

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

3

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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+ Γ 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. 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*_{TEM-1} 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*_{TEM-1},

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_{TEM-1}*, *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)
Acquired AMR genes / QRDR mutations	74 (89.16%)	0 (0.00%)	74 (36.63%)
<i>bla</i> _{TEM-1} , <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> _{TEM-1} , <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> _{TEM-1} , <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> _{TEM-1} , <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> _{TEM-1} , <i>sul2</i> , <i>qnrS</i> , <i>tetA</i> / <i>gyrA</i> -S83Y	9 (10.84%)	0 (0.00%)	9 (4.46%)
QRDR mutations only	83 (100%)	102 (85.71%)	185 (91.6%)
<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%)
Susceptible to all antibiotics	0 (0.00%)	17 (16.67%)	17 (8.42%)

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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636 multidrug-resistant Salmonella enterica serotype Typhi with decreased ciprofloxacin
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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

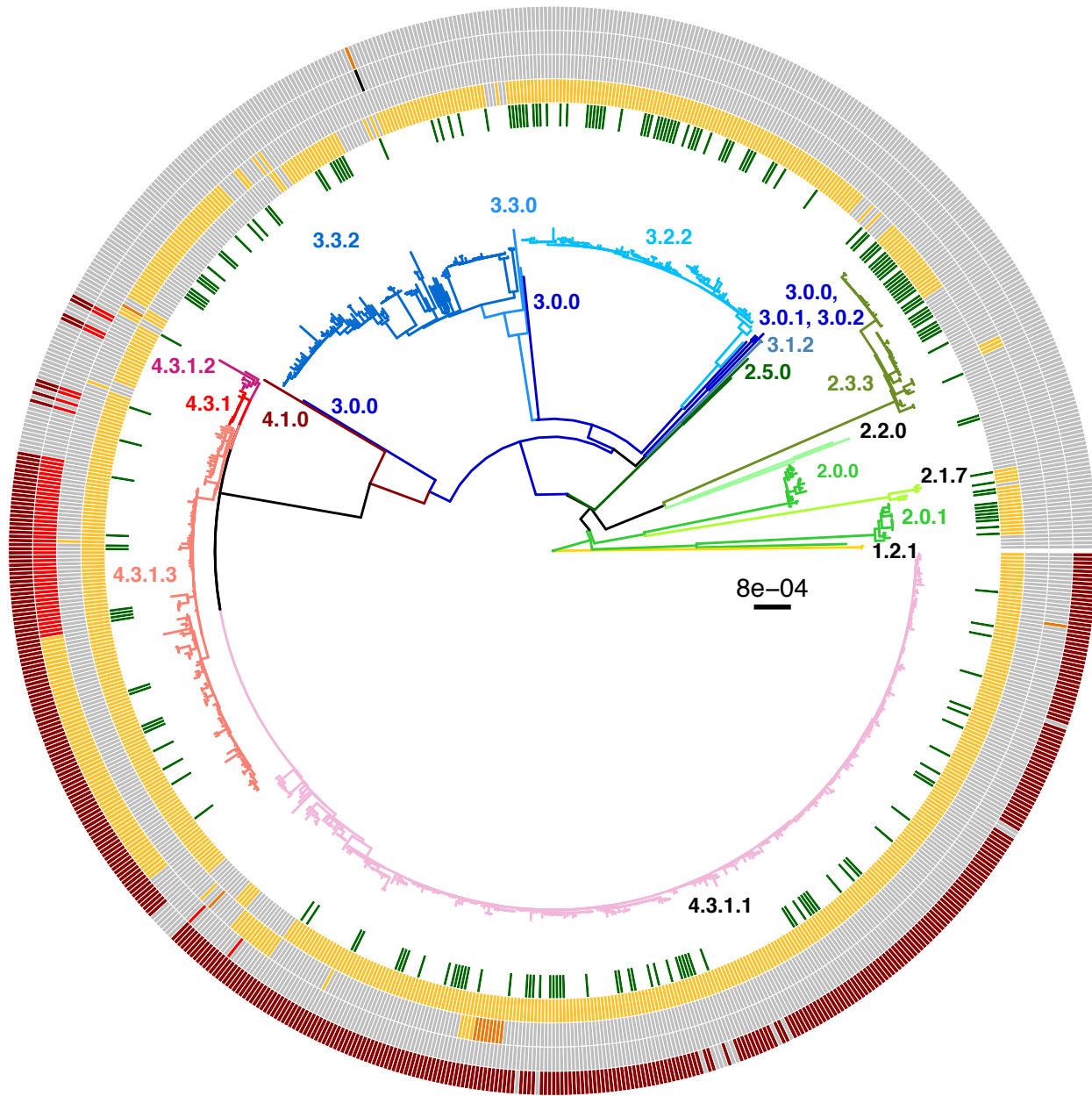
659 **S3 Table. Genotyping results for the 818 S. Typhi isolates from Bangladesh.**

