

1 **Title:** Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in  
2 Bangladesh from 2004 to 2016

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21

## 22 **Abstract**

### 23 **Background**

24 Multi-drug resistant typhoid fever remains an enormous public health threat in low and middle-  
25 income countries. However, we still lack a detailed understanding of the epidemiology and  
26 genomics of *S. Typhi* in many regions. Here we have undertaken a detailed genomic analysis of  
27 typhoid in Bangladesh to unravel the population structure and antimicrobial resistance patterns in  
28 *S. Typhi* isolated in between 2004-2016.

29

### 30 **Principal findings**

31 Whole genome sequencing of 202 *S. Typhi* isolates obtained from three study locations in urban  
32 Dhaka revealed a diverse range of *S. Typhi* genotypes and AMR profiles. The bacterial  
33 population within Dhaka were relatively homogenous with little stratification between different  
34 healthcare facilities or age groups. We also observed evidence of transmission of Bangladeshi  
35 genotypes with neighboring South Asian countries (India, Pakistan and Nepal) suggesting these  
36 are circulating throughout the region. This analysis revealed a decline in H58 (genotype 4.3.1)  
37 isolates from 2011 onwards, coinciding with a rise in a diverse range of non-H58 genotypes and  
38 a simultaneous rise in isolates with reduced susceptibility to fluoroquinolones, potentially  
39 reflecting a change in treatment practices. We identified a novel *S. Typhi* genotype, subclade  
40 3.3.2 (previously defined only to clade level, 3.3), which formed two localised clusters  
41 (3.3.2.Bd1 and 3.3.2.Bd2) associated with different mutations in the Quinolone Resistance  
42 Determining Region (QRDR) of gene *gyrA*.

43

## 44 **Significance**

45 Our analysis of *S. Typhi* isolates from Bangladesh isolated over a twelve year period identified a  
46 diverse range of AMR profiles and genotypes. The observed increase in non-H58 genotypes  
47 associated with reduced fluoroquinolone susceptibility may reflect a change in treatment practice  
48 in this region and highlights the importance of continued molecular surveillance to monitor the  
49 ongoing evolution of AMR in Bangladesh. We have defined new genotypes and lineages of  
50 Bangladeshi *S. Typhi* which will facilitate identification of these emerging AMR clones in future  
51 surveillance efforts.

52

## 53 **Author Summary**

54 Typhoid fever, caused by *Salmonella enterica* serovar Typhi, is an acute and often life-  
55 threatening febrile illness in developing countries. Until recently, there have been limited studies  
56 focusing on the epidemiology and disease burden of typhoid in poor resource settings including  
57 Bangladesh. This study highlights the urgent need for sustained genomics based surveillance  
58 studies to monitor the population structure and ongoing evolution of AMR. Our data revealed a  
59 diverse range of *S. Typhi* genotypes and AMR patterns among 202 isolates collected from three  
60 urban areas in Dhaka. Moreover, we defined a new genotype, subclade 3.3.2 (previously typed  
61 only to clade level, 3.3) with two Bangladesh-localised clades 3.3.2.Bd1 and 3.3.2.Bd2 showing  
62 reduced susceptibility to fluoroquinolones. Our data shows a significant increase in non-H58  
63 genotypes carrying QRDR mutations from 2012 onwards, replacing MDR H58 genotypes. Our  
64 data suggest that a shift in treatment practice towards third generation cephalosporins to control

65 typhoid might be beneficial, in addition to the introduction of vaccination programs and  
66 improvements in water sanitation and hygiene (WASH) in Bangladesh.

67

## 68 **Introduction**

69 *Salmonella enterica* serovar Typhi (*S. Typhi*), the causative agent of typhoid fever, is a  
70 facultative intracellular and human restricted pathogen predominantly transmitted by the fecal-  
71 oral route. Typhoid remains an enormous public health threat in many developing countries due  
72 to inadequate access to safe water, poor sanitation systems and inappropriate use of antimicrobial  
73 drugs. It is estimated that typhoid fever affects 12-27 million people globally each year whereas  
74 in Bangladesh the overall incidence is estimated at between 292-395 cases per 100,000 people  
75 per year [1-5]. Multi-drug resistant (MDR) *S. Typhi*, defined as resistance to the first-line  
76 antibiotics ampicillin, chloramphenicol and trimethoprim-sulfamethoxazole, was first observed  
77 in the 1970s [6-9]. The more recent emergence of MDR *S. Typhi* with nalidixic acid resistance  
78 and reduced susceptibility to fluoroquinolones complicates treatment options. However, third  
79 generation cephalosporins such as ceftriaxone and cefixime have proven to be effective choices  
80 for treatment as resistance to cephalosporin in *S. Typhi* is still relatively rare. The first major  
81 outbreak with ceftriaxone resistance (extensively drug resistant, defined as resistant to three first-  
82 line drugs, fluoroquinolones and third-generation cephalosporin) was observed in Pakistan from  
83 2016 onwards [7, 10, 11].

84 The acquisition of antimicrobial resistance (AMR) genes by *S. Typhi* was historically associated  
85 with self-transmissible IncHI1 plasmids that harbor composite transposons [9, 12]. The global  
86 burden of MDR typhoid is driven to a significant degree by the dissemination of the highly

87 clonal, expanding haplotype H58 (genotype 4.3.1), which is now dominant in many endemic  
88 settings throughout Africa and Asia [7, 9, 13, 14]. H58 *S. Typhi* encoding nonsynonymous  
89 mutations in the quinolone resistance determining region (QRDR) of DNA gyrase genes *gyrA*  
90 and *gyrB* and DNA topoisomerase IV genes *parC* and *parE* have shown reduced susceptibility to  
91 fluoroquinolones [7, 15]. Studies on typhoid in Nepal have reported the evolution of a  
92 fluoroquinolone resistant H58 lineage II isolates carrying three QRDR mutations (*gyrA-S83F*,  
93 *gyrA-D87N*, and *parC-S80I*) responsible for the failure of a gatifloxacin treatment trial [6, 15].  
94 More recently, in Bangladesh, a new H58 lineage I triple QRDR mutant carrying three different  
95 mutations (*gyrA-S83F*, *gyrA-D87G*, and *parC-E84K*) has been observed [14]. This study also  
96 defined a H58 “lineage Bd” containing the sublineage “Bdq” that is characterized by a high  
97 median minimal inhibitory concentration (MIC) to ciprofloxacin (4 µg/mL) potentially involving  
98 a *qnrS* gene in addition to a *gyrA-S83Y* mutation [14].

99

100 The lack of credible surveillance data representing the actual disease burden of typhoid fever in  
101 Bangladesh presents a barrier to the implementation of control strategies. Thus, there is an urgent  
102 need for sustained genomics-based surveillance studies in poor resource settings like Bangladesh  
103 to monitor the pathogen population structure, transmission dynamics, AMR patterns and the  
104 impacts of control strategies such as vaccination programs. Here, we have used whole genome  
105 sequencing (WGS) to understand the population structure of *S. Typhi* isolated from three  
106 different urban areas of Dhaka, Bangladesh, between 2004 and 2016.

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109

## 110 **Methods**

### 111 **Ethics statement**

112 Ethical approval was obtained from Research Review Committee (RRC) and the Ethical Review  
113 Committee (ERC) of the International Centre for Diarrhoeal Disease Research, Bangladesh  
114 (icddr,b). Informed written consent and clinical information were taken from legal guardians of  
115 child participants and adult participants.

116

### 117 **Study settings and blood sample collection**

118 Dhaka is the capital city of Bangladesh and the most densely populated city with a population of  
119 over 18 million [16]. icddr,b is an international health research organization located at the  
120 Mohakhali area in Dhaka which runs two urban field sites at Kamalapur and Mirpur. Kamalapur  
121 is situated in southeast part of Dhaka, whereas Mirpur is located in northeast part of Dhaka  
122 metropolitan area. Both of these field sites are frequented by typhoid fever patients where  
123 sanitation systems are poor and access of safe drinking water is limited. This study was designed  
124 with these three urban areas inside Dhaka city: icddr,b Kamalapur field site, icddr,b Mirpur field  
125 site and icddr,b Dhaka hospital, Mohakhali. Suspected typhoid fever patients were enrolled from  
126 the three sites based on the criteria of fever at least 38°C with a minimum duration of 3 days.  
127 Blood samples (3 mL for children <5 years of age and 5 mL for others) from typhoid suspected  
128 patients were collected between 2004 to 2016 and were cultured using the automated BacT/Alert  
129 method [17, 18] for confirmation of typhoid fever.

