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

Tharani Priya T[1#], Jobin John Jacob[1#], Aravind V[1], Monisha Priya T[1], Yesudoss M[1], Bhavini Shah[2], Veena Iyer[3], Anand Shah[4], Anushree Gaigawale[5], Pooja Patel[6], Ankur Mutreja[7], Jacob John[1], Gagandeep Kang[1], Balaji Veeraraghavan[1*]

[1] Christian Medical College, Vellore, Tamilnadu, India

[2] Neuberg Supratech Reference Laboratories, Ahmedabad, Gujarat, India

[3] Indian Institute of Public Health Gandhinagar, Gujarat, India

[4] Zydus Hospitals, Ahmedabad, Gujarat, India

[5] Suburban Diagnostics India Pvt. Ltd, Andheri (w), Mumbai, India

[6] Pathocare Pathology Laboratory, Vadodara, Gujarat, India

[7] Cambridge Institute of Therapeutic Immunology and Infectious Disease, Jeffrey Cheah Biomedical Centre, Cambridge, UK

[#]Contributed equally to this work

*Address correspondence to

Dr. Balaji Veeraraghavan
Professor
Department of Clinical Microbiology
Christian Medical College, Vellore – 632 004
Tamil Nadu, India
Ph: +91 9442210555
E-mail: vbalaji@cmcvellore.ac.in

Abstract

The emergence and spread of *Salmonella* Typhi resistant to third generation cephalosporins are a serious global health concern. In this study, we have genomically characterized twelve cephalosporin resistant *S*. Typhi strains isolated from India. Comparative genome analysis of study isolates revealed the emergence of a new clone of ceftriaxone-resistant *S*. Typhi containing 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* and *tetA*. Phylogenetic analysis of strains revealed that a single isolate belongs to a clade corresponding to genotype 4.3.1 and isolates from Ahmedabad (*n=11*) belong to a distinct subclade within genotype 4.3.1.2 (H58 lineage II). SNP-based phylogenetic analysis of the core genes in IncFIB(K) revealed the plasmid backbone is closely related to that of IncFIB(K) from other Enterobacterales. The findings suggest that H58 lineage II can acquire MDR plasmids from other Enterobacteriales if compensatory evolution balances the cost of carrying the plasmids. Though, like previously reported, exposure to the third generation cephalosporins during the treatment may have selected these variants, this could indicate the beginning 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 between 28,000 and 161,000 deaths annually (WHO, 2023; Mogasale et al 2014). In particular, high-burden countries in South Asia contribute approximately 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 one of the biggest concerns around the world (Marchello et al., 2020). Consequently, fluoroquinolones became the drugs of choice in the treatment of MDR *S*. Typhi. However, reports 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 of *S*. Typhi isolates. The current population structure of *S*. Typhi strains identifies 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 IncHI1 plasmid carrying multiple antimicrobial resistance determinants (*bla*$_{TEM}$, *cat*, *dhfr*, *sul2*) (Holt et al., 2011; da Silva et al., 2022). Similarly, following the introduction of ciprofloxacin as the drug of choice to treat typhoid, a new sublineage (genotype 4.3.1.2 or H58 lineage 2) of fluoroquinolone non-susceptible (FQNS) strains carrying double/ triple-point mutations in the quinolone resistance determining regions (QRDRs) have emerged (Pham et al., 2016). Further, the recent emergence of a new sub-lineage P1 (4.3.1.1.P1) of *S*. Typhi in Pakistan, was of concern due to its resistance to third-generation cephalosporins. XDR isolates belonging to this sub-lineage carried *bla*$_{CTX-M-15}$ mobilized by IncY plasmids (Klemm et al., 2018). To make it worse, emergence of point mutations in the *acrB* gene (R717Q/L) have caused resistance to azithromycin, the last oral therapy option (Hooda et al., 2019; Duy et al., 2020).

