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1 **Title:** Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in

2 Bangladesh from 2004 to 2016

3

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20

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-localiased 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

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.

107

108

109

Methods

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

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

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 colonie*s* 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

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

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 IS*1* (accession number J01730) and IS*26* (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

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

260

Intra-country transmission dynamics within Bangladesh

Geographical location data were available for the 202 novel *S.* Typhi, which were collected from three different sites inside Dhaka (icddr,b Kamalapur field site, icddr,b Mirpur field site and icddr,b Dhaka hospital Mohakhali). Detailed genotypic distribution of 202 isolates from these three study sites are shown in **Fig. 2**, and an interactive version of the phylogeny and map are available online at https://microreact.org/project/sP2Uwk_DI). Our data showed that genotypes 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 intermingled in the phylogeny (**Fig. 2B)** suggesting circulation across the city. Two genotypes, 2.0.1 and 2.1.7, were restricted to two of the three study locations (Kamalapur and icddr,b hospital, and Mirpur and icddr,b hospital, respectively); genotype 2.0.0, 3.1.2 and genotype 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 IS*1* transposition associated with an 8 bp target site duplication (GGTTTAGA;

329 see **Fig 5**). However as IS*1* 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 *cyaA* 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 (0.0%)**	**74 (36.63%)**
*bla*_{TEM-1} , *catA1, dfrA7, sul1, sul2, strAB / gyrA*-S83F	47 (56.63%)	0 (0.00%)	47 (23.27%)
*bla*_{TEM-1} , *catA1, dfrA7, sul1, sul2, strAB, tetB / gyrA*-S83Y	8 (9.64%)	0 (0.00%)	8 (3.96%)
*bla*_{TEM-1} , *catA1, dfrA7, sul1, sul2, strAB / gyrA*-S83F, *parC*-E84K	1 (1.20%)	0 (0.00%)	1 (0.50%)
*bla*_{TEM-1} , *catA1, dfrA7, sul1 / gyrA*-S83F	1 (1.20%)	0 (0.00%)	1 (0.50%)
catA1, dfrA7, sul1 / gyrA-S83F	8 (9.64%)	0 (0.00%)	8 (3.96%)
*bla*_{TEM-1}, *sul2, qnrS, tetA / gyrA*-S83Y	9 (10.84%)	0 (0.00%)	9 (4.46%)
QRDR mutations only	**83 (100%)**	**102 (85.71%)**	**185 (91.6%)**
gyrA-S83F	62 (74.70%)	85 (83.33%)	147 (72.77%)
gyrA-S83Y	19 (22.90%)	3 (2.94%)	22 (10.89%)
gyrA-D87G	1 (1.20%)	0 (0.00%)	1 (0.50%)
gyrA-D87N	0 (0.00%)	12 (11.76%)	12 (5.94%)
gyrA-D87Y	0 (0.00%)	2 (1.96%)	2 (0.99%)
gyrA-S83F, *parC*-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

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

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

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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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

