islands.

*Salmonella* Typhi sequence accession number for this manuscript is: PRJEB21992.

Two supplementary tables are available with the online version of this article.
became the preferred choice for its oral formulation and affordable cost, compared with ceftriaxone. Typhoid cases are often treated empirically, with oral antibiotics in the community, and referred to hospital for parenteral therapies only when the patient fails to respond [15]. The widespread use of fluoroquinolones however led to the global emergence of strains with reduced susceptibility and then high-level resistance [16]. Recommendations for the treatment of fluoroquinolone-resistant S. Typhi are ceftriaxone or azithromycin. Azithromycin, a macrolide, is popular because of its oral formulation and single daily dose. However, rapid emergence of resistance to macrolides during treatment of other infections [17, 18] has triggered opposition to its use for typhoid fever [19]. This leaves third generation cephalosporins as the most common, acceptable treatment, but concern is growing that widespread resistance to this last line of treatment for typhoid fever will emerge. The relentless spread of extended spectrum beta-lactamases (ESBLs) in the Enterobacteriaceae, in particular CTX-M type ESBLs [20] predicts that these concerns will be realised and an H58-like ceftriaxone-resistant S. Typhi will most likely, when it emerges, spread globally. To date, only sporadic reports of ceftriaxone-resistant S. Typhi have been published, mainly from Asia (including Japan) but also from West and southern Africa [21–26].

In S. Typhi, plasmid-encoded resistance to cephalosporins remains rare, especially mediated by the successful blaCTX-M -group of enzymes [27]. However, the diverse nature of the mobile elements now encoding ceftriaxone resistance combined with the selective pressure exerted by the widespread use of ceftriaxone across the sub-continent suggest there is a real risk of an outbreak of ceftriaxone-resistant typhoid fever.

Here we report a comprehensive analysis of the full genome sequence of ceftriaxone-resistant S. Typhi from Bangladesh and place both plasmid and chromosome into context.

**METHODS**

**Bacterial isolation and identification**

In 2000, an S. Typhi was isolated from a child’s blood (PRJEB21992) sample in the microbiology laboratory of the Popular Diagnostic Center, Dhaka, Bangladesh, and sub-cultural on MacConkey agar. Identification was confirmed using API20E (bioMérieux, USA) and agglutination with Salmonella-specific antisera (Ramel, Thermo Fisher Scientific, USA).

**Antimicrobial resistance profile**

Disk diffusion antibiotic susceptibility tests were carried out and interpreted according to the Clinical and Laboratory Standards Institute guidelines [28]. The following discs were used: ampicillin (10 µg), cotrimoxazole (25 µg), chloramphenicol (30 µg), ciprofloxacin (5 µg), levofloxacin (5 µg), nalidixic acid (30 µg), azithromycin (15 µg), gentamicin (10 µg), ceftazidime (30 µg), cefixime (5 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg) and tetracycline (30 µg). MIC was determined for clinically relevant antibiotics using ETEST strips (bioMérieux-USA) (Table 1). The ability of the strain to produce beta-lactamase was determined via nitrocefin disc (Oxoid-UK).

**Whole genome sequencing and in silico analysis**

DNA extracted from the S. Typhi isolate was converted into a Nextera XT library for sequencing on an Illumina NextSeq 500 platform according to the manufacturer’s instructions. The S. Typhi library was diluted to 4 nM (as determined by analysis on an Agilent Technologies 2200 Tapestation and using the Qubit High Sensitivity dsDNA assay) and pooled in equimolar amounts with other barcoded libraries. The entire library pool was then diluted to 1.8 pM and sequenced using the NextSeq 500 v2 2×150 bp paired-end protocol.