The genomic epidemiology of *S*. Typhi has already 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., 2022). Contrary to this, the population structure of *S*. Typhi in Africa consists primarily of non-H58 lineages or regional variants of H58 (Park et al., 2018). Though XDR lineage (4.3.1.1P1) has become dominant in Pakistan (70% in 2020), it has not yet been established elsewhere. For instance, ceftriaxone resistant (Cef^{R}) *S*. Typhi strains reported from India primarily belonged to sub-clade of 4.3.1.2 (recently renamed as 4.3.1.2.1) 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 (Inc) types have been reported from *S*. Typhi strains 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 twelve cases of ESBL-positive *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 settings

The isolates used in this study were obtained from Neuberg Supratech Reference Laboratory (NSRL) and three other clinical diagnostic laboratories located in Ahmedabad and Mumbai, India between September 2022 and March 2023 (Suppl Fig. 1). NSRL served as a reference laboratory for hospitals, clinics, and diagnostic centers in the region and received blood samples from them. Additionally, the site also collected data from the labs at three other private-sector, tertiary care hospitals in the city. Samples, once confirmed as *S.* Typhi by conventional biochemical tests were shipped to the central laboratory at Department of Clinical Microbiology, Christian Medical College, Vellore for confirmation and further testing.

Phenotypic characterization

S. Typhi isolates transferred to the central laboratory were re-identified by conventional biochemical tests and serotyping using commercial anti-sera (BD Difco) based on the Kauffmann-White scheme according to the manufacturer's instructions. The phenotypical antimicrobial susceptibility testing was performed by 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 include ampicillin (10µg), Chloramphenicol (30µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), ciprofloxacin (5µg), pefloxacin (5µg), ceftriaxone (30µg) and cefixime (5µg). 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) 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 an Illumina iSeq-100 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 three isolates were subjected to sequencing using Nanopore Technology (ONT). The libraries for sequencing were prepared using Ligation Sequencing Kit (SQK-LSK114) (Oxford Nanopore Technologies, 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 v2. and subsequent reads were used to estimate sequence coverage. The high quality reads 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 complete genome hence generated 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=412*), 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). 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/). Genome collection of *S*. Typhi genotypes were further characterized using the genotyphi scheme as described on the GitHub repository (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 AMR finder plus database v3.11.14. Plasmids were identified and typed using PlasmidFinder

(https://cge.cbs.dtu.dk/services/PlasmidFinder/) and Mobtyper pipelines. The complete list of genomic sequence data is available in the Supplementary Data File.

Phylogenetic analysis

The assembled contigs (*n=424*) 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-NG v1.2.0 under the GTR+G4 model (200 bootstraps). The resulting phylogenetic tree was visualised and annotated using the Interactive Tree of Life software (iTOL v.3).

Plasmid characterization

We manually collected a total of 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 comprehensive genome metadata reported elsewhere (Carey et al., 2022). 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

S. Typhi isolates (*n=12*) examined here were collected from febrile patients attending private health care facilities in Ahmedabad and Mumbai in India. Eleven of the 12 isolates came from diagnostic laboratories located in Ahmedabad, and one isolate was collected from Mumbai Supplementary Fig 1. The isolates were re-confirmed as *S.* Typhi by biochemical testing and serotyping upon arrival at the clinical microbiology lab at CMC Vellore. The antimicrobial susceptibility profiling showed all the study isolates from Ahmedabad being resistant to ampicillin, ciprofloxacin, ceftriaxone and trimethoprim-sulfamethoxazole and susceptible to chloramphenicol, and azithromycin. The Mumbai isolate showed similar AST profile except that it was susceptible to trimethoprim-sulfamethoxazole. In addition, the minimum inhibitory concentration of ceftriaxone, was determined and found to be >512 µg/ml.

Population structure of *S*. Typhi

The population structure of ceftriaxone resistant *S.* Typhi (*n=12*) 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=412*) revealed positioning of isolates in clades corresponding to genotype 4.3.1 (H58) (Fig. 1). Sublineage level analysis indicated that *S.* Typhi isolates from Ahmedabad are closely related and were positioned in a distinct subclade within genotype 4.3.1.2 (H58 lineage II). The *S*. Typhi isolate from Mumbai belonged to the subclade 4.3.1 and were closely related to ceftriaxone resistant *S*. Typhi isolates reported from Gurgaon, India (Fig 2**)**.