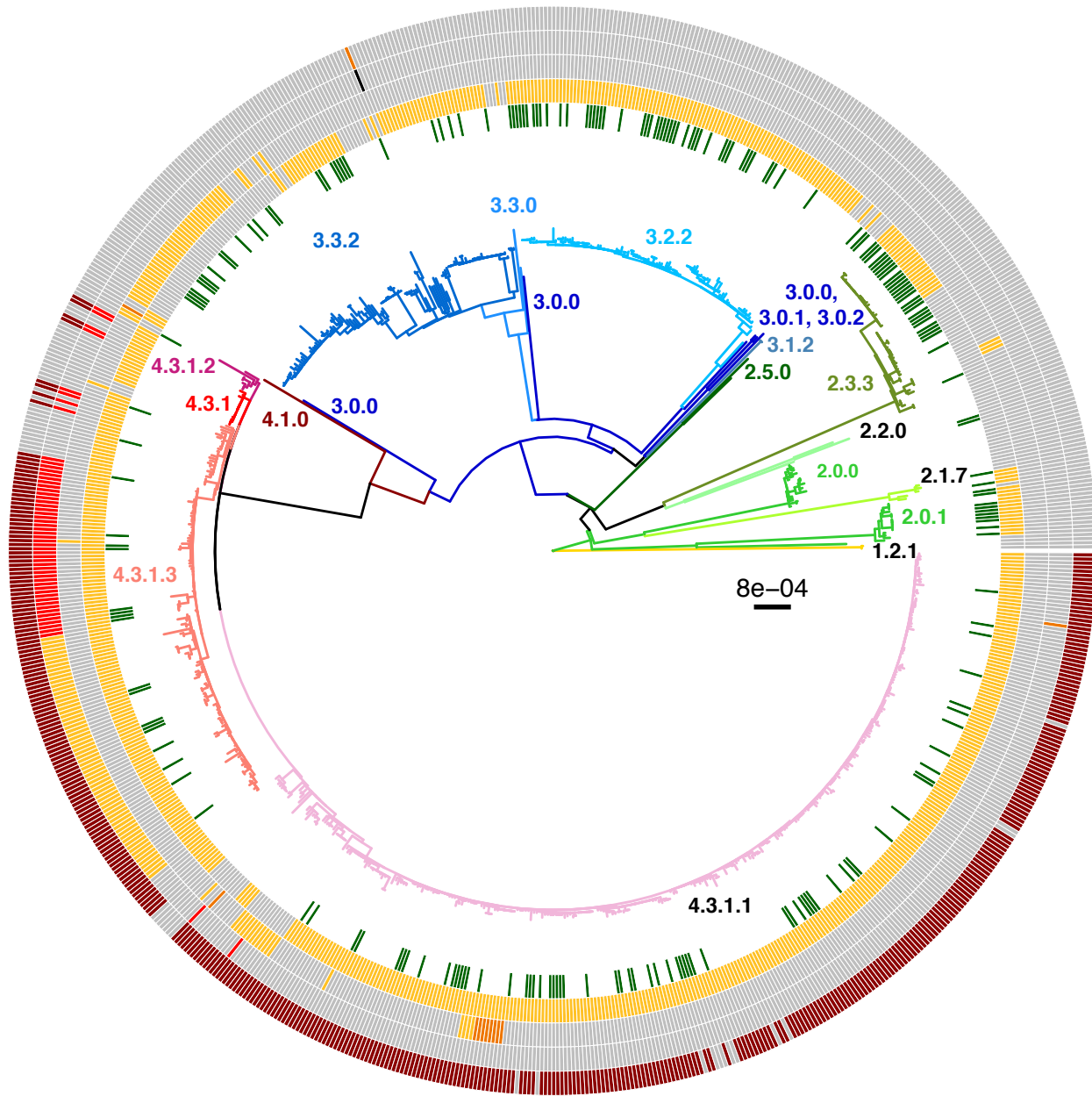656 **S2 Table. Data of 818 S. Typhi isolates from Bangladesh including previous studies**