130

131

## 132 **Bacterial isolation from blood culture**

133 Specimens from positive blood culture bottles were sub-cultured on MacConkey agar plate and  
134 incubated at 37°C for 18-24 hours. *S. Typhi* colonies were identified using standard biochemical  
135 test and slide agglutination test with *Salmonella*-specific antisera (Denka Sieken Tokyo, Japan)  
136 [17-19]. On the basis of blood culture confirmation result, we included all the available stored  
137 202 *S. Typhi* isolated from 2004 to 2016 in this study and subjected these to whole genome  
138 sequencing analysis.

139

## 140 **DNA extraction and whole genome sequencing**

141 Genomic DNA was extracted using the Wizard Genomic DNA Kit (Promega, Madison, WI,  
142 USA) according to the manufacturer's instructions. Index-tagged paired-end Illumina sequencing  
143 libraries with an insert size of 500 bp were prepared as previously described [20] and combined  
144 into pools each containing 96 uniquely tagged libraries. WGS was performed at the Wellcome  
145 Trust Sanger Institute using the Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA) to  
146 generate 125 bp paired-end reads. Sequence data quality was checked using FastQC  
147 (<http://www.bioinformatics.babraham.ac.uk/projects/fastqc>) to remove adapter sequences and  
148 low quality reads. Illumina sequence data was submitted to the European Nucleotide Archive and  
149 a full list of accession numbers for each isolate is summarised in **S1 Table**. Sequence data from  
150 88 Bangladeshi *S. Typhi* from Wong *et al.* 2016 [21], and a further 528 from Tanmoy *et al.* 2018  
151 [14], were also included for context in this study (raw sequence data are available in European  
152 Nucleotide Archive under study accessions ERP001718 and PRJEB27394, respectively).

153

## 154 **Read alignment and SNP analysis**

155 *S. Typhi* Illumina reads were mapped to the CT18 (accession no. AL513382) reference  
156 chromosome sequence [22] using the RedDog mapping pipeline (v1beta.10.3;  
157 <https://github.com/katholt/reddog>). RedDog uses Bowtie (v2.2.9) [23] to map reads to the  
158 reference genome, and SAMtools (v1.3.1) [24, 25] to identify SNPs that have a phred quality  
159 score above 30, and to filter out those SNPs supported by less than five reads, or with 2.5x the  
160 average read depth that represent putative repeated sequences, or those that have ambiguous base  
161 calls. For each SNP that passes these criteria in any one isolate, consensus base calls for the SNP  
162 locus were extracted from all genomes mapped, with those having phred quality scores under 20  
163 being treated as unknown alleles and represented with a gap character. These SNPs were then  
164 used to assign isolates to previously defined genotypes according to an extended *S. Typhi*  
165 genotyping framework using the GenoTyphi python script, available at  
166 <https://github.com/katholt/genotyphi> [6, 21]. Unique SNPs defining novel genotypes and  
167 lineages detected in the present study were manually extracted from allele tables output by  
168 RedDog using R, with SNPs responsible for non-synonymous mutations in highly conserved  
169 genes prioritised for lineage definitions.

170

171 Chromosomal SNPs with confident homozygous calls (phred score above 20) in >95% of the  
172 genomes mapped (representing a ‘soft’ core genome) were concatenated to form an alignment of  
173 alleles at 6,089 variant sites. SNPs called in prophage regions and repetitive sequences (354 kb;  
174 ~7.4% of bases in the CT18 reference chromosome, as defined previously) [21] or in



175 recombinant regions as detected by Gubbins (v2.3.2) [26] were excluded resulting in a final  
176 alignment of 4,395 chromosomal SNPs out of a total alignment length of 4,462,203 bp for 818  
177 Bangladeshi isolates. SNP alleles from *S. Paratyphi A* AKU1\_12601 (accession no: FM2000053)  
178 were included for the purpose of outgroup rooting the phylogenetic tree.

179

180 To provide global context, 1,560 additional *S. Typhi* genomes belonging to the genotypes found  
181 in Bangladesh [6, 7, 9, 14, 15, 21, 27] were subjected to both SNP calling and genotyping as  
182 described above, resulting in an alignment of 14,852 chromosomal SNPs.

183

184 SNP distances were calculated using the `dna.dist()` function in the Analysis of Phylogenetics and  
185 Evolution (ape) R package [28]. Shannon diversity was calculated using the `diversity()` function  
186 in the R package `vegan` [29]. Unless otherwise stated all statistical analyses were carried out in  
187 R [30].

188

## 189 **Phylogenetic analysis**

190 Maximum likelihood (ML) phylogenetic trees were inferred from the aforementioned  
191 chromosomal SNP alignments using RAxML (v8.2.8) [31]. A generalized time-reversible model  
192 and a Gamma distribution was used to model site-specific rate variation (GTR+  $\Gamma$  substitution  
193 model; GTRGAMMA in RAxML) with 100 bootstrap pseudoreplicates used to assess branch  
194 support for the ML phylogeny. The resulting phylogenies were visualized and annotated using  
195 Microreact [32] and the R package `ggtree` [33].

196

197

## 198 **Antimicrobial resistance gene identification and plasmid replicon analysis**

199 The mapping based allele typer SRST2 [34] was used in conjunction with the ARGannot [35]  
200 and PlasmidFinder [36] databases to detect acquired AMR genes and plasmid replicons,  
201 respectively. Where detected, isolates possessing IncHI1 plasmids were subjected to plasmid  
202 multilocus sequence typing (PMLST) [37] using SRST2 [34]. Where AMR genes were present  
203 without evidence of a known resistance plasmid replicon, raw sequence reads were assembled *de*  
204 *novo* using Unicycler (v0.3.0b) [38] and examined visually using the assembly graph viewer  
205 Bandage (0.8.1) [39] to inspect the composition and insertion sites of resistance-associated  
206 composite transposons. ISMapper (v2.0) [40] was run with default parameters to screen for  
207 insertion sites the transposases *IS1* (accession number J01730) and *IS26* (accession number  
208 X00011), relative to the CT18 reference sequence in order to identify the location of these in the  
209 chromosome of each Bangladeshi *S. Typhi* genome. Point mutations in the QRDR of genes *gyrA*  
210 and *parC* associated with reduced susceptibility to fluoroquinolones [15] were determined using  
211 GenoTyphi [6, 21].

212

## 213 **Results**

### 214 **Population structure of *S. Typhi* in Bangladesh**

215 A total collection of 202 Bangladeshi *S. Typhi* isolated between 2004 and 2016 (**S1 Table**) were  
216 subjected to Illumina whole genome sequencing, and were analysed together with 616 additional

217 Bangladeshi *S. Typhi* whole genome sequences from previous studies (isolated between 1998  
218 and 2016, see **S2 Table**) to provide a robust genomic context. The *S. Typhi* genomes were  
219 subjected to phylogenetic and GenoTyphi analysis, revealing that the pathogen population  
220 structure in Bangladesh is diverse with 17 distinct genotypes as summarised in **S3 Table** and  
221 **Fig. 1**. Eight *S. Typhi* subclades (genotypes 2.0.1, 2.1.7, 2.3.3, 3.0.1, 3.1.2, 3.2.2, 3.3.0, 4.3.1)  
222 and two undifferentiated isolates of major lineage 2 (genotype 2.0.0) were observed among our  
223 samples collected in Bangladesh between 2004 and 2016 (**Table 1**). The majority of our isolates  
224 (n=83, 41.1%) belonged to genotype 4.3.1 (haplotype H58), which has rapidly expanded in  
225 South Asia from the early 1990s. Previously defined major sublineages of H58 (lineage I and  
226 lineage II) [9] were present among the Bangladeshi isolates, with H58 lineage I (genotype  
227 4.3.1.1) isolates appearing dominant (n=63, 31.2%). Only a single H58 lineage II (genotype  
228 4.3.1.2) isolate (n=1, 0.50%) was present; this is somewhat surprising as this lineage is common  
229 in neighboring countries Nepal and India [6, 15]. In addition to H58 lineage I and II isolates, 19  
230 genetically undifferentiated H58 isolates (genotype 4.3.1 according to the framework of Wong *et*  
231 *al* 2016) were also observed. Further analysis of these revealed close clustering with 119 “H58  
232 lineage Bd” isolates reported by Tanmoy *et al.* 2018 [14], forming a monophyletic sister clade to  
233 H58 lineages I and II that we herein define as 4.3.1.3 (see **Fig. 1**). The 19 4.3.1.3 isolates from  
234 our collection possess the characteristic SNPs STY0054-C711A (position 561056 in CT18) and  
235 STY2973-A1421C (position 2849843 in CT18) that Tanmoy *et al* used to define this lineage  
236 [14]. We have therefore added the SNP STY0054-C711A to the GenoTyphi script (available at:  
237 <http://github.com/katholt/genotyphi>), to facilitate the detection of this lineage (genotype 4.3.1.3)  
238 in future WGS surveillance studies.

