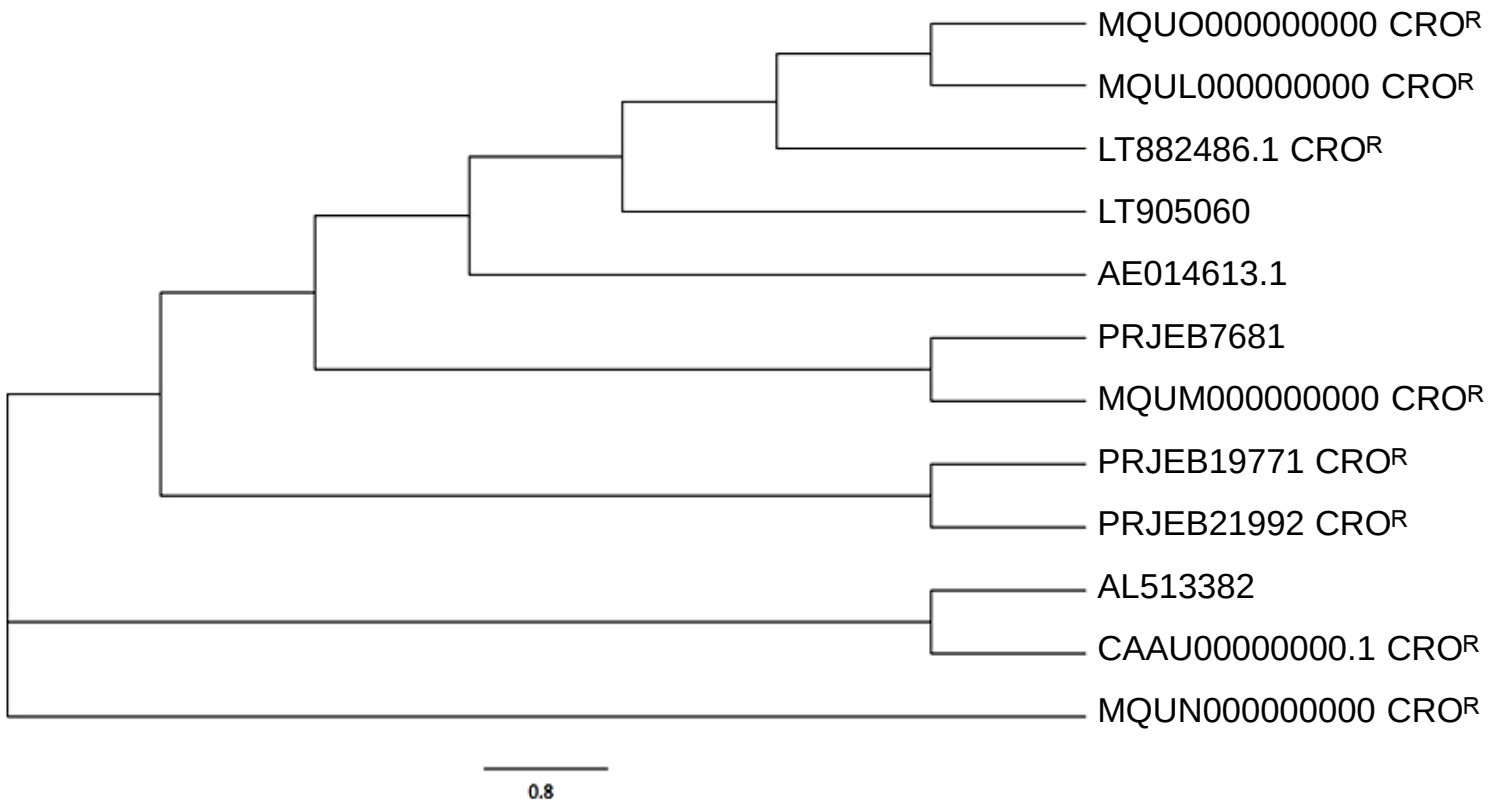