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)
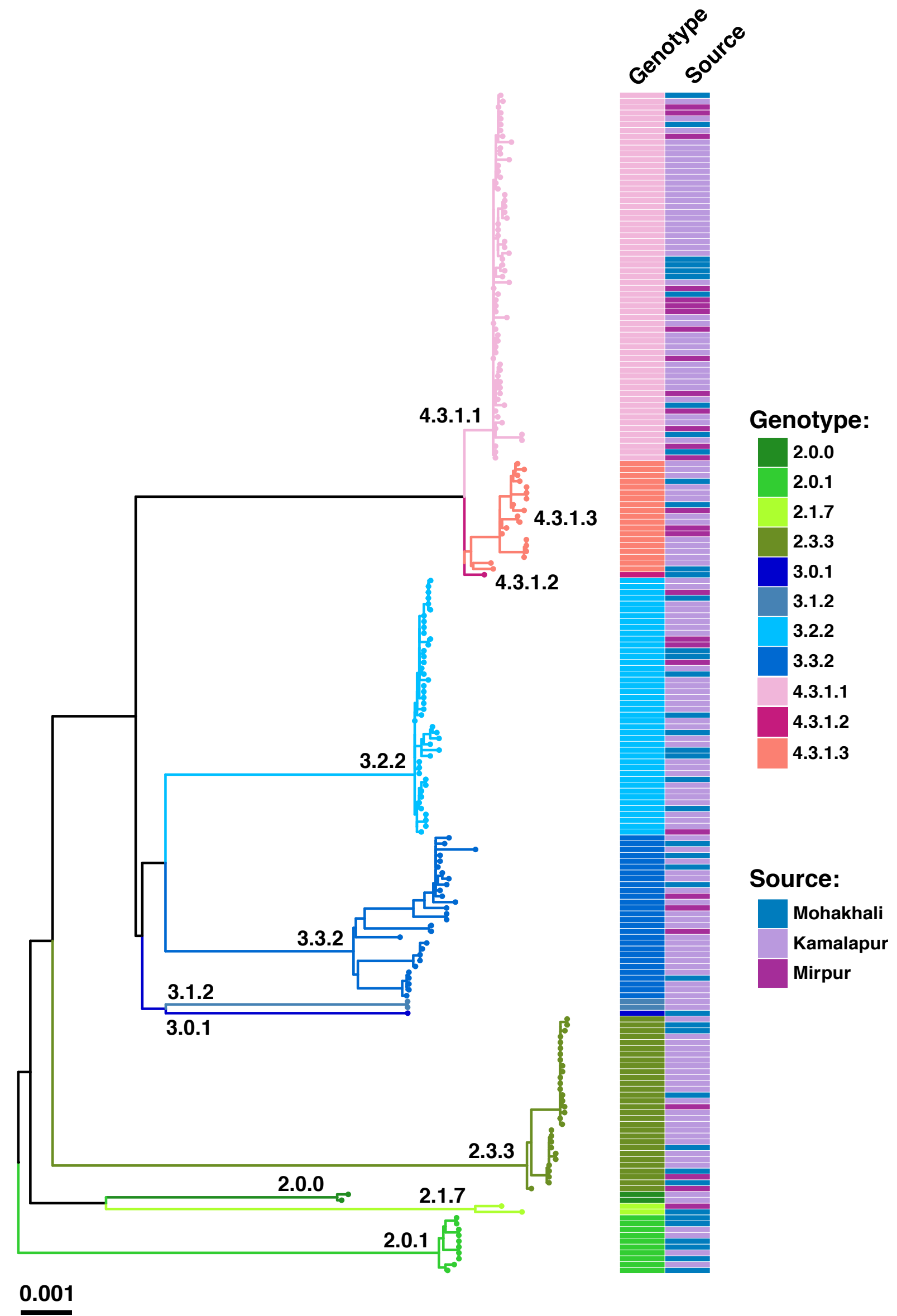- No mutation