Further AMR gene analysis revealed that the Mumbai isolate carried $bla_{\text{CTX-M-15}}$, $bla_{\text{TEM-1B}}$, *qnrS1*, and *sul2* on the IncY plasmid. On the other hand, the cluster of isolates from Ahmedabad carried $bla_{\text{CTX-M-15}}$, *qnrS1*, *sul2*, *dfrA14*, *tet(A)* on IncFIB(K) plasmid. Mutation analysis on the QRDR region showed single point mutations in *gyrA*: S83Y (Mumbai isolate) and *gyrA*:S83F (Ahmedabad cluster) 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 consist of IncFIB(K) plasmids (*n=276*) representing major Enterobacterales 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 eleven newly sequenced IncFIB(K) plasmids, a final set of n=288 plasmid sequence 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 3**)**. Within the plasmid phylogeny, IncFIB(K) plasmids carried by the study isolates belonged to cluster 1 represented by multiple bacterial host. 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 4).

Discussion:

One of the salient features of the SEFI phase 2 surveillance was to spot the emergence of a new ceftriaxone resistant *S*. Typhi clone from India. Phenotypic characterization revealed that the emergent clone is also resistant to ampicillin, ciprofloxacin and trimethoprim-sulfamethoxazole but susceptible to chloramphenicol (Table 1). The resistance profile of the study isolates differs from that of the classical XDR strains, which are resistant to the first-line agents, fluoroquinolones and ceftriaxone (Klemm at al., 2018). Here we attempted to uncover the population structure of the emerging *S*. Typhi clone as well as the genetic background of the plasmid transfer by whole genome sequencing (WGS) and comparative genome analysis.

Analysis of *S*. Typhi genome sequences reveal that ceftriaxone resistant strains from Ahmedabad (*n=11*) were of H58 lineage II (genotype 4.3.1.2) that is characterized by

chromosome-mediated quinolone resistance (Fig. 1 and 2). The predominance of the same genotype in India has already been reported by multiple genome surveillance studies (Wong et al., 2015; Pragasam et al., 2020; Britto et al., 2020; Pragasam et al., 2021). However, circulating *S*. Typhi strains in western India, particularly in Ahmedabad, were not adequately represented in these genomic surveillance studies. Therefore, ten contextual (ceftriaxone-susceptible) Ahmedabad isolates were sequenced over a comparable period of time for the purposes of comparison. Nine of the ten contextual isolates were located closer to the ceftriaxone resistant strains and separated by 7 SNPs (Suppl Fig: 2). This suggests that the endemic H58 lineage II clone circulating locally may have acquired an ESBL-encoding AMR plasmid. Meanwhile, isolates closer to the Mumbai strain were ceftriaxone resistant *S.* Typhi belonging to genotype 4.3.1 previously detected in circulation in Delhi NCR area, India (Sah et al., 2020).

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 harboured 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* and *tetA* (Fig. 4) Considering the restricted host access of *S.* Typhi, and high fitness cost imposed by plasmid to the host bacterium this was an unexpected event.

Evolutionary history of *S.* Typhi has been attributed to genome degradation events, as evidenced by the accumulation of deletions and inactivating mutations within coding sequences (Holt et al., 2008; Wong et al., 2015). The findings of these studies were supported by comparative genomic studies (McClelland et al., 2004; Holt et al., 2009) as well as very recently by a pan-genome analysis (Laing et al., 2017) where genome degradation events

rather than gene acquisition were considered as the primary driver behind the evolution of *S*. Typhi. Additionally, fitness costs associated with plasmid carriage have also been considered to be a contributing factor to *S*. Typhi's genetic stability (Jacob et al., 2021). Hence, in the absence of antibiotic selection pressure, *S*. Typhi has shown to be genetically conserved with minimal genomic variation due to SNPs, recombination or plasmid acquisitions (Baddam et al., 2014).

However, the trend and understanding of evolution of *S*. Typhi has been changed with the emergence of resistant phenotypes. Both MDR and XDR strains acquired large plasmids (such as IncHI1 and IncY), which are commonly believed to impose a fitness burden on the organism (Wain et al., 2003; Klemm et al., 2018). But the plasmid-carrying clones have undergone compensatory evolution, thereby conferring a fitness advantage over their plasmid-free ancestors and resulting in expansion of these phylogenetic lineages (Wong et al., 2015). For instance, the global expansion of MDR *S.* Typhi (genotype 4.3.1.1) following the acquisition of IncHI1 plasmid has given the strains a selective advantage over other genotypes (Park et al., 2018). Subsequently, the chromosomal integration of the MDR composite transposon has enabled the strains to become plasmid-free, therefore stabilizing the MDR phenotype for the acquisition of new plasmids (Dyson et al., 2019). Later in Pakistan, the stabilized MDR genotype (4.3.1.1) has become XDR clone (4.3.1.1.P1) by acquiring IncY plasmid harbouring $bla_{\text{CTX-M-15}}$ and *qnrS1* (Klemm et al., 2018). Therefore, it is hypothesized that H58 lineage I, with a chromosomal MDR transposon and a single QRDR mutation (S83F) can acquire and stably maintain plasmids harboring AMR determinants.

