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The molecular basis of extensively drug-resistant *Salmonella* Typhi isolates from pediatric septicemia patients

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Abstract

Sepsis is a syndromic response to infections and is becoming an emerging threat to the public health sector, particularly in developing countries. *Salmonella* Typhi (*S.* Typhi), the cause of typhoid fever, is one primary cause of pediatric sepsis in typhoid endemic areas. Extensively drug-resistant (XDR) *S.* Typhi is more common among pediatric patients, which is responsible for over 90% of the reported XDR typhoid cases, but the majority of antibiotic resistance studies available have been carried out using *S.* Typhi isolates from adult patients. Here, we characterized antibiotic-resistance profiles of XDR *S.* Typhi isolates from a medium size cohort of pediatric typhoid patients (n = 45, 68.89% male and 31.11% female) and determined antibiotic-resistance-related gene signatures associated with common treatment options to typhoid fever patients of 18 XDR *S.* Typhi representing all 45 isolates. Their ages were 1–13 years old: toddlers aging 1–2 years old (n = 9, 20%), pre-schoolers aging 3–5 years old (n = 17, 37.78%), school-age children aging 6–12 years old (n = 17, 37.78%), and adolescents aging 13–18 years old (n = 2, 4.44%). Through analyzing bla_{TEM1}, *dhfR7*, *sul1*, and *catA1* genes for multidrug-resistance, *qnrS*, *gyrA*, *gyrB*, *parC*, and *parE* for fluoroquinolone-resistance, $bla_{CTX-M-15}$ for XDR, and *macAB* and *acrAB* efflux pump system-associated genes, we showed the phenotype of the XDR *S.* Typhi isolates matches with their genotypes featured by the acquisitions of the genes bla_{TEM1}, *dhfR7*, *sul1*, *catA1*, *qnrS*, and $bla_{CTX-M-15}$ and a point mutation on *gyrA*. This study informs the molecular basis of antibiotic-resistance among recent *S.* Typhi isolates from pediatric septicemia patients, therefore providing insights into the development of molecular detection methods and treatment strategies for XDR *S.* Typhi.

Introduction

Sepsis is a syndromic response to infections and is becoming an emerging threat to the public health sector. The World Health Organization (WHO) estimated that 48.9 million cases of sepsis have been reported and that one person dies every 2.58 seconds around the world [1]. Furthermore, 20 million cases were detected among children, with 2.9 million deaths worldwide and 85% of these deaths in developing countries [1]. *Salmonella enterica* serovar Typhi (*S.* Typhi), the causative agent of typhoid fever, is one primary cause of pediatric sepsis in typhoid endemic areas including Pakistan [1–3]. Antibiotics are the primary treatment options for typhoid fever, but *Salmonella* are continuously evolving to acquire plasmid, prophage, transposon, or chromosomal gene mutation to attain resistance against antibiotics. A myriad of reports indicated the global spread of *S.* Typhi that is resistant to all of the first-line antibiotics, ampicillin, chloramphenicol, and co-trimoxazole, collectively known as multidrug-resistant (MDR) [4–8]. All of the identified MDR *S.* Typhi carry the IncHI1 region located on either the plasmid or chromosome, which encodes several antibiotic-resistance genes, including *catA1* (conferring resistance to chloramphenicol), $bla_{\text{TEM-1}}$ (resistance to ampicillin), *dhfR7*, and *sul1* (resistance to co-trimoxazole), among other antibiotics-resistance-related genes found in MDR *S.* Typhi [4,9–11].

Fluoroquinolones were used to treat MDR cases but became largely ineffective in some endemic regions. Fluoroquinolone-resistant *S.* Typhi encodes the quinolone resistance gene *qnrS* and point mutations in the quinolone resistance determining region (QRDR) harboring the genes for gyrase/topoisomerase II *gyrA* and *gyrB* and topoisomerase IV *parC* and *parE*. For instance, several point mutations occurred in *gyrA* have been correlated to resistance to fluoroquinolones, including M52L, G81C, D82G, S83F/Y/L, D87N/G/A/Y/H, and A119E [10,12–16]. Point mutations in *gyrB*, *parC*, and *parE* have also been reported, while some variants have been reported only from certain geographical locations [13,15–20]. Some of those mutation sites are near the quinolone binding site, which in many cases results in the inhibition of the binding of antibiotics to topoisomerases [21]. *S.* Typhi strains resistant to chloramphenicol, ampicillin, co-trimoxazole, fluoroquinolones, and third-generation cephalosporins were first reported in Hyderabad, Sindh, Pakistan, affecting over 300 cases in 2016 [9], collectively known as extensively drug-resistant (XDR) *S.* Typhi [8,22]. XDR *S.* Typhi isolates commonly harbor an IncY plasmid carrying the extended-spectrum β-lactamase resistance gene $bla_{\text{CTX-M-15}}$ and quinolone resistance gene *qnrS*, among others [9].

Drug efflux pump systems also play a significant role in resistance to a wide range of antibiotics. *Salmonella spp*. possess five efflux pump families, including the ATP-binding cassette (ABC) MacAB-TolC system and resistance-nodulation-cell division (RND) AcrAB-TolC system [23]. Members of the other families of drug transporters, major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), and small multidrug resistance (SMR), are located in the inner membrane (IM) of gram-negative bacteria [24]. They usually function as independent units in the IM to translocate antibiotics across the membrane bilayer, followed by their cooperation with RND-type efflux pumps to pump out antibiotics across the entire cell envelope [24]. In typhoidal *Salmonella*, point mutations at amino acid position 717 (R717Q or R717L) on AcrB, the antibiotic-binding subunit of the RND-type AcrAB-TolC efflux pump, have been correlated with resistance to azithromycin in *S.* Typhi and *S.* Paratyphi A, respectively [19,25,26].

Macrolides (e.g., azithromycin) and carbapenems (e.g., imipenem, meropenem) remain to be "last resort" oral and injectable antibiotics for treating *S.* Typhi infection, respectively. *S.* Typhi strains resistant to macrolide azithromycin have emerged [27]. *S.* Typhi strains resistant to carbapenem antibiotic meropenem, have also been reported, and many cases of invasive

nontyphoidal Salmonellae (NTS) resistant to carbapenems have been reported [25,28–30]. Given that typhoid fever vaccines and treatment options have limitations [31–35], there is an urgent need for closely monitoring drug-resistance profiles of *S.* Typhi strains at the point-of-care to provide valuable insights into the development of control strategies against drug-resistant *S.* Typhi among pediatric septicemia patients. Note that the majority of antibiotic resistance studies available have been carried out using *S.* Typhi isolates from adult patients, although antibiotic-resistant *S.* Typhi infection is more common among children. More than 90% of the XDR typhoid cases are currently from children younger than 15 years old of age [9,36,37].

Materials and methods

Ethics statement

Before initiating this research, ethical approval was obtained following the Declaration of Helsinki from the Institutional Review Board (IRB# FMH-03-2020-IRB-774-F), the Fatima Memorial Hospital Lahore. In addition, informed consent was obtained from a legal guardian of each study participant. Informed consent was read to the person in the language they understood and signed appropriately. They were willing to provide a sample and utilize the isolates for research. They were assured that the samples would be used solely for research purposes and that personal information would be kept confidential. Before samples were transferred to researchers, all XDR *S.* Typhi samples were de-identified, number-based identification codes were assigned to samples (S1–S3 Tables). The data were analyzed anonymously throughout the study.

S. Typhi isolation from patient specimens and minimum inhibitory concentration (MIC) determination

Blood samples from children (1–13 years) diagnosed with suspected septicemia were collected for the study. Blood samples were taken directly prior to antibiotics being administered. The patients showing two or more of the following signs and symptoms were included in the study: body temperature >38˚C or <35˚C, pulse rate >90 beats/minute, and respiratory rate >20 breathes/minute. This study is the continuation of the previous research. We identified or selected 45 XDR *S.* Typhi for further investigation as part of this study. Both sexes were included in the study.