Ring 3: Other QRDR mutations
- 1 other QRDR mutation
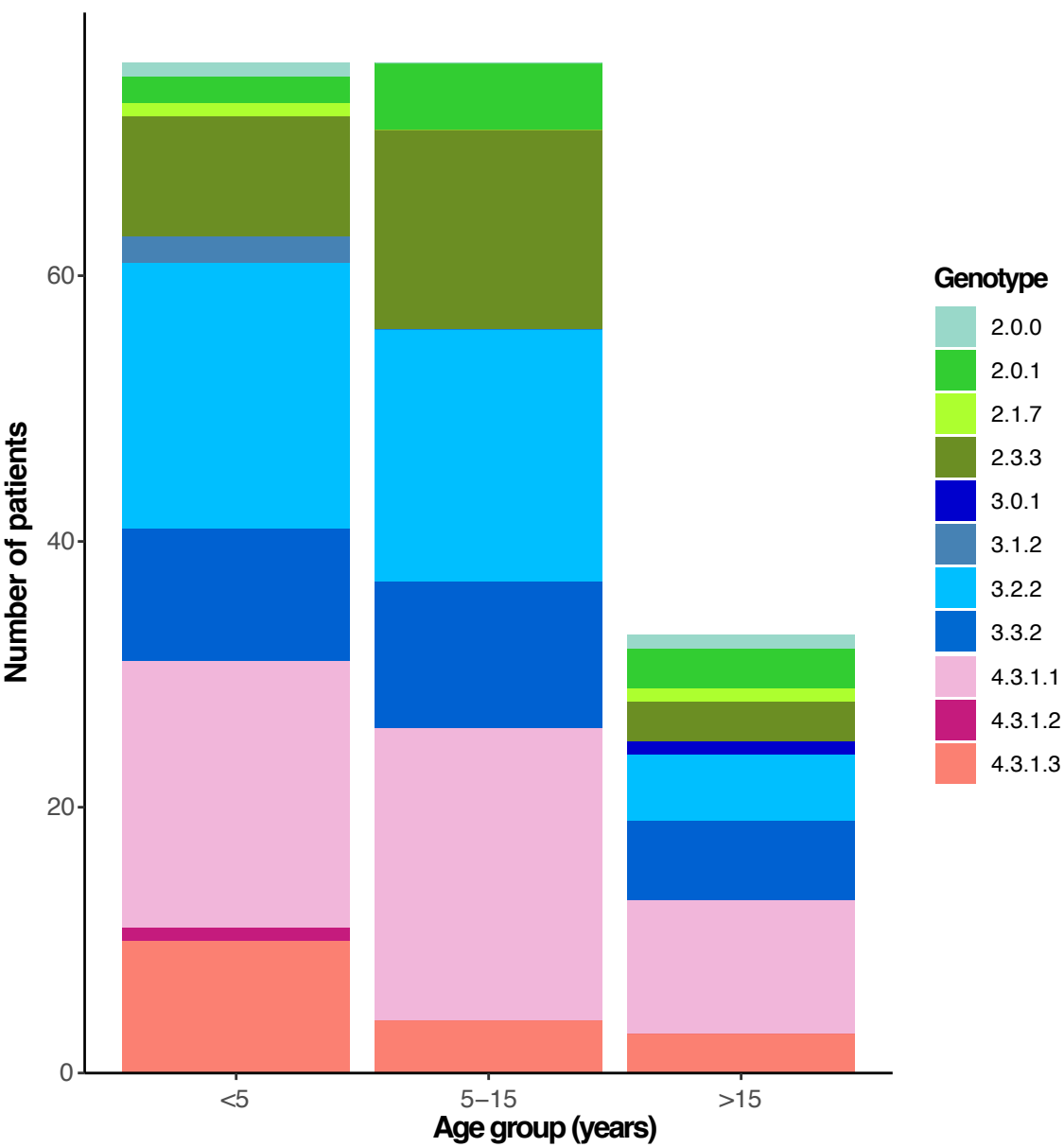- 2 other QRDR mutations
- No mutation

