Emergence and molecular basis of azithromycin resistance in typhoidal *Salmonella* in Dhaka, Bangladesh

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Abstract

With rising fluoroquinolone and ceftriaxone-resistant *Salmonella* Typhi, azithromycin, a macrolide, has become the last oral drug available against typhoid. Between 2009-2016, we isolated 1,082 *Salmonella* Typhi and Paratyphi A strains in Bangladesh, 13 (12 Typhi and 1 Paratyphi A) of which were azithromycin-resistant. When compared to 462 previously sequenced Typhi strains, the genomes of the 12 azithromycin-resistant Typhi strains (4.3.1 sub-clade, H58) harbored an exclusive non-synonymous single-point mutation R717Q in AcrB, an RND-efflux pump. Expression of AcrB-R717Q in *E. coli* and Typhi strains increased its minimum inhibitory concentration (MIC) for azithromycin by 11- and 3-fold respectively. The azithromycin-resistant Paratyphi A strain also contained a mutation at R717 (R717L), whose introduction in *E. coli* and Paratyphi A strains increased MIC by 7- and 3-fold respectively, confirming the role of R717 mutations in conferring azithromycin resistance. With increasing azithromycin use, strains with R717 mutations may spread leading to treatment failures, making antibiotic stewardship and vaccine introduction imperative.

Introduction

Typhoid and paratyphoid, collectively known as enteric fever, are among the most common bacterial causes of morbidity worldwide, with the greatest burden in low- and middle-income countries (GBD 2017 Typhoid and Paratyphoid Collaborators, 2019). *Salmonella enterica* subspecies *enterica* serovars Typhi (*Salmonella* Typhi) and Paratyphi (A, B and C), etiologies of enteric fever, cause an estimated 14 million illnesses and 136,000 deaths annually.

In the pre-antibiotic era, enteric fever mortality rates exceeded 20% in many areas, but ampicillin, chloramphenicol and co-trimoxazole were instrumental in reducing the rates to <1%. Resistance to all three antibiotics (referred to as multidrug resistance, MDR) emerged in late 1980's (Mirza et al., 1996), predominantly due to the rise and subsequent continental migration of H58 haplotype (now referred to as 4.3.1), which contained the resistance genes either on IncH1 plasmids or integrated within the chromosome (Holt et al., 2011; Wong et al., 2015, 2016). Fluoroquinolones soon became the most-commonly prescribed antibiotic (White et al., 1996), but since the 2000's there have been increasing reports of decreased fluoroquinolone susceptibility due to the acquisition of chromosomal mutations in the DNA gyrase and topoisomerase IV genes (Roumagnac et al., 2006; Chau et al., 2007, Dimitrov et al., 2007; Pham Thanh et al., 2016). In Bangladesh, >99% of all Typhi and Paratyphi strains exhibit decreased susceptibility to ciprofloxacin (Saha et al., 2018b). In 2011, WHO recommended ceftriaxone or azithromycin for treating *Salmonella* Typhi non-susceptible to fluroquinolones (Balasegaram et al., 2012).

There have been sporadic reports of ceftriaxone-resistant *Salmonella* Typhi strains, (Saha et al., 1999; Djeghout et al., 2018), but in 2016, an outbreak of extensively drug-resistant (XDR) *Salmonella* Typhi, resistant to chloramphenicol, ampicillin, cotrimoxazole, fluoroquinolones, and third-generation cephalosporins was recognized in Pakistan and to date >1000 cases have been confirmed (Andrews et al., 2018). Cephalosporin resistance of the XDR strains was caused by the acquisition of a broad-spectrum beta-lactamase (bla-CTX-M-15) on an IncY plasmid found in other enteric species. Typhoid patients in Pakistan are primarily being treated with the last available oral option, the macrolide azithromycin, resistance to which is rare (Klemm et al., 2018, Parry et al., 2015). This increasing use of azithromycin places selective pressure for the emergence and spread of azithromycin-resistant isolates, raising concerns of untreatable infections and increased mortality rates. Little is known about azithromycin resistance in typhoidal *Salmonella*; while there are some sporadic reports on azithromycin treatment failures (Molloy et al., 2010; Sjölund-Karlsson et al., 2011; Wong et al., 2015; Patel et al., 2017), there are no data on the molecular mechanism of resistance.

In Bangladesh, *Salmonella* Typhi and Paratyphi A are the most common causes of bloodstream infections in children >2 months of age and comprise of two-third of blood-culture positive isolates in microbiology laboratories (Saha et al., 2017). Leveraging our surveillance system in place for enteric fever, here we describe the emergence of azithromycin resistance among typhoidal *Salmonella* in Bangladesh and identify the molecular basis behind this resistance.