239

240 Genotypes 2.3.3 (n=30, 14.85%), 3.2.2 (n=44, 21.80%), 3.3.0 (n=28, 13.86%) were also  
241 relatively common in Bangladesh. Of the 818 Bangladeshi *S. Typhi* analysed, 119 (14.5%)  
242 formed a monophyletic sublineage within clade 3.3 that did not belong to any of the previously  
243 defined subclades (i.e. assigned genotype 3.3.0 in the existing scheme) and were closely related  
244 to other clade 3.3 isolates found in Nepal (median distance of ~70 SNPs) [6, 15] (see **S2 Fig** and  
245 interactive phylogeny available at <http://microreact.org/project/5GzJ7Umoz>). We herein assign  
246 these Bangladesh and Nepal *S. Typhi* to a novel genotype, subclade 3.3.2 (labeled in **Fig. 1**),  
247 which can be identified by the presence of a marker SNP STY3641-A224G (position 3498544 in  
248 CT18) that confers an amino acid mutation (Q75G) within the ST3641 encoded protein. This  
249 genotype has also been added to the GenoTyphi script to facilitate detection of subclade 3.2.2  
250 from WGS data in future studies.

251

252 **Table 1:** Genotypes present among 202 novel *S. Typhi* isolates from Dhaka, Bangladesh.

253 \*Novel genotypes described in this manuscript.

Genotype	No. of isolates (%)
2.0.0	2 (0.99%)
2.0.1	10 (4.95%)
2.1.7	2 (0.99%)
2.3.3	30 (14.85%)
3.0.1	1 (0.50%)
3.1.2	2 (0.99%)
3.2.2	44 (21.80%)
3.3.2*	28 (13.86%)

4.3.1.3*	19 (9.41%)
4.3.1.1	63 (31.19%)
4.3.1.2	1 (0.50%)

254

255 **Fig. 1. Bangladesh *S. Typhi* population structure with antimicrobial resistance.** Maximum  
256 likelihood outgroup-rooted tree of 816 Bangladeshi *S. Typhi* isolates. Branch colours indicate the  
257 genotype (as labelled). Inner ring indicates the position of the 202 Bangladesh *S. Typhi* from  
258 this study, the second and third rings indicate the presence of QRDR mutations, fourth ring  
259 indicates plasmids and the outer fifth ring indicates AMR genes coloured as per the inset legend.

260

#### 261 **Intra-country transmission dynamics within Bangladesh**

262 Geographical location data were available for the 202 novel *S. Typhi*, which were collected from  
263 three different sites inside Dhaka (icddr,b Kamalapur field site, icddr,b Mirpur field site and  
264 icddr,b Dhaka hospital Mohakhali). Detailed genotypic distribution of 202 isolates from these  
265 three study sites are shown in **Fig. 2**, and an interactive version of the phylogeny and map are  
266 available online at [https://microreact.org/project/sP2Uwk\\_DI](https://microreact.org/project/sP2Uwk_DI). Our data showed that genotypes  
267 2.3.3, 3.3.2, 3.2.2, 4.3.1.1 and 4.3.1.3 were present in all three study sites (**Fig. 2A**) and were  
268 intermingled in the phylogeny (**Fig. 2B**) suggesting circulation across the city. Two genotypes,  
269 2.0.1 and 2.1.7, were restricted to two of the three study locations (Kamalapur and icddr,b  
270 hospital, and Mirpur and icddr,b hospital, respectively); genotype 2.0.0, 3.1.2 and genotype  
271 3.0.1, 4.3.1.2 were found only in Kamalapur and icddr,b hospital, respectively.

272

273 Information on patient ages were available for 185 (91.6%) of the 202 *S. Typhi*, facilitating  
274 stratification by age groups (young children under 5 years of age, older children from 5 to 15  
275 years of age, and adults above 15 years of age). This stratification did not reveal any significant  
276 differences ( $p=0.344$  using Chi-squared test) as all age groups appeared to be infected with a  
277 diverse range of *S. Typhi* genotypes (**Fig. 3**).

278

279 **Fig. 2. Spatial analysis of 202 *S. Typhi* from this study.** (A) Map of Dhaka indicating the  
280 prevalence of *S. Typhi* genotypes present at each of the three study sites of icddr,b. (B)  
281 Maximum likelihood tree of 202 Bangladeshi *S. Typhi* isolates from the three study sites. Branch  
282 colors indicate the genotype (as labelled) and the colored heatmap (on the right) shows, for each  
283 isolate, its genotype, and source of the isolates coloured as per the inset legend.

284

285 **Fig. 3. Bangladesh genotypes observed in pediatric and adult patients.** Individual *S. Typhi*  
286 genotypes are colored as described in the inset legend.

287

### 288 **Inter-country transmission patterns and population structure of Bangladeshi *S. Typhi***

289 To provide a global context for the 818 Bangladeshi *S. Typhi* and to better understand inter-  
290 country transmission patterns, we constructed a global phylogeny including an additional 1,560  
291 *S. Typhi* from over 30 countries [6, 7, 14, 15, 21, 27] from the global WGS collection, belonging  
292 to the 17 genotypes that were detected in Bangladesh (**Fig. 4**, interactive phylogeny available at  
293 <https://microreact.org/project/5GzJ7Umoz>). A single South African *S. Typhi* isolated in 2010

294 was intermingled among the genotype 4.3.1.3 isolates, ~2 SNPs away from its closest  
295 Bangladesh relative, suggesting that the H58 lineage Bd has been transferred from South Asia to  
296 Africa on at least one occasion. Predominantly, the Bangladeshi H58 and non-H58 *S. Typhi* were  
297 related to isolates from India, Pakistan, and Nepal (see **Fig. 4** and interactive phylogeny at  
298 <https://microreact.org/project/5GzJ7Umoz>), suggesting regional circulation of these genotypes  
299 throughout South Asia. Notably, we found that Bangladesh isolates formed several unique  
300 monophyletic lineages within this tree, consistent with local establishment and ongoing clonal  
301 transmission within Bangladesh; e.g. 2.3.3, 3.2.2, 3.3.2 (**Fig. 4A**) and 4.3.1.1, 4.3.1.3 (**Fig. 4B**).

302

303 **Fig. 4. Global population structure of Bangladesh *S. Typhi*.** (A) Global population structure  
304 of Non-H58 (4.3.1) Bangladesh genotypes. (B) Global population structure of H58. Branch  
305 colours indicate country/region of origin, as do the inner two rings. The third ring indicates  
306 mutations in codon 83 of gene *gyrA*, fourth ring indicate the number of additional QRDR  
307 mutations. The fourth ring indicates the presence of any plasmids and the sixth ring indicates the  
308 presence of AMR genes. All branches and rings are coloured as per the inset legend. Arrows  
309 indicate localised lineages of Bangladeshi isolates.

310

### 311 **Antimicrobial resistance and plasmid replicons in *S. Typhi* in Bangladesh**

312 To better understand the AMR burden among *S. Typhi* in Bangladesh, we subjected our 202  
313 isolates (**Table 2**) and the additional 616 *S. Typhi* from previous studies [9, 14, 21] (**S4 Table**) to  
314 screening for both genes and mutations associated with AMR. Only 17 of our *S. Typhi* (9.42%)

315 lacked any known molecular determinants of AMR and were thus predicted to be fully  
316 susceptible to antibiotics (**Fig. 1** and **Table 2**).