Ring 4: Plasmids
- IncHI1
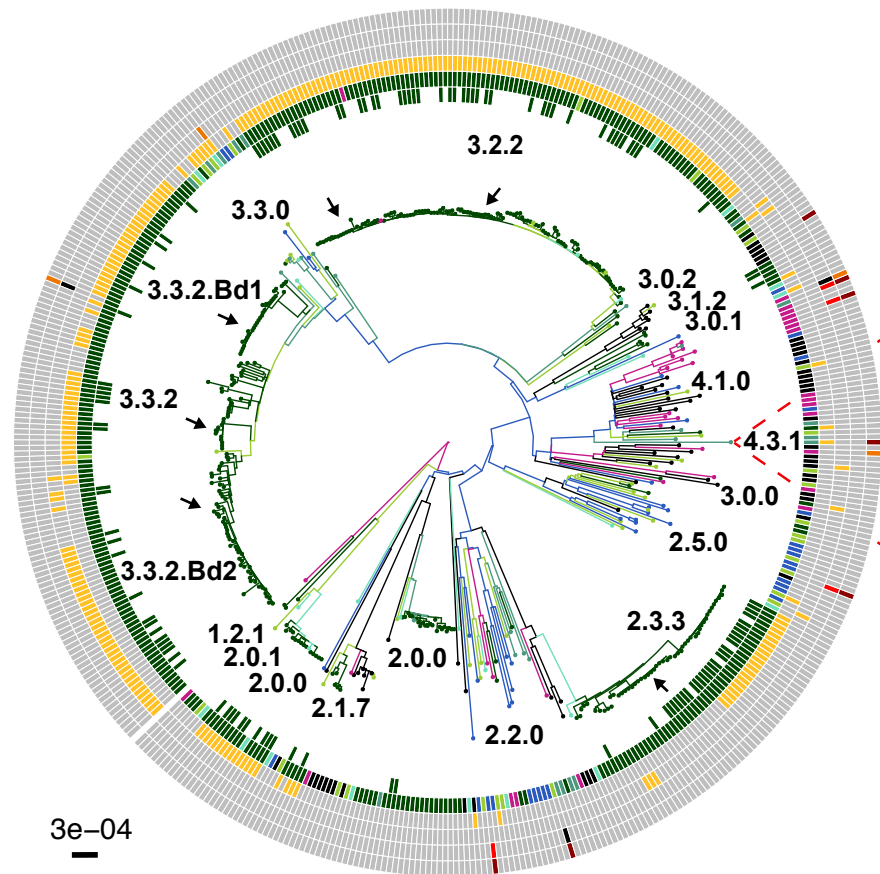- IncFIB(K) & MDR transposon
- IncFIB(K)
- IncI plamid
- No plasmid

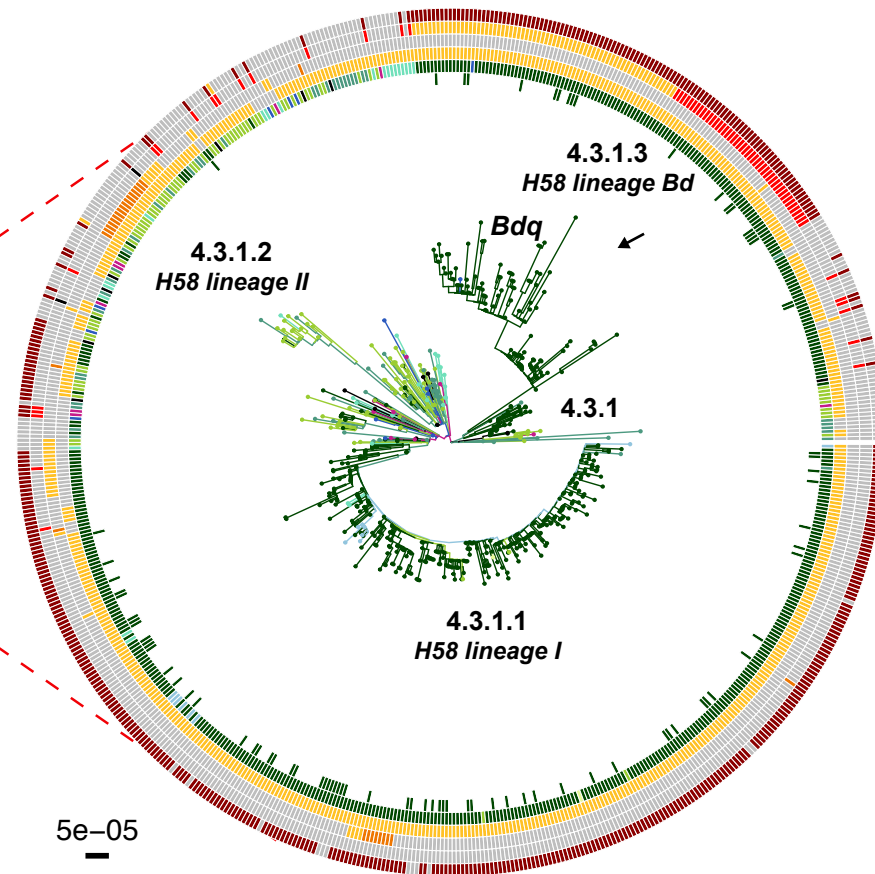Ring 5: AMR genes
- >=3 AMR genes
- 2 AMR genes
- No AMR genes