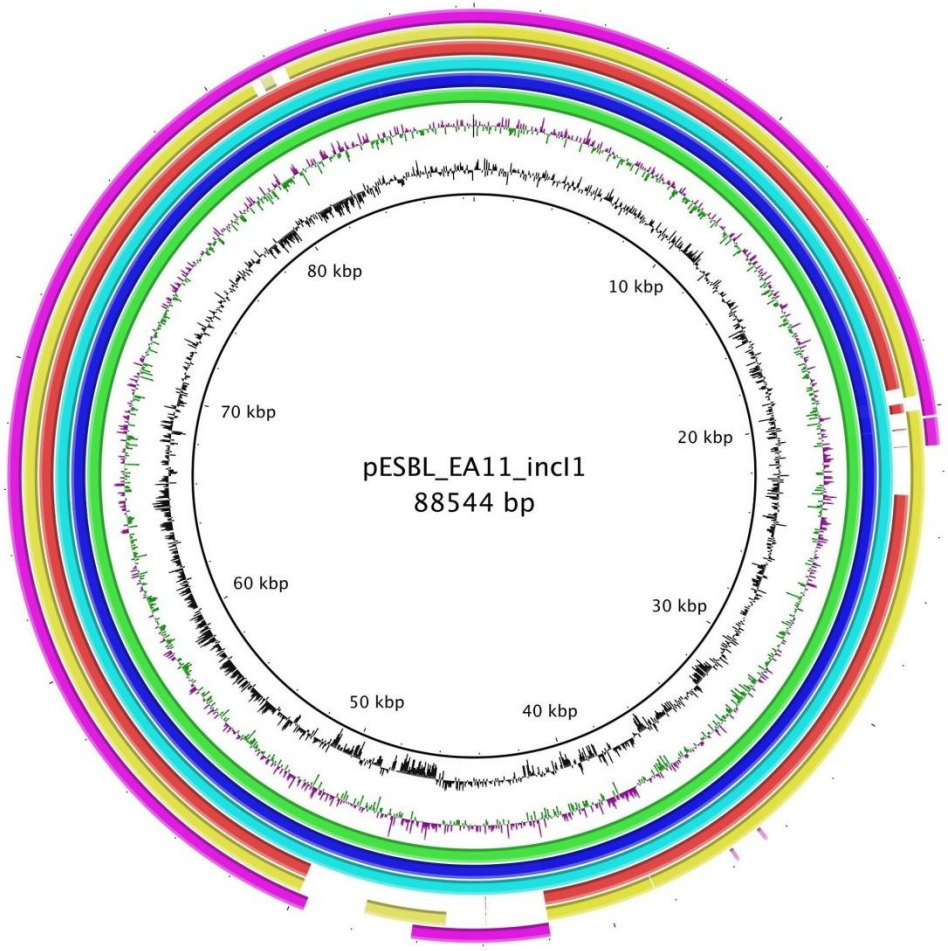