The BacT/ALERT® 3D Microbial Detection System with PF/PF Plus culture bottles (bioMérieux, France), an automated bacterial culture and antibiotic-resistance test system capable of incubating, agitating, and continuously monitoring aerobic and anaerobic media inoculated with patient specimens was used in this study. If microorganisms were present in the test sample, carbon dioxide is produced as the microorganisms metabolize the substrates in the culture medium. When growth of the microorganisms produces CO_2, the color of the sensor in the bottom of each culture bottle changes from dark to light. A light-emitting diode (LED) projects light onto the sensor. The light reflected was measured by a photodetector. As more CO_2 was generated, more light was reflected. This information was compared to the initial sensor reading. If there is a high initial CO_2 content, an unusually high rate of CO_2 production, and/or a sustained production of CO_2, the sample was determined to be positive. If the CO_2 level does not change significantly after a specified number of days at optimal conditions, the sample was determined to be negative.

Forty-five samples were collected between October 2019 to January 2020. In brief, 1–4 mL blood samples of each septicemia suspected child based on their age and bodyweight were

taken and placed in BacT/ALERT PF/PF Plus bottles for up to 5 days. The bottles contained BacT/Alert FAN Plus media with Adsorbent Polymeric Beads (APB) that neutralized antimicrobials [38]. Positive blood culture bottles were sub-cultured on blood and MacConkey agar plates and incubated overnight at 37˚C aerobically. Preliminary identification of the isolates was conducted according to colony morphology and culture characteristics. *S*. Typhi colonies characteristics on growth plates were smooth, low convex, colorless and transparent. Standard laboratory testing available, such as widal tests, was performed. MIC determinations were made using the automatic VITEK 2 compact system (bioMérieux, France) and antibiotics interpretation was carried out as per the clinical laboratory standards institute (CLSI) 2018 guidelines (https://clsi.org) (S2 Table). The MIC determination was repeated in duplicate by the Vitek 2 compact system. All 45 *S*. Typhi isolates are determined to be XDR.

XDR *S*. Typhi samples selection for detailed molecular characterization

Of 45 XDR *S*. Typhi isolates, 18 isolates were selected for detailed molecular characterization based on their MIC results, gender, age, and hospital wards, representing all 45 XDR isolates (S1–S3 Tables). As indicated in S1 Table, the following antibiotics are tested: ampicillin (AMP), co-trimoxazole or trimethoprim-sulfamethoxazole (SXT), ciprofloxacin (CIP), cefotaxime (CTX), ceftriaxone (CRO), azithromycin (AZM), piperacillin (PIP), tazobactam (TZB), amoxicillin/clavulanic acid (AMC), imipenem (IPM), and meropenem (MEM).

Polymerase chain reaction (PCR)-based detection of antibiotic-resistance-related genes among XDR *S*. Typhi isolates

Bacterial genomic DNA was prepared using a DNeasy bacterial DNA extraction kit (QIAGEN, Hilden, Germany) following the vendor's recommendation. *S*. Typhi ISP2825 (GenBank number CP080960), a drug-susceptible clinical isolate, was used as a negative control for PCR reactions [39]. The PCR primer sequences and reaction conditions used are summarized in S4 Table. Green *Taq* DNA polymerase with provided buffers (GenScript, USA Inc., Piscataway, NJ, USA) was used for *pltB*, *bla*$_{TEM1}$, *dhfR7*, *sul1*, *catA1*, *parC*, *parE*, *bla*$_{CTX-M-15}$, *macA*, *acrB*, and *acrR*. Phusion high fidelity DNA polymerase with the provided GC buffer (New England BioLabs, Ipswich, MA, USA) was used for *gyrA*, *gyrB*, and *qnrS*. PCR reaction steps were: predenaturation at 95˚C for 3 min, 34 cycles of denaturation at 95˚C for 30 sec, annealing (see S4 Table), and extension at 72˚C for 1 min/kb (see S4 Table for amplicon size), and final extension at 72˚C for 7 min using a C1000 Touch Thermal Cycle (BIO-RAD, Hercules, CA, USA). PCR results were run on 1% agarose gels, along with GeneRuler 1 kb plus DNA ladder (ThermoFisher Scientific, catalog # SM1333, USA), and imaged using an iBright CL1500 Imaging system (ThermoFisher Scientific, Waltham, MA, USA).

Sanger sequencing of PCR amplicons

When indicated, PCR amplicons were extracted from agarose gels for sequencing analysis by using the QIAEX II gel extraction system (QIAGEN, cat # 20051), followed by standard Sanger sequencing (The Cornell Institute of Biotechnology or Eton Bioscience Inc). The primer sequences used for Sanger sequencing are summarized in S4 Table.

Whole-genome sequencing (WGS) analysis for efflux pump-related genes

The latest NCBI RefSeq dataset of the fully assembled complete genome of *S*. Typhi (107 in total; S5 Table) was collected on Feb 26, 2021. The 107 complete whole-genome sequences were utilized to analyze the sequence variations for *acrR* (NP_459472.1) using 'General Feature

Formats (gff)' files with a bash script (grep acrR *.gff | grep pseudo = true). Two whole genome sequences (GCF_001121865.2 and GCF_900205275.1) that have an *acrR* variant were further analyzed with CLC Main Workbench 8.1.3 (QIAGEN) for multidrug efflux pump-related genes: *macA* (NP_459918.1), *acrA* (NP_459471.1), *acrB* (NP_459470.1), *marA* (WP_000091194.1), and *robA* (NP_463442.1).

Results

XDR *S*. Typhi isolates from children at various developmental stages

We obtained *S*. Typhi isolates from 45 typhoid fever-suspected pediatric septicemia patients with 1–13 years of age (68.89% male and 31.11% female) who have visited the Fatima Memorial Hospital, Lahore, Punjab, Pakistan, between October 2019 to January 2020. Punjab is the most populous province approximately 1,044 km away from Hyderabad, Sindh, Pakistan, where the first XDR *S*. Typhi was reported (Fig 1A). *S*. Typhi samples were de-identified and analyzed anonymously throughout the study (S1 Table). Positive blood culture bottles from the initial step using a fully automated culture and test system for patient blood specimens were sub-cultured on blood and MacConkey agar plates and incubated overnight at 37˚C. Preliminary identification of the isolates was conducted according to colony morphology and culture characteristics, followed by PCR and Sanger sequencing-based molecular determination (Fig 1B and 1C). These results indicate that all suspected patient specimens carried *S*. Typhi
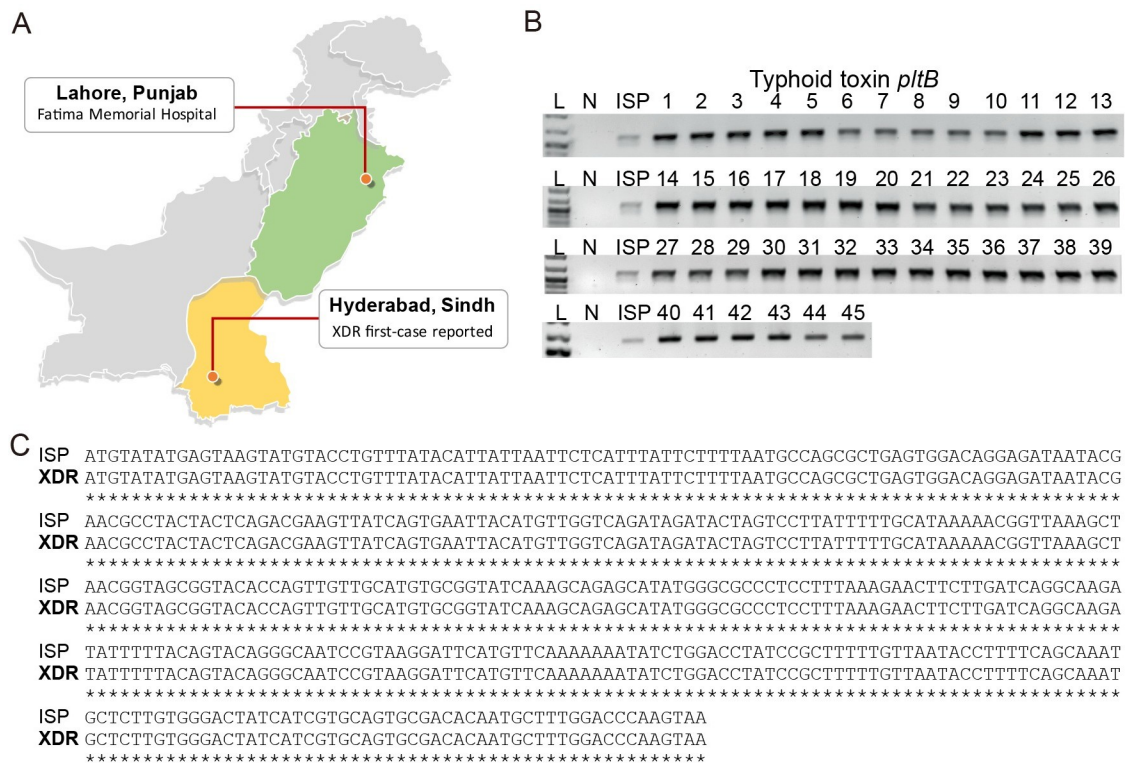