A

icddr, b
Mirpur Field Site

icddr, b
Dhaka Hospital Mohakhali

icddr, b
Kamalapur Field Site

Leaflet | Map data © OpenStreetMap contributors, CC-BY-SA, Imagery © Mapbox

B

Genotype   Source

4.3.1.1
4.3.1.3
4.3.1.2
3.2.2
3.3.2
3.1.2
3.0.1
2.3.3
2.0.0   2.1.7
2.0.1

0.001

Genotype:
- 2.0.0
- 2.0.1
- 2.1.7
- 2.3.3
- 3.0.1
- 3.1.2
- 3.2.2
- 3.3.2
- 4.3.1.1
- 4.3.1.2
- 4.3.1.3

Source:
- Mohakhali
- Kamalapur
- Mirpur

A

3e−04

3.2.2
3.3.0
3.3.2.Bd1
3.3.2
3.3.2.Bd2
1.2.1
2.0.1
2.0.0
2.1.7
2.0.0
2.2.0
2.3.3
2.5.0
3.0.0
4.1.0
3.0.1
3.1.2
3.0.2
4.3.1

B

5e−05

4.3.1.3
H58 lineage Bd
4.3.1.2
H58 lineage II
Bdq
4.3.1
4.3.1.1
H58 lineage I

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
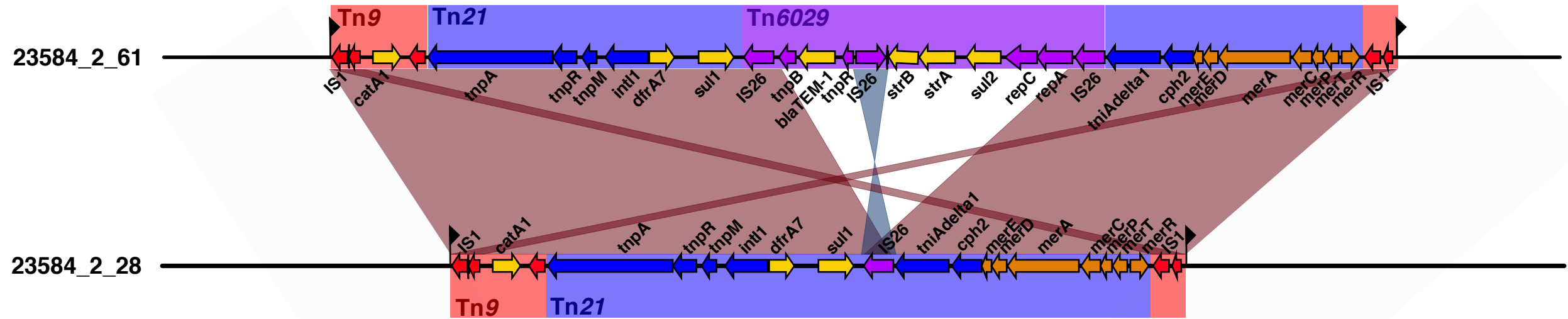- 2 other QRDR mutations
- No mutation