MQUO000000000 CRO^{R}
MQUL000000000 CRO^{R}
LT882486.1 CRO^{R}
LT905060
AE014613.1
PRJEB7681
MQUM000000000 CRO^{R}
PRJEB19771 CRO^{R}
PRJEB21992 CRO^{R}
AL513382
CAAU00000000.1 CRO^{R}
MQUN000000000 CRO^{R}
0.8



pESBL-EA11 (*E. coli*) - 2011

GC Skew

GC Content

pPRJEB21992 (*S.* Typhi) - This study

100% identity
90% identity
70% identity
Ring 4

pEC_Bactec (*E. coli*) - 2009

100% identity
90% identity
70% identity
Ring 5

pHUSEC2011-1 (*E. coli*) - 2011

100% identity
90% identity
70% identity
Ring 6

pMVAST0167_2 (*E. coli*) - 2016

100% identity
90% identity
70% identity
Ring 7

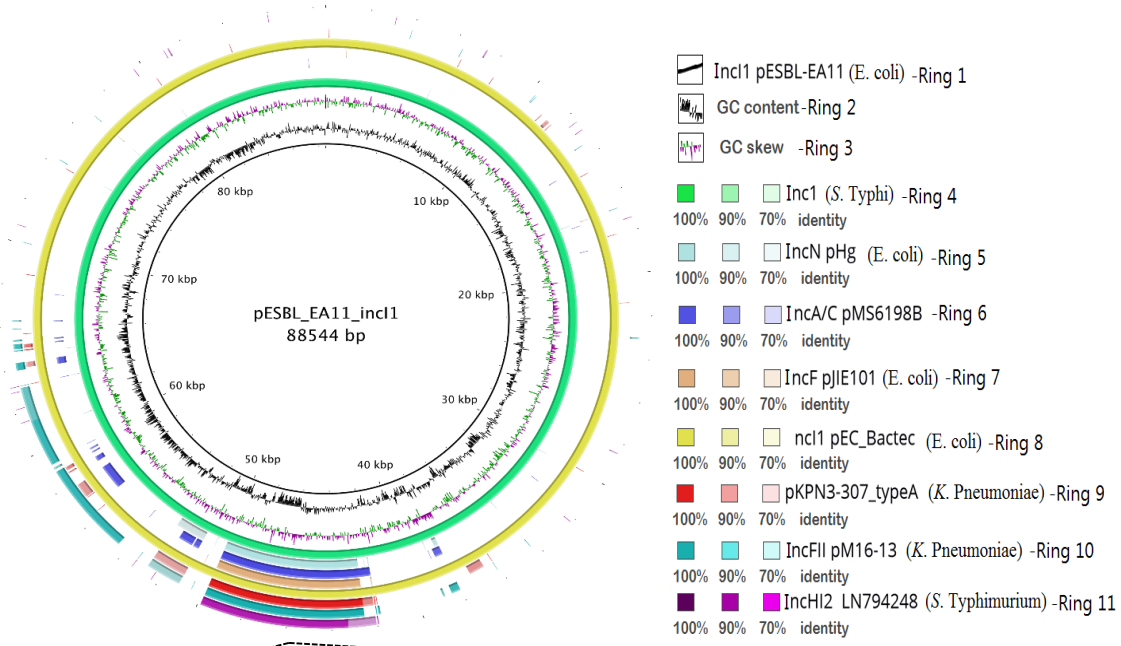
pSE115 (*S.* Enteritidis) - 2015

100% identity
90% identity
70% identity
Ring 8

pV150-a (*E. coli*) - 2013

100% identity
90% identity
70% identity
Ring 9

A.


pESBL_EA11_incI1
88544 bp
10 kbp
20 kbp
30 kbp
40 kbp
50 kbp
60 kbp
70 kbp
80 kbp
IncI1 pESBL-EA11 (E. coli) -Ring 1
GC content-Ring 2
GC skew -Ring 3
Inc1 (S. Typhi) -Ring 4
IncN pHg (E. coli) -Ring 5
IncA/C pMS6198B -Ring 6
IncF pJIE101 (E. coli) -Ring 7
ncI1 pEC_Bactec (E. coli) -Ring 8
pKPN3-307_typeA (K. Pneumoniae) -Ring 9
IncFII pM16-13 (K. Pneumoniae) -Ring 10
IncHI2 LN794248 (S. Typhimurium) -Ring 11
100% 90% 70% identity

B.