Fig 1. XDR *S*. Typhi strains isolated from children ages 1–13 years old. **A**, Illustration showing the geographical location that XDR *S*. Typhi characterized in this study was isolated compared to the location that the first case of XDR *S*. Typhi was reported. **B**, PCR reactions of 45 XDR isolates for typhoid toxin *pltB*. **C**, *pltB* sequences from all 45 XDR isolates (XDR, one representative is shown) were identical to typhoid toxin *pltB* sequence of *S*. Typhi ISP2825 (ISP). ISP, antibiotic-susceptible *S*. Typhi ISP2825, GenBank accession number CP080960. N, negative control containing all PCR components except for *S*. Typhi genomic DNA. See S1 and S2 Tables for sample information and antibiotic breakpoints.

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and all isolates are XDR since they are resistant to both first- and second-line antibiotics (S1 and S2 Tables). XDR *S.* Typhi isolates exhibit similar MIC values across 45 isolates with some variations, indicating that antibiotic resistance phenotypes do not correlate with age, sex, or hospital wards (S1 Table). Both sexes, males (n = 31, 68.89%) and females (n = 14, 31.11%), are included in this study (S1 Table). Their ages were 1–13 years old: toddlers aging 1–2 years old (n = 9, 20%), pre-schoolers aging 3–5 years old (n = 17, 37.78%), school-age children aging 6–12 years old (n = 17, 37.78%), and adolescents aging 13–18 years old (n = 2, 4.44%) (S1 Table). *S.* Typhi was isolated from all wards included in the study, indicating that wards were not an important factor for the odds of seeing *S.* Typhi-infected patients (S1 Table).

Molecular basis of resistance to first-line antibiotics among XDR *S.* Typhi isolates

We hypothesized that resistance to first- and second-line antibiotics among XDR *S.* Typhi isolates is primarily due to the acquisition and/mutation of antibiotic-resistance-related genes. To carry out a series of molecular characterization via PCR and/or PCR amplicon sequencing, we selected 18 *S.* Typhi samples based on sex, age, hospital wards, antibiotic-resistance profile, and MIC (S3 Table). According to child development milestones defined by the Centers for Disease Control and Prevention (CDC), age groups were split into toddlers (ages 1 to 2), pre-schoolers (ages 3 to 5), school-age children (ages 6 to 12), and adolescents (ages 13 to 18) (Fig 2A). To understand the molecular basis of the MDR phenotype resistant to all the first-line antibiotics with clinical relevance, we have designed primers specific to *catA1*, *bla*$_{TEM1}$, *dhfR7*, and *sul1*, MDR-related genes encoded in the IncHI1 region (S4 Table). Using these primers,
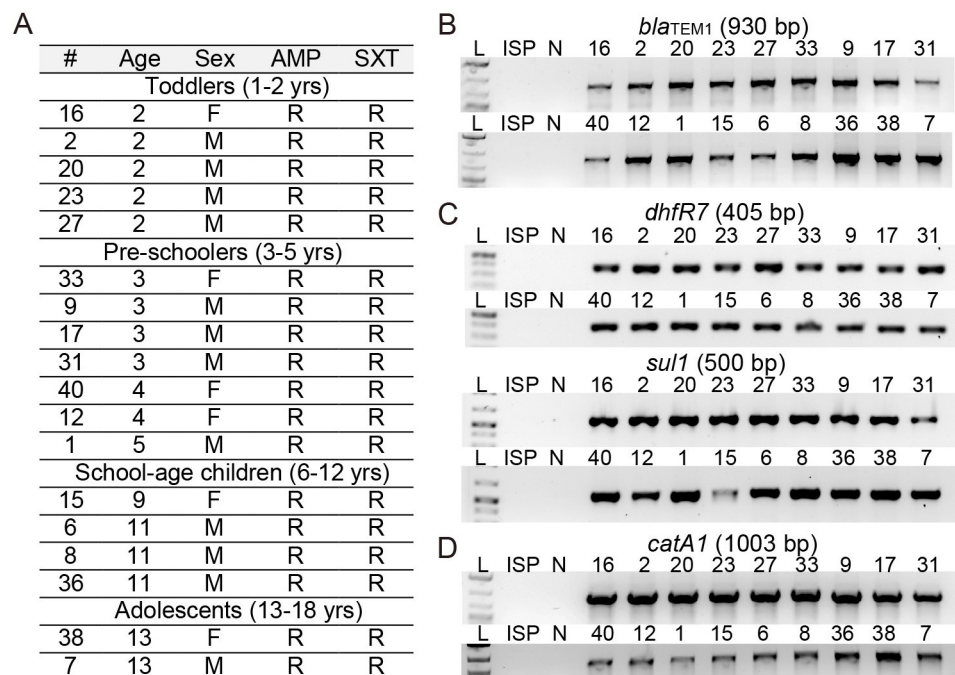
Fig 2. Molecular basis of resistance to first-line antibiotics among XDR *S.* Typhi isolates. A, Sample information and antibiotic resistance profiles of select of XDR *S.* Typhi isolates for molecular characterization. See S3 Table for details. **B**, PCR reactions for *bla*$_{TEM1}$. **C**, PCR reactions for *dhfR7* and *sul1*. **D**, PCR reactions for *catA1*. ISP, antibiotic-susceptible *S.* Typhi ISP2825. N, negative control containing all PCR components except for *S.* Typhi genomic DNA. See S4 Table for details.

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we evaluated the presence of these MDR-related genes in 18 selected *S.* Typhi isolates via PCR analysis. All PCR reactions resulted in amplicons with expected size for specific genes except for two controls, antibiotic-susceptible *S.* Typhi ISP2825 (ISP) and a control PCR reaction mixture that did not contain any *S.* Typhi genomic DNA (N) (Fig 2B–2D). Drug-susceptible *S.* Typhi ISP clinical isolate available in the laboratory was used as a control since all *S.* Typhi isolates from this cohort were XDR (S1 Table). These results indicate that the MDR phenotype exhibited by these XDR *S.* Typhi isolates is most likely due to MDR-related genes encoded in the IncHI1 region.

