Recent emergence of cephalosporin resistant *Salmonella* Typhi in India due to the endemic clone acquiring IncFIB(K) plasmid encoding *bla*$_{\text{CTX-M-15}}$ gene

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Abstract

The emergence and spread of *Salmonella* Typhi (*S*. Typhi) resistant to third generation cephalosporins are a serious global health concern. In this study, we have genomically characterized 142 cephalosporin resistant *S*. Typhi strains isolated from Gujarat, India. Comparative genome analysis of study isolates revealed the emergence of a new clone of ceftriaxone-resistant *S*. Typhi harboring three plasmids of the incompatibility group IncFIB(K), IncX1 and IncFIB(pHCM2). Among the three, IncFIB(K) plasmid confers resistance to third-generation cephalosporins by means of $bla_{\text{CTX-M-15}}$ gene, as well as other resistance determinants such as *aph(3'')*, *aph(6')*, *sul2*, *dfrA14*,*qnrS* and *tetA*. Phylogenetic analysis of strains revealed that the isolates from Gujarat (*n=140/142*) belong to a distinct subclade (genotype 4.3.1.2.2) within genotype 4.3.1.2 (H58 lineage II). SNP-based phylogenetic analysis of the core genes in IncFIB(K) suggested a close relatedness of the plasmid backbone to that of IncFIB(K) from other Enterobacteriales. This implies that H58 lineage II can acquire MDR plasmids from other Enterobacteriales provided compensatory evolution balances the associated fitness costs. Although, as previously reported, exposure to the third generation cephalosporins during treatment may have selected for these variants, this could indicate the potential onset of a new wave of ceftriaxone-resistant *S*. Typhi in India. The implementation of control measures such as vaccination, improved water, sanitation, etc., could be undertaken in areas where MDR or XDR *S*. Typhi strains are prevalent.

Introduction

Enteric fever is a systemic febrile illness caused by human-restricted pathogens *Salmonella enteri*ca serovar Typhi and Paratyphi A, B, and C (Crump and Mintz, 2010). It continues to

be a major cause of morbidity and mortality in low and middle-income countries in South Asia, Southeast Asia, and Africa (Antillón et al., 2017). In most cases, typhoid infection is contracted from contaminated food or water, rather than through direct contact with an infected person. Globally, typhoid fever is estimated to cause approximately 11 to 20 million cases and result in between 28,000 and 161,000 deaths annually (WHO, 2023; Mogasale et al 2014). Another estimate from the Institute for Health Metrics and Evaluation (IHME) in 2019 suggested a global burden of 9.24 million cases of typhoid fever, associated with around 110,000 deaths (https://www.healthdata.org/results/gbd_summaries/2019/typhoid-fever-level-4-cause). Notably, region-wise data indicates that high-burden countries in South Asia account for roughly 70% of the global burden of enteric fever (Stanaway et al 2019).

Historically, antimicrobial therapy was the most effective method of combating typhoid; however, this has been threatened by the emergence of antimicrobial resistant (AMR) strains (Browne et al., 2020). During the late 1970s and early 1980s, multidrug-resistant (MDR) strains of *S.* Typhi became major concerns globally (Marchello et al., 2020). Consequently, fluoroquinolones became the drugs of choice in the treatment of MDR *S.* Typhi. However, emergence of fluoroquinolone non-susceptible *S.* Typhi have shifted the treatment options to azithromycin and third-generation cephalosporins (3GC) (Britto et al., 2018; Parry et al., 2019). These two drugs have continued as the 'last resort' antibiotics for treating enteric fever in the endemic regions of South Asia. A recent outbreak of extensive drug resistant (XDR) *S.* Typhi strains (resistant to three first-line antibiotics, quinolones and 3GCs) in Pakistan has caused treatment recommendations to shift in favor of azithromycin (Klemm et al., 2018).

The introduction of genome-based surveillance for typhoid has greatly improved our knowledge of the origins, transmission, and antibiotic resistance profiles of *S.* Typhi isolates. The current population structure of *S.* Typhi strains includes 85 haplotypes (H1 to H85), of which haplotype 58 (genotype 4.3.1) is highly pathogenic as well as multidrug resistant

(Wong et al., 2015; 2016). During the past few decades, H58 *S.* Typhi has become the global dominant lineage due to its efficient transmission and persistence within human populations (Pragasam et al., 2020; Carey et al., 2022). The intercontinental spread of MDR *S.* Typhi (genotype 4.3.1.1 or H58 lineage 1) was largely driven by the acquisition of an incompatibility (Inc)HI1 plasmid carrying multiple antimicrobial resistance determinants (bla_{TEM}, *cat*, *dhfr*, *sul2*) (Holt et al., 2011; da Silva et al., 2022). Similarly, with the introduction of ciprofloxacin as the primary treatment for typhoid, a distinct sublineage (genotype 4.3.1.2 or H58 lineage 2) has emerged, characterized by fluoroquinolone non-susceptibility (FQNS) and the presence of double/triple-point mutations in the quinolone resistance determining regions (QRDRs) (Pham et al., 2016). Further, the emergence of a new sub-lineage P1 (4.3.1.1.P1) of *S.* Typhi in Pakistan has raised concerns due to its resistance to third-generation cephalosporins in addition to first-line antimicrobials and fluoroquinolones. Extensively drug-resistant (XDR) isolates belonging to this sub-lineage carry $bla_{\mathrm{CTX\text{-}M\text{-}15}}$, which is mobilized by IncY plasmids (Klemm et al., 2018). Complicating matters further, the development of point mutations in the *acrB* gene (R717Q/L) has given rise to azithromycin-resistant *S.* Typhi, posing a significant threat to the last available oral therapy option (Hooda et al., 2019; Duy et al., 2020).