The genome was assembled using the Velvet default parameter [29]. The web-based tool SeqSero 1.0 was used to check the genetic serotype of the isolate [30]. The genome assembly was then subjected to sequence type (ST) analysis using **Table 1. Antimicrobial susceptibility phenotype and MIC of the ceftriaxone-resistant S. Typhi isolated in Bangladesh**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Zone diameter of inhibition (mm)</th>
<th>MICs Interpretation [28]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>00</td>
<td>&gt;256 µg ml⁻¹</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>26</td>
<td>4 µg ml⁻¹</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>32</td>
<td>0.023 µg ml⁻¹</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>36</td>
<td>0.012 µg ml⁻¹</td>
</tr>
<tr>
<td>Levofloxacin</td>
<td>35</td>
<td>Not done</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>28</td>
<td>Not done</td>
</tr>
<tr>
<td>Azithromycin</td>
<td>18</td>
<td>4 µg ml⁻¹</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>22</td>
<td>0.75 µg ml⁻¹</td>
</tr>
<tr>
<td>Ceftazidime</td>
<td>15</td>
<td>8 µg ml⁻¹</td>
</tr>
<tr>
<td>Cefixime</td>
<td>00</td>
<td>Not done</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>00</td>
<td>&gt;256 µg ml⁻¹</td>
</tr>
<tr>
<td>Imipenem</td>
<td>32</td>
<td>Not done</td>
</tr>
<tr>
<td>Meropenem</td>
<td>34</td>
<td>&lt;0.125 µg ml⁻¹</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>26</td>
<td>1 µg ml⁻¹</td>
</tr>
</tbody>
</table>

Sensitive

Resistant
the Salmonella in silico Typing Resource platform [31]. The assembly was interrogated for acquired resistance genes [32], Salmonella pathogenicity islands (SPI) [32], plasmids and incompatibility group using Res.Finder [32], SPI-Finder-1.0 (CGE online platform – checked with Geneious R10 [33]) and Plasmid-Finder-1.3 [34], respectively. The presence of SPI-7 was confirmed by mapping the genome against a published SPI-7 sequence (accession number NC_004631.1) to detect the tviA gene 540 bp; the first gene in SPI-7 and sopE, the major type three secretion system (TTSS) effector protein. Comparison to the major extant haplotypes of S. Typhi was carried out using Geneious Tree Builder, neighbour-joining default parameters [33]. The following genome sequences were used to build a rooted phylogenetic tree, H is used to define clonal groups or ‘haplotypes’ of S. Typhi: H10 (accession number AE014613.1), H55 (CAAUU0000000.1) and (PJREB19771), H58 isolates, (NZ_LT8882486.1), (LT905060) and (PJREB7681), CT18, H1 (root) (AL513382). These strains were selected to represent the major S. Typhi strains currently circulating. This allowed us to define the chromosomal background of the PRJEB21992 ceftriaxone-resistant S. Typhi which acquired the plasmid described here.

Plasmid MLST profiling for the IncI1 group of plasmids was performed using the PMLST-1.4 Server [34]. Geneious R10 software [33] was used for plasmid mapping with the map to reference functions. The plasmid sequences listed in Table S1 and described in Table S2 (available in the online version of this article) were obtained from NCBI and used as references for comparison. Plasmids were selected using either of two criteria: (1) the presence of the same mobile element and resistance gene (ISEcp1 and blaCTX-M15) as on the plasmid investigated and (2) a top five hit based in core genome similarity with the plasmid investigated. A nucleotide BLAST was performed on the BLAST Ring Image Generator [35] in order to build a circular comparative figure with different selected references against the S. Typhi plasmid found in this study.

**RESULTS AND DISCUSSION**

**Microbiological identification**

The isolate PRJEB21992 was agglutinated with specific antisera O9 and Hd and was confirmed as S. Typhi by API 20E (profile: 4404540).