Interestingly the Indian isolates have evolved through the expansion of H58 lineages in India has mainly been driven by lineage II isolates (genotype 4.3.1.2) which carries resistance mutations in the fluroquinolone target genes (*gyrA* and *parC*). The dominance of lineage with reduced susceptibility to fluoroquinolones can be attributed to the high fluoroquinolone

exposure in the region (Dahiya et al., 2014). Notably, these QRDR double/ triple mutant strains rapidly displaced other lineages as a result of the fitness advantage gained during the evolutionary process (Baker et al., 2013). As a result, H58 lineage II isolates are likely to limit the acquisition of plasmids due to the high fitness cost imposed during the plasmid carriage (Jacob et al., 2021). Although previously H58 lineage II isolates were reported to carry plasmids that conferred ceftriaxone resistance, the plasmids were subsequently removed by purifying selection. Specifically, there have been sporadic isolates of the IncX3 and IncN plasmids carrying H58 lineage II isolates between 2015 and 2019. However, the plasmid-free isolates likely outcompeted plasmid-bearing isolates because of the fitness cost. Despite our expectations of rare plasmid carriage in H58 lineage II, a newly circulating clone of ceftriaxone-resistant isolates carrying three plasmids has been reported from Ahmedabad India.

We hypothesize that the emergence of ceftriaxone-resistant isolates from Ahmedabad India could be the result of a recent event of acquisition of multiple plasmids from other Enterobacteriaceae donor. The SNP-based phylogeny of the core genes in IncFIB(K) plasmid backbone identified six major clusters within the collection (Fig. 3). Based on a closer examination of cluster 2, 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. 4). 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 confer resistance to last-line antibiotics poses a serious problem on typhoid treatment and management. In view of the increase in resistance

to third generation cephalosporins, azithromycin is the only 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. Among areas where MDR or XDR *S*. Typhi strains are endemic, the roll-out of typhoid conjugate vaccines (TCVs) is an additional effective method of preventing typhoid fever.

Conclusion

Comparative genome analysis of twelve 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 which are related to antibiotic resistance, it becomes apparent that *S*. Typhi continues to improve its ability to remodel its genome. Our data adds more shreds of evidence of how H58 lineage II *S*. Typhi previously considered to have a stable genome may have undergone rapid evolution by acquiring MDR plasmids. Though exposure of third generation cephalosporins during the treatment may have selected 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: Phylogenetic structure of *S* Typhi showing ceftriaxone resistant isolate sequences in a global context. Maximum likelihood core genome phylogenetic tree inferred for n=425 *S* Typhi sequences including n=12 from this study and representative global genomes collection. The tree was derived from 9091 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 Red (#EA9999). Ring refer to major *S*. Typhi genotypes Non-H58, 4.3.1, 4.3.1.1, 4.3.1.1EA1, 4.3.1.1P1, 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. The scale bar indicates substitutions per site. The tree was visualized and labeled using iTOL (https://itol.embl.de/).

Fig 2: 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 3: (a) **An unrooted Maximum likelihood phylogenetic tree of global IncFIB(K) plasmids based on alignment of core genes.** Phylogenetic clustering of different lineages are 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 Ahmedabad 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.