Molecular basis of fluoroquinolone-resistance among XDR *S.* Typhi isolates

The acquisition of an IncY region harboring *qnrS* and one or more point mutations on the genes *gyrA*, *gyrB*, *parC*, and/or *parE*, referred to as the quinolone resistance determining region (QRDR), have been correlated to fluoroquinolone-resistance among typhoidal *Salmonella* strains. A PCR primer set specific for *qnrS* was designed and used to investigate whether XDR *S.* Typhi isolates encode this fluoroquinolone-resistance-related gene. We found that, unlike antibiotic-susceptible *S.* Typhi ISP2825, all XDR *S.* Typhi isolates tested encode *qnrS* (Fig 3A). Fluoroquinolone-resistant *Salmonella* strains have been reported to carry point mutations in *gyrA*, *gyrB*, *parC*, and/or *parE*, which exhibits variations depending on geographical locations [10,12–20] (S6 Table). Specific primer sets for the known mutations on these
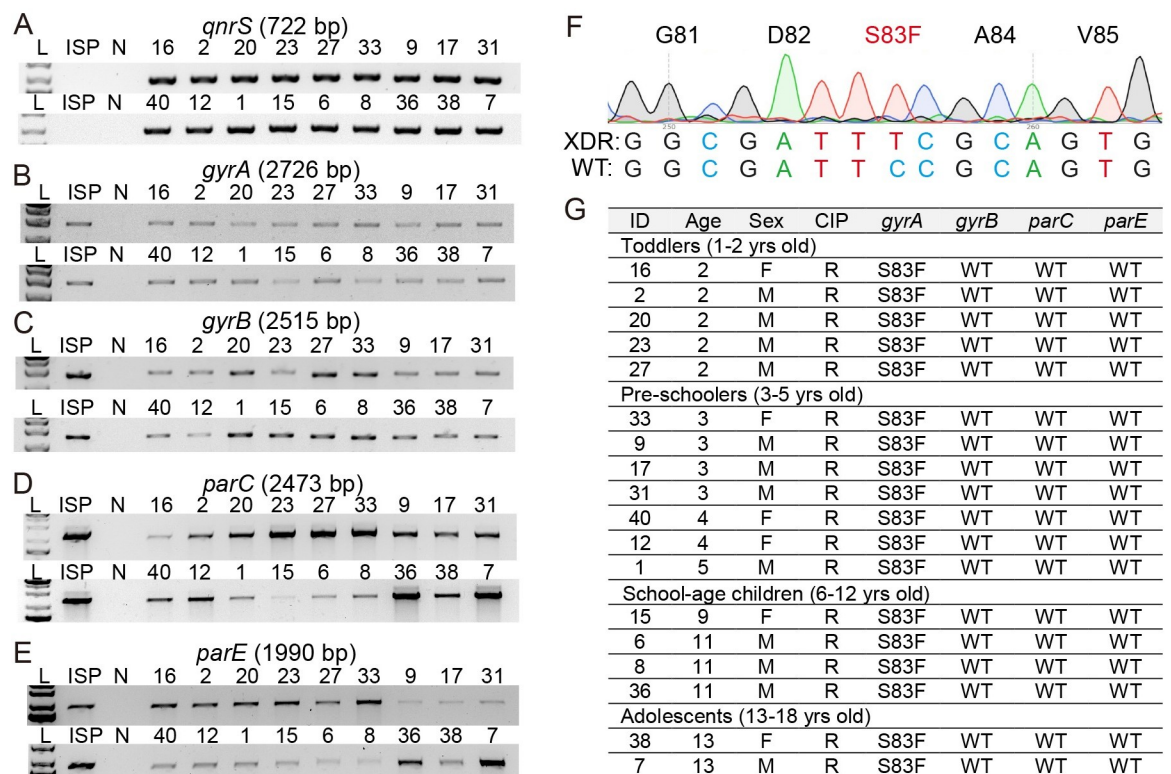
Fig 3. Molecular basis of fluoroquinolone-resistance among XDR *S.* Typhi isolates. A-E, PCR reactions for *qnrS* (**A**), *gyrA* (**B**), *gyrB* (**C**), *parC* (**D**), and *parE* (**E**). See S4 Table for details. ISP, antibiotic-susceptible *S.* Typhi ISP2825. N, negative control containing all PCR components except for *S.* Typhi genomic DNA. **F,** Representative sequencing chromatogram showing GyrA S83F mutation. **G,** Summary of PCR amplicon sequencing analysis for *gyrA*, *gyrB*, *parC*, and *parE*. See S6 Table for additional information. WT, wild type for the known mutations.

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four genes were designed for PCR and PCR amplicon sequencing (S4 Table). Consistent with their essential roles in bacterial cell replication, both antibiotic-susceptible *S.* Typhi ISP2825 and all XDR *S.* Typhi isolates resulted in PCR products with expected size for the four topo-isomerase genes (Fig 3B–3E). To determine whether XDR *S.* Typhi isolates resistant to fluoro-quinolones have point mutations in these topoisomerase genes, we carried out Sanger sequencing of PCR amplicons. Consistent with the antibiotic-susceptible phenotype, *S.* Typhi ISP2825 carries wild-type topoisomerases. In contrast, we found that all the XDR *S.* Typhi iso-lates carry a mutant form of *gyrA* encoding for GyrA^{Ser83Phe} (Fig 3F and 3G). GyrA^{Ser83Phe} has been found most commonly among XDR *S.* Typhi identified from other endemic regions. We also found that these XDR *S.* Typhi isolates from pediatric septicemia patients encode wild-type *gyrB*, *parC*, and *parE* (Fig 3G and S6 Table).

Molecular basis of third-generation cephalosporin-resistance among XDR *S.* Typhi isolates

In addition to GyrA^{Ser83Phe}, resistance to second-line antibiotics among XDR *S.* Typhi in Paki-stan has been associated with an IncY region carrying the quinolone resistance gene *qnrS* (Fig 3A) and extended-spectrum β-lactamase resistance gene *bla*$_{CTX-M-15}$ [9,40]. Consistent with resistance to second-line antibiotics, fluoroquinolones and cephalosporins, among these XDR *S.* Typhi isolates from pediatric septicemia patients, we found the acquisition of *bla*$_{CTX-M-15}$ among all XDR *S.* Typhi tested (Fig 4). In contrast, antibiotic-susceptible *S.* Typhi ISP2825 did not result in PCR amplicon for *bla*$_{CTX-M-15}$, indicating the specificity of the primers used.

Effects of efflux pumps on antibiotic resistance among XDR *S.* Typhi isolates

XDR *S.* Typhi isolates from pediatric typhoid patients exhibit some variations in their antibi-otic-resistance/susceptibility profiles across antibiotics tested in S1 Table. These results led us to investigate drug efflux pumps in XDR *S.* Typhi isolates since drug efflux pump systems are associated with resistance/susceptibility to a wide range of antibiotics. The recent worrisome trend among some XDR *S.* Typhi includes a correlation between efflux pump mutations and azithromycin resistance. For instance, a point mutation(s) on the antibiotic-binding subunit AcrB of the tripartite AcrAB-TolC efflux pumps (e.g., R717Q or R717L) has recently been cor-related to azithromycin-resistance among some *S.* Typhi and *S.* Paratyphi A clinical isolates [19,25,26]. In addition, *Salmonella* encodes another tripartite efflux pump, ABC-type MacAB-TolC, and three other small efflux pumps, major facilitator superfamily (MFS), multidrug and toxin extrusion (MATE), and small multidrug resistance (SMR), spanning in the inner mem-brane of the bacteria and therefore need to cooperate with another tripartite efflux pump such
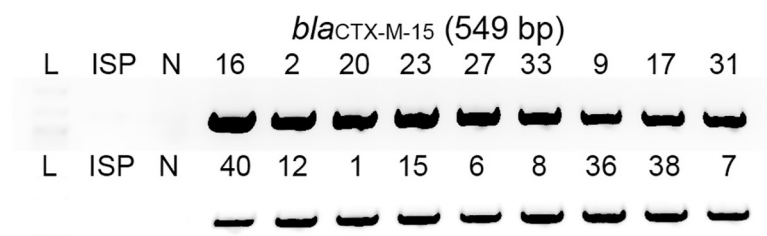