Genomic epidemiology studies of *S.* Typhi have demonstrated the evolution and spread of multiple distinct lineages on a regional and global scale (Dyson and Holt, 2021). The regional dominance of H58 sublineages such as 4.3.1.1 (MDR) and 4.3.1.1P1 (XDR) in Pakistan, 4.3.1.2 (FQNS strains) in India and Nepal, and IncFIB(K) carrying genotype 4.3.1.3 in Bangladesh are well documented (Carey et al., 2023). Contrary to this, the population structure of *S.* Typhi in Africa shows H58 in eastern and southern Africa while west and central Africa consists primarily of non-H58 lineages or distinct genotypes of H58 (Park et al., 2018; Carey et al., 2023). While the XDR lineage (4.3.1.1P1) has become dominant in

Pakistan (70% in 2020), it has not yet become established elsewhere. Conversely, ceftriaxone resistant (Cef^R) *S*. Typhi strains reported from India primarily belonged to sub-clade 4.3.1.2 (recently renamed as 4.3.1.2.1.1) and carry bla_{SHV-12} mobilized by IncX3 plasmid backbone (Jacob et al., 2021, Argimón et al., 2022). Over the past few years, ESBL-harbouring plasmids belonging to different incompatibility types have been observed in *S*. Typhi isolates in India, however, its long-term persistence and stability was not completely understood (Jacob et al., 2021). In this study, we aimed to analyze the genomic epidemiology and evolutionary origin of 142 cases of ceftriaxone resistant *S*. Typhi in India. We also explored the recent plasmid transfer events and gene flow that led to the emergence of IncFIB(K) carrying cephalosporin resistant *S*. Typhi in India.

Methodology

Ethical Statement

This study was approved by the Institutional Review Board (IRB) of Christian Medical College, Vellore (IRB Min No: 13489 dated 28.10.2020).

Study setting and design

The Surveillance of Enteric Fever in India (SEFI) study, which concluded its first phase from 2018 - 2020 monitored the changes in the antimicrobial susceptibility of *S*. Typhi and *S*. Paratyphi A in India. To continue tracking the antimicrobial resistance patterns, we initiated a smaller-scale second phase of the SEFI network's surveillance in 8 cities, commencing in March 2022. In Ahmedabad, Gujarat, Neuberg Supratech Reference Laboratories (NSRL), in collaboration with the Indian Institute of Public Health, Gandhinagar, functioned as a reference laboratory for hospitals, clinics, and diagnostic centers in the region, undertaking the culturing and preliminary identification of isolates. Initial cases of ceftriaxone resistant *S*. Typhi were reported from NSRL followed by laboratory confirmation at the Department of

Clinical Microbiology, Christian Medical College, Vellore. After issuing an alert to clinicians and laboratories about the new strain, isolates from other part of the state (Vadodara) were largely identified by Toprani Advanced Lab Systems (*n=86*) and Unipath Speciality Laboratory (*n=31*). Once confirmed as *S.* Typhi by conventional biochemical tests, samples were shipped to the central laboratory at the Department of Clinical Microbiology, Christian Medical College, Vellore for confirmation and further testing. The present study examined a collection of 142 ceftriaxone resistant *S*. Typhi isolates obtained from five clinical diagnostic laboratories located in Ahmedabad and Vadodara, India between June 2022 and September 2023 (Suppl Fig. 1).

Phenotypic characterization

Identification of *S*. Typhi isolates transferred to the central laboratory was re-confirmed using conventional biochemical tests and serotyping was conducted using commercial anti-sera (BD Difco, USA) based on the Kauffmann-White scheme according to the manufacturer's instructions. Phenotypic antimicrobial susceptibility testing was performed using the Kirby-Bauer disc diffusion method and the zone diameter was measured and interpreted based on Clinical Laboratory Standards Institute (CLSI, 2023) guidelines. The tested antimicrobial agents included ampicillin (10µg), Chloramphenicol (30µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), ciprofloxacin (5µg), pefloxacin (5µg), ceftriaxone (30µg), cefixime (5µg) and azithromycin (15µg). In addition, minimum inhibitory concentration (MIC) for ceftriaxone and azithromycin was determined using broth microdilution (BMD) and the results were interpreted using CLSI guidelines (CLSI, 2023).

DNA extraction and whole genome sequencing (WGS)

Genomic DNA was extracted using QIAamp® Mini Kit (250) (QIAGEN, Hilden, Germany) following the manufacturer's instructions. The purity and concentration of the extracted DNA

was measured using Nanodrop One (Thermo Fisher, Waltham, USA) and Qubit Fluorometer using dsDNA HS Assay Kit (Life Technologies, Carlsbad, USA). For short read sequencing, genomic DNA is fragmented and the paired-end library is prepared using Illumina Nextera DNA Flex Library Kit and Nextera DNA CD Indexes (Illumina, Massachusetts, MA, USA). The libraries were pooled at equal molar concentration and sequenced on Illumina Novaseq 6000 platform yielding 2×150 bp paired-end reads (Illumina, San Diego, CA, USA). DNA tagmentation, library amplification, and clean-up were performed according to the manufacturer's protocol.

A subset of five isolates were subjected to sequencing using Oxford Nanopore Technology (ONT). The libraries for sequencing were prepared using Ligation Sequencing Kit (SQK-LSK114) (ONT, UK) and sequenced on an ONT MinION device using R10.4.1 flow cell and Q20 chemistry according to the instructions of the manufacturer. Duplex ONT read pairs were prepared for basecalling using ONT's duplex tools (https://github.com/nanoporetech/duplex-tools) and raw nanopore reads were basecalled with Guppy (https://pypi.org/project/ont-pyguppy-client-lib/).

QC, Assembly

Short reads generated from Illumina platform were checked for quality using FastQC v0.12.1 and adapters and indexes were trimmed using Trimmomatic, v0.39. Contamination detection and filtering has been performed by Kraken v 1.1.1 (https://github.com/DerrickWood/kraken) followed by sequence coverage. The high-quality reads (Phred score >30) hence generated were assembled using SPAdes (https://github.com/ablab/spades) with default settings.

Long read data from ONT sequencer were subsampled using Rasusa v0.7.1 to depths of 100x coverage (average) and reads shorter than 5000 bp were also removed using filtlong v0.2.1. The reads were subjected to hybrid assembly using Unicycler and short reads were then used

to polish further using polypolish v0.5.0. The resulting complete genome assemblies were assessed by QUAST v5.2.0 and seqkit v2.4.0. Default parameters were used unless otherwise mentioned.