Result and Discussion

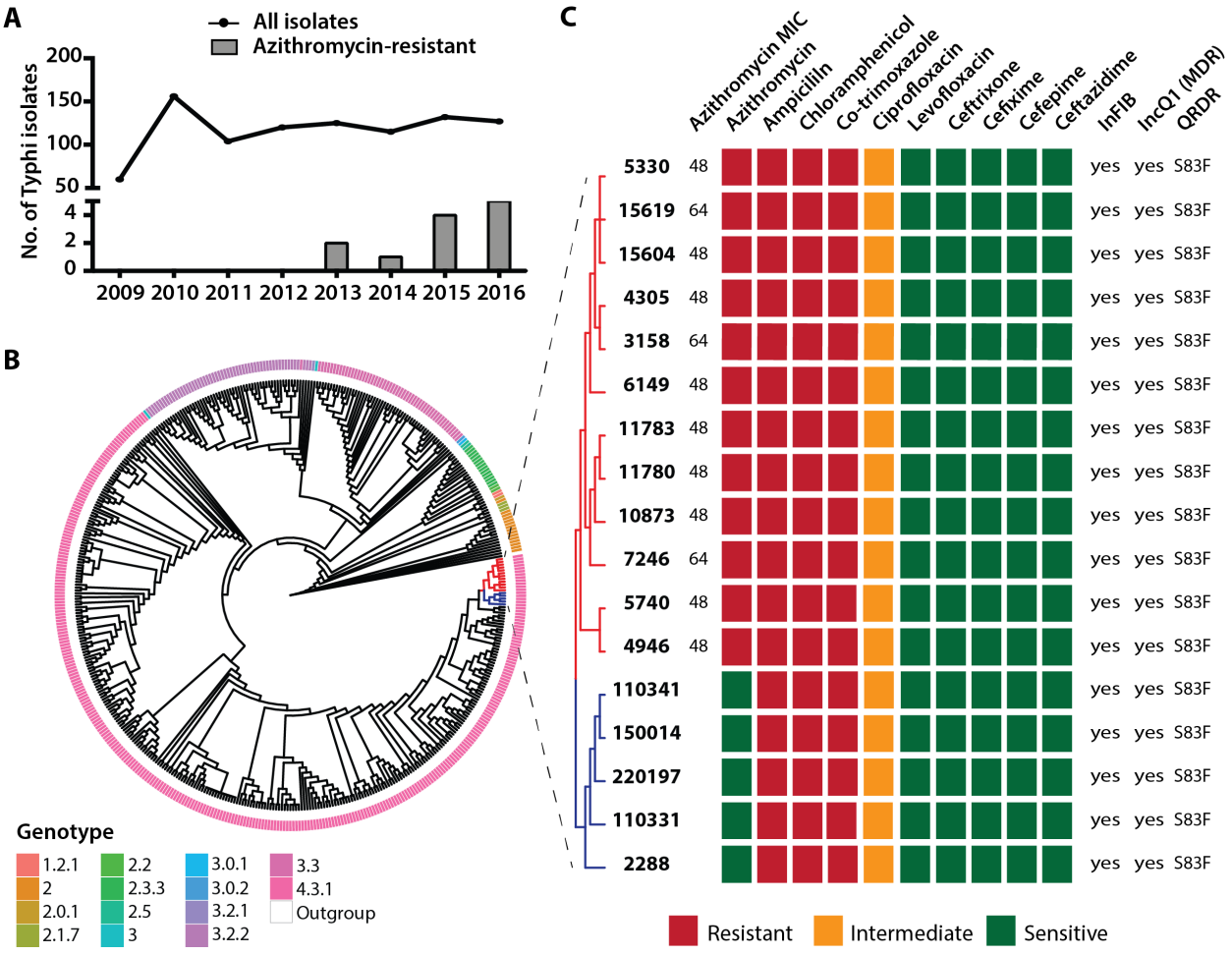
Emergence of azithromycin-resistant *Salmonella* Typhi and Paratyphi A

Between 2009 and 2016, through our enteric fever surveillance (Saha et al., 2017) in the inpatient departments of the two largest pediatric hospitals of Bangladesh, we isolated 939 *Salmonella* Typhi and 143 Paratyphi A strains. Twelve of the Typhi and one of the Paratyphi A strains were resistant to azithromycin, with disc diameters of ≤12 mm, and minimum inhibitory concentration (MIC) of ≥32 µg/ml (Parry et al., 2015). All 12 azithromycin-resistant *Salmonella* Typhi strains were also MDR and were increasingly isolated since 2013 (Fig 1A), marking gradual emergence of azithromycin-resistant *Salmonella* Typhi in Bangladesh. All patients lived in Dhaka city, known to be endemic for typhoid (Figure S1).

Azithromycin resistant *Salmonella* Typhi harbors a mutation in the AcrB efflux pump

We sequenced the 12 azithromycin-resistant Typhi strains and found that all azithromycin-resistant strains belonged to genotype 4.3.1 (H58), the most common genotype found in South Asia (Tanmoy et al., 2018; Wong et al., 2015). In a whole-genome single nucleotide polymorphism (SNP) tree, the 12 strains clustered together indicating that they are genetically similar to one another and potentially arose for a single common parental strain (Figure 1B). To identify the genetic basis of azithromycin resistance, we used three bioinformatic tools: SRST2 (Inouye et al., 2014), Resfinder (Zankari et al., 2012) and CARD (Jia et al., 2017) and to evaluate the results obtained from these tools, we tested antimicrobial susceptibility against a panel of nine other antibiotics (Figure 1C). While the tools successfully predicted the observed susceptibility patterns for the nine antibiotics, no known azithromycin resistance mechanism was identified (Figure 1C). Using PlasmidFinder (Carattoli et al., 2014) we identified two plasmids found in these *Salmonella* Typhi strains: (i) IncQ1 (12/12 strains), containing genes for ampicillin, co-trimoxazole and chloramphenicol resistance, and (ii) InFIB (12/12 strains), a

106 plasmid commonly found in *Salmonella* Typhi strains (Kidgell et al., 2002; Park et al., 2018).
107 Both these plasmids were also present in closely related azithromycin-sensitive strains. The lack
108 of known azithromycin-resistance genes indicated a novel mechanism of azithromycin resistance
109 in these strains.
110