Fig 4. Molecular basis of third-generation cephalosporin-resistance among XDR *S.* Typhi isolates. PCR reactions for *bla*$_{CTX-M-15}$. ISP, antibiotic-susceptible *S.* Typhi ISP2825. N, negative control containing all PCR components except for *S.* Typhi genomic DNA. Note that all XDR *S.* Typhi isolates are resistant to cephalosporin.
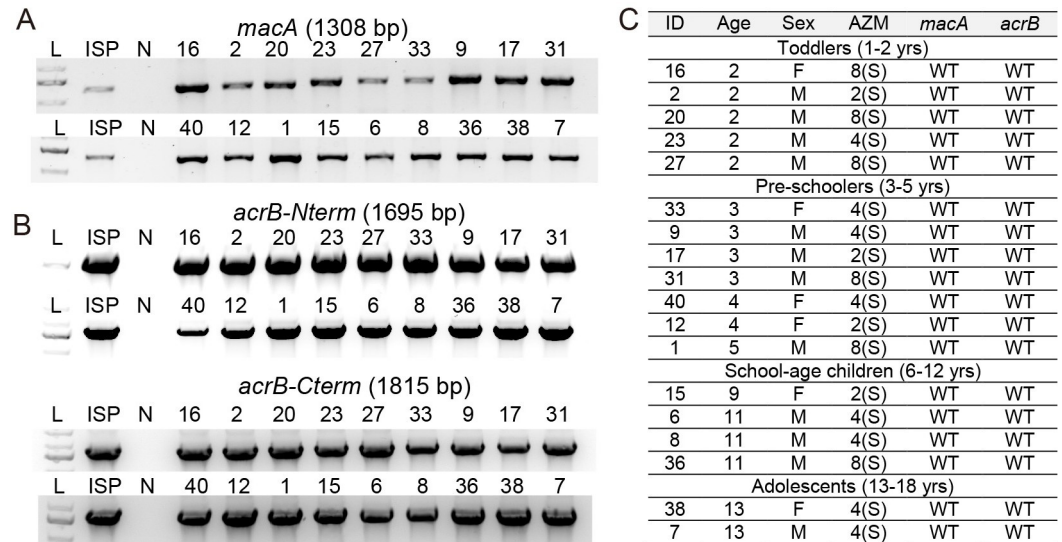
https://doi.org/10.1371/journal.pone.0257744.g004

Fig 5. *macA* and *acrB* sequence analysis among XDR *S.* Typhi isolates. A-B, PCR reactions for *macA* (**A**) and *acrB* (**B**). *acrB* was split into two pieces for more productive PCR reaction outcomes (*acrB*-Nterm and *acrB*-Cterm). ISP, antibiotic-susceptible *S.* Typhi ISP2825. N, negative control containing all PCR components except for *S.* Typhi genomic DNA. See S4 Table for details. **C**, Summary of PCR amplicon sequencing analysis for *macA* and *acrB*. WT, wild type for the known mutations. S, susceptible to azithromycin.

as RND-type AcrAB-TolC in exporting antibiotics [24]. In *Neisseria gonorrhoeae* that, like *S.* Typhi, is also a human-adapted gram-negative bacterial pathogen, point mutations on the promoter of *macA* of the tripartite MacAB-TolC efflux pump have been correlated to azithromycin-resistance [41].

To assess whether point mutations on tripartite efflux pumps have occurred among XDR *S.* Typhi isolates from pediatric patients, we have determined *macA* promoter and *acrB* sequences via PCR and PCR amplicon sequencing using specific primer sets summarized in S4 Table. We found that all XDR *S.* Typhi isolates tested carry wild-type -10 promoter sequence in the *macA* promoter and wild-type Arg at position 717 on the AcrB protein (Fig 5A–5C). These results are consistent with azithromycin-susceptibility (2–8 μg/ml) among XDR *S.* Typhi isolates from our pediatric septicemia patient cohort (Fig 5C and S1 Table). As indicated in S2 Table, azithromycin-susceptibility breakpoint is ≤16 μg/ml.

The expression of drug-efflux pumps is tightly regulated. For instance, AcrR represses *acrAB* gene expression by binding to the operator and inhibiting the transcription of *acrAB*. In 3-dimensional protein structure, wild-type AcrR protein monomer forms nine α-helices crucial for homodimer assembly, DNA-binding, and ligand-binding for its function in repressing *acrAB* expression [42,43]. Through whole-genome sequencing analysis of the latest NCBI RefSeq dataset of the fully assembled complete genome of *S.* Typhi (107 in total; S5 Table), we found that two *S.* Typhi strains (RefSeq assembly accession IDs GCF_001121865.2 and GCF_900205275.1) carry a variant form of AcrR with 48 amino acid difference at the C-terminus (Fig 6A). Unlike the repressor AcrR variant, these two *S.* Typhi strains still carry wild-type RobA (NP_463442.1) and MarA (WP_000091194.1), activators for the *acrAB* gene expression, and wild-type AcrA (NP_459471.1), AcrB (NP_459470.1), and MacA (NP_459918.1). These results led us to investigate whether our XDR *S.* Typhi isolates carry a variant form of AcrR. Consistent with our azithromycin-susceptibility data, we found that all the XDR *S.* Typhi isolates from our pediatric septicemia patient cohort carry wild-type AcrR (Fig 6B and 6C).
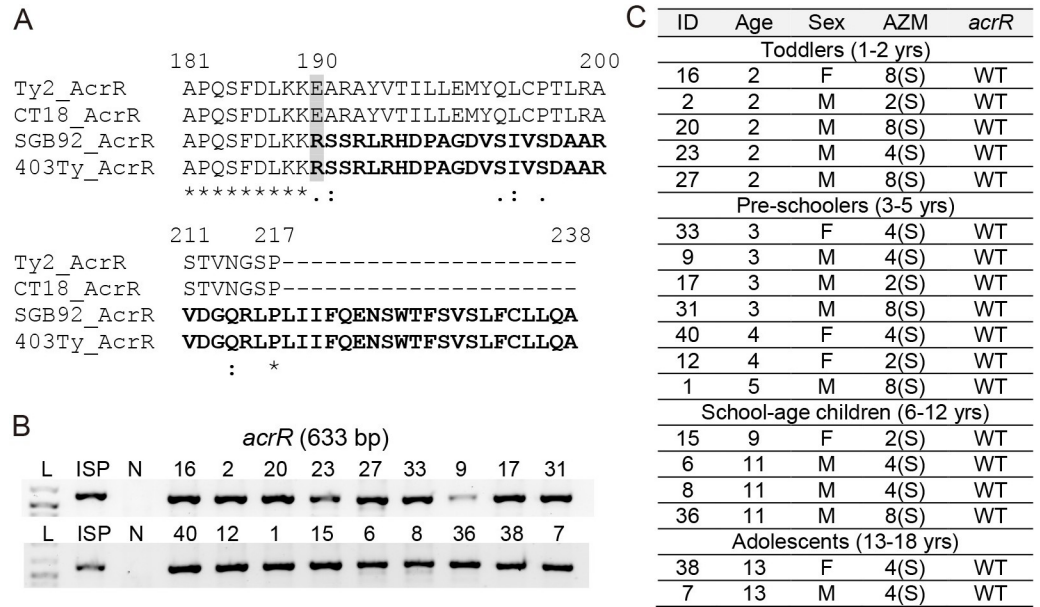
A

```
              181       190               200
Ty2_AcrR     APQSFDLKKEARAYVTILLEMYQLCPTLRA
CT18_AcrR    APQSFDLKKEARAYVTILLEMYQLCPTLRA
SGB92_AcrR   APQSFDLKKRSSRLRHDPAGDVSIVSDAAR
403Ty_AcrR   APQSFDLKKRSSRLRHDPAGDVSIVSDAAR
             *********.:            .: .

              211  217             238
Ty2_AcrR     STVNGSP--------------------
CT18_AcrR    STVNGSP--------------------
SGB92_AcrR   VDGQRLPLIIFQENSWTFSVSLFCLLQA
403Ty_AcrR   VDGQRLPLIIFQENSWTFSVSLFCLLQA
             :    *
```