**Antimicrobial resistance phenotype**

The isolate (PRJEB21992) was sensitive to chloramphenicol, cotrimoxazole, ciprofloxacin, levofloxacin, nalidixic acid, azithromycin, imipenem, gentamicin, meropenem and tetracycline. It was resistant to ampicillin, ceftazidime, cefixime and ceftriaxone. Zone diameters and MICs are provided in Table 1. Ceftriaxone is the mainstay of typhoid fever treatment in Bangladesh, and indeed globally, and so the resistance of this isolate was immediately reported to the treating physician and tracking of this strain given a high priority – comparison of resistance profiles was the only method available in the hospital until recently and no similar isolate has been identified since isolation. This is the second ceftriaxone-resistant S. Typhi described in this study.
resistant S. Typhi reported from Bangladesh, but the first isolate (found in 1999 [22]) had been lost, preventing direct comparison. Ceftriaxone-resistant S. Typhi is rarely reported in the literature but cases are increasing. So, to allow tracking of this strain and to identify clonal expansion (i.e. emergence of an outbreak strain) whole genome sequencing was performed. We seek to genome sequence any ceftriaxone-resistant strain isolated subsequently to allow comparison.

In silico analysis of the chromosome

The isolate PRJEB21992 was identified as S. Typhi sequence type 2 (ST2). The isolate contained the pathogenicity islands (SPIs) normally associated with S. Typhi: SPI-1, 2, 5, 7 and 8, required for systemic infection and intracellular pathogenesis. The full genome sequence is available for comparison with other ceftriaxone-resistant S. Typhi at the ENA (EMBL-EBI) (project PRJEB21992). Analysis for antimicrobial resistance genes revealed that aminoglycoside resistance genes were present and that clinically important beta-lactam resistance was mediated by blaTEM-1B and blaCTX-M-15 genes. Comparison with other published ceftriaxone-resistant isolates of S. Typhi revealed high diversity (Fig. 1). Of the genomes used in building the phylogenetic tree the nearest neighbour to the isolate described here (PRJEB21992) was an isolate from the Democratic Republic of the Congo isolated in 2015 [36] (PRJEB19771). However, looking at 95 % variant frequency single nucleotide polymorphism (SNPs) [relative to CT18 with quality >20, approximately 70 % of SNPs] the Democratic Republic of the congo isolate and our strain is not closely related since ~1600 SNPs separate them. Relative to CT18, these two strains share 1052 SNPs. This highlights the importance of using both chromosomal background and plasmid content.

The isolate reported in this study harboured one plasmid of incompatibility group I1. IncI1 plasmids are normally associated with E. coli, only rarely seen in Salmonella. We therefore investigated the origins of this plasmid and associated mobile elements.

Genetic characterization of the IncI1-ST31 plasmid identified in S. Typhi

The plasmid in this study (pPRJEB21992) was identified as IncI1-ST31. Comparison with another IncI1 Salmonella plasmid carrying blacTX-M-15: pS115 from S. Enteritidis, revealed high sequence similarity (90 %) over large stretches of DNA (Fig. 2). Subsequent searching of the NCBI databases revealed...
full length, 100% matches of plasmid sequence in NCBI (see Fig. 2) S. Typhi plasmid with plasmid IncI1 pESBL-EA11 from E. coli (CP003290.1) – present in the shiga-toxin positive enteroaggregative E. coli from a large outbreak in Germany in 2011 [37]. Two more E. coli plasmids: pEC_Bactec (GU371927.1) from E. coli isolated from the joint of a horse suffering from arthritis in Belgium [38]; and pHUSEC2011 (HE610900.2) (reported as epidemic plasmid in E. coli strain HUSEC2011 in Germany, but not published), appeared to be identical plasmids (Fig. 2) from remote sources, suggesting...
that the plasmid has been widely transmitted between *E. coli* and has now entered the *S. Typhi* population.

The *blaCTX-M-15* was detected within a mobile element which mapped to an IncHI2 plasmid from *S. Typhimurium* and other plasmids listed in Table S1, and was adjacent to the insertion sequence ISEcp-1 (Fig. 3). The mobile element ISEcp-1 is commonly associated with CTX-M-15 [39] and is present on many plasmid backbones [38, 40], so we interrogated the databases for the mobile element. IncN pHg from *Klebsiella pneumoniae* (CP006662), IncF pHIE101 from *E. coli* (EU418922), incFII pM16-13 from *Klebsiella pneumoniae* (KY751925) and incHI2 pKSt313 from *S. Typhimurium* (LN794248) belonged to different incompatibility groups, but all carried the *blaTEM-CTX-M-15* gene complex associated with ISEcp-1 (Fig. 2), showing the widespread nature this mechanism of CTX-M-15 dissemination can have.