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

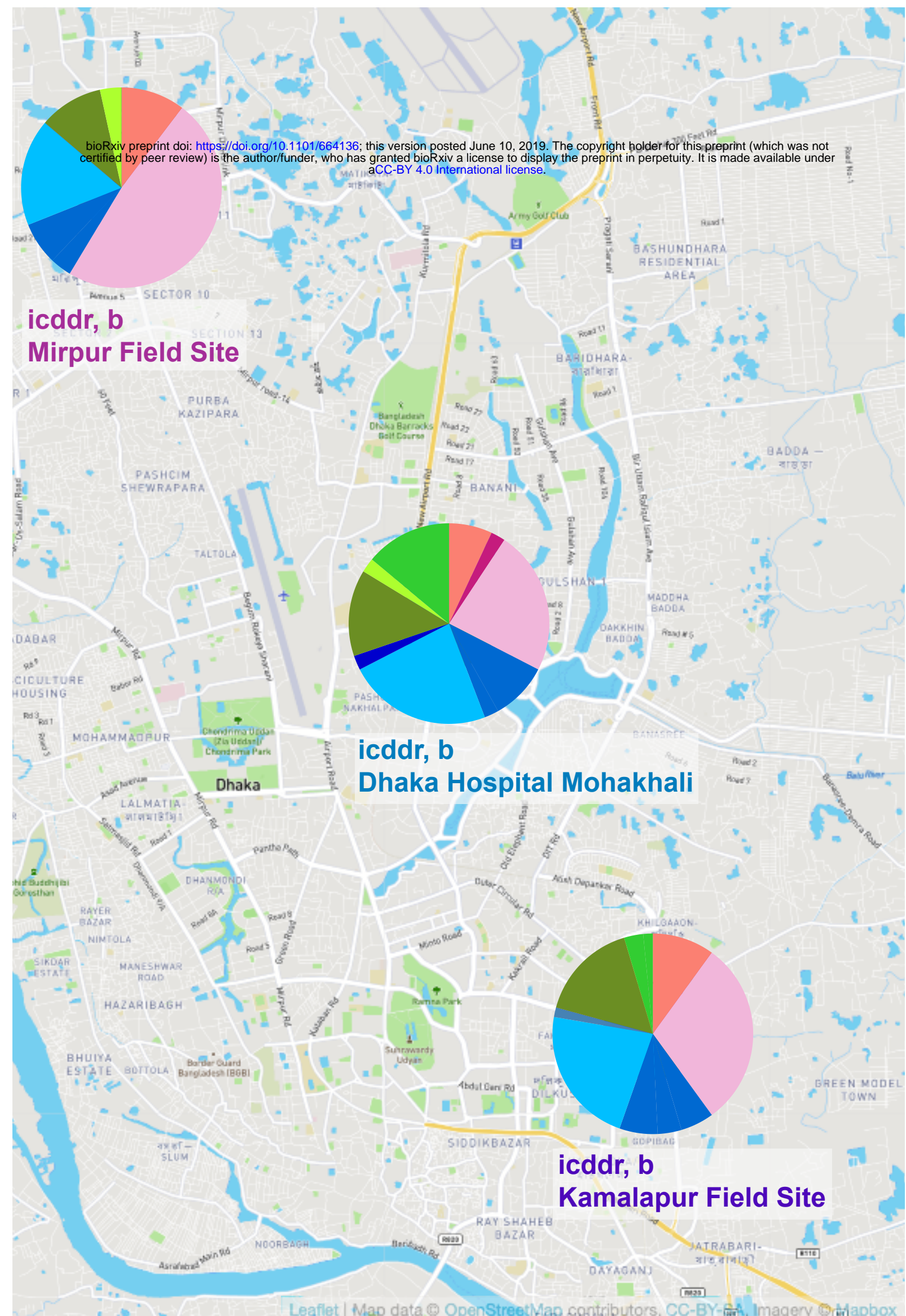