Comparative genomics

From the global collection of *S.* Typhi genomes (*n=415*), reads representing all major genotypes were obtained from the European Nucleotide Archive (ENA; http://www.ebi.ac.uk/ena) or the Sequence Read Archive (SRA) based on the curated data provided by the Global Typhoid Genomics Consortium (https://bridges.monash.edu/articles/dataset/Global_Typhoid_Genomics_Consortium_2022_-_Genome_Assemblies/21431883) (Carey ME et al., 2023). Genomes were classified according to their sequence types using the Multilocus Sequence Typing (MLST) pipeline offered by the Center for Genomic Epidemiology (https://cge.cbs.dtu.dk/services/MLST/). *S.* Typhi genotypes were determined using the GenoTyphi scheme (https://github.com/katholt/genotyphi). Antimicrobial resistance genes were identified from the genome sequences using the BLASTn-based ABRicate v0.8.10 program (https://github.com/tseemann/abricate) with the AMRFinderPlus database v3.11.14. Plasmids were identified and typed using PlasmidFinder (https://cge.cbs.dtu.dk/services/PlasmidFinder/) and MOB-suite pipelines (https://github.com/phac-nml/mob-suite). The complete list of raw sequence data is available in the Supplementary Data File.

Phylogenetic analysis

The assembled contigs (*n=574*) were mapped to the reference genome of *S.* Typhi CT18 (GenBank: AL513382.1) using Snippy v4.6.0. Single nucleotide polymorphisms (SNPs) were called from the core multiple-sequence alignment file using snp-sites v2.5.1 and maximum-

likelihood phylogeny were inferred from the alignment using RAxML-HPC v1.2.0 under the GTR+GAMMA model (200 bootstraps). The resulting phylogenetic tree was visualized and annotated using the Interactive Tree of Life software (iTOL v.3).

Plasmid characterization

We identified 288 plasmid sequences typed as IncFIB(K) from public databases including NCBI's RefSeq database (https://www.ncbi.nlm.nih.gov/), the PLSDB database (Schmartz et al., 2022) and COMPASS reference database (Douarre et al., 2020). IncFIB(K) plasmids previously reported from *S*. Typhi were retrieved from the curated data provided by the Global Typhoid Genomics Consortium (Carey et al., 2023). Contigs belonged to plasmids were extracted from the draft assembly using Plaforest v1.4 as previously described (Gomi et al., 2021). Incomplete and redundant sequences were removed manually and plasmids were reconfirmed as IncFIB(K) using abricate v1.01 with PlasmidFinder database (Giménez et al., 2022). Plasmid taxonomic units (PTUs) were assigned by COPLA (Redondo-Salvo et al., 2021) and plaSquid v1.0.0 (Giménez et al., 2022). Near-identical plasmids were detected with Mash tree v1.2.0 and outliers/ singletons were removed from the analysis.

The plasmid sequences were annotated with Prokka (https://github.com/tseemann/prokka) using the default parameters. Annotated assemblies in the GFF3 format were used as input for pan-genome analysis using Panaroo (https://github.com/gtonkinhill/panaroo) in its "Strict" mode with core threshold 80. The core genome alignment hence generated were used to construct plasmid core gene phylogeny using IQ-Tree v2.2.2.6 with parameters -m GTR+F+I+G4. We performed RhierBAPS to define the IncFIB(K) population structure and phylogenetic cluster carrying IncFIB(K) were further redefined to fully understand plasmid evolution. The gene presence or absence in each genome obtained were grouped according to

the phylogenetic lineages using twilight scripts (https://github.com/ghoresh11/twilight) with default parameters.

Data availability

Raw read data were deposited in the European Nucleotide Archive (ENA) under project accession number: PRJEB62461

Results

Phenotypic characterization

The ceftriaxone resistant *S.* Typhi isolates *(n=142)* investigated in this study were collected from febrile patients attending private health care facilities in Ahmedabad and Vadodara, India. Among the study cohort, 29 isolates were obtained from diagnostic laboratories in Ahmedabad, 113 isolates were collected from Vadodara, while one isolate was obtained from a diagnostic facility in Mumbai (Supplementary Fig 1). Antimicrobial susceptibility profiling revealed that all except one (*n=141/142*) study isolates exhibited resistance to ampicillin, co-trimoxazole, ciprofloxacin, and ceftriaxone, while showing susceptibility to chloramphenicol and azithromycin. The isolate from Mumbai (*n=1*) was susceptible to co-trimoxazole but resistant to ceftriaxone. The minimum inhibitory concentration of ceftriaxone was determined and found to be >512 µg/ml.

Genotype assigning:

The genomes of 140/142 *S*. Typhi isolates were characterized by a unique genotype, identified through a synonymous Single Nucleotide Polymorphism (SNP) in the highly conserved *thyA* gene, as assigned by the GenoTyphi scheme. All study isolates belonged to the prevalent subclade 4.3.1 genotype (H58), further specified as a unique genotype 4.3.1.2.2

based on the marker G3014988A. This novel genotype has been incorporated into the GenoTyphi scheme, enhancing its utility for early detection in future surveillance studies.

Population structure of *S*. Typhi

The population structure of ceftriaxone resistant *S.* Typhi from the study collection was inferred from a core gene SNP based phylogeny. The phylogenetic relationship of *S.* Typhi isolates in comparison to a curated set of global genome collection (*n=415*) and contextual isolates from Gujarat (*n=16*) highlighted the placement of isolates within clades corresponding to genotype 4.3.1 (H58) (Suppl. Fig. 2). Further analysis at the sublineage level revealed that majority of *S*. Typhi isolates (*n=140/142*) from Gujarat are closely related, forming a distinct subclade (4.3.1.2.2) within genotype 4.3.1.2 (H58 lineage II). The SNP analysis identified 16 core SNP differences between 4.3.1.2.2 the nearest neighbor. The sole isolate obtained from Mumbai, which was susceptible to co-trimoxazole, was assigned to the parent clade 4.3.1 while another belonged to genotype 4.3.1.1 (Fig. 1).

Further AMR gene analysis revealed that the study isolates carried $bla_{\text{CTX-M-15}}$, *qnrS1*, *sul2*, *dfrA14*, *tet(A)* on an IncFIB(K) plasmid. Mutation analysis on the QRDR region showed single point mutations in *gyrA*: S83F conferring non-susceptibility to fluoroquinolones. This collection exhibits an accumulation of acquired AMR genes, in contrast to the mostly susceptible contemporaneous *S*. Typhi collections from India. No known mutations associated with azithromycin resistance were detected among any of the *S*. Typhi isolates

Population structure of IncFIB(K) plasmids

Our curated dataset consists of IncFIB(K) plasmids (*n=276*) representing major Enterobacteriales genera. The dataset includes plasmids from a diverse of bacterial hosts, with 53.9% from *K. pneumoniae*, 19.9% from *S*. Typhi, 9.6% from *E. coli* and 7.9% from other *Klebsiella* spp. Among the selected plasmids that are assigned as IncFIB(K) replicon

types, MOB typing was performed and successfully classified into three MOB types, of which MOBV were the dominant MOB type. Notably twelve plasmids were assigned to multiple MOB types.