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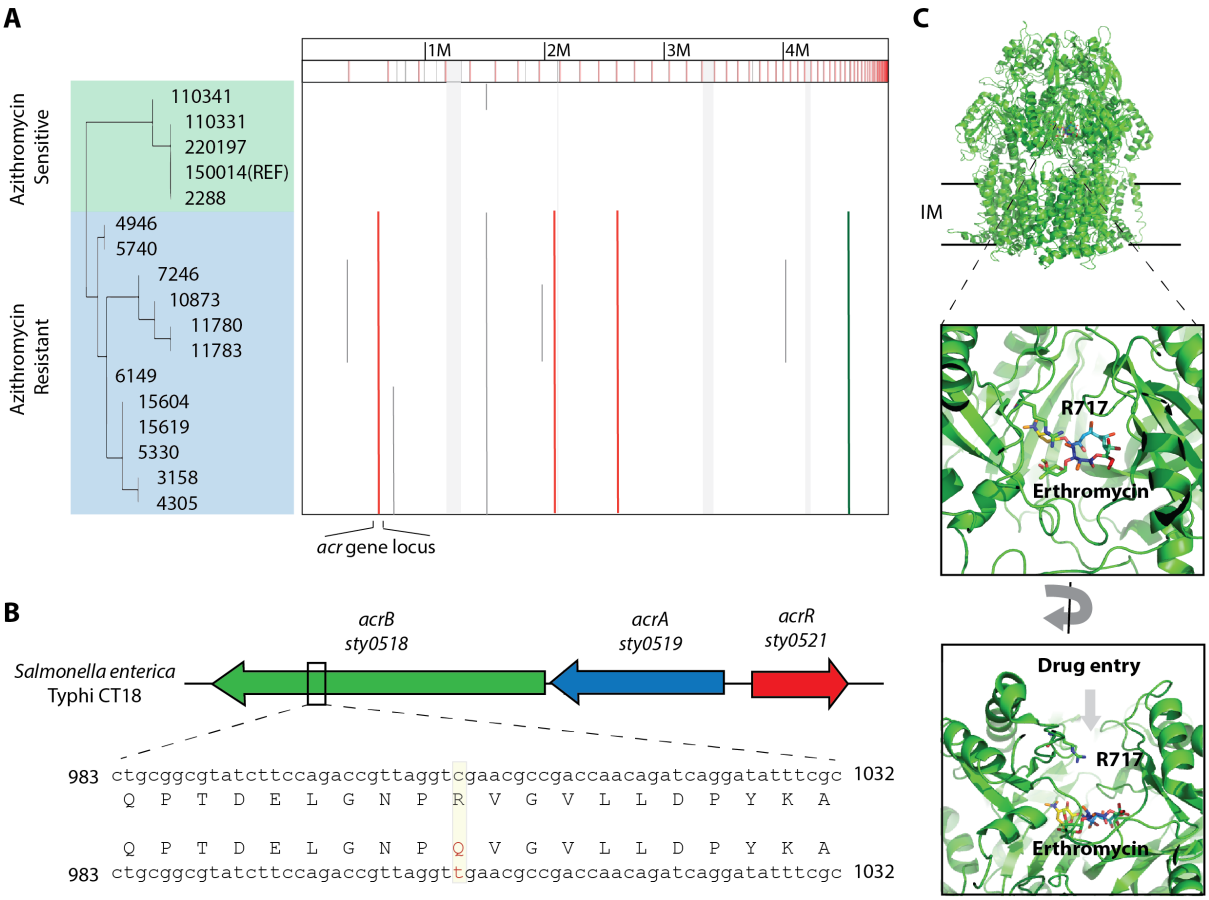
112 **Figure 1: Emergence of azithromycin-resistant strains of *Salmonella* Typhi in Bangladesh**
113 **and their genomic analysis. A)** Temporal distribution of 939 *Salmonella* Typhi isolates
114 included in the study. The number of isolates is shown as the line plot from 2009-2016. The
115 numbers of azithromycin-resistant strains isolated each year is shown in the bar plot.
116 Azithromycin-resistant strains were first isolated in 2013. **B)** Whole-genome SNP tree of 474
117 *Salmonella* Typhi strains isolated in Bangladesh previously (Tanmoy et al., 2018). The tree
118 highlights the different genotypes that are found in Bangladesh including the most prevalent
119 genotype 4.3.1 (H58 haplotype). The 12 azithromycin-resistant strains (colored in red) clustered

together within the genotype 4.3.1. *Salmonella* typhimurium strain LT2 was used as an outgroup. **C)** Predicted and experimentally determined antimicrobial susceptibility pattern of azithromycin-resistant *Salmonella* Typhi strains and the most-closely related five azithromycin-sensitive strains. The antimicrobial susceptibility was experimentally determined through disc diffusion assay against a panel of 10 antibiotics. The predicted transmissible elements and antimicrobial resistance markers are also shown.

We compared the sequences of these 12 azithromycin-resistant strains to that of 462 Typhi strains that we had previously sequenced and genetically characterized (Tanmoy et al., 2018). In the WGS SNP tree, we identified four unique SNPs, present only in the 12 azithromycin-resistant strains, three of which were non-synonymous: STY2741 (codes for purN, a glycinamidine ribonucleotide transformyltransferase), STY1399 (codes for a hypothetical protein) and STY0519 (codes for AcrB, an inner membrane permease) (Figure 2A, Figure S2). For the first two candidates, there is no evidence of their involvement in mediating antimicrobial resistance in the literature. However, the third gene, *acrB* is part of a trans-envelope resistance-nodulation-division (RND) efflux pump that has been previously reported to transport macrolides including azithromycin across the bacterial cell envelope, making it the most promising candidate (Nakashima et al., 2011). Mutations affecting expression of AcrB have been implicated in macrolide resistance in *Neisseria gonorrhoeae* (Wadsworth et al., 2018). Furthermore, laboratory mutagenesis studies in *Escherichia coli* have shown that mutations in *acrB* can lead to higher macrolide efflux thereby contributing to resistance (Ababou and Koronakis, 2016). The SNP observed in the 12 azithromycin-resistant *Salmonella* Typhi strains changed the arginine residue (R) at position 717 to a glutamine (Q) (Figure 2B). R717 is a conserved residue on the periplasmic cleft that acts as the entry portal for most drugs in AcrB (Figure 2C). In a previous mutagenesis study, substitution of the arginine residue with an alanine (R717A) was found to partially increase efflux of the macrolide erythromycin in *E. coli* (Yu et al., 2005).