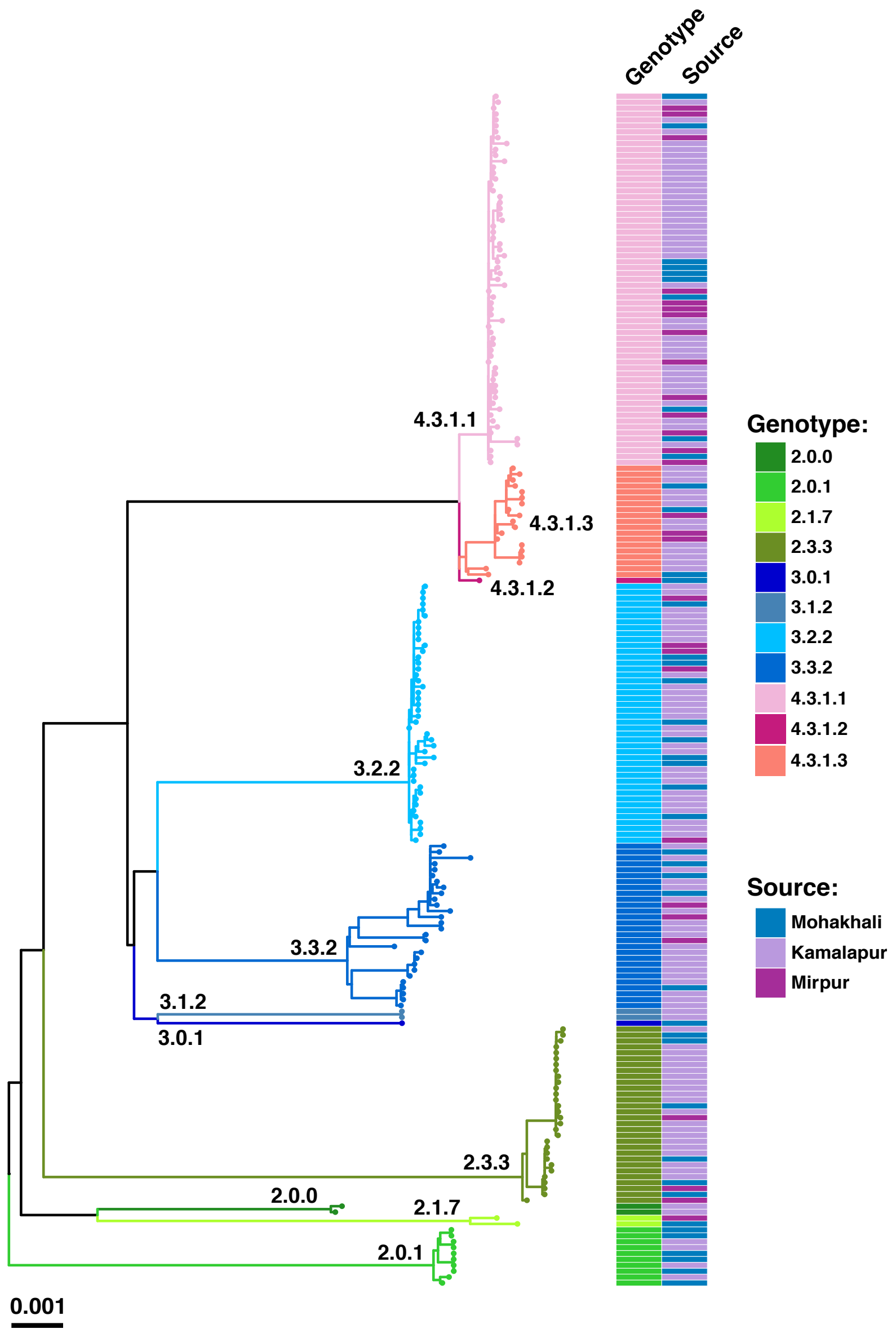
- █ 1 other QRDR mutation
- █ 2 other QRDR mutations
- █ No mutation