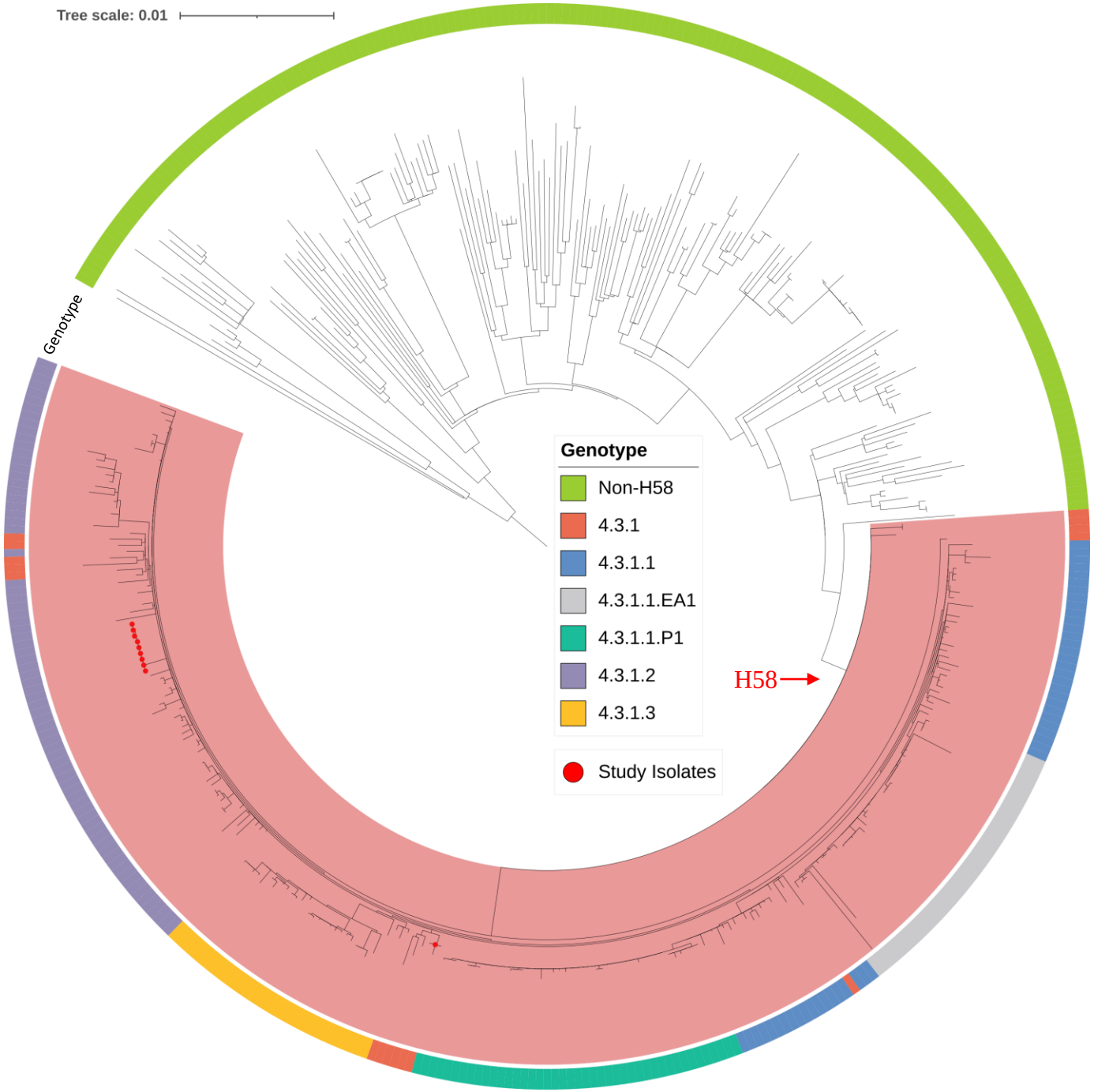
Fig 4: Comparison of IncFIB(K) plasmid. IncFIB(K) plasmid from ceftriaxone resistant *S*. Typhi isolates from Ahmedabad is compared to plasmid of same incompatibility group from other Enterobacteriales using Proksee (https://proksee.ca/). GC-content and GC-skew of the reference are depicted in the two 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 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)	