317

318 Many H58 isolates (n=57, 68.67%) were predicted to be MDR, carrying genes associated with  
319 resistance to the first-line drugs chloramphenicol, trimethoprim-sulfomethoxazole and  
320 ampicillin. The majority of these were H58 lineage I isolates (genotype 4.3.1.1, n=48, 64.86%)  
321 carrying genes *catA1*, *dfrA7*, *sul1*, *sul2*, *bla*<sub>TEM-1</sub> and *strAB* (**S1 Fig**) in a composite transposon  
322 (**Fig. 5**) conferring resistance to all three first-line drugs and also streptomycin. These isolates  
323 lacked the IncHI1 plasmid, suggesting that the AMR genes have been integrated into the  
324 chromosome. A small proportion of genotype 4.3.1.1 *S. Typhi* (n=8, 10.8%) carried an  
325 alternative form of the typical transposon encoding just three AMR genes (*catA1*, *dfrA7*, *sul1*;  
326 see **Fig. 5**). Examination of the assembly graphs and nucleotide sequence comparisons [41] of  
327 the genomes carrying the 3-gene vs 7-gene locus revealed that integration of both transposons  
328 were mediated by *IS1* transposition associated with an 8 bp target site duplication (GGTTTAGA;  
329 see **Fig 5**). However as *IS1* was present at multiple locations in the chromosome sequences of  
330 these isolates, we were unable to resolve the precise location of the MDR integration site, and  
331 insertions at either of two previously reported chromosomal integration sites (near *cyoA* or  
332 within *yidA* [9]) were equally possible in the Bangladeshi isolates.

333

334 Of the 19 H58 lineage Bd isolates detected in our collection, two different plasmid mediated  
335 AMR patterns emerged (**Fig. S2**). The first pattern (n=8) was characterized by the presence of  
336 the IncHI1 plasmid (plasmid sequence type, PST6) [9] carrying eight AMR genes (*bla*<sub>TEM-1</sub>,



337 *catA1*, *dfrA7*, *sul1*, *sul2*, *strAB*, and *tetB*) conferring resistance to the first line drugs plus  
338 streptomycin and tetracyclines. The second pattern (n=9) was characterized by the presence of an  
339 IncFIB(K) plasmid carrying the AMR genes (*bla<sub>TEM-1</sub>*, *sul2*, *tetA*) conferring resistance to  
340 ampicillin, sulfonamides, tetracyclines, and also *qnrS* together with the *gyrA*-S83Y mutation  
341 confers resistance to fluoroquinolones. These isolates were intermingled with IncFIB(K)-  
342 carrying isolates described by Tanmoy *et al* 2018 (which they termed “sublineage Bdq”). This  
343 IncFIB(K)-carrying cluster appears to have emerged from the main 4.3.1.3 group that typically  
344 carries the IncHI1 plasmid (separated by a median of ~11 SNPs), but the IncHI1 MDR plasmid  
345 has been replaced in this group by the IncFIB(K) fluoroquinolone resistance plasmid (see **S2**  
346 **Figure**). The IncFIB(K)-containing Bdq cluster appears to have undergone a clonal expansion in  
347 Bangladesh, but has not replaced the IncHI1 form of 4.3.1.3 (see below).

348  
349 Overall, non-synonymous mutations in the QRDR of genes *gyrA* and *parC* associated with  
350 reduced susceptibility to fluoroquinolones (FQ) were common among our Bangladeshi *S. Typhi*  
351 isolates (n=185, 91.6%) harboring at least one QRDR mutation (**Table 2**). Unlike the acquisition  
352 of MDR genes, the QRDR mutations (mainly in gene *gyrA* at codon 83) were common in both  
353 non-H58 (n=102, 85.71%) as well as H58 isolates (n=83, 100%). Examination of genotype 3.3.2  
354 revealed two distinct monophyletic lineages of Bangladeshi *S. Typhi* of this genotype each with  
355 a different QRDR mutation. Here we defined these two Bangladeshi lineages as 3.3.2.Bd1 and  
356 3.3.2.Bd2, carrying the *gyrA*-S83F and *gyrA*-D87N mutations, respectively (**Fig. 4A**). Markers  
357 for these two lineages (SNPs STY2588-G378A, position 2424394 in CT18; and STY2441-  
358 G439A, position 2272144 in CT18; respectively) have been added to the GenoTyphi script to  
359 facilitate their detection. No QRDR triple mutants were observed among our collection;

360 however, a single double mutant *S. Typhi* of genotype 4.3.1.1 was identified carrying both *gyrA*-  
 361 S83F and *parC*-E84K mutations. Hence, the only isolates we predict to be ciprofloxacin resistant  
 362 are the IncFIB(K) group carrying *qnrS* and *gyrA*-S83Y.

363

364 **Table 2:** Genetic determinants of antimicrobial resistance in 202 *S. Typhi* isolates from Dhaka,  
 365 Bangladesh

Resistance patterns	H58 isolates (n=83)	Non H58 isolates (n=119)	Total isolates (n=202)
<b>Acquired AMR genes / QRDR mutations</b>	<b>74 (89.16%)</b>	<b>0 (0.0%)</b>	<b>74 (36.63%)</b>
<i>bla</i> <sub>TEM-1</sub> , <i>catA1</i> , <i>dfrA7</i> , <i>sul1</i> , <i>sul2</i> , <i>strAB</i> / <i>gyrA</i> -S83F	47 (56.63%)	0 (0.00%)	47 (23.27%)
<i>bla</i> <sub>TEM-1</sub> , <i>catA1</i> , <i>dfrA7</i> , <i>sul1</i> , <i>sul2</i> , <i>strAB</i> , <i>tetB</i> / <i>gyrA</i> -S83Y	8 (9.64%)	0 (0.00%)	8 (3.96%)
<i>bla</i> <sub>TEM-1</sub> , <i>catA1</i> , <i>dfrA7</i> , <i>sul1</i> , <i>sul2</i> , <i>strAB</i> / <i>gyrA</i> -S83F, <i>parC</i> -E84K	1 (1.20%)	0 (0.00%)	1 (0.50%)
<i>bla</i> <sub>TEM-1</sub> , <i>catA1</i> , <i>dfrA7</i> , <i>sul1</i> / <i>gyrA</i> -S83F	1 (1.20%)	0 (0.00%)	1 (0.50%)
<i>catA1</i> , <i>dfrA7</i> , <i>sul1</i> / <i>gyrA</i> -S83F	8 (9.64%)	0 (0.00%)	8 (3.96%)
<i>bla</i> <sub>TEM-1</sub> , <i>sul2</i> , <i>qnrS</i> , <i>tetA</i> / <i>gyrA</i> -S83Y	9 (10.84%)	0 (0.00%)	9 (4.46%)
<b>QRDR mutations only</b>	<b>83 (100%)</b>	<b>102 (85.71%)</b>	<b>185 (91.6%)</b>
<i>gyrA</i> -S83F	62 (74.70%)	85 (83.33%)	147 (72.77%)
<i>gyrA</i> -S83Y	19 (22.90%)	3 (2.94%)	22 (10.89%)
<i>gyrA</i> -D87G	1 (1.20%)	0 (0.00%)	1 (0.50%)
<i>gyrA</i> -D87N	0 (0.00%)	12 (11.76%)	12 (5.94%)
<i>gyrA</i> -D87Y	0 (0.00%)	2 (1.96%)	2 (0.99%)
<i>gyrA</i> -S83F, <i>parC</i> -E84K	1 (1.20%)	0 (0.00%)	1 (0.50%)
<b>Susceptible to all antibiotics</b>	<b>0 (0.00%)</b>	<b>17 (16.67%)</b>	<b>17 (8.42%)</b>

366

367 **Fig. 5. Insertion sites of transposons observed in *S. Typhi* from Bangladesh.** Genes and  
368 transposons are indicated according to the inset legend. TSD indicates target site duplication,  
369 and *Tn* indicates transposons.

370

### 371 **Temporal trends in genotypic distribution and AMR over time**

372 We examined the genotypic distribution and AMR patterns over time during our study period of  
373 2004 to 2016; note our sampling includes all available blood culture positive *S. Typhi* from the  
374 three sites in urban Dhaka. Prior to 2011, H58 lineages (4.3.1.1 and 4.3.1.3) dominated the  
375 population across the three sites sampled (median 66.67% per year; see **Fig. 6A**). During this  
376 time most isolates were MDR (median 55.56% per year, see **Fig. 6B**). After this period, there  
377 appears to be a relative decrease in the frequency of H58 genotypes (4.3.1.1, 4.3.1.2, 4.3.1.3;  
378 median 28.03% per year in 2011-2016) and an increase in non-H58 genotypes 2.3.3, 3.2.2, 3.3.2  
379 (from median 22.2% per year in 2004-2010 to 65.2% per year in 2011-2016; overall diversity  
380 increasing from 1.34 Shannon diversity in 2004-2010 to 1.83 in 2011-2016). The decline in non-  
381 H58 *S. Typhi* coincided with a decline in MDR (median 11.88% per year in 2011-2016), with the  
382 most common profile being non-MDR with a single QRDR mutation (*gyrA*-S83F being the most  
383 prevalent). Notably, the IncFIB(K)/*qnrS* lineage Bdq remained at low frequency throughout the  
384 study period (detected from 2007 to 2014, median 4.78% per year).