Ring 5: Plasmids
- IncHI1
- IncFIB(K) & MDR transposon
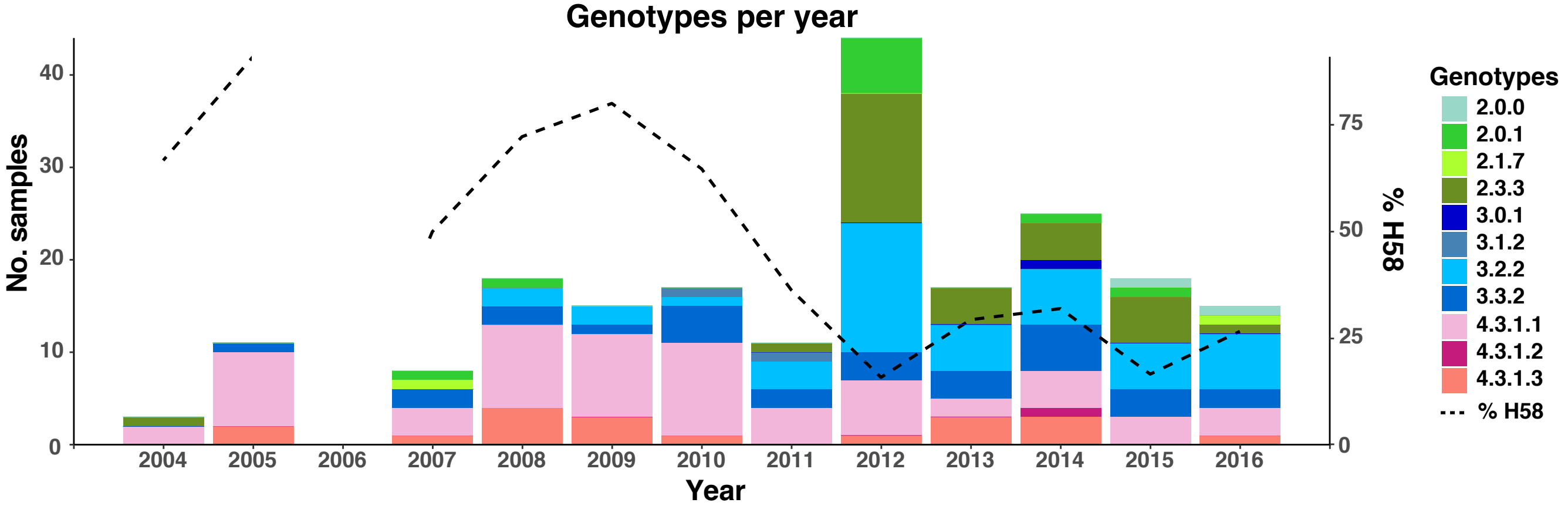- IncFIB(K)
- Other MDR plasmid
- No plasmid

Ring 6: AMR genes
- >=3 AMR genes
- 2 AMR genes
- 1 AMR gene
- No AMR genes

A Genotypes per year

B AMR profiles per year

Genotypes
- 2.0.0
- 2.0.1
- 2.1.7
- 2.3.3
- 3.0.1
- 3.1.2
- 3.2.2
- 3.3.2
- 4.3.1.1
- 4.3.1.2
- 4.3.1.3
- - - % H58

AMR Profile
- **1 QRDR mutation**
- **1 QRDR mutation & AMR**
  catA1, dfrA7, sul1
- **1 QRDR mutation & MDR**
  catA1, dfrA7, sul1, sul2, strAB, blaTEM-1
  catA1, dfrA7, sul1, sul2, strAB, blaTEM-1, tetB
- **1 QRDR mutation & PMQR/AMR**
  qnrS blaTEM-1, sul1, tetA
- **2 QRDR mutation & MDR**
  catA1, dfrA7, sul1, sul2, strAB, blaTEM-1
- **Sensitive**
- - - % MDR