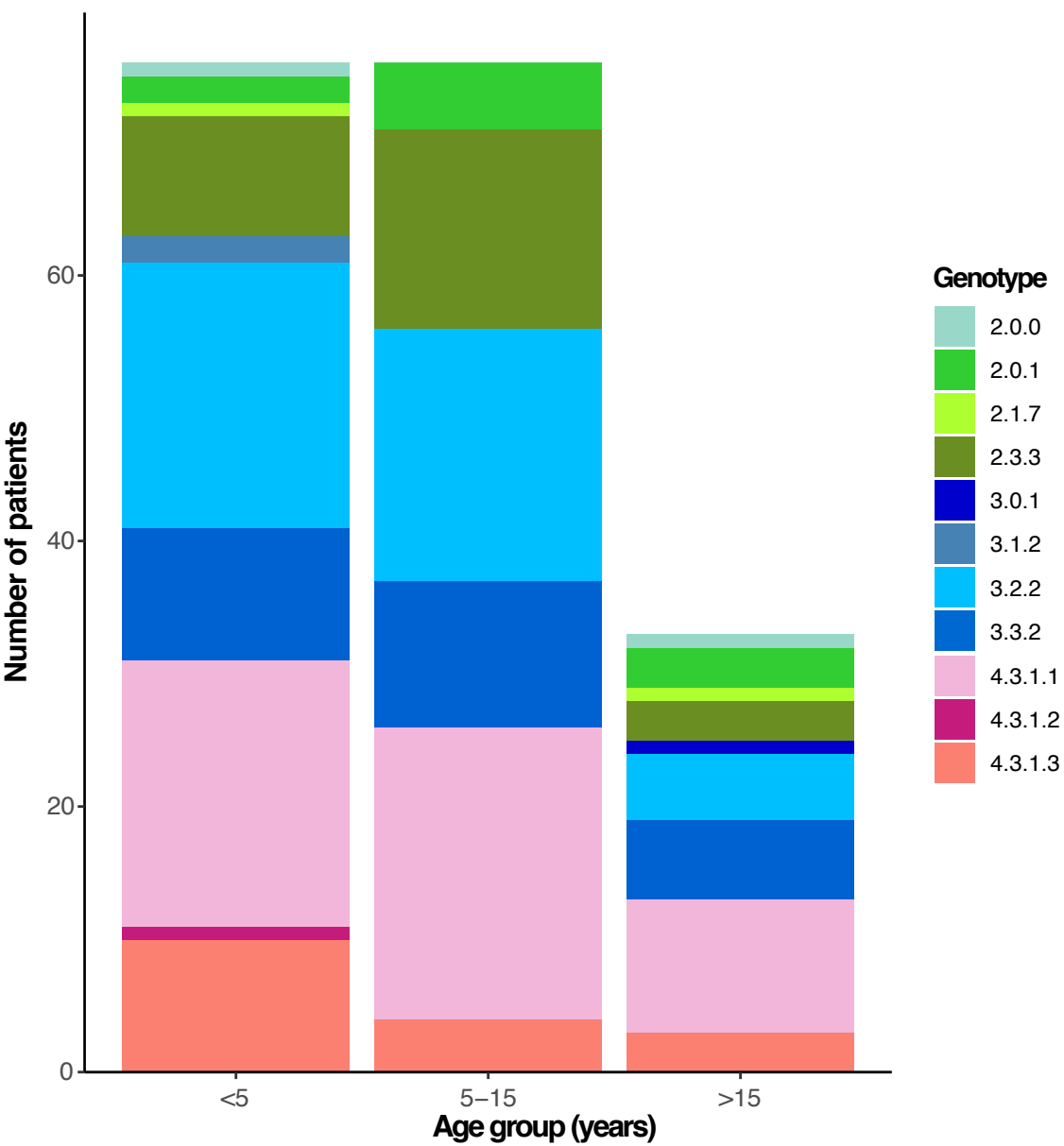
Ring 4: Plasmids

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

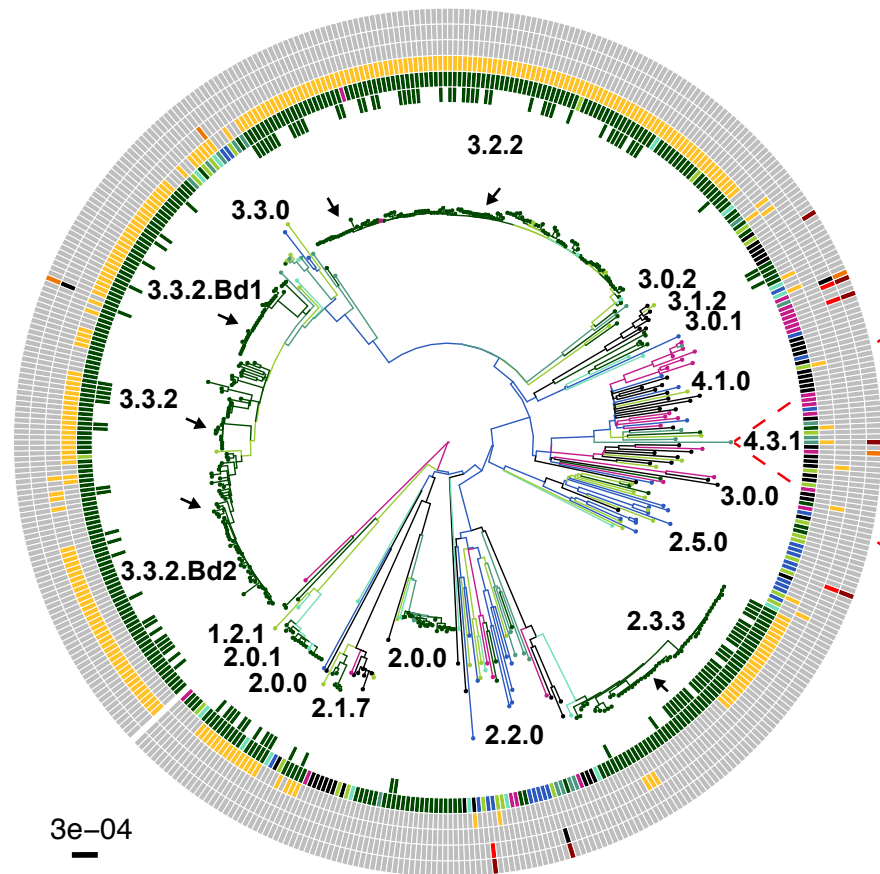
Ring 5: AMR genes

- █ ≥ 3 AMR genes