Pangenome analysis IncFIB(K) plasmid sequence showed a well conserved backbone consisting of four genes (*repB*, *sopA*, *umuC*, *umuD*) which formed a typical core gene set. After removing outliers and including our five newly sequenced IncFIB(K) plasmids, a final set of n=288 plasmid sequences were used for constructing a plasmid core gene phylogeny. The genetic relatedness of selected IncFIB(K) plasmids revealed a diverse population structure with six major (level 1) BAPS clusters (Fig. 2**)**. Within the plasmid phylogeny, IncFIB(K) plasmids carried by the study isolates (Accession number: CP144681) belonged to cluster 1 represented by multiple bacterial hosts. To identify the near identical IncFIB(K) plasmids that were similar to those reported from study isolates, the entire plasmid sequences within BAPS cluster 1 (*n=76*) were examined. Detailed inspection revealed that IncFIB(K) plasmids carried by the study isolates were closer to plasmids hosted by *S*. Typhi isolates previously reported from Eastern Africa (LT904889) (Fig. 3).

Discussion:

From June 2022 to September 2023, we identified 142 isolates of third-generation cephalosporin-resistant *S*. Typhi in India. The outbreak was initially detected as part of the SEFI phase 2 surveillance program for typhoid fever, which focuses on monitoring drug-resistant strains. Phenotypic analysis revealed resistance to ampicillin, trimethoprim-sulfamethoxazole, ciprofloxacin, and ceftriaxone, with susceptibility to chloramphenicol and azithromycin (Table 1). The resistance profile differs from classical extensively drug-resistant (XDR) strains, which are resistant to all first-line agents, fluoroquinolones and ceftriaxone (Klemm at al., 2018). Our study aimed to elucidate the population structure of the

emerging *S*. Typhi clone and investigate the genetic background of plasmid transfer through whole genome sequencing (WGS) and comparative genome analysis.

Analysis of *S*. Typhi genome sequences revealed that the majority of ceftriaxone-resistant strains from Gujarat (*n=140/142*) belonged to genotype 4.3.1.2 and further subtyped to 4.3.1.2.2 (Fig. 1). The genotype 4.3.1.2 has been previously reported in multiple genome surveillance studies in India (Wong et al., 2015; Pragasam et al., 2020; Britto et al., 2020; Pragasam et al., 2021). However, genomic surveillance studies did not adequately represent circulating *S*. Typhi strains in western India, particularly in Ahmedabad or Vadodara. To address this gap, we sequenced 16 ceftriaxone-susceptible isolates (contextual) from Ahmedabad over the same period for comparative analysis. Five of these isolates were found to be closely related to the ceftriaxone-resistant strains, differing by only 26 SNPs (Suppl Fig: 3). This suggests that the endemic H58 lineage II clone circulating locally may have acquired an ESBL-encoding AMR plasmid. Meanwhile, the single isolate of ceftriaxone-resistant *S*. Typhi from Mumbai belonged to genotype 4.3.1, which has been previously detected in circulation in Northern India. (Sah et al., 2020; Dahiya et al., 2023).

One of the key observations was the plasmid profile of the emergent clone, which carries three plasmids that have not been previously reported in *S*. Typhi isolates. Among them, only IncFIB(K) carried resistance genes, while no resistance gene was found in IncFIB(pHCM2) and IncX1 plasmids. In particular, IncFIB(K) plasmid confers resistance to third-generation cephalosporins by means of $bla_{\text{CTX-M-15}}$ gene, as well as other resistance determinants such as *aph(3")*, *aph(6')*, *sul2*, *dfrA14*, *qnrS a*nd *tetA* (Fig. 3) Considering the restricted host access of *S*. Typhi, and high fitness cost imposed by plasmid to the host bacterium, this was an unexpected event.

The evolutionary history of *S*. Typhi is characterized by genome degradation events, including deletions and inactivating mutations within coding sequences (McClelland et al., 2004; Holt et al., 2008; Wong et al., 2015). This trend has been corroborated by comparative genomic studies (Holt et al., 2009) and recent pan-genome analyses (Laing et al., 2017), which identify genome degradation, rather than gene acquisition, as the primary driver of *S*. Typhi evolution. Additionally, the genetic stability of *S*. Typhi is influenced by fitness costs resulting in minimal genomic variation in the absence of antibiotic selection pressure (Jacob et al., 2021; Baddam et al., 2014). However, the emergence of resistant phenotypes has altered the evolutionary trajectory. MDR and XDR strains have acquired large plasmids such as IncHI1 and IncY, initially associated with fitness burdens (Wain et al., 2003; Klemm et al., 2018). Yet, plasmid-carrying clones have undergone compensatory evolution, gaining a fitness advantage and expanding phylogenetic lineages (Wong et al., 2015). For example, IncHI1 plasmid acquisition drove the emergence of MDR S. Typhi (H58 lineage I/genotype 4.3.1.1) in South Asia (Holt et al., 2011; Carey et al., 2023). Subsequent chromosomal integration of the MDR composite transposon facilitated plasmid-free strains, stabilizing the MDR phenotype across lineages (Dyson et al., 2019; Carey et al., 2023). In Pakistan, the stabilized MDR genotype (4.3.1.1) evolved into an XDR clone (4.3.1.1.P1) via IncY plasmid acquisition harboring blaCTX-M-15 and qnrS1 (Klemm et al., 2018). This suggests that H58 lineage I, with a chromosomal MDR transposon and a single QRDR mutation (S83F), can acquire and maintain plasmids carrying AMR determinants.