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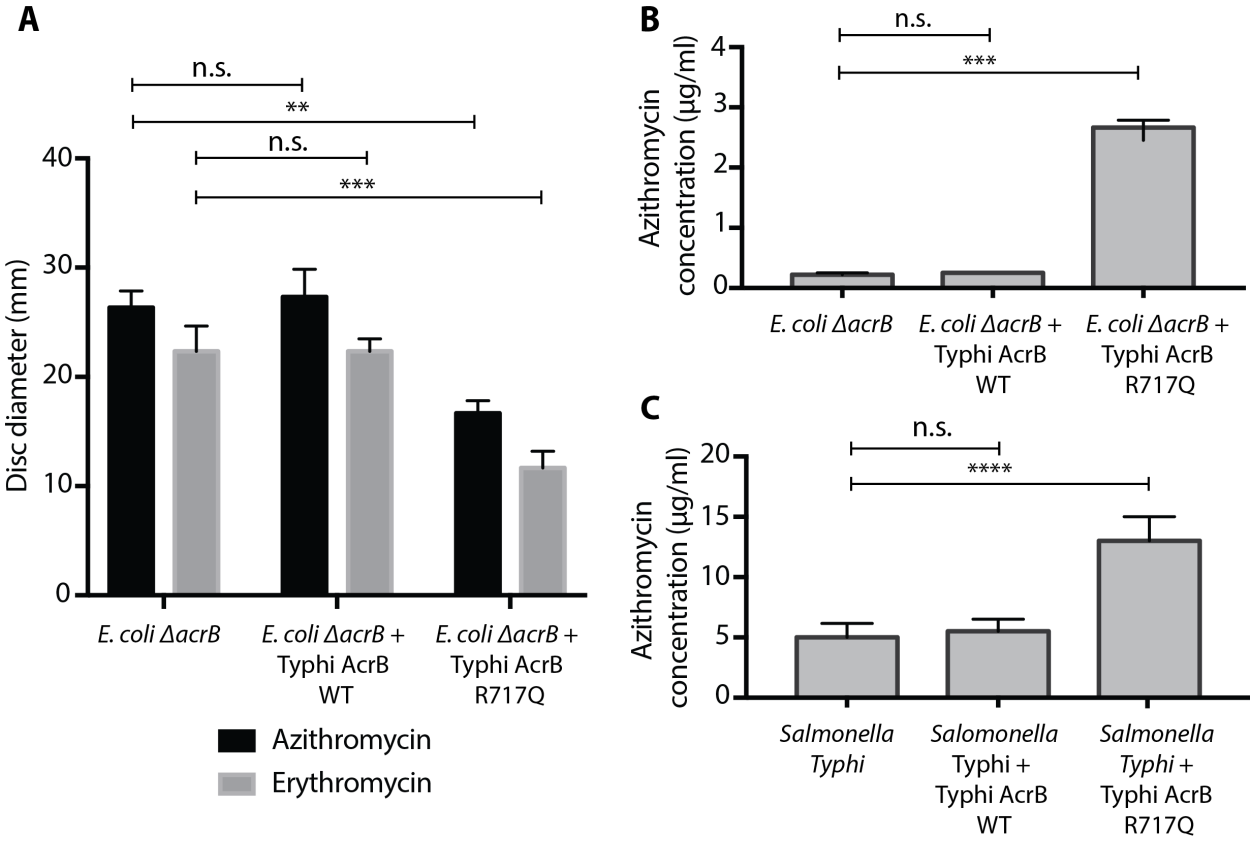
147 **Figure 2: Identification of R717Q mutation on AcrB efflux pump as a cause of**
148 **azithromycin resistance in *Salmonella* Typhi. A)** Whole genome sequence alignment of 12
149 azithromycin-resistant and 5 genetically related azithromycin-sensitive *Salmonella* Typhi strains.
150 Whole genome SNP detection and alignment was done using ParSNP and results were visualized
151 in GinGR (Treangen et al., 2014). The single nucleotide polymorphisms (SNPs) unique to the
152 resistant strains are highlighted with vertical lines. Four SNPs were identified: 3 non-
153 synonymous (shown as a red line) and 1 synonymous SNPs (shown as a brown line) that are
154 exclusive to the azithromycin resistant strains. **B)** The *acr* gene cluster in *Salmonella* Typhi
155 reference strain CT18. One of the SNPs found exclusively in azithromycin-resistant strains was
156 mapped to the gene cluster composed of: *acrA* (STY0519) and *acrB* (STY0518), that encodes a
157 periplasmic and inner membrane protein component of the RND-efflux pumps respectively, and
158 *acrR* (STY0521), a transcriptional regulator of AcrA/B protein synthesis. The SNP was present
159 on the *acrB* gene and resulted in the change of an arginine (R) at position 717 to a glutamine (Q)
160 residue on the encoded AcrB protein. **C)** R717Q mutation is present at the periplasmic cleft of
161 the proximal binding pocket on AcrB. Structure of *E. coli* AcrB (PDB ID: 3AOC) is shown in
162 green with the macrolide erythromycin bound in the proximal drug binding pocket. AcrB is

present in the inner membrane of the bacterial cells and drug molecules, including macrolides, enter the AcrB pump through a periplasmic opening that leads to a proximal binding pocket. The drug molecules are shuttled outside the cells through the proximal binding pocket with the help of the proton motive force. R717 lines the entry the periplasmic opening.

R717 mutations in AcrB confer azithromycin resistance

We cloned *acrB* from azithromycin susceptible and resistant *Salmonella* Typhi strains into an *E. coli* plasmid and introduced them into an *E. coli* strain that lacks the endogenous *acrB* (*E. coli ΔacrB*). Compared to *E. coli* strains containing empty plasmid or wild type *acrB*, the strain expressing AcrB-R717Q showed a smaller zone of disc clearance for both azithromycin (26.3 mm vs 16.7 mm, $p = 0.0013$) and erythromycin discs (22.3 mm vs 11.7 mm, p *value* $= 0.0009$) and exhibited a 11-fold increase in azithromycin MIC (0.22 µg/ml vs 2.7 µg/ml, $p = 0.0002$) (Figure 3A, B). Certain AcrB mutations have previously been shown to effect transport of other antibiotics such as ciprofloxacin (Blair et al., 2015), but the R717Q mutation did not change the susceptibility patterns for any other nine antibiotics we tested (Figure S3). For further confirmation of the effects of this mutation in *Salmonella* Typhi, we introduced the plasmids in an azithromycin-sensitive Typhi strain and observed a 3-fold increase in MIC (5 µg/ml vs 13 µg/ml, $p < 0.0001$) in the presence of AcrB-R717Q (Figure 3C). The difference here is lower compared to that seen in *E. coli ΔacrB* plausibly because the Typhi strain contains endogenous wild-type AcrB competing against the exogenous AcrB-R717Q that we artificially introduced. Taken together, these results confirm that AcrB-R717Q leads to increased macrolide resistance.