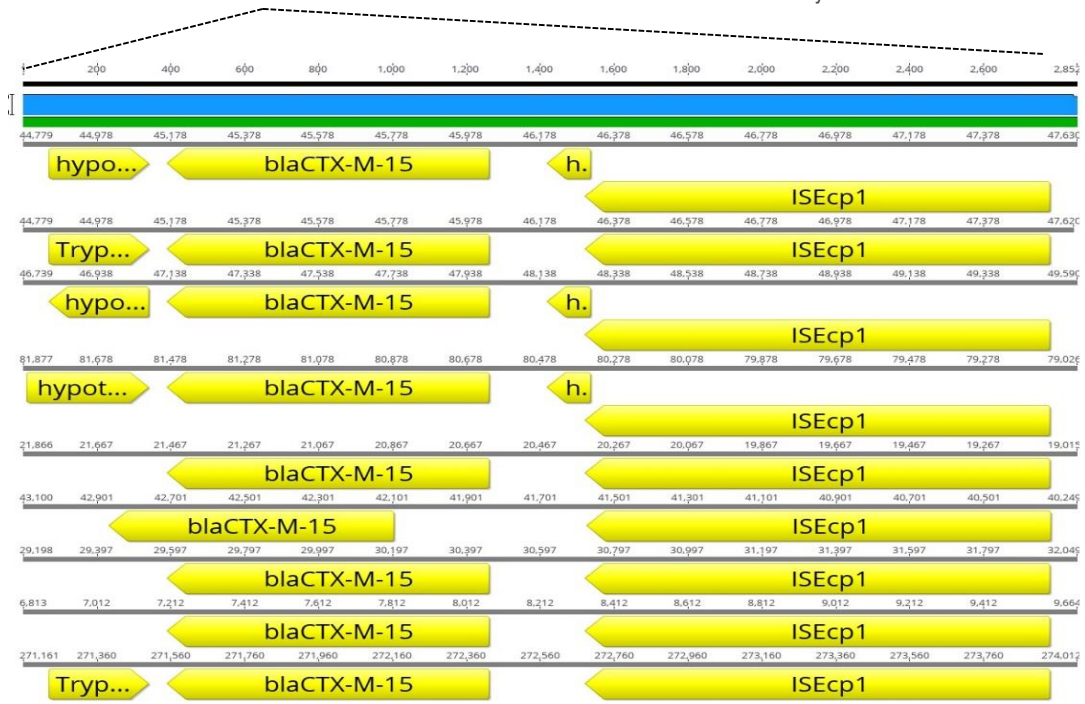

hypo...
blaCTX-M-15
h.
ISEcp1
Tryp...
hypot...

Table. Antimicrobial susceptibility phenotype and minimum inhibitory concentration (MIC) of the ceftriaxone-resistant *S.* Typhi isolated in Bangladesh.

Antibiotic	**Zone diameter of inhibition (mm)**	**MICs**	**Interpretation (CLSI, 2016)**
Ampicillin	00	>256 μg/mL	Resistant
Chloramphenicol	26	4 μg/mL	Sensitive
Co-trimoxazole	32	0.023 μg/mL	Sensitive
Ciprofloxacin	36	0.012 μg/mL	Sensitive
Levofloxacin	35	Not done	Sensitive
Nalidixic acid	28	Not done	Sensitive
Azithromycin	18	4 μg/mL	Sensitive
Gentamicin	22	0.75 μg/mL	Sensitive
Ceftazidime	15	8 μg/mL	Resistant
Cefixime	00	Not done	Resistant
Ceftriaxone	00	>256 μg/mL	Resistant
Imipenem	32	Not done	Sensitive
Meropenem	34	<0.125 μg/mL	Sensitive
Tetracycline	26	1 μg/mL	Sensitive

Sup. 1. Plasmids used as references for comparison in this study, selected by BLASTn against NCBI database.

Plasmid	Accession number	Inc-group	Strain	Percentage of Identity (%)/ Detection of blaCTX-M15 + ISEcp1
pESBL-EA11	CP003290.1	I1	*Escherichia coli* O104:H4 str. 2011C-3493	99% (100% coverage)
pEC_Bactec	GU371927.1	I1	*Escherichia coli*	99% (99% coverage)
pHUSEC2011-1	HE610900.2	I1	*Escherichia coli* HUSEC2011	99% (100% coverage)
pMVAST0167_2	CP014494.1	*	*Escherichia coli*str. MVAST0167	99% (87% coverage)
pSE115	KT868530.1	I1	*Salmonella* Enteritidis str. SE115	99% (98% coverage)
pV150-a	LC056403.1	I1	*Escherichia coli*str. V150	99% (72% coverage)
pHg	CPOO6662	N	*Klebsiella* pneumoniae str. ATCC BAA-2146	Mobile element detected
pMS6198B	CP015836.1	A/C	*Escherichia coli*str. MS6198	Mobile element detected
pJIE101	EU418922	F	*Escherichia coli*str. Tx101	Mobile element detected
pKPN3-307	KY271404.1	*	*Klebsiella*pneumoniaestr. Kp-48	Mobile element detected
pM16-13	KY751925	FII	*Klebsiella*pneumoniaestr. M16-13	Mobile element detected
*	LN794248	HI2	*Salmonella* Typhimurium	Mobile element detected