Interestingly, the expansion and regional dominance of H58 lineage II (genotype 4.3.1.2) in India have been driven by mutations in the *gyrA* and *parC* genes, which confer non-susceptibility to fluoroquinolones (Pragasam et al., 2020). This dominance of lineages with reduced susceptibility to fluoroquinolones is linked to the high fluoroquinolone exposure in the region (Dahiya et al., 2014). Notably, these QRDR double/triple mutant strains rapidly

outcompeted other lineages due to the fitness advantage gained during evolution (Baker et al., 2013). As a result, H58 lineage II isolates are hypothesized to less likely acquire plasmids due to the initial cost associated with plasmid carriage (Jacob et al., 2021). Nevertheless, H58 lineage II isolates were previously reported to carry plasmids encoding cephalosporin resistance genes. Sporadic isolates of IncX3 and IncN plasmids carrying H58 lineage II isolates were reported between 2015 and 2019 (Jacob et al., 2021; Argimón et al., 2022). However, plasmid-free isolates likely outcompeted those bearing plasmids due to the associated fitness cost. Contrary to our expectations of rare plasmid carriage in H58 lineage II, the newly circulating clone of ceftriaxone-resistant isolates surprisingly carried three plasmids, one of which harbored resistance determinants

We hypothesize that the emergence of ceftriaxone-resistant isolates from Gujarat, India could be the result of a recent event of acquisition of multiple plasmids from another Enterobacteriaceae donor. The SNP-based phylogeny of the core genes in IncFIB(K) plasmid backbone identified six major clusters within the collection (Fig. 2). Based on a closer examination of cluster 1, where IncFIB(K) plasmids of study isolates were located, we were able to identify sequences of plasmids from *S*. Typhi, *S. flexneri, E. coli* and other *Salmonella* sp. with a pairwise distances of 0 to 1 core SNPs (Fig. 2 & 3). In particular, the IncFIB(K) plasmid carried by study isolates is closely related to the plasmid identified in a previously sequenced Tanzanian strain of *S*. Typhi (LT904889.1) (Ingle at al., 2019). The findings suggest that H58 lineage II can acquire MDR plasmids from other Enterobacteriaceae if compensatory evolution balances the cost of carrying the plasmids.

The emergence of new *S*. Typhi clones that are resistant to last-line antibiotics poses a serious problem for typhoid treatment and management. In view of the increased prevalence of resistance to third generation cephalosporins, azithromycin is the only oral treatment option, as per the current regimen. Though azithromycin and carbapenems remain effective for the

treatment uncomplicated and complicated typhoid fever, sporadic reports of resistance to these antibiotics have already been reported from the endemic areas (Octavia et al., 2021; Sajib et al., 2021; Carey et al., 2021; Ain et al., 2022). As a result, clinicians are faced with a challenging situation when choosing antibiotics for the treatment of typhoid fever. Considering the burden of typhoid fever, Indian NITAGs have advised the nationwide introduction of typhoid conjugate vaccine in June 2022. In order to mitigate the emergence of drug-resistant bacteria and the occurrence of untreatable typhoid cases, it is imperative that we take immediate action to widely implement Typhoid Conjugate Vaccines (TCVs), with a primary emphasis on introducing them in regions where antimicrobial resistance (AMR) is more prevalent. **Conclusion**

Comparative genome analysis of 142 ceftriaxone resistant *S.* Typhi strains revealed the population structure and resistance plasmid dynamics in India. With the acquisition of various mobile genetic elements and different genetic structures that are related to antibiotic resistance, it becomes apparent that *S*. Typhi continues to improve its ability to remodel its genome. Our data provide additional evidence of how H58 lineage II *S*. Typhi, which previously appeared to have a stable genome, may have undergone rapid evolution by acquiring MDR plasmids. Though exposure of third generation cephalosporins during treatment may have selected for these variants, this could indicate the beginning of a new wave of ceftriaxone resistant *S.* Typhi in India. Finally, the introduction of new typhoid conjugate vaccines as well as other control measures such as improved water, sanitation, and hygiene (WASH) systems could potentially reduce the selection pressure that drives the emergence of increasingly resistant strains of *S*. Typhi.

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Figure Legend

Fig 1: Population structure of H58 *S*. Typhi isolates. Maximum likelihood phylogenetic tree based on core genome sequence SNPs of 247 global H58 *S*. Typhi mapped to *S*. Typhi CT18 and rooted to outgroup isolates (Genotype 4.3.1). Major H58

sub-lineages such as 4.3.1.1EA1, 4.3.1.1P1, 4.3.1.3 and 4.3.1.2.1 are shaded in different colours. Red-colored dots at the tip of the branches indicates the position of this study isolates. Metadata are labeled as color strips and key for each variable were mentioned. Strip 1, 2 and 3 indicate the Genotype, Region of isolation and location in South Asia of each genomes. Heatmap represents the plasmid mediated resistance genes, QRDR mutations that confer resistance to fluoroquinolone and the presence of plasmids. The scale bar indicates substitutions per site. Color keys for all the variables are given in the inset legend. The tree was visualized and labeled using iTOL (https://itol.embl.de/).

Fig 2: (a) **An unrooted Maximum likelihood phylogenetic tree of global IncFIB(K) plasmids based on alignment of core genes.** Phylogenetic clustering of different lineages is represented by RhierBAPS population clustering (level 1). Clusters are shaded in different colours. (b) A higher-resolution diagram of cluster 2 where IncFIB(K) plasmids from Gujarat were located. Metadata are labeled as color strips and key for each variable were mentioned. Strip 1 and 2 indicate the MOB type, and host organism. Heatmap represents the plasmid mediated resistance genes.

Fig 3: Comparison of IncFIB(K) plasmid. IncFIB(K) plasmid from ceftriaxone resistant *S*. Typhi isolates from Ahmedabad (Accession number: CP144681) is compared to plasmid of same incompatibility group from other Enterobacteriales using Proksee (https://proksee.ca/). GC-content and GC-skew of the reference are depicted in the two innermost circles.