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185 **Figure 3: AcrB R717Q increases efflux of macrolides in *E. coli* and *Salmonella* Typhi**
186 **strains.** Quantification of results obtained from three biological replicates for **(A)** disc diffusion
187 and **(B)** E-strip assays are shown for *E. coli* BW25113 *ΔacrB* transformed with different
188 plasmids. **(C)** Quantification of results obtained from at least three biological replicates from
189 azithromycin E-strip assay in Typhi strain 4119. One way-ANOVA with multiple comparisons
190 was used to test statistical significance. ns: $p>0.05$; **: $p \leq 0.01$; ***: $p \leq 0.001$, ****: $p \leq$
191 0.0001
192

193 We conducted an extensive BLAST search to identify other typhoidal *Salmonella* strains with
194 mutations in AcrB the NCBI database and found only one *Salmonella* Typhi strain isolated in
195 Oceania in 2008 that contained the same R717Q mutation, however no AMR data were available
196 for this strain (Wong et al., 2015). Interestingly, whole genome sequencing of the one
197 azithromycin-resistant *Salmonella* Paratyphi A strain identified during our surveillance showed
198 that this strain also contained a mutation in *acrB* which changed R717 to a leucine (L) (Figure
199

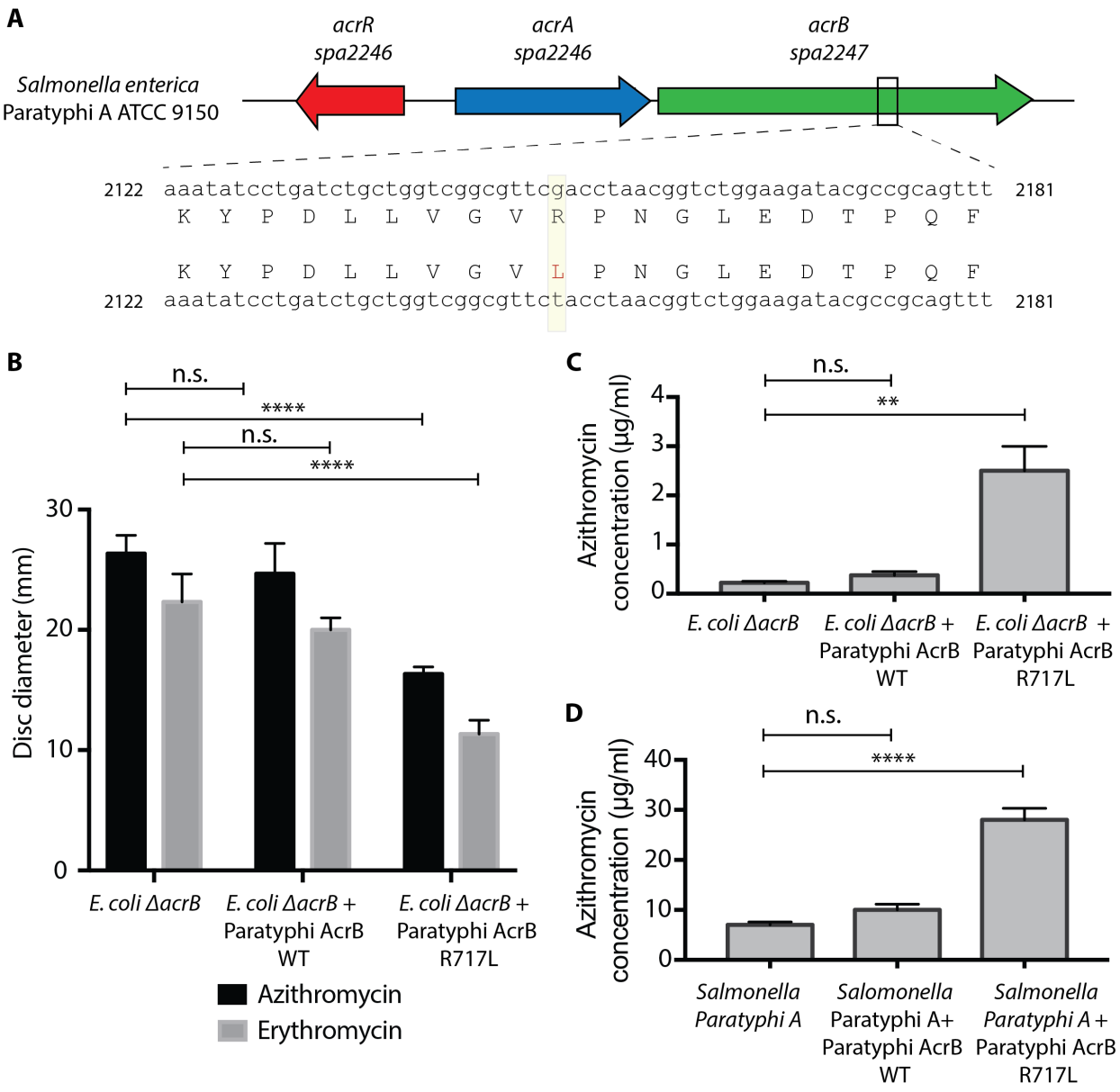

Figure 4: Identification of R717L mutation in AcrB protein in *Salmonella* Paratyphi A strains. (A) Sequence alignment of *acrB* gene from the azithromycin-resistant *Salmonella* Paratyphi A strain to the reference strain ATCC 9150. A SNP was identified that changed the R717 to a leucine (L) residue. Quantification of results obtained from three biological replicates for **(B)** disc diffusion and **(C)** E-strip assays in *E. coli* are shown. **(D)** Quantification of results obtained from at least three biological replicates from azithromycin E-strip assay in Paratyphi strain 4071 is shown. One way-ANOVA with multiple comparisons was used to test statistical significance. ns: $p>0.05$; **: $p \leq 0.01$; ****: $p \leq 0.0001$.