385

386 **Fig 6. Timeline of Bangladesh *S. Typhi* genotypes and AMR profiles from 2004-2016.** (A)  
387 Genotypes observed per annum. Overlaid line indicates the percentage of H58 isolates per year.

388 (B) AMR profiles observed per annum. Overlaid line indicates the percentage of MDR isolates  
389 per year. Genotypes and AMR profiles coloured as per inset legend.

390

391

392

### 393 **Discussion**

394 We show here that the population structure of *S. Typhi* in Bangladesh is diverse, harboring 17  
395 distinct genotypes, with 9 genotypes circulating within urban Dhaka between 2004-2016 (**Fig 1**).  
396 There was little evidence of any local geographic restriction of *S. Typhi* genotypes within the  
397 city of Dhaka (**Fig 2**) and no obvious stratification of *S. Typhi* genotypes by patient age (**Fig 3**),  
398 consistent with what has been observed in other South Asian settings [6, 15, 42].

399 *S. Typhi* circulating in Bangladesh are closely related to isolates from neighboring India,  
400 Pakistan and Nepal suggesting circulation throughout South Asia. However, the formation of  
401 multiple localised lineages indicates establishment and ongoing local transmission of multiple  
402 genotypes in parallel within Bangladesh; and warranted definition of novel Bangladesh-specific  
403 subclades for future tracking via WGS surveillance. Firstly, the most common genotype in our  
404 collection was H58 lineage I (31.2%) and H58 lineage II was rare (0.5%), in contrast to  
405 neighboring India and Nepal where lineage II are highly prevalent and lineage I is rarely reported  
406 [6, 9, 15]. Secondly, this distinction between Bangladeshi and Indian/Nepali pathogen  
407 populations was also evident for the newly defined H58 lineage Bd (4.3.1.3) [14], which so far  
408 has been almost exclusively found in Bangladesh (the exceptions being singleton isolates  
409 reported in Nepal and South Africa). Thirdly, we identified a novel subclade of *S. Typhi*,

410 genotype 3.3.2, which included a Bangladesh-specific monophyletic group with relatives in  
411 Nepal, that we further divided into 3.3.2.Bd1 and 3.3.2.Bd2 based on distinct QRDR mutations  
412 conferring reduced susceptibility to fluoroquinolones (**Fig 4A**). These novel Bangladesh-  
413 associated lineages (4.3.1.3, 3.3.2.Bd1, 3.3.2.Bd2) have been added to the GenoTyphi  
414 genotyping scheme, which will facilitate their detection and tracking in future surveillance  
415 efforts, and over time will reveal whether they remain localized to Bangladesh or being to  
416 disseminate through Asia and Africa as has been observed for H58 lineages I and II [6, 13, 27,  
417 43].

418

419 The sustained, very high frequency of *S. Typhi* carrying mutations associated with reduced  
420 susceptibility to fluoroquinolones that we detected in this study (>66% per year; **see Fig 6B**) is  
421 likely the result of an increase in over-the-counter sale (without prescription) of this antibiotic  
422 class over the last decade [44]. This, along with a decrease in MDR is similar to reports from  
423 both India and Nepal [6, 9]. However, while the prevalence of reduced susceptibility was very  
424 high, we found limited evidence of evolution towards full resistance, with the *qnrS*-positive  
425 clade (associated with ciprofloxacin MIC of 4 µg/mL, [14]) remaining at low frequency  
426 throughout the study period. An H58 lineage I (4.3.1.1) QRDR triple mutant has been previously  
427 reported in Bangladesh [14], however, this was not observed among our collection; we only  
428 detected a single QRDR double mutant in H58 lineage I in 2008 (see **Table 2** and **Fig. 6**).

429

430 Notably, the reduced prevalence of MDR coincided with a reduction in H58 (4.3.1) isolates  
431 across our 3 study sites and a significant diversification in the pathogen population, particularly

432 driven by increased prevalence of QRDR single-mutant *S. Typhi* genotypes 2.3.3, 3.2.2 and  
433 3.3.2. This unexpected change in population structure that cannot be explained by selection for  
434 AMR suggests unknown selective pressures may be influencing the pathogen population in  
435 Bangladesh, and highlights the need for ongoing genomic surveillance. Further, while MDR was  
436 less frequent after 2010, the presence of multiple forms of the MDR chromosomal insertion is  
437 highly concerning, as such insertions may facilitate more stable transmission of the MDR  
438 phenotype [6, 9]. Similarly, the persistent presence of plasmid-mediated quinolone resistance  
439 (PMQR) via an IncFIB(K) plasmid carrying a *qnrS1* gene in Bangladesh is concerning, despite  
440 the relatively low frequency (4.5%) at which it is observed currently.

441

## 442 **Conclusion**

443 This study demonstrates the importance of molecular based surveillance studies in endemic  
444 regions, especially in Bangladesh, where the disease burden is high and many different AMR  
445 phenotypes were observed. The change in both population structure and AMR patterns over  
446 twelve years (2004 to 2012) shows increased prevalence of populations with reduced  
447 susceptibility fluoroquinolones, emphasizing the ongoing evolution of AMR in this setting as  
448 well as the urgent need for WGS based surveillance in Bangladesh to inform both treatment  
449 guidelines and control strategies.

450

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466

#### 467 **Author contributions**

468 GD, FQ, ZAD, SIAR contributed to the design of the study and GD, FQ supervised the study.  
469 SIAR, ZAD, EJK and KEH performed the genomic data analysis. SIAR, ZAD, KEH, FQ and  
470 GD wrote the manuscript. All authors contributed to the interpretation of results, editing of the  
471 manuscript and approved the final version.

472

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638

639

## 640 **Supporting Information**

641 **S1 Fig. Bangladesh *S. Typhi* population structure showing acquired AMR genes, and**  
642 **plasmids.** Branches are coloured by genotype as labelled, the heatmap the molecular  
643 determinants of antimicrobial resistance and the presence of plasmids coloured as per the inset  
644 legend.

645

646 **S2 Fig. Population structure and country of origin of genotype 3.3.2 isolates.** Maximum  
647 likelihood phylogeny of genotype 3.3.2 isolates. Coloured bar indicates country of origin as per  
648 the inset legend.

649

650

651

652

653 **S1 Table. Data of 202 S. Typhi isolates from 3 different study sites in Dhaka, Bangladesh**  
654 **between 2004-2016**

655

656 **S2 Table. Data of 818 S. Typhi isolates from Bangladesh including previous studies**  
657 **between 1998-2016**