Suppl Fig: 1 Monthwise distribution of ceftriaxone resistant *S*. Typhi isolates received at the reference laboratory from April 2022 to March 2023

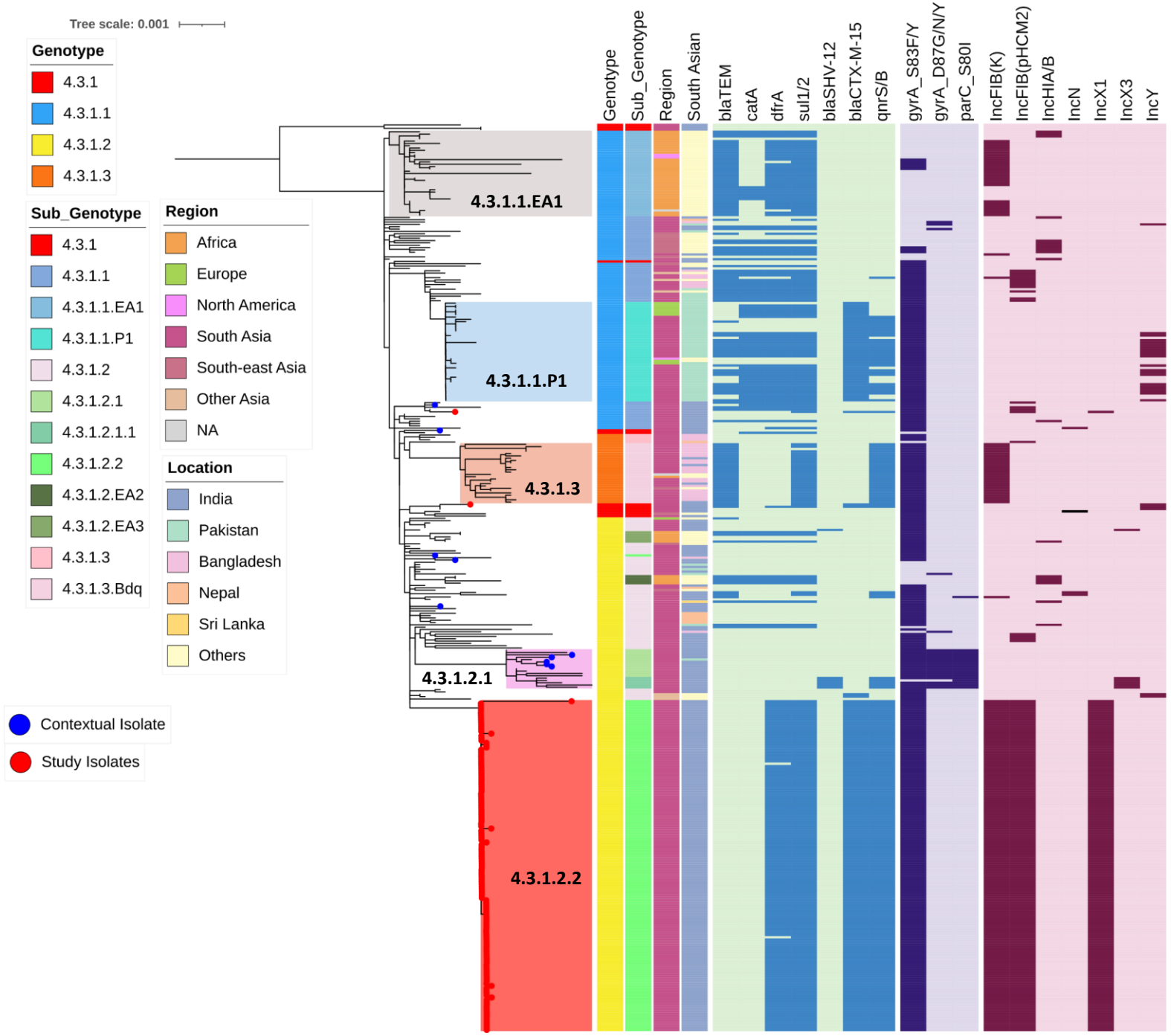
Suppl. Fig 2: Phylogenetic structure of *S* Typhi showing ceftriaxone resistant isolate sequences in a global context. Maximum likelihood core genome phylogenetic tree

inferred for n=571 *S* Typhi sequences (including n=142 from this study and n=11 contextual isolates). The tree was derived from 8980 core genome SNPs mapped against the reference genome of S Typhi CT18 (Accession No: AL513382.1) using Snippy and rooted to the outgroup isolates (Genotype 0.1). Isolates belong to H58 lineage are shaded in Grey (#dcd4d4). Ring refer to major *S*. Typhi genotypes Non-H58, 4.3.1, 4.3.1.1, 4.3.1.2, 4.3.1.3 and respective colour codes for all the variables are given in the inset legend. Red-coloured branch symbols reflect the position of study isolates. Blue-coloured branch symbols reflect the position of contextual isolates The scale bar indicates substitutions per site. The tree was visualized and labeled using iTOL (https://itol.embl.de/).

Suppl Fig: 3 An unrooted maximum-likelihood phylogenetic tree of 247 global H58 *S*. Typhi depicting the phylogenetic position of ceftriaxone resistant *S*. Typhi isolates from Ahmedabad. Lineages are represented by various colored branches. (b) A higher-resolution diagram of H58 *S*. Typhi depicting the core SNP variation between branches. The ceftriaxone resistant isolates (red branches) are separated by 7 SNPs from the rest of the branch (black).

Table: 1: Molecular fingerprint of cephalosporin resistant *S*. Typhi in India.