B

acrR (633 bp)

| L | ISP | N | 16 | 2 | 20 | 23 | 27 | 33 | 9 | 17 | 31 |
| L | ISP | N | 40 | 12 | 1 | 15 | 6 | 8 | 36 | 38 | 7 |

C

ID	Age	Sex	AZM	*acrR*
Toddlers (1-2 yrs)				
16	2	F	8(S)	WT
2	2	M	2(S)	WT
20	2	M	8(S)	WT
23	2	M	4(S)	WT
27	2	M	8(S)	WT
Pre-schoolers (3-5 yrs)				
33	3	F	4(S)	WT
9	3	M	4(S)	WT
17	3	M	2(S)	WT
31	3	M	8(S)	WT
40	4	F	4(S)	WT
12	4	F	2(S)	WT
1	5	M	8(S)	WT
School-age children (6-12 yrs)				
15	9	F	2(S)	WT
6	11	M	4(S)	WT
8	11	M	4(S)	WT
36	11	M	8(S)	WT
Adolescents (13-18 yrs)				
38	13	F	4(S)	WT
7	13	M	4(S)	WT

Fig 6. AcrR sequence analysis among XDR *S*. Typhi isolates. A, AcrR amino acid sequence comparison analysis of the latest RefSeq dataset of the fully assembled complete genome of *S*. Typhi (107 in total) collected from NCBI as of Feb 26, 2021. Ty2, *S*. Typhi Ty2 (RefSeq assembly accession: GCF_000007545.1, assembly name: ASM754v1, strain: Ty2, submitter: University of Wisconsin). CT18, *S*. Typhi CT18 (RefSeq assembly accession: GCF_000195995.1, assembly name: ASM19599v1, strain: CT18, submitter: Sanger Institute). SGB92, *S*. Typhi SGB92 (RefSeq assembly accession: GCF_001121865.2, assembly name: 404Ty, strain name: SGB92, submitter: Wellcome Sanger Institute). 403Ty, *S*. Typhi 403Ty-sc-1979084 (RefSeq assembly accession: GCF_900205275.1, assembly name: 403Ty, isolate: 403Ty-sc-1979084, submitter: Wellcome Sanger Institute). See S5 Table for details. **B**, PCR reactions for *acrR*. ISP, antibiotic-susceptible *S*. Typhi ISP2825. N, negative control containing all PCR components except for *S*. Typhi genomic DNA. **C**, Summary of PCR amplicon sequencing analysis for *acrR*. WT, wild type for the known mutations. S, susceptible to azithromycin.

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Discussion

XDR *S*. Typhi is more common among pediatric patients but the majority of antibiotic resistance studies available have been carried out using *S*. Typhi isolates from adult patients [9]. Here, we characterized *S*. Typhi isolates from a medium size cohort of pediatric typhoid patients to determine antibiotic-resistance-related gene signatures associated with their drug-resistant profiles. This study provides a valuable overview of the recent (2019–2020) populations in the setting of Lahore, Pakistan, among a septic pediatric cohort and provides insights into the development of simple, cost-effective molecular detection methods with point-of-care testing potential.

Biochemical method-mediated identification assisted by the streamlined automated system was further validated via molecular typing for typhoidal *Salmonella* specific gene sequences [44–46]. Antibiotic resistance profiles were obtained through the automated test system that followed the CLSI 2018 guidelines. To determine the molecular basis of the antibiotic resistance profiles among these XDR *S*. Typhi, we have designed primer sets and optimized PCR reaction conditions for antibiotic resistance genes harbored in the IncHI1 (*catA1*, *bla*$_{TEM1}$, *dhfR7*, and *sul1*) and IncY (*qnrS* and *bla*$_{CTX-M-15}$) regions, contributing to resistance to first-line and second-line antibiotics, respectively. Overall findings across the XDR *S*. Typhi isolates for these antibiotic resistance genes are in agreement with our MIC results and other reports correlated to molecular determinants of MDR and XDR phenotypes among recent XDR *S*. Typhi isolates from adult typhoid patients [9].

Besides *qnrS*, mutations on *gyrA*, *gyrB*, *parC*, and *parE* also contribute to fluoroquinolone-resistance, which exhibits more diverse patterns depending on geographical locations. Our

XDR *S.* Typhi isolates from pediatric patients in Northern Pakistan carry GyrA^{S83F} across all samples tested, while the remaining genes were found to be wild-type (Fig 3 and S6 Table). This result indicates that XDR *S.* Typhi circulating in this geographical location is different from the ones prevalent in other locations that exhibit different QRDR mutation signatures [9]. Future investigations on XDR *S.* Typhi isolates from adult patients in the same geographical location would inform us about whether a divergent host adaptation process has occurred in pediatric and adult patients.

Macrolides such as azithromycin are considered the only remaining oral antibiotic option in treating XDR *S.* Typhi resistant to both first-line and second-line antibiotics. An additional treatment option against XDR *S.* Typhi, although requiring injection, are carbapenems such as imipenem and meropenem. Our XDR *S.* Typhi isolates from pediatric patients are susceptible to azithromycin, imipenem, and meropenem (S1 Table). In contrast, 5% and 48% of recent *S.* Typhi isolates (n = 81) from a mid-size adult cohort in Northern Punjab were resistant to azithromycin and meropenem, respectively [29], supporting the concept of a divergent host adaptation and/or transmission process in child and adult groups. These results also support a possibility that, with time, molecular determinants for azithromycin-resistance and meropenem-resistance would likely be adopted in nearly all *S.* Typhi circulating locally and globally.

Current analysis indicates that our XDR *S.* Typhi isolates from pediatric septicemia patients in Punjab do not carry molecular determinants that have been correlated to azithromycin-resistance in typhoidal *Salmonella*, *S.* Typhi and *S.* Paratyphi A, and another human-adapted Gram-negative pathogen *N. gonorrhoeae* [41] (Fig 5). Besides wild-type AcrB and wild-type -10 promoter sequence in the *macA* promoter across our XDR *S.* Typhi isolates, WGS analysis of the latest NCBI RefSeq dataset of the fully assembled complete genome of *S.* Typhi (107 in total), conducted as part of this study, indicates the emergence of *S.* Typhi strains carrying the frameshifted AcrR variant, the repressor of the *acrAB* efflux pump components. Although further investigations are required to understand the consequence of having the AcrR frameshifted variant in antibiotic-resistance, it is intriguing to hypothesize that the AcrR variant is less effective in repressing the expression of *acrAB*, therefore contributing to antibiotic-resistance such as azithromycin. We also found that those *S.* Typhi strains carrying the AcrR variant carry wild-type RobA and MarA, activators for the *acrAB* gene expression, and wild-type AcrAB, collectively supporting the hypothesis that possession of the AcrR frameshifted variant is an adaptation/evolution outcome, rather than a stochastic event outcome.

The emergence and spread of *S.* Typhi resistant to macrolides and carbapenems are a serious global health concern, deserving close surveillance for local and global spread. We envision that some of the methods detecting key molecular determinants for *S.* Typhi antibiotic resistance used in the current study could be developed as a surveillance strategy and point-of-care testing strategy. The detection and analysis methods for resistance to first-line and second-line antibiotics described in the study are straightforward. Besides efflux pump related molecular determinants described in the study, the future surveillance strategy could include additional molecular traits predicted to be associated with resistance to macrolides and carbapenems in *S.* Typhi. For instance, in *Enterobacteriaceae*, *erm* genes encoding for methylases to modify target sites, *ere* genes for esterase transferases, and *mph* genes for phosphor transferases are known to confer macrolide-resistance by altering the structure of antibiotics [47]. Similarly, carbapenem resistance in *S.* Typhi can be acquired by mutational events or gene acquisition via horizontal gene transfer, leading to the overexpression of efflux pumps that expel carbapenems and the acquisition of carbapenemases. The most effective carbapenemases known that hydrolyze carbapenem and spread across many bacterial pathogens are KPC, VIM, IMP, NDM and OXA-48 types [48].

In summary, this study informs the molecular basis of antibiotic-resistance among recent *S.* Typhi isolates from pediatric septicemia patients and provides insights into the development of molecular detection and treatment strategies for XDR *S.* Typhi.