658

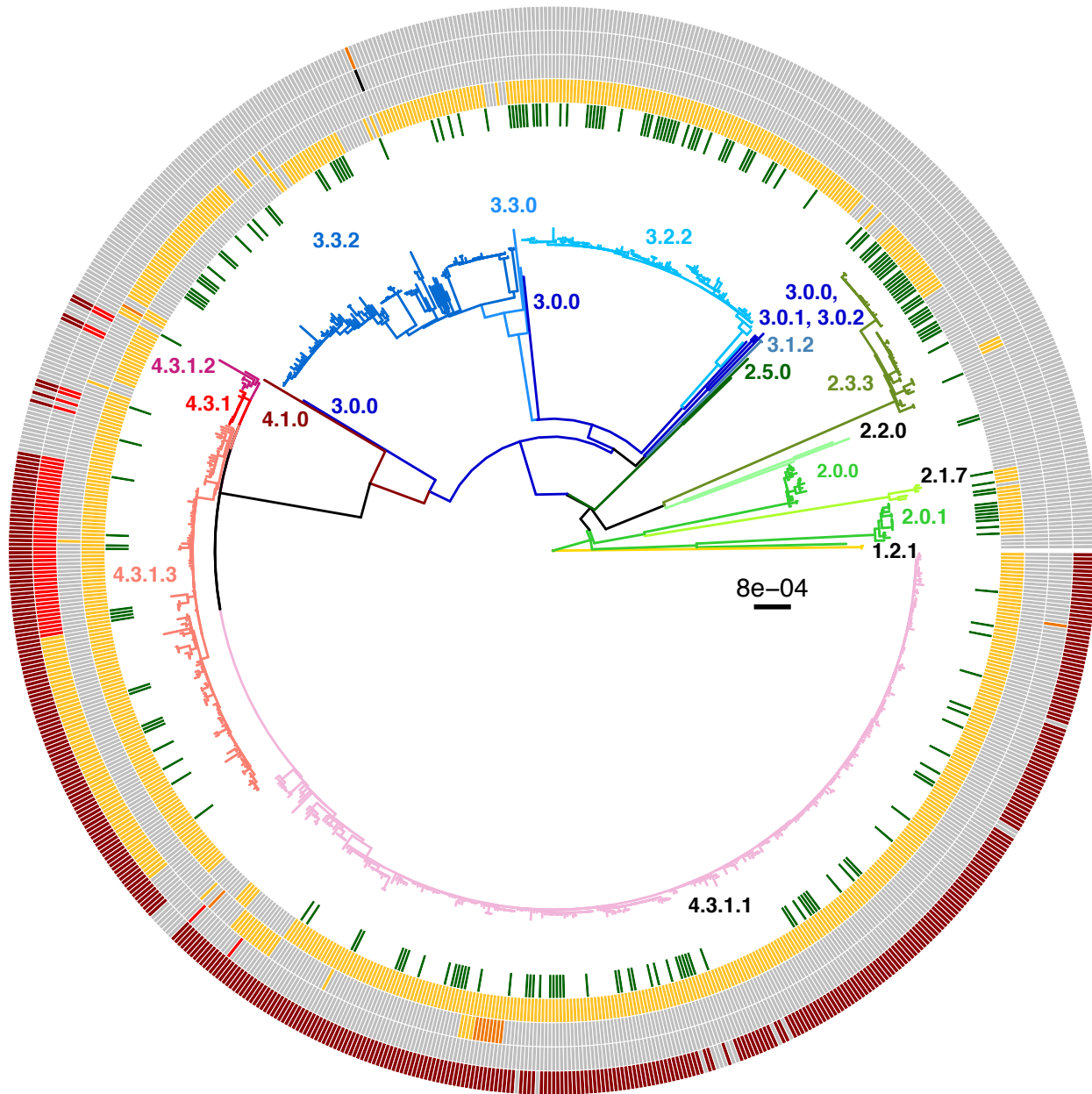
659 **S3 Table. Genotyping results for the 818 S. Typhi isolates from Bangladesh.**

660

661 **S4 Table. Genetic determinants of antimicrobial resistance in S. Typhi isolates from**  
662 **Bangladesh**

663

664



**Ring 1: Study**

- This study
- Previous study

**Ring 2: QRDR *gyrA* codon 83**

- QRDR mutation (S83F or S83Y)
- No mutation

**Ring 3: Other QRDR mutations**

- 1 other QRDR mutation
- 2 other QRDR mutations
- No mutation

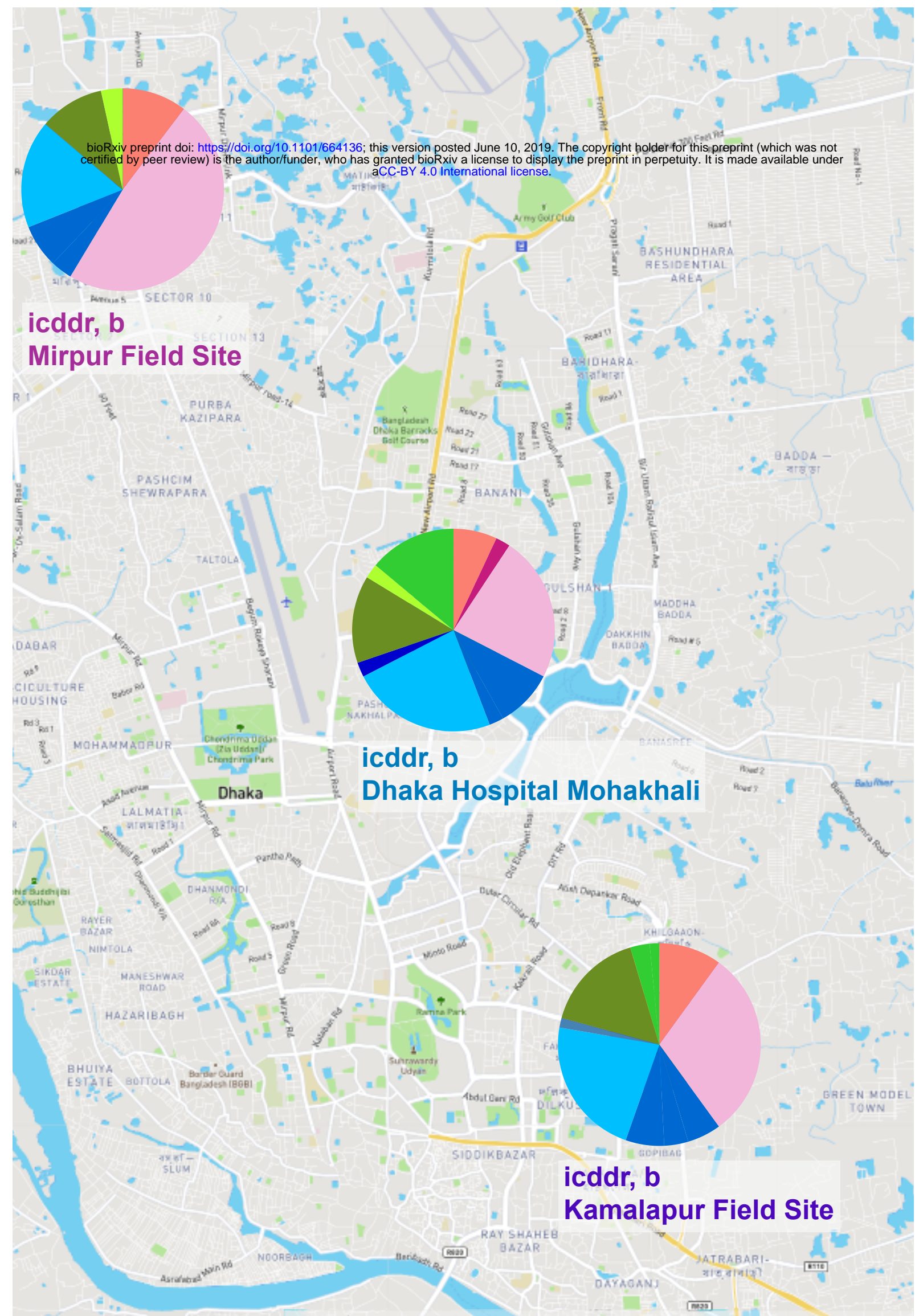
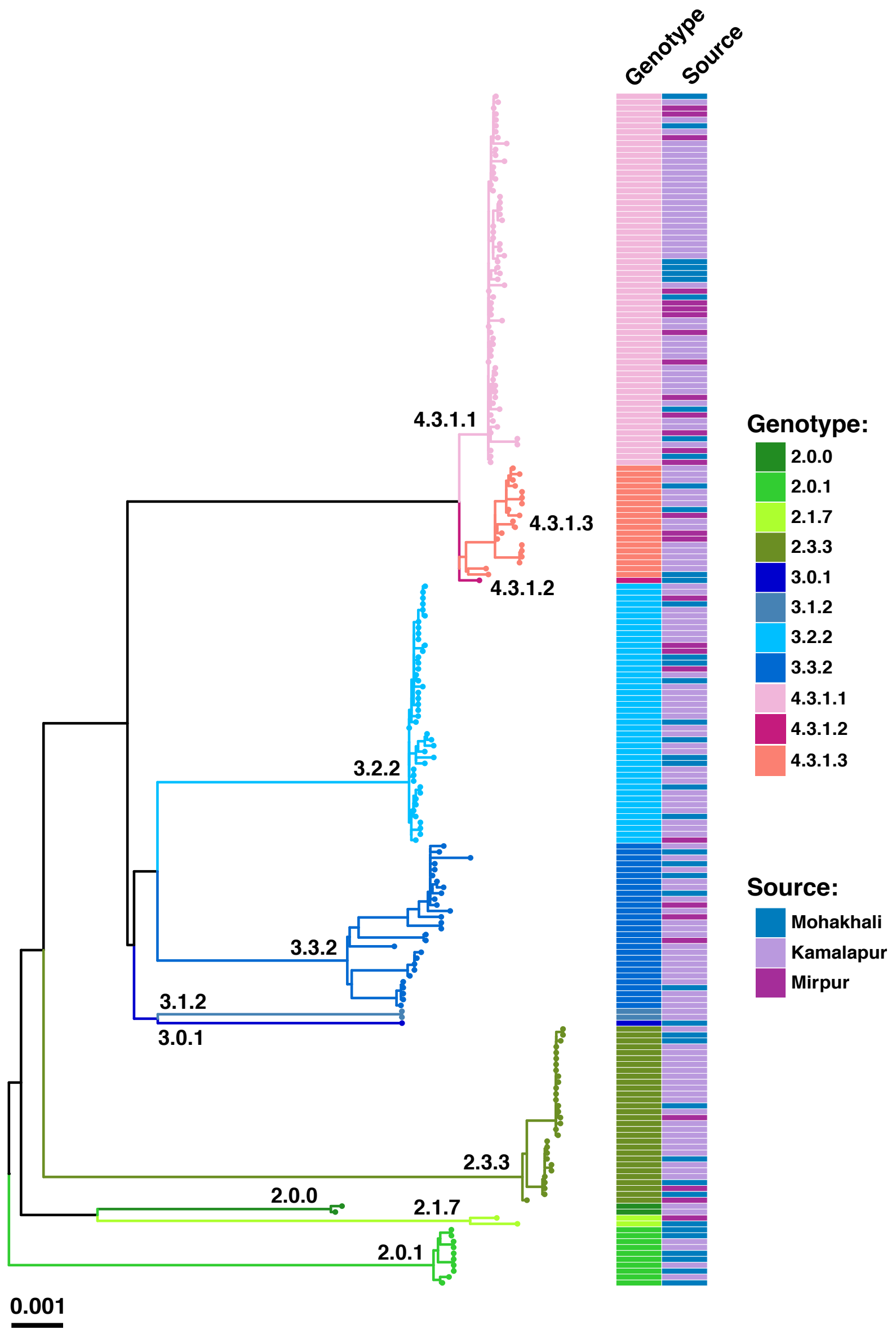
**Ring 4: Plasmids**