Country of origin	Lineage (Genotype)	Location	Location Isolate Id & Accession No	Year	Acquired resistance			Chromosomal resistance (QRDR)	Ceftriaxone MIC (µg/ml)
					Betalactamase	Other AMR determinants	Plasmids		
India	H58 Lineage II (4.3.1.2)	Vellore (n = 1)	CMCST_CEPR_1 CP053702 and CP053703	2015	TEM-1B, DHA	qnrB4, aac6', sul1	IncN	S83F, E84K	>16
	H58 Lineage II (4.3.1.2.1)	Mumbai (n = 16) & Kolkata (n=1)	458,426 (MQUO00000000) 430,040 (MQUM00000000) 429,038 (MQUL00000000) LHST_2018 (CP052767 and CP052768)	2016-2019	blaSHV-12	aac(6')-laa, qnrB7	IncX3	S83F, D87N, S80I	16
			PRJEB29740						4
			KOL585(JABWHV000000000)		blaSHV-12	aac(6')-laa	IncX3	S83Y	>32
	H58 Lineage (4.3.1)	Gurgaon, Haryana (n = 2)	Gurgaon01 (ERR3527963)	2019	blaTEM-1B, blaCTX-M-15	dfrA14, sul1/2, tetA, qnrS1, aac(6')-laa, aph6', aph3'	IncY	S83Y	–
			Gurgaon02 (ERR3527964)			aac(6")laa, tetA	IncY (lost while sub culturing)		–
		Mumbai (n=1)	1CTRMumbai*	2022		aac(6')-laa, qnrS1, sul2, aph3, aph6	IncY		>512
	H58 Lineage II (4.3.1.2.2)	Ahmedabad & Vadodara (n=141)	PRJEB62461	2022 – 2023	blaCTX-M-15	aac(6')-laa, qnrS1, sul2, dfrA14, tet(A), aph3, aph6	IncFIB(pHCM2), IncFIB(K), IncX1	S83F	>512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4.3.1.1
4.3.1.2
4.3.1.3
Sub_Genotype
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4.3.1.1
4.3.1.1.EA1
4.3.1.1.P1
4.3.1.2
4.3.1.2.1
4.3.1.2.1.1
4.3.1.2.2
4.3.1.2.EA2
4.3.1.2.EA3
4.3.1.3
4.3.1.3.Bdq
Region
Africa
Europe
North America
South Asia
South-east Asia
Other Asia
NA
Location
India
Pakistan
Bangladesh
Nepal
Sri Lanka
Others
Contextual Isolate
Study Isolates
4.3.1.1.EA1
4.3.1.1.P1
4.3.1.3
4.3.1.2.1
4.3.1.2.2
Genotype
Sub_Genotype
Region
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catA
dfrA
sul1/2
blaSHV-12
blaCTX-M-15
qnrS/B
gyrA_S83F/Y
gyrA_D87G/N/Y
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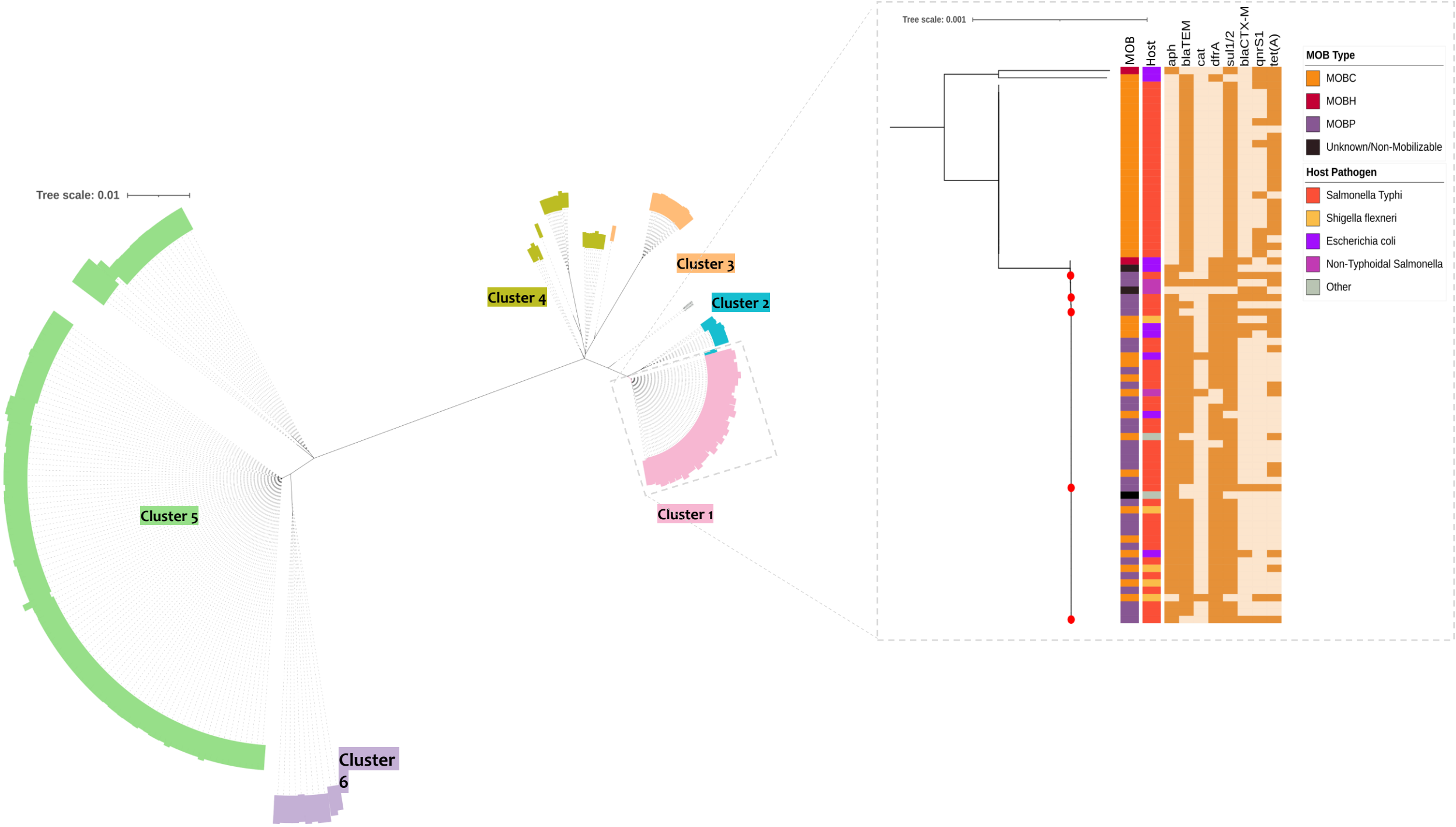
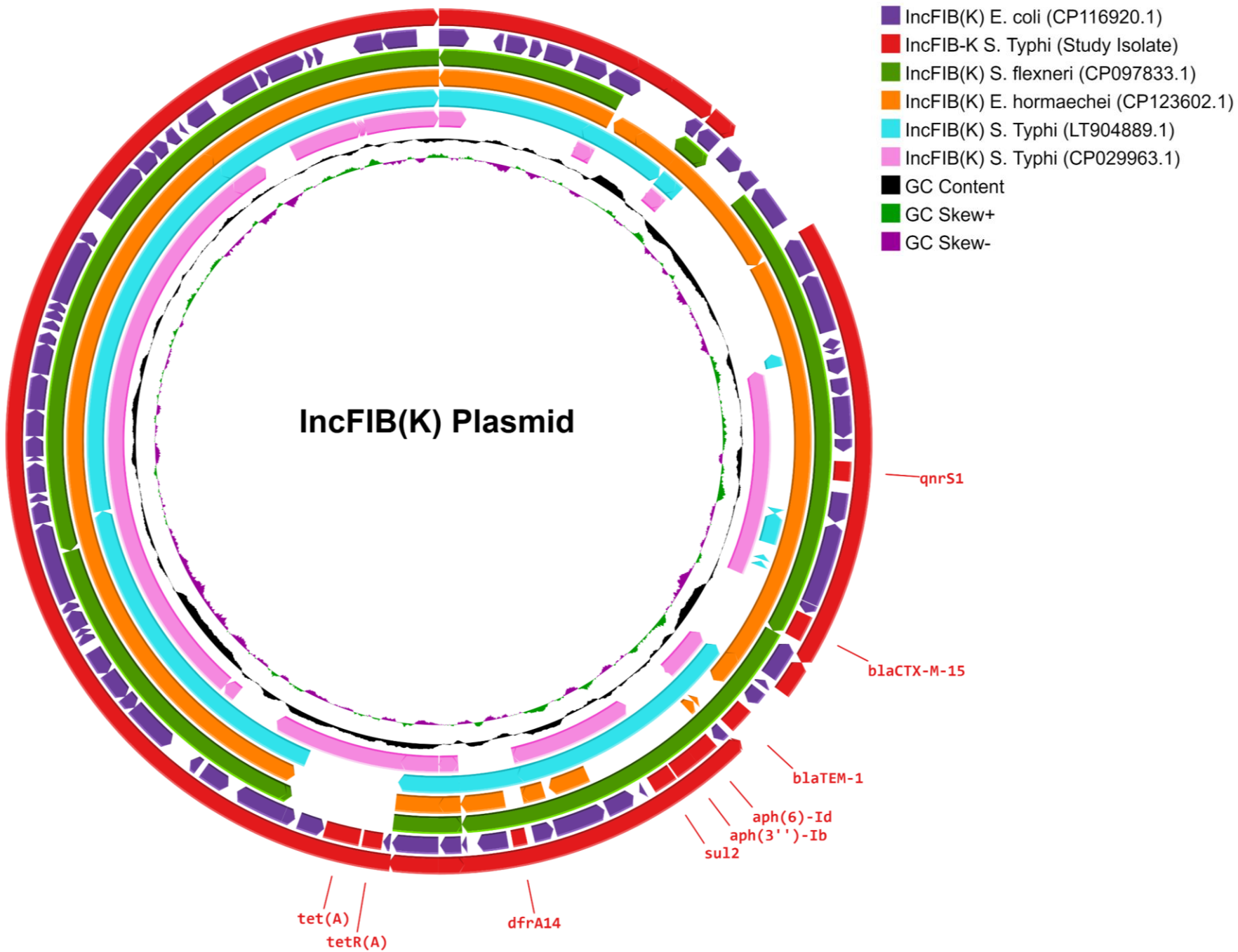
IncFIB(K)
IncFIB(pHCM2)
IncHIA/B
IncN
IncX1
IncX3
IncY