210 4A). This mutation was absent in the genomes of the Paratyphi A strains in the NCBI database.
211 To determine to effect of R717L mutation, we expressed Paratyphi wild-type AcrB and AcrB-
212 R717L in *E. coli ΔacrB* (Figure 4B, C). As seen for Typhi AcrB R717Q, Paratyphi AcrB-R717L
213 leads to a smaller disc clearance for azithromycin (26.3 mm vs 16.3 mm, $p = 0.0001$) and
214 erythromycin (22.4 mm vs 11.4 mm, $p = 0.0001$) and 10-fold higher azithromycin MIC (0.22
215 µg/ml vs 2.5 µg/ml, $p = 0.003$). When these plasmids were introduced in a sensitive Paratyphi A
216 strain, we observed 4-fold change in MIC (7 µg/ml vs 28 µg/ml, $p = 0.0001$) in the presence of
217 the R7171L mutation, confirming that mutations in R717 lead to macrolide resistance.
218
219 Rising antimicrobial resistance threatens the progress made so far in management of enteric
220 fever. The rate of azithromycin resistance of typhoidal *Salmonella* in Bangladesh is low and the
221 genetic basis is a chromosomal SNP. However, in light of the outbreak in Pakistan, increasing
222 azithromycin use can place selective pressure on strains such as the ones with R717 mutations to
223 spread. Although no azithromycin resistant XDR isolate has been reported to date, the increasing
224 use of azithromycin and the clear historical record of widespread dissemination of resistance to
225 all previously widely used antimicrobials by *Salmonella* Typhi and Paratyphi, suggest we will
226 soon face strains resistant to all oral antibiotics. An azithromycin-resistant XDR strain would
227 shift enteric fever treatment from outpatient departments, where patients are currently treated
228 with oral azithromycin, to inpatient departments to be treated with injectable antibiotics like
229 carbapenems, thereby weighing down already struggling health systems in endemic regions
230 (Andrews et al., 2018; Saha et al., 2018a). Moreover, with the dearth of novel antimicrobials in
231 the horizon, we risk losing our primary defense against widespread mortality from enteric fever.
232 In addition to continued surveillance and antimicrobial stewardship, it is imperative to roll-out

233 the recent WHO prequalified typhoid conjugate vaccine in endemic areas and decrease the
234 overall burden of typhoid.
235
236 **Materials and Methods**
237 **Study site and population**
238 In this study, we report data from enteric fever surveillance conducted in the inpatient
239 departments of the two largest pediatric hospitals of Bangladesh, Dhaka Shishu (Children)
240 Hospital, DSH, and Shishu Shasthya (Child Health) Foundation Hospital, SSFH. These are
241 sentinel sites of the World Health Organization supported Invasive Bacterial Vaccine
242 Preventable Diseases surveillance platform in Bangladesh.
243
244 **Patient enrollment, etiology detection and antibiogram**
245 Blood culture was performed at the discretion of the treating physicians. We enrolled patients
246 with positive blood cultures for *Salmonella* Typhi or Paratyphi A. Blood cultures were
247 performed using standard methods (Saha et al., 2017). We aseptically obtained 2–3 milliliters of
248 blood, which was inoculated into trypticase soy broth supplemented with sodium polyanethole
249 sulphonate (0.25%) and isovitalex (1%). Incubated blood culture bottles were sub-cultured on the
250 second, third, and fifth days of incubation. Identification of *Salmonella* Typhi/Paratyphi isolates
251 was confirmed using standard biochemical tests and agglutination with *Salmonella* species and
252 serovar-specific antisera (Ramel, Thermo Fisher Scientific). Laboratory methods for blood
253 culture and organism identification were consistent over the reporting period.
254

We used disc diffusion methods for determining antibiotic susceptibility patterns for azithromycin, ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, levofloxacin, ceftriaxone, cefepime, cefixime and ceftazidime (Oxoid, Thermo Scientific, MA, USA). Azithromycin e-strips (bioMérieux, France) were used to determine the minimum inhibitory concentration (MIC) and confirm azithromycin resistance for strains that exhibited zone of clearance ≤12 mm in the presence of azithromycin discs. All results were interpreted according to the latest Clinical and Laboratory Standards Institute guidelines 2018.

DNA extraction and whole genome sequencing

We conducted whole genome sequencing on all identified azithromycin-resistant strains (12 *Salmonella* Typhi and 1 Paratyphi A). Isolates were grown in MacConkey agar (Oxoid, UK) overnight and DNA was extracted from a suspension of the overnight culture using the QIAamp DNA minikit (Qiagen, Hilden, Germany). Whole genome sequencing was performed on the Illumina HiSeq 4000 platform to generate 150 bp paired-end reads (Novogene Co. Ltd., Beijing, China). We used SPAdes (Bankevich et al., 2012) to assemble the short paired-end reads into contigs for downstream analyses. All the sequences have been submitted to EnteroBase and NCBI (BioProject ID: PRJNA528114).

Bioinformatics analysis

For comparative genomic analysis, we compared the 12 azithromycin-resistant *Salmonella* Typhi strains with 462 strains that were previously isolated and genetically characterized by our group in Bangladesh (Tanmoy et al., 2018). Using the ParSNP tool (Treangen et al., 2014), we constructed whole-genome SNP tree using all 475 genomes and determined the genotypes using

srst2 package (Inouye et al., 2014). Ggtree was used to make the phylogenetic tree and overlay the genotype data (Yu et al., 2017). Srst2, ResFinder (Zankari et al., 2012) and CARD (Jia et al., 2017) were used to predict antimicrobial resistance markers, and PlasmidFinder (Carattoli et al., 2014) to identify the putative plasmids present in these strains. Finally, we compared the resistant strains to all sensitive *Salmonella* Typhi strains manually to find SNPs exclusive to the resistant strains (comparison to the most closely related 5 genomes are shown in Figure 1C) using the GinGR tool from the Harvest suite (Treangen et al., 2014). To predict the function of the SNPs on protein function, we examined the protein sequence and conducted structural analyses (Figure 2).