- █ 2 AMR genes
- █ No AMR genes

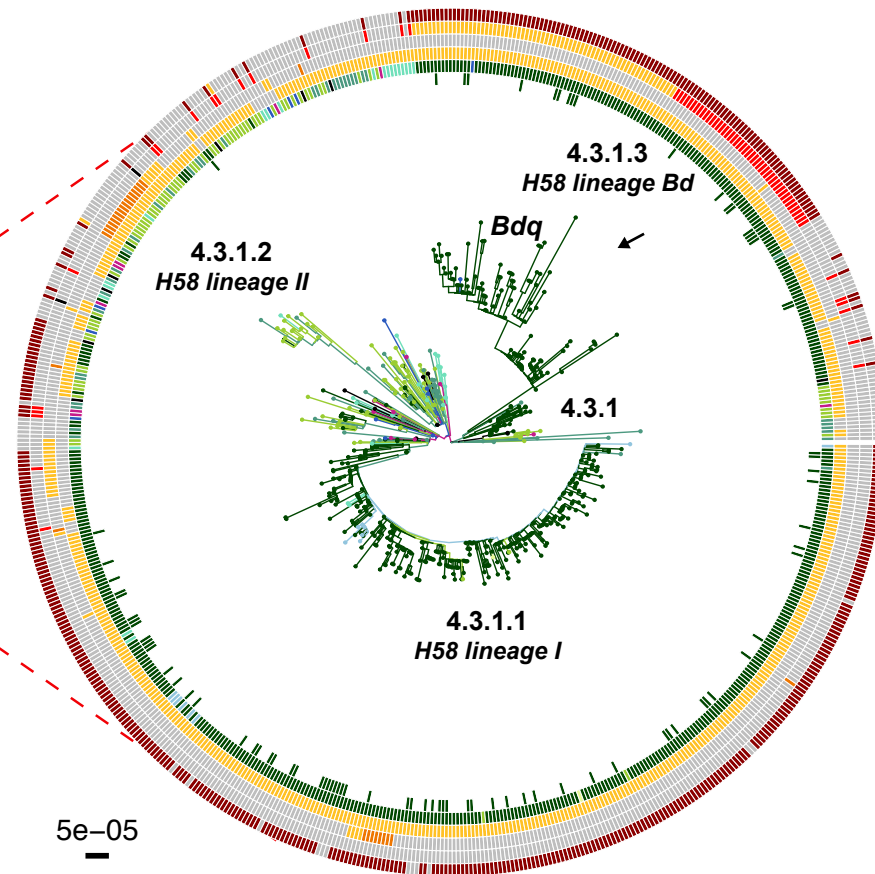
A**B**



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

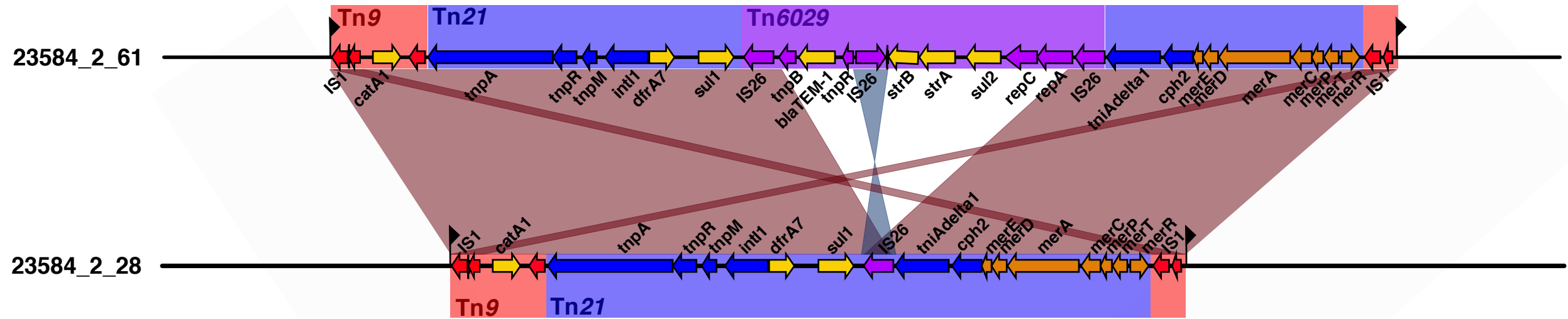