Tree scale: 0.01
Cluster 1
Cluster 2
Cluster 3
Cluster 4
Cluster 5
Cluster 6
Tree scale: 0.001
MOB
Host
aph
blaTEM
cat
dfrA
sul1/2
blaCTX-M
qnrS1
tet(A)
MOB Type
MOBC
MOBH
MOBP
Unknown/Non-Mobilizable
Host Pathogen
Salmonella Typhi
Shigella flexneri
Escherichia coli
Non-Typhoidal Salmonella
Other


IncFIB(K) E. coli (CP116920.1)
IncFIB-K S. Typhi (Study Isolate)
IncFIB(K) S. flexneri (CP097833.1)
IncFIB(K) E. hormaechei (CP123602.1)
IncFIB(K) S. Typhi (LT904889.1)
IncFIB(K) S. Typhi (CP029963.1)
GC Content
GC Skew+
GC Skew-
IncFIB(K) Plasmid
qnrS1
blaCTX-M-15
blaTEM-1
aph(6)-Id
aph(3'')-Ib
sul2
dfrA14
tet(A)
tetR(A)

Country of origin	Lineage (Genotype)	Location	Location Isolate Id & Accession No	Year	Acquired resistance			Chromosomal resistance (QRDR)	Ceftriazone MIC (µg/ml)
					Betalactamase	Other AMR determinants	Plasmids		
India	H58 Lineage II (4.3.1.2)	Vellore (n = 1)	CMCST_CEPR_1 CP053702 and CP053703	2015	TEM-1B, DHA	qnrB4, aac6', sul1	IncN	S83F, E84K	>16
	H58 Lineage II (4.3.1.2.1)	Mumbai (n = 16) & Kolkata (n=1)	458,426 (MQUO00000000) 430,040 (MQUM00000000) 429,038 (MQUL00000000) LHST_2018 (CP052767 and CP052768)	2016-2019	blaSHV-12	aac(6')-laa, qnrB7	IncX3	S83F, D87N, S80I	16
			PRJEB29740						4
			KOL585(JABWHV000000000)		blaSHV-12	aac(6')-laa	IncX3	S83Y	>32
	H58 Lineage (4.3.1)	Gurgaon, Haryana (n = 2)	Gurgaon01 (ERR3527963) Gurgaon02 (ERR3527964)	2019	blaTEM-1B, blaCTX-M-15	dfrA14, sul1/2, tetA, qnrS1, aac(6')-laa, aph6', aph3'	IncY	S83Y	–
						aac(6")laa, tetA	IncY (lost while sub culturing)		–
		Mumbai (n=1)	1CTRMumbai*	2022		aac(6')-laa, qnrS1, sul2, aph3, aph6	IncY		>512
	H58 Lineage II (4.3.1.2)	Ahmedabad n=141	2CTRGujarat*	2022 – 2023	blaCTX-M-15	aac(6')-laa, qnrS1, sul2, dfrA14, tet(A), aph3, aph6	IncFIB(pHCM2), IncFIB(K), IncX1	S83F	>512
			116_NSRL* 120_NSRL, 153_NSRL, 154_NSRL, 19_NSRL, 22_NSRL, 42_NSRL						
			Zydus 1 (ZY2304100302) Zydus 2 (ZY2304120004) Zydus 3 (ZY230502087)						