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)	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)	Ahmedabad n=11	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)						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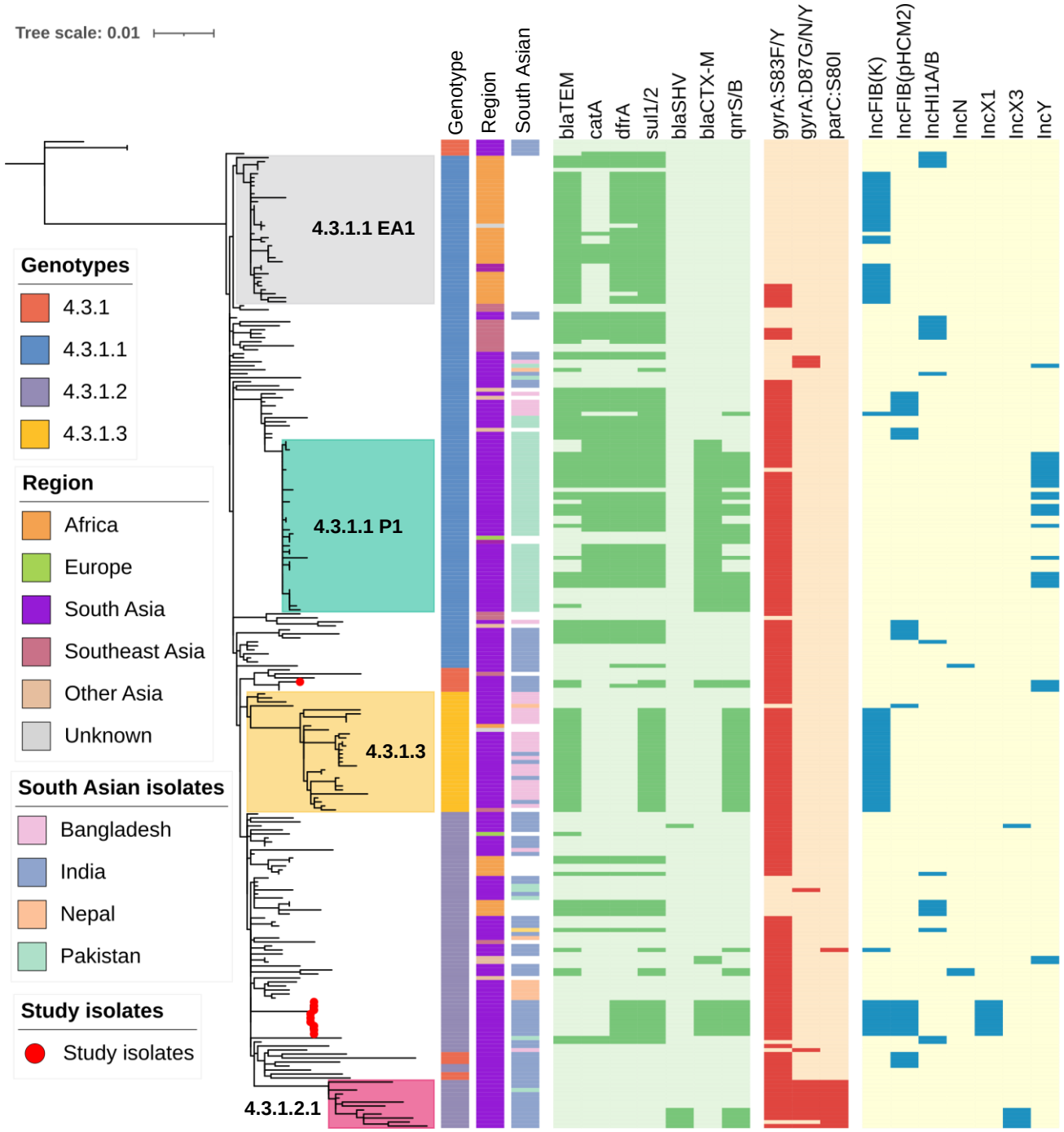
Genotype
Genotype
Non-H58
4.3.1
4.3.1.1
4.3.1.1.EA1
4.3.1.1.P1
4.3.1.2
4.3.1.3
Study Isolates
H58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Genotype
Region