Macrolide susceptibility test in *E. coli* and *Salmonella* Typhi

We amplified the *acrB* genes from azithromycin-resistant *Salmonella* Typhi strain 5003 and Paratyphi A strain 3144 and azithromycin-sensitive Typhi strain 4119 and Paratyphi A strain 4071 for downstream cloning. The genes were inserted into the multiple cloning site of pHERD30T using Gibson assembly (Gibson et al., 2009). We verified the sequences of all inserted genes through Sanger sequencing. The plasmids were chemically transformed into $CaCl_2$-competent *E. coli* BW25112 *ΔacrB* strains, and electroporated into Typhi strain 4119 and Paratyphi A strain 4071. *E. coli* strains with plasmids (with or without an insert) were tested for susceptibility patterns for erythromycin, azithromycin, ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, levofloxacin, ceftriaxone, cefepime, cefixime and ceftazidime using the disc diffusion method, and MIC was determined using azithromycin E-strips. Typhi and Paratyphi strains with plasmids (with or without an insert) were tested for susceptibility patterns for azithromycin using E-strips.

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Conflict of interest

Authors declare no conflict of interest pertaining to the work presented here.

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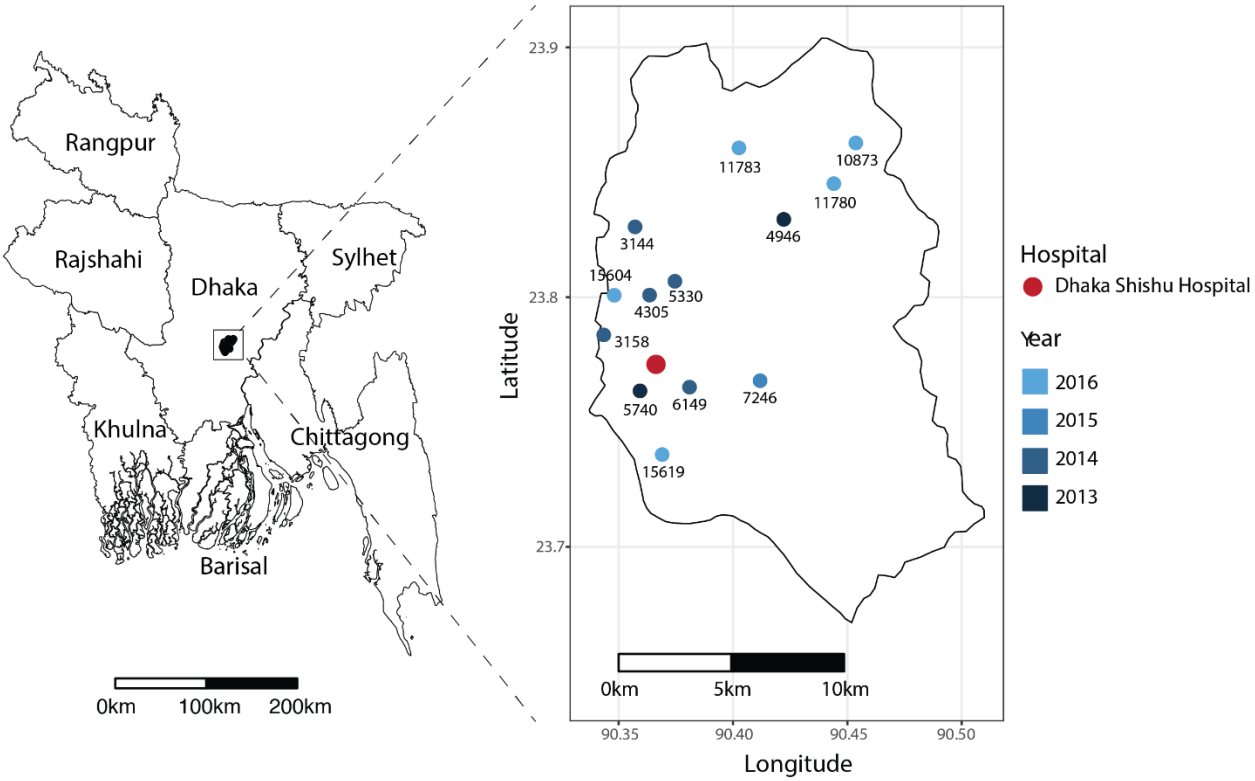
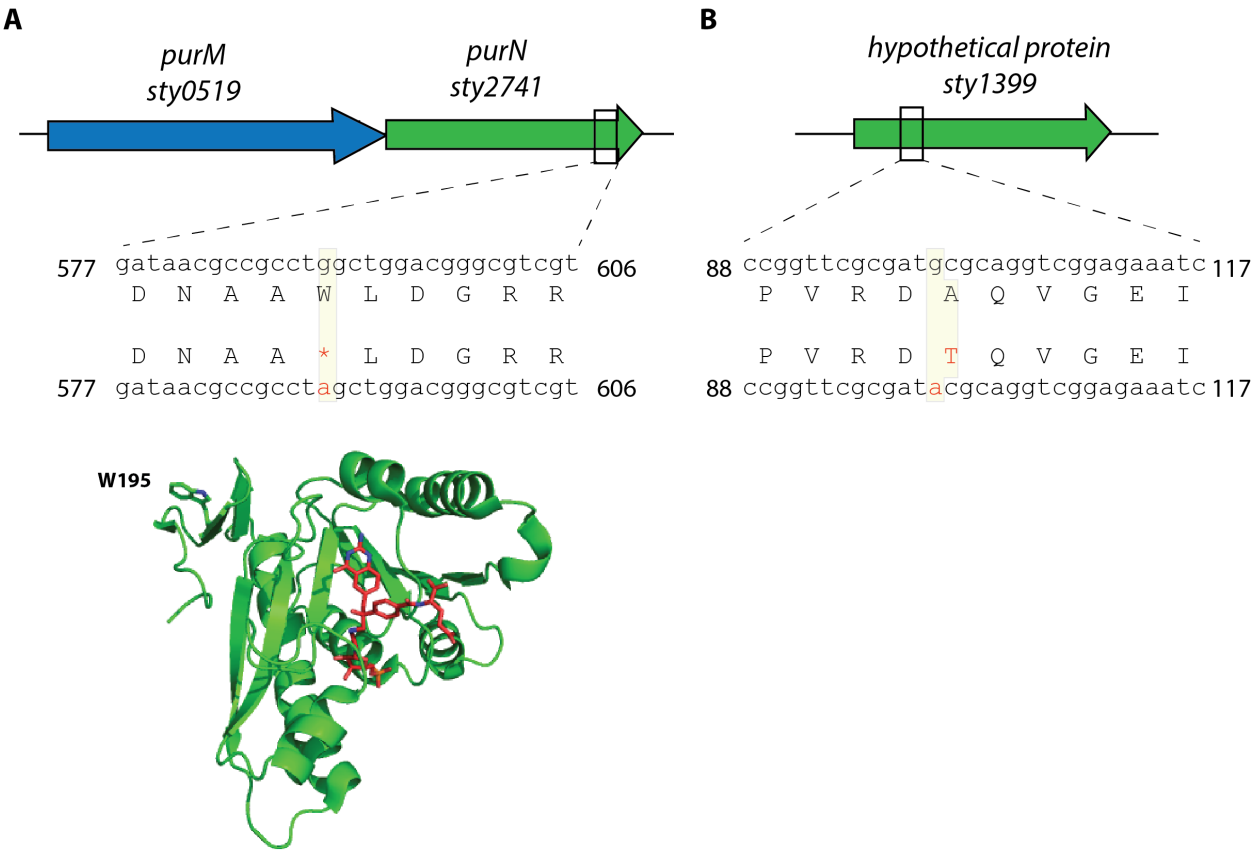
Supplementary Figures