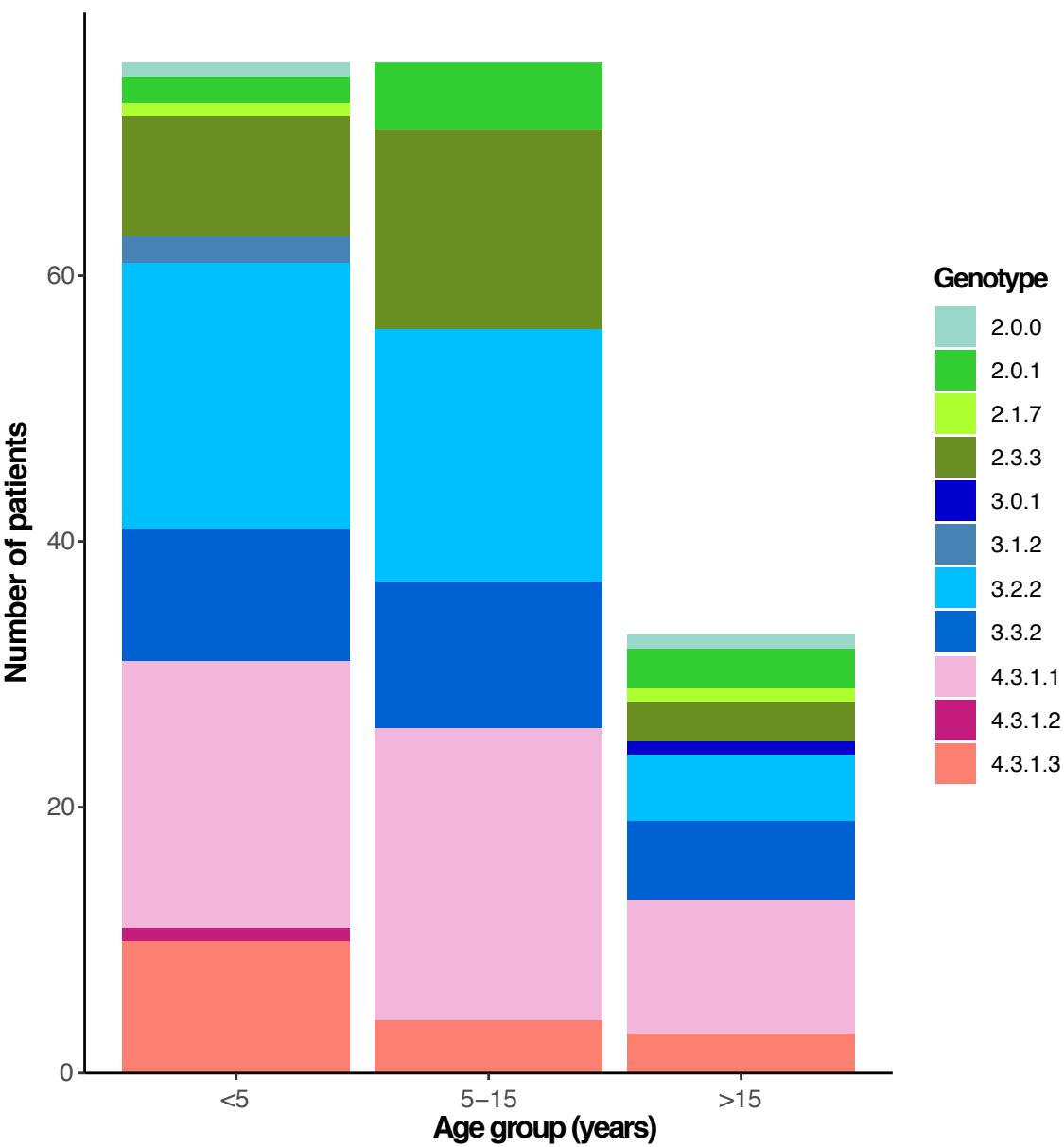
- IncHI1
- IncFIB(K) & MDR transposon
- IncFIB(K)
- IncI plamid
- No plasmid

**Ring 5: AMR genes**

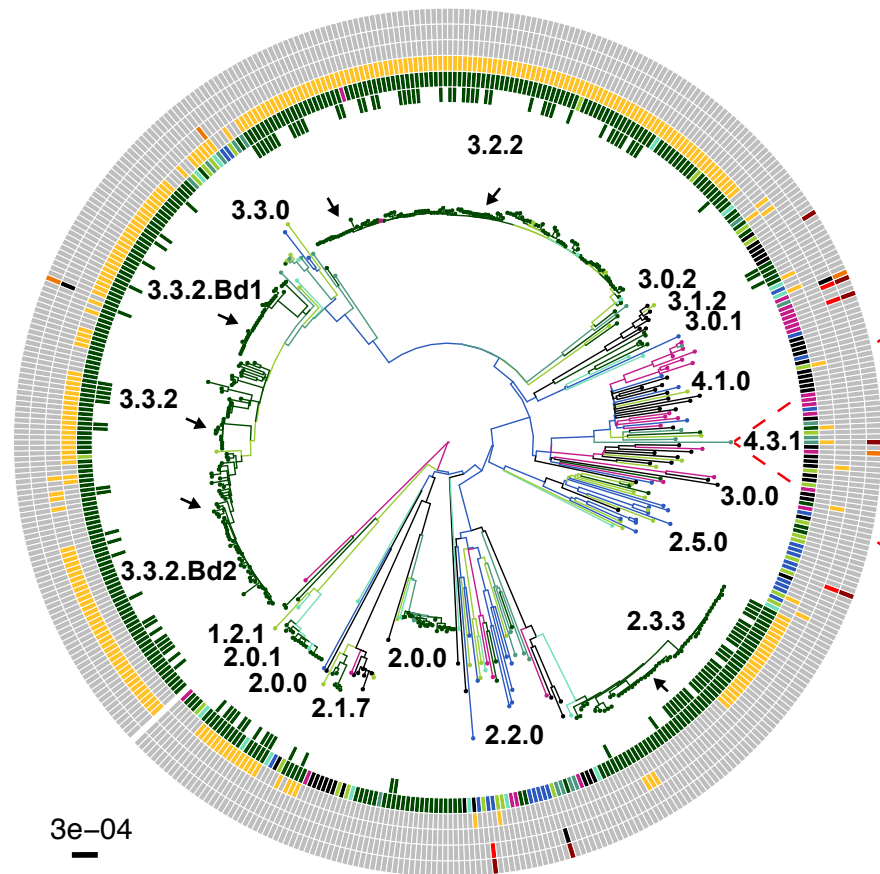
- $\geq 3$  AMR genes
- 2 AMR genes
- No AMR genes