South Asian
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catA
dfrA
sul1/2
blaSHV
blaCTX-M
qnrS/B
gyrA:S83F/Y
gyrA:D87G/N/Y
parC:S80I
IncFIB(K)
IncFIB(pHCM2)
IncHI1A/B
IncN
IncX1
IncX3
IncY
4.3.1.1 EA1
4.3.1.1 P1
4.3.1.3
4.3.1.2.1
Genotypes
4.3.1
4.3.1.1
4.3.1.2
4.3.1.3
Region
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Europe
South Asia
Southeast Asia
Other Asia
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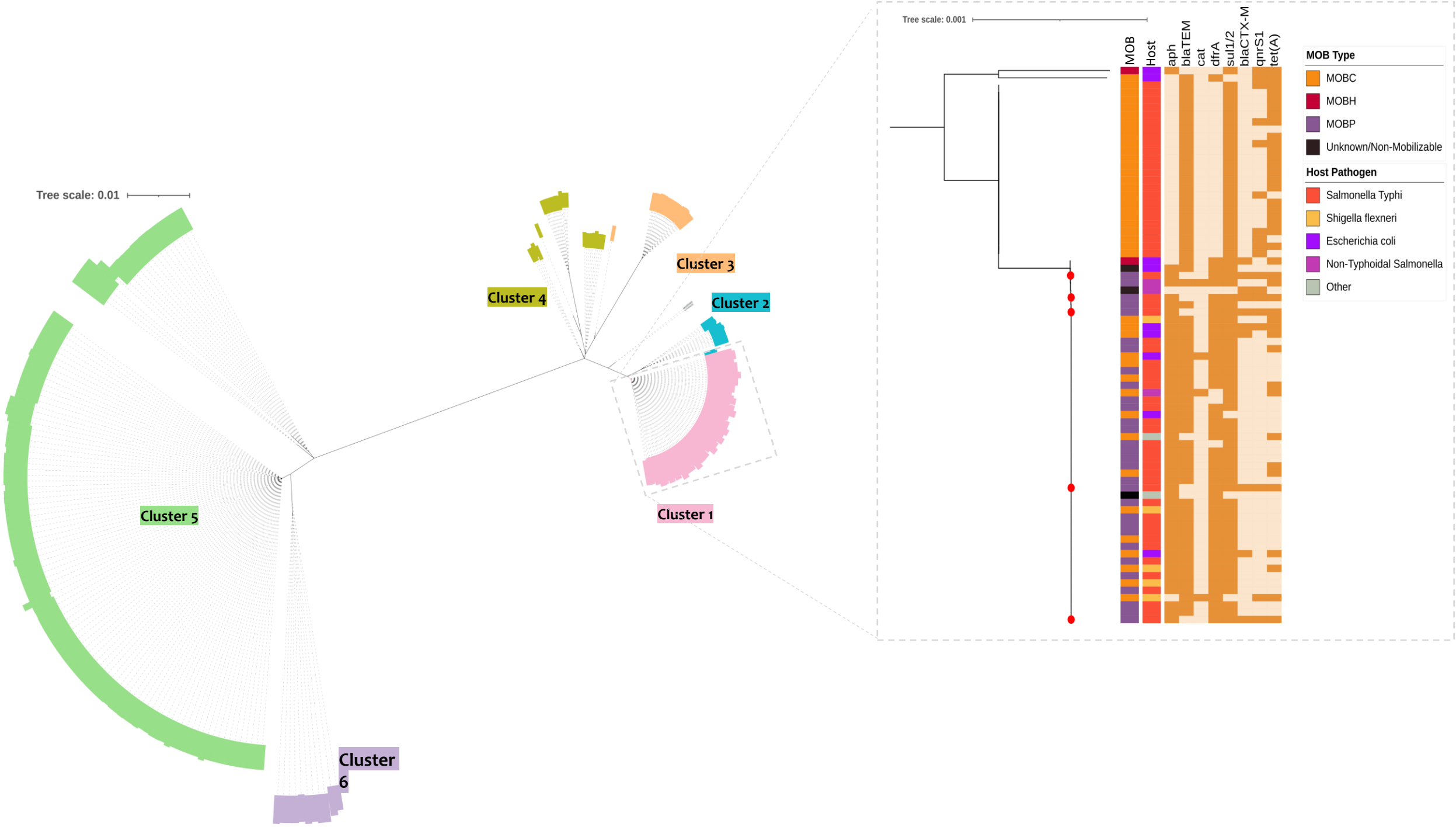