Ring 5: Plasmids

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

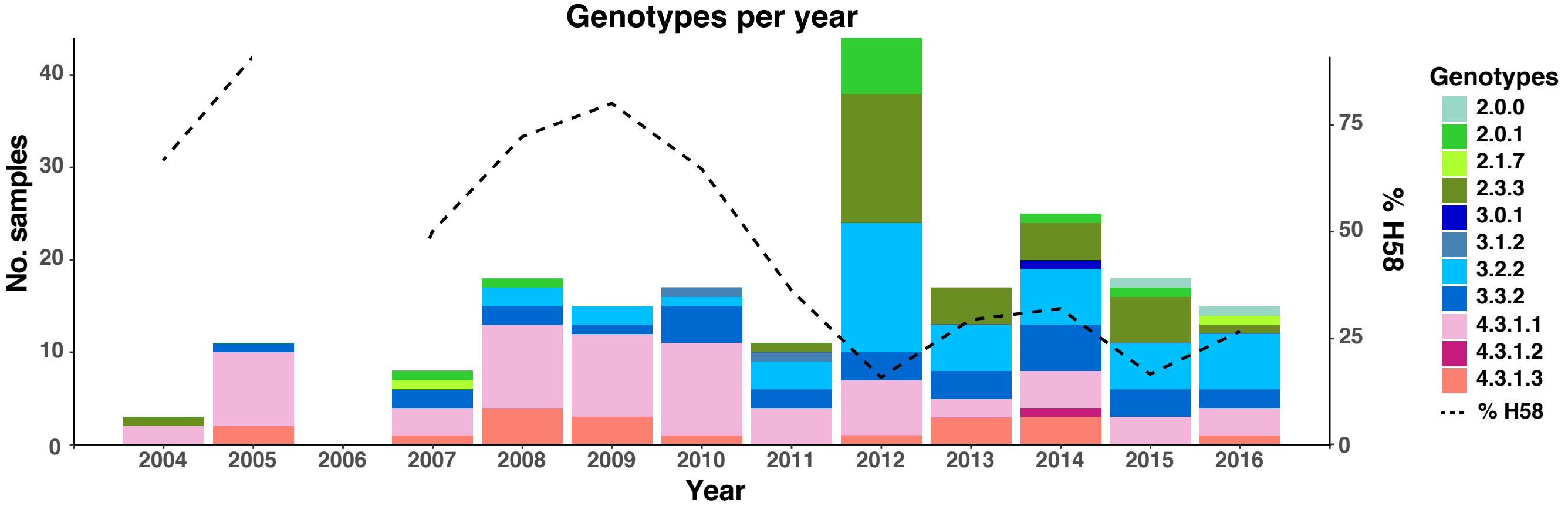
Ring 6: AMR genes

- ≥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**