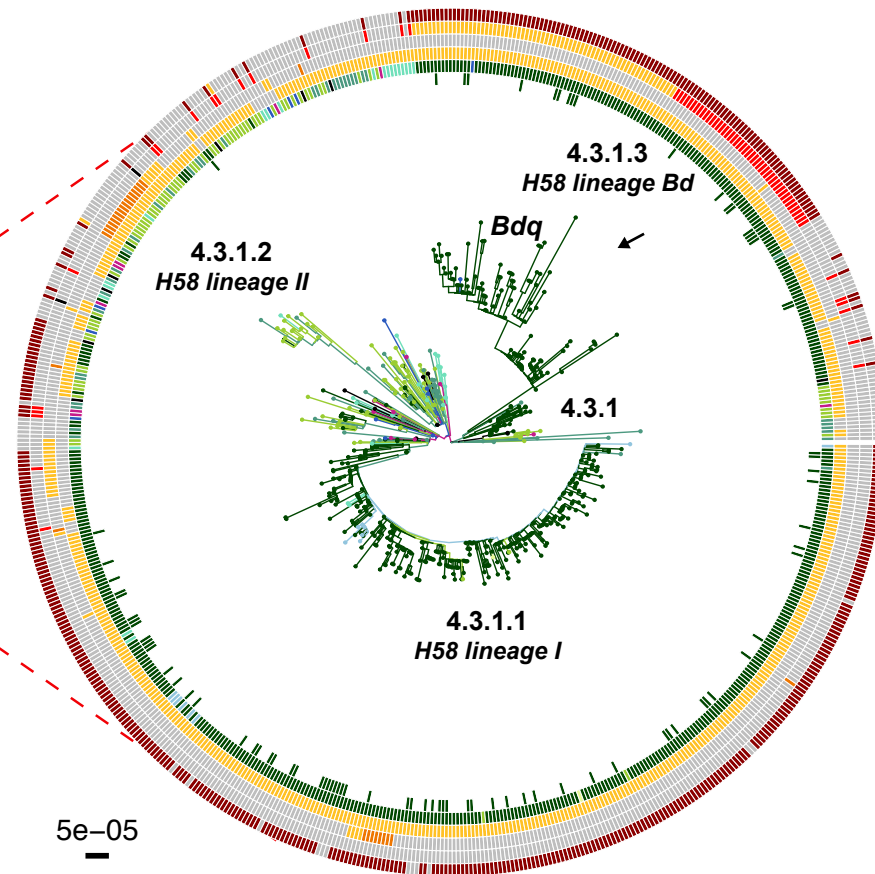
**A****B**



A



B



## Ring 1: Study

- This study
- Previous study

## Ring 2: Location

- Bangladesh
- Nepal
- Pakistan
- India
- Palestine
- Iraq
- South East Asia
- Africa
- Other

Ring 3: QRDR *gyrA* codon 83

- QRDR mutation (S83F or S83Y)
- No mutation

## Ring 4: Other QRDR mutations

- 1 other QRDR mutation
- 2 other QRDR mutations
- No mutation







## Ring 5: Plasmids

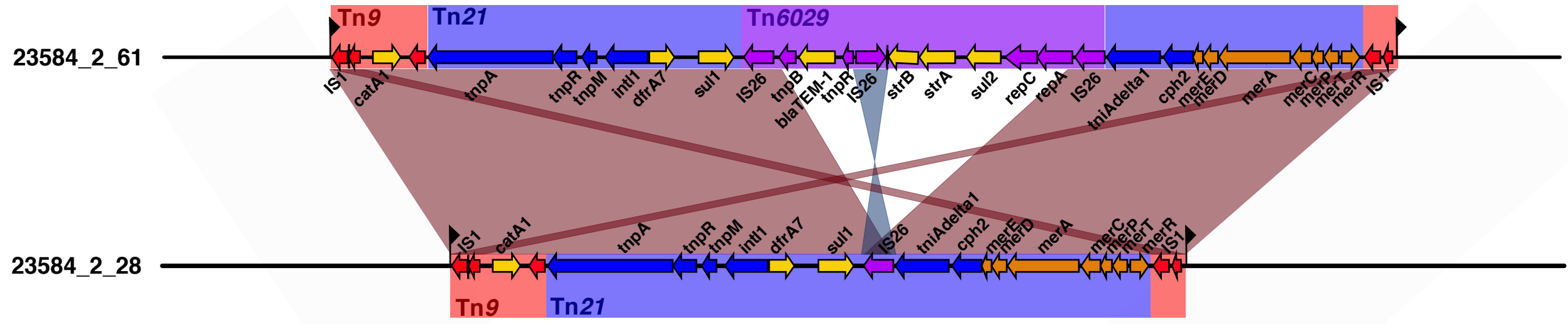
- IncHI1
- IncFIB(K) & MDR transposon
- IncFIB(K)
- Other MDR plasmid
- No plasmid

## Ring 6: AMR genes

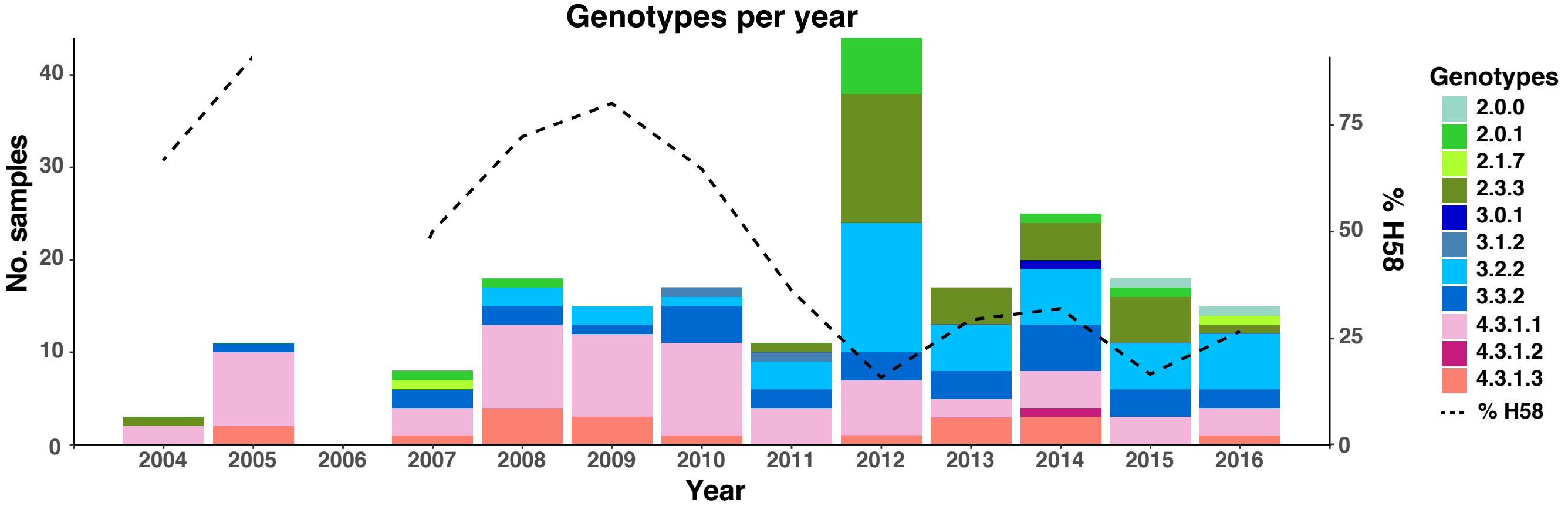
- ≥3 AMR genes
- 2 AMR genes
- 1 AMR gene
- No AMR genes



-  Tn9 backbone
-  Tn21 backbone
-  Tn6029 backbone
-  Antimicrobial Resistance
-  Mercury Resistance
-  8-bp TSD



5 kbp

**A****B**