* No inc group detectable, no plasmid name

Sup. 2. *Salmonella* Typhi used for comparison

Accession number	Year of isolation	Country of isolation	Ceftriaxone susceptibility	Plasmid
MQUL00000000	2016	India	R	IncX3
MQUM00000000	2016	India	R	IncX3
MQUN00000000	2016	India	R	IncA/C2
MQUO00000000	2016	India	R	IncX3
LT882486.1	*	Pakistan	S	*
LT905060	*	*	S	*
AE014613.1	Ty2	Russia	S	No plasmids
PRJEB7681	*	*	R	*
PRJEB19771	2015	DRC	R	*
CAAU00000000.1	1998	Kenya	R	*
AL513382	CT18	Vietnam	S	IncIH1

*Data not found

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

Bilal Djeghout[1], Senjuti Saha[2, 3], Mohammad Saiful Islam Sajib[2, 3], Maksuda Islam[2, 3], Gemma L. Kay[4], Gemma C. Langridge[4], John Wain[4,*], Samir K. Saha[2, 3 *].

1. Laboratory of Microbiology and Virology, Department of Biomedical Sciences, University of Sassari, V. le San Pietro 43/B, 07100 Sassari, Italy
2. Child Health Research Foundation, Department of Microbiology, Dhaka Shishu Hospital, Dhaka, Bangladesh;
3. Bangladesh Institute of Child Health, Dhaka Shishu Hospital, Dhaka, Bangladesh
4. Medical Microbiology Research Laboratory, Norwich Medical School, University of East Anglia, Norwich, NR4 7UQ, United Kingdom

Running title: Ceftriaxone resistant *S.* Typhi

Key words: *Salmonella* Typhi; ceftriaxone resistance; antibiotic resistance. CTX-M-15; IncI1-ST31 plasmid; Bangladesh

*Co-corresponding Authors

Samir K. Saha (samirk.sks@gmail.com)

Bangladesh Institute of Child Health, Department of Microbiology, Dhaka Shishu Hospital, Sher E Bangla Nagar, Dhaka, BD 1207

Tel No. +8801713461254

John Wain* (j.wain@uea.ac.uk) ;

[1,*]Norwich Medical School, Room 2.28, Bob Champion Research and Education Building, James Watson Road, University of East Anglia, Norwich Research Park, Norwich, NR4 7UQ. Tel: +44 (0)1603 597567.

Other e-mail addresses:

bdjeghout@uniss.it (B. Djeghout); senjutisaha@gmail.com (S. Saha); saiful.i.sajib@gmail.com (M. S. I. Sajib); maksuda.chrf@gmail.com (M. Islam); g.langridge@uea.ac.uk (G. C. Langridge); g.kay@uea.ac.uk (G. L. Kay).

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 prioritised. 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 2011 at the Popular Diagnostic Center, Dhaka, Bangladesh was subjected to whole genome sequencing, using an Illumina NextSeq 500 and analysis using Geneious software.

Results: 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 $bla_{\text{CTX-M-15}}$ (ceftriaxone 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 main stay 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 revolutionised 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 1950's and 60's, was replaced in the 1970's by cotrimoxazole and amoxicillin after chloramphenicol-resistant strains of *S*. Typhi emerged [7]. In the 1990's 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) 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.

Materials and methods

Bacterial Isolation and identification

In 2001, an *S*. Typhi was isolated from a child's blood sample in the microbiology laboratory of the Popular Diagnostic Center, Dhaka, Bangladesh, and sub-cultured 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 (CLSI - 2016) [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), cefixine (5 µg), ceftriaxone (30 µg), imipenem (10 µg), meropenem (10 µg) and tetracycline (30 µg). Minimum inhibitory concentration (MIC) was determined 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 (WGS) 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 4nM (as determined by analysis on an Agilent Technologies 2200 Tapestation and using the Qubit HS dsDNA assay) and pooled in equimolar amounts with other barcoded libraries. The entire library pool was then diluted to 1.8pM and sequenced using the NextSeq 500 v2 2x150bp paired-end protocol.