Supporting information

S1 Fig. The original uncropped images obtained using an iBright CL1500 imager. (TIF)

S1 Table. The minimum inhibitory concentration (MIC) results (μg/ml) of all samples used in this study, related to Fig 1. (DOCX)

S2 Table. Antibiotic breakpoints, related to Fig 1 and S1 Table. (DOCX)

S3 Table. Select of samples for molecular characterization, related to Figs 2–6. (DOCX)

S4 Table. PCR and sequencing primers and PCR conditions used in this study, related to Methods. (DOCX)

S5 Table. Details of the 107 completed *S.* Typhi genomes used in the study, related to Fig 6. (DOCX)

S6 Table. Sequencing results associated with fluoroquinolone resistance, related to Fig 3. (DOCX)

S1 Raw images. (PDF)

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References

1. Rudd KE, Johnson SC, Agesa KM, Shackelford KA, Tsoi D, Kievlan DR, et al. Global, regional, and national sepsis incidence and mortality, 1990–2017: analysis for the Global Burden of Disease Study. Lancet. 2020; 395(10219):200–11. Epub 2020/01/20. https://doi.org/10.1016/S0140-6736(19)32989-7 PMID: 31954465; PubMed Central PMCID: PMC6970225.

2. Wain J, Hendriksen RS, Mikoleit ML, Keddy KH, Ochiai RL. Typhoid fever. Lancet. 2015; 385 (9973):1136–45. Epub 2014/12/03. https://doi.org/10.1016/S0140-6736(13)62708-7 PMID: 25458731.

3. Crump JA, Mintz ED. Global trends in typhoid and paratyphoid Fever. Clin Infect Dis. 2010; 50(2):241–6. Epub 2009/12/18. https://doi.org/10.1086/649541 PMID: 20014951; PubMed Central PMCID: PMC2798017.

4. Wong VK, Baker S, Pickard DJ, Parkhill J, Page AJ, Feasey NA, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. Nat Genet. 2015; 47(6):632–9. Epub 2015/05/12. https://doi.org/10.1038/ng.3281 PMID: 25961941.

5. Baker S, Holt KE, Clements AC, Karkey A, Arjyal A, Boni MF, et al. Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. Open Biol. 2011; 1(2):110008. Epub 2012/05/31. https://doi.org/10.1098/rsob.110008 PMID: 22645647; PubMed Central PMCID: PMC3352080.

6. Holt KE, Dolecek C, Chau TT, Duy PT, La TT, Hoang NV, et al. Temporal fluctuation of multidrug resistant salmonella typhi haplotypes in the mekong river delta region of Vietnam. PLoS Negl Trop Dis. 2011; 5(1):e929. Epub 2011/01/20. https://doi.org/10.1371/journal.pntd.0000929 PMID: 21245916; PubMed Central PMCID: PMC3014949.

7. Yan M, Li X, Liao Q, Li F, Zhang J, Kan B. The emergence and outbreak of multidrug-resistant typhoid fever in China. Emerg Microbes Infect. 2016; 5(6):e62. Epub 2016/06/23. https://doi.org/10.1038/emi.2016.62 PMID: 27329848; PubMed Central PMCID: PMC4932652.

8. Ingle DJ, Nair S, Hartman H, Ashton PM, Dyson ZA, Day M, et al. Informal genomic surveillance of regional distribution of Salmonella Typhi genotypes and antimicrobial resistance via returning travellers. PLoS Negl Trop Dis. 2019; 13(9):e0007620. Epub 2019/09/13. https://doi.org/10.1371/journal.pntd.0007620 PMID: 31513580; PubMed Central PMCID: PMC6741848.

9. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, et al. Emergence of an Extensively Drug-Resistant Salmonella enterica Serovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. MBio. 2018; 9(1):e00105–18. https://doi.org/10.1128/mBio.00105-18 PMID: 29463654.

10. Parkhill J, Dougan G, James KD, Thomson NR, Pickard D, Wain J, et al. Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. Nature. 2001; 413(6858):848–52. https://doi.org/10.1038/35101607 PMID: 11677608

11. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic Resistance and Typhoid. Clin Infect Dis. 2019; 68(Suppl 2):S165–s70. Epub 2019/03/08. https://doi.org/10.1093/cid/ciy1111 PMID: 30845331; PubMed Central PMCID: PMC6405283.

12. Dutta S. Fitness gains hamper efforts to tackle drug resistance. Elife. 2013; 2:e01809. Epub 2013/12/12. https://doi.org/10.7554/eLife.01809 PMID: 24327563; PubMed Central PMCID: PMC3857915.

13. Veeraraghavan B, Anandan S, Muthuirulandi Sethuvel DP, Puratchiveeran N, Walia K, Devanga Ragupathi NK. Molecular Characterization of Intermediate Susceptible Typhoidal Salmonella to Ciprofloxacin, and its Impact. Mol Diagn Ther. 2016; 20(3):213–9. Epub 2016/03/10. https://doi.org/10.1007/s40291-016-0191-6 PMID: 26951258.

14. Capoor MR, Nair D, Walia NS, Routela RS, Grover SS, Deb M, et al. Molecular analysis of high-level ciprofloxacin resistance in Salmonella enterica serovar Typhi and S. Paratyphi A: need to expand the QRDR region? Epidemiol Infect. 2009; 137(6):871–8. Epub 2008/08/09. https://doi.org/10.1017/S0950268808001076 PMID: 18687156.

15. Gopal M, Elumalai S, Arumugam S, Durairajpandian V, Kannan MA, Selvam E, et al. GyrA ser83 and ParC trp106 Mutations in Salmonella enterica Serovar Typhi Isolated from Typhoid Fever Patients in Tertiary Care Hospital. J Clin Diagn Res. 2016; 10(7):Dc14–8. Epub 2016/09/16. https://doi.org/10.7860/JCDR/2016/17677.8153 PMID: 27630841; PubMed Central PMCID: PMC5020279.

16. Ling JM, Chan EW, Lam AW, Cheng AF. Mutations in topoisomerase genes of fluoroquinolone-resistant salmonellae in Hong Kong. Antimicrob Agents Chemother. 2003; 47(11):3567–73. Epub 2003/10/25. https://doi.org/10.1128/AAC.47.11.3567-3573.2003 PMID: 14576119; PubMed Central PMCID: PMC253778.

17. Accou-Demartin M, Gaborieau V, Song Y, Roumagnac P, Marchou B, Achtman M, et al. Salmonella enterica Serotype Typhi with nonclassical quinolone resistance phenotype. Emerg Infect Dis. 2011; 17(6):1091–4. Epub 2011/07/14. https://doi.org/10.3201/eid1706.101242 PMID: 21749778; PubMed Central PMCID: PMC3358197.

18. Qian H, Cheng S, Liu G, Tan Z, Dong C, Bao J, et al. Discovery of seven novel mutations of gyrB, parC and parE in Salmonella Typhi and Paratyphi strains from Jiangsu Province of China. Sci Rep. 2020; 10(1):7359. Epub 2020/05/02. https://doi.org/10.1038/s41598-020-64346-0 PMID: 32355184; PubMed Central PMCID: PMC7193621.

19. Cheng MW, Lee CM, Wang NY, Wu AY, Lin CC, Weng LC, et al. Clinical characteristics in adult patients with Salmonella bacteremia and analysis of ciprofloxacin-nonsusceptible isolates. J Microbiol Immunol

Infect. 2015; 48(6):692–8. Epub 2015/11/07. https://doi.org/10.1016/j.jmii.2015.09.001 PMID: 26542649.

20. Matono T, Morita M, Yahara K, Lee KI, Izumiya H, Kaku M, et al. Emergence of Resistance Mutations in Salmonella enterica Serovar Typhi Against Fluoroquinolones. Open Forum Infect Dis. 2017; 4(4): ofx230. Epub 2017/12/20. https://doi.org/10.1093/ofid/ofx230 PMID: 29255729; PubMed Central PMCID: PMC5726467.