The IncI1 plasmid reported here in *S. Typhi*, not commonly seen in *Salmonella*, was contained in Bangladesh (and so may have a biological cost in the *S. Typhi* bacterial host) but the clear transmissibility of the ISEcp-1 element is a matter of concern [41]. When chloramphenicol resistance first emerged in *S. Typhi*, the phenotype was associated with a cost [42] but the continued selective pressure allowed the co-evolution of plasmids and large outbreaks of MDR typhoid fever [43]. It is likely that MDR ceftriaxone-resistant *S. Typhi* will also emerge. Biological fitness is currently an unpredictable phenotype but is more likely to evolve if the numbers of ceftriaxone-resistant *S. Typhi* increase and so preventing the spread of ceftriaxone-resistant *S. Typhi* is critical.

Plasmids belonging to incompatibility group I1 are widely spread in Enterobacteriaceae. They are known to have carried β-lactamase genes and type IV pili-encoded genes, responsible for resistance to beta lactams and virulence respectively [44]. Bacteria carrying plasmids of this group are known to be more pathogenic than commensal strains [44, 45] Inc I1 plasmids carrying *blaCTX-M-15* were previously identified in England [46], Pakistan, Honduras (in *E. coli* of human and animal origin), *S. Anatum*, *S. Infantis*, *S. Ohio* and *S. Typhimurium* [47], Inc FII plasmids carrying the *blaCTX-M-15* gene is present in *S. Enteritidis* [47]. Given this diversity of opportunity for *S. Typhi* to pick up CTX-M-15 it seems likely that if we continue the widespread use of ceftriaxone for the treatment of typhoid fever then a plasmid carrying resistance, probably encoded by *blaCTX-M-15*, will find a compatible MDR *S. Typhi* host and this combination will eventually emerge and cause outbreaks. Were this to happen, it would leave us with very few treatment options for typhoid fever. It is of great clinical importance that sequence data from ceftriaxone-resistant *S. Typhi* is shared and that efforts to limit the spread of ceftriaxone-resistant strains are supported. In particular, treatment trials with alternative antibiotics should be prioritized.

**Conclusion**

Here we report the whole genome sequence of a second ceftriaxone-resistant *S. Typhi* strain isolated in Bangladesh in 2000. It has, again, been contained, but the emergence of two different strains shows that selective pressure is widespread. A similar strain in the DRC several years later (2015) with the same mobile element on a different plasmid also demonstrates the pressure of antibiotic selection and also suggests that the ISEcp1–CTX-M-15 mobile element is becoming established in the *S. Typhi* population. It is only a matter of time until the CTX-M-15 gene appears in a successful plasmid-chromosome background as happened with *S. Typhi* H58 and pST6. Given the dependence of typhoid treatment on ceftriaxone, the tracking of ceftriaxone-resistant *S. Typhi* is crucial and we encourage any laboratory isolating ceftriaxone-resistant *S. Typhi* to report to both physician and public health authorities as swiftly as possible to allow the potential outbreak to be contained. Rapid sequencing of genomes is now well-established in many countries and comparison of any new isolates with results from others is routine for accredited reference labs such as the UK’s Gastrointestinal Bacteriology Reference Laboratory. Sequence data however, needs to be generated in a useful time frame.

A global outbreak, as with the MDR-ciprofloxacin-resistant *S. Typhi* H58, of MDR-ceftriaxone-resistant *S. Typhi* has the potential to be catastrophic and must be identified as quickly as possible so that patients can be treated with active antibiotics (e.g. the carbapenems or penems) but these expensive drugs are not yet licenced for typhoid fever; they must be tested and made available for treatment of ceftriaxone-resistant typhoid fever. Any ceftriaxone treatment failure must be dealt with promptly using alternatives – treatment trials with alternative agents are urgently needed.

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**Conflicts of interest**

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