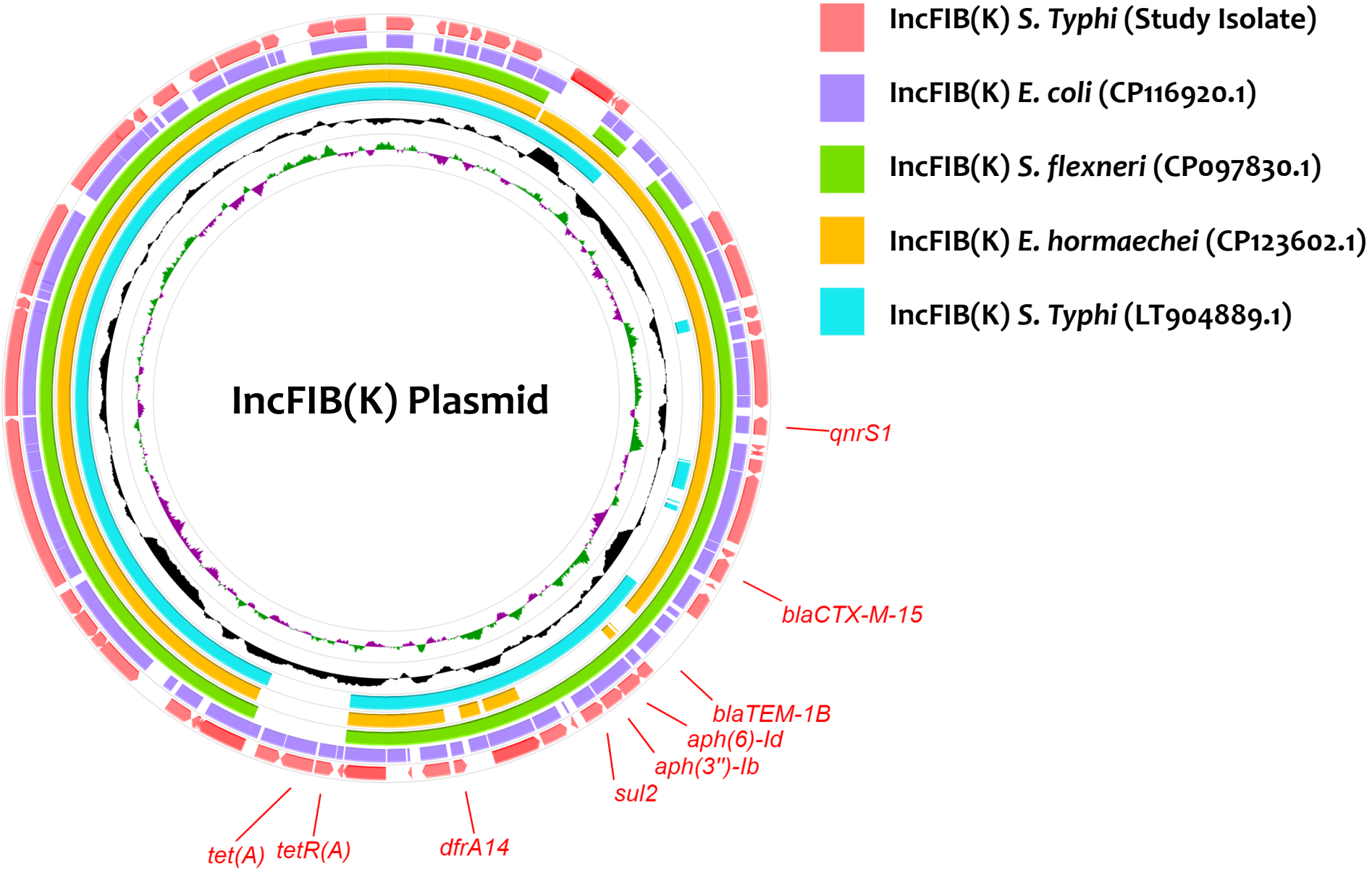
South Asian isolates
Bangladesh
India
Nepal
Pakistan
Study isolates
Study isolates


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) S. Typhi (Study Isolate)
IncFIB(K) E. coli (CP116920.1)
IncFIB(K) S. flexneri (CP097830.1)
IncFIB(K) E. hormaechei (CP123602.1)
IncFIB(K) S. Typhi (LT904889.1)
IncFIB(K) Plasmid
qnrS1
blaCTX-M-15
blaTEM-1B
aph(6)-Id
aph(3")-Ib
sul2
dfrA14
tetR(A)
tet(A)