The genome was assembled using Velvet defaults 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 *Salmonella* in Silico Typing Resource platform (SISTR) [31]. We interrogated the

assembly for acquired resistance genes [32], *Salmonella* Pathogenicity Islands (SPI) [32], plasmids and incompatibility group using Res.Finder [32], SPIFinder-1.0 (CGE online platform – checked with Geneious R10 [33]) and PlasmidFinder-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 was necessary. Comparison of the background strain 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 [CAAU00000000.1] and [PJREB19771], H58 isolates, [NZ_LT8882486.1], [LT905060] and [PRJEB7681], CT18, H1 (root) [AL513382],

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 2 were obtained from NCBI and used as references for comparison. A nucleotide BLAST was performed on BLAST Ring Image Generator (BRIG) [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 agglutinated with specific antisera O9 and Hd and was confirmed as *S.* Typhi by API 20E (Profile: 4404540).

Antimicrobial Resistance Phenotype

The isolate 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 minimum inhibitory concentrations (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 flagged 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. This is the second ceftriaxone-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.

In silico analysis of the chromosome

The isolate was *S*. Typhi multi-locus Sequence Type 2 (ST2) (Table 3). 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 *bla*TEM-1B and *bla*CTX-M-15 genes (Table 3). Comparison with other published ceftriaxone resistant isolates of *S*. Typhi revealed a close relationship only with an isolate from the Democratic Republic

of the Congo [36] (Fig. 1); so this isolate represents a sporadic case. Four Isolates recently reported from India [26] highlight the importance of using chromosomal background and plasmid content – of three ceftriaxone resistant isolates of *S*. Typhi reported as harbouring IncX plasmids two are closely related but one is very different – thus an outbreak definition using plasmids only would falsely include the isolate annotated MQUN in Figure 1.

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 [pPRJEB21992] was identified as IncI1-ST31. Comparison with another IncI1 salmonella plasmid carrying *bla*CTX-M-15: pS115 from *S*. Enteritidis, revealed high sequence similarity 70-100% over large stretches of DNA (Fig. 2). Subsequent searching revealed full length, near 100% matches of the new *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 is transmitted between *E. coli*.

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 2, 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] and so we interrogated the databases for the mobile element. IncN pHg from *Klebsiella pneumoniae* [CP006662], IncF pJIE101 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].

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. Bacteria carrying plasmids of this group are known to be more pathogenic than commensal strains [44, 45] Inc I1 plasmids carrying bla-CTX-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 plamids 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 *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 funded immediately.

Conclusion

Here we report the whole genome sequence of a second ceftriaxone-resistant *S.* Typhi strain isolated in Bangladesh. 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 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 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 *S*. Typhi H58, of MDR-ESBL-expressing *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 containment. Any ceftriaxone treatment failure must be dealt with promptly using alternatives - treatment trials with alternative agents and control of outbreaks through vaccination are urgently needed.

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Transparency declaration

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Figure 1. Phylogenetic tree based on single nucleotide polymorphism of whole-genome alignments of *S*. Typhi strains. Strains are annotated with the accession number of the sequencing data. The tree was carried out using Geneious Tree Builder, Neighbour-Joining default parameters. Juke-Cantor was used as distance model calculator [33]. The scale below the tree represents numbers of substitutions divided by the length of the sequence

Figure 2. Pairwise comparisons of IncI1 plasmid identified in *S*. Typhi in this study (ring 4, green). This figure shows plasmid pESBL_EA11 compared against six other plasmids (the full list of plasmid sequences is described in table 2). Ring 2 and 3 show GC and skew content respectively. The remaining rings show nucleotide BLAST comparison of the six plasmids against pESBL_EA11.

Figure 3. Pairwise comparisons of plasmids from different Incompatibility groups encoding blaCTX-M-15 and ISEcp1 genes. (**A**) This figure shows plasmid pESBL_EA11 compared against eight other plasmids (the full list of plasmid sequences is described in table 2). Ring 2 and 3 show GC and skew content respectively. The remaining rings show nucleotide BLAST comparison of the eight plasmids against pESBL_EA11. **(B**) Focus on the sequence region encoding blaCTX-M-15 plus the insertion sequence ISEcp1; on the same plasmids from (A) respectively. Geneious R10 software was used for multiple alignment of these plasmids. The black thick line represents the consensus sequence. The blue bar represents the coverage. The green thick line represents the identity of the alignment. Sequences of each plasmid are represented in grey tick lines with base pairs numbers on. In yellow are the annotated genes.