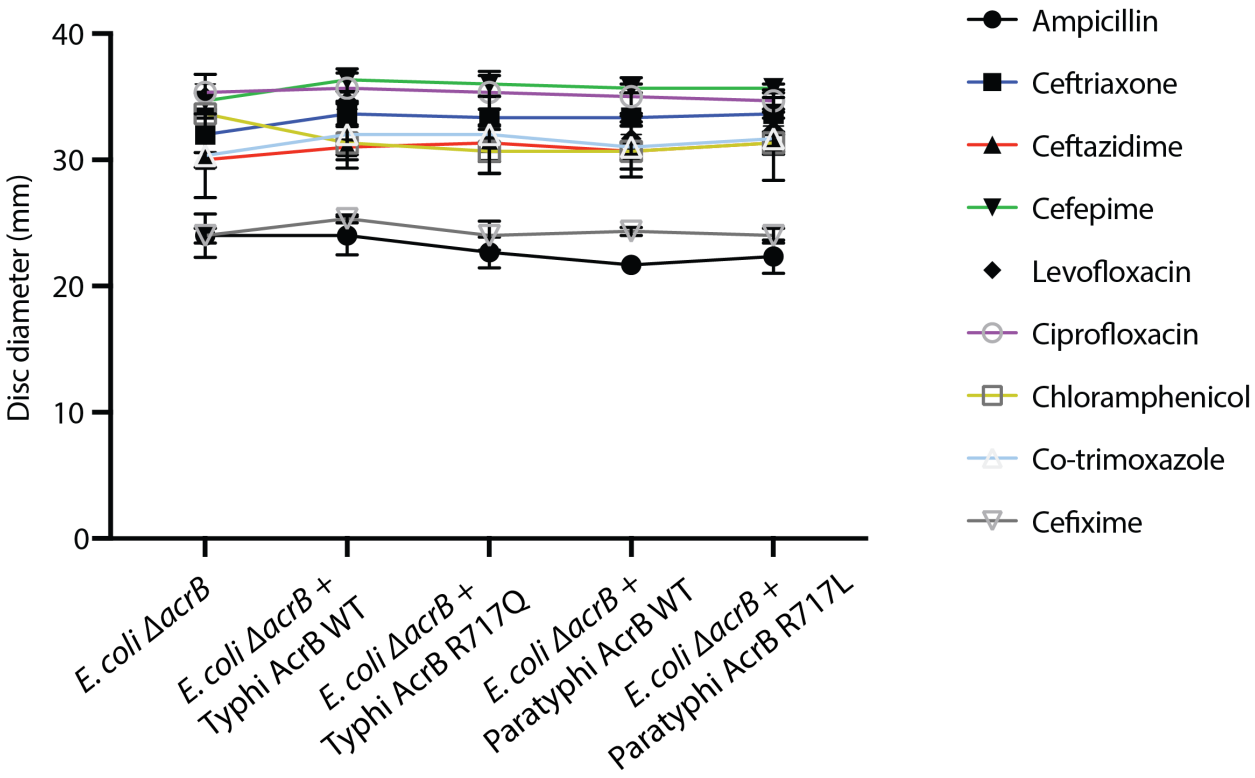
494

495 **Figure S1: Spatiotemporal distribution of azithromycin-resistant *Salmonella* Typhi and**
496 **Paratyphi A strains.** The 13 azithromycin-resistant typhoidal *Salmonella* strains were isolated
497 from Dhaka Shishu Hospital (shown in red). All the patients lived within the Dhaka municipal
498 area.



499

500 **Figure S2: Genetic and structural analysis of 2 other non-synonymous SNPs. A)** SNP on
501 *sty2741* gene (also known as *purN*) that encodes a glycinamidine ribonucleotide
502 transformyltransferase (GAR-Tfase) enzyme. The SNP leads to change in W195 to a stop codon,
503 leading to premature termination of the protein sequence. The structure of *E. coli* GAT-Tfase
504 (green, PDB ID: 1C3E) in complex with an inhibitor (shown in red) highlighting the active site is
505 shown. The W195 is present close to the C-terminus and premature termination at this position is
506 predicted to not affect protein function. **C)** SNP on *sty1399* that encodes a hypothetical protein
507 that is proposed to contain a B3/B4 tRNA-binding domain. The function of this protein is not
508 known and the SNP results in conversion of an alanine residue at position 34 to a threonine
509 residue. None of these two genes have been previously implicated in macrolide resistance.



510

511 **Figure S3 – figure supplement 3: AcrB mutations do not affect efflux of other families of**
512 **antibiotics.** Susceptibility of *E. coli* strains with empty, *Salmonella* Typhi AcrB WT/R717Q and
513 Paratyphi A AcrB WT/R717L were tested against a panel of 9 different antibiotic including 5
514 beta-lactams, 2 fluoroquinolones, 1 phenicol and 1 diaminopyrimidine /sulphonamide. The data
515 are shown as mean and standard error from 3 different biological replicates.