21. Vashist J, Vishvanath, Kapoor R, Kapil A, Yennamalli R, Subbarao N, et al. Interaction of nalidixic acid and ciprofloxacin with wild type and mutated quinolone-resistance-determining region of DNA gyrase A. Indian J Biochem Biophys. 2009; 46(2):147–53. Epub 2009/06/13. PMID: 19517991.

22. Chiou CS, Lauderdale TL, Phung DC, Watanabe H, Kuo JC, Wang PJ, et al. Antimicrobial resistance in Salmonella enterica Serovar Typhi isolates from Bangladesh, Indonesia, Taiwan, and Vietnam. Antimicrob Agents Chemother. 2014; 58(11):6501–7. Epub 2014/08/20. https://doi.org/10.1128/AAC.03608-14 PMID: 25136011; PubMed Central PMCID: PMC4249406.

23. Buckner MM, Blair JM, La Ragione RM, Newcombe J, Dwyer DJ, Ivens A, et al. Beyond Antimicrobial Resistance: Evidence for a Distinct Role of the AcrD Efflux Pump in Salmonella Biology. mBio. 2016; 7 (6):e01916. Epub 2016/11/24. https://doi.org/10.1128/mBio.01916-16 PMID: 27879336; PubMed Central PMCID: PMC5120143.

24. Du D, Wang-Kan X, Neuberger A, van Veen HW, Pos KM, Piddock LJV, et al. Multidrug efflux pumps: structure, function and regulation. Nat Rev Microbiol. 2018; 16(9):523–39. Epub 2018/07/14. https://doi.org/10.1038/s41579-018-0048-6 PMID: 30002505.

25. Hooda Y, Sajib MSI, Rahman H, Luby SP, Bondy-Denomy J, Santosham M, et al. Molecular mechanism of azithromycin resistance among typhoidal Salmonella strains in Bangladesh identified through passive pediatric surveillance. PLoS Negl Trop Dis. 2019; 13(11):e0007868. Epub 2019/11/16. https://doi.org/10.1371/journal.pntd.0007868 PMID: 31730615; PubMed Central PMCID: PMC6881056.

26. Sajib MSI, Tanmoy AM, Hooda Y, Rahman H, Andrews JR, Garrett DO, et al. Tracking the Emergence of Azithromycin Resistance in Multiple Genotypes of Typhoidal Salmonella. mBio. 2021; 12(1):e03481–20. Epub 2021/02/18. https://doi.org/10.1128/mBio.03481-20 PMID: 33593966.

27. Ahsan S, Rahman S. Azithromycin Resistance in Clinical Isolates of Salmonella enterica Serovars Typhi and Paratyphi in Bangladesh. Microb Drug Resist. 2019; 25(1):8–13. Epub 2018/07/18. https://doi.org/10.1089/mdr.2018.0109 PMID: 30016183.

28. Wattal C, Goel N. Pediatric Blood Cultures and Antibiotic Resistance: An Overview. Indian J Pediatr. 2020; 87(2):125–31. Epub 2019/12/22. https://doi.org/10.1007/s12098-019-03123-y PMID: 31863394; PubMed Central PMCID: PMC6974494.

29. Ali Shah SA, Nadeem M, Syed SA, Fatima Abidi ST, Khan N, Bano N. Antimicrobial Sensitivity Pattern of Salmonella Typhi: Emergence of Resistant Strains. Cureus. 2020; 12(11):e11778. Epub 2021/01/08. https://doi.org/10.7759/cureus.11778 PMID: 33409025; PubMed Central PMCID: PMC7779132.

30. Fernández J, Guerra B, Rodicio MR. Resistance to Carbapenems in Non-Typhoidal Salmonella enterica Serovars from Humans, Animals and Food. Vet Sci. 2018; 5(2):40. Epub 2018/04/13. https://doi.org/10.3390/vetsci5020040 PMID: 29642473; PubMed Central PMCID: PMC6024723.

31. Levine MM, Simon R. The Gathering Storm: Is Untreatable Typhoid Fever on the Way? mBio. 2018; 9 (2):e00482–18. https://doi.org/10.1128/mBio.00482-18 PMID: 29559573.

32. Yang YA, Chong A, Song J. Why Is Eradicating Typhoid Fever So Challenging: Implications for Vaccine and Therapeutic Design. Vaccines (Basel). 2018; 6(3):E45. Epub 2018/07/26. https://doi.org/10.3390/vaccines6030045 PMID: 30042307.

33. WHO. Typhoid vaccines: WHO position paper, March 2018—Recommendations. Vaccine. 2018. Epub 2018/04/18. https://doi.org/10.1016/j.vaccine.2018.04.022 PMID: 29661581.

34. Voysey M, Pollard AJ. Seroefficacy of Vi Polysaccharide-Tetanus Toxoid Typhoid Conjugate Vaccine (Typbar TCV). Clin Infect Dis. 2018; 67(1):18–24. Epub 2018/01/20. https://doi.org/10.1093/cid/cix1145 PMID: 29351594.

35. Wain J, House D, Zafar A, Baker S, Nair S, Kidgell C, et al. Vi antigen expression in Salmonella enterica serovar Typhi clinical isolates from Pakistan. J Clin Microbiol. 2005; 43(3):1158–65. Epub 2005/03/08. https://doi.org/10.1128/JCM.43.3.1158-1165.2005 PMID: 15750077; PubMed Central PMCID: PMC1081282.

36. Feasey NA, Gaskell K, Wong V, Msefula C, Selemani G, Kumwenda S, et al. Rapid emergence of multidrug resistant, H58-lineage Salmonella typhi in Blantyre, Malawi. PLoS Negl Trop Dis. 2015; 9(4): e0003748. https://doi.org/10.1371/journal.pntd.0003748 PMID: 25909750.

37. Qamar FN, Yousafzai MT, Sultana S, Baig A, Shakoor S, Hirani F, et al. A Retrospective Study of Laboratory-Based Enteric Fever Surveillance, Pakistan, 2012–2014. J Infect Dis. 2018; 218(suppl_4):S201–S5. https://doi.org/10.1093/infdis/jiy205 PMID: 30060168.

38. Mitteregger D, Barousch W, Nehr M, Kundi M, Zeitlinger M, Makristathis A, et al. Neutralization of anti-microbial substances in new BacT/Alert FA and FN Plus blood culture bottles. J Clin Microbiol. 2013; 51 (5):1534–40. Epub 2013/03/15. https://doi.org/10.1128/JCM.00103-13 PMID: 23486710; PubMed Central PMCID: PMC3647900.

39. Galan JE, Curtiss R, 3rd. Distribution of the invA, -B, -C, and -D genes of Salmonella typhimurium among other Salmonella serovars: invA mutants of Salmonella typhi are deficient for entry into mammalian cells. Infect Immun. 1991; 59(9):2901–8. https://doi.org/10.1128/iai.59.9.2901-2908.1991 PMID: 1879916.

40. Rasheed F, Saeed M, Alikhan NF, Baker D, Khurshid M, Ainsworth EV, et al. Emergence of Resistance to Fluoroquinolones and Third-Generation Cephalosporins in Salmonella Typhi in Lahore, Pakistan. Microorganisms. 2020; 8(9). Epub 2020/09/05. https://doi.org/10.3390/microorganisms8091336 PMID: 32883020; PubMed Central PMCID: PMC7564241.

41. Rouquette-Loughlin CE, Balthazar JT, Shafer WM. Characterization of the MacA–MacB efflux system in Neisseria gonorrhoeae. Journal of Antimicrobial Chemotherapy. 2005; 56(5):856–60. https://doi.org/10.1093/jac/dki333 PMID: 16162665

42. Kang SM, Kim DH, Jin C, Ahn HC, Lee BJ. The crystal structure of AcrR from Mycobacterium tuberculosis reveals a one-component transcriptional regulation mechanism. FEBS Open Bio. 2019; 9 (10):1713–25. Epub 2019/08/02. https://doi.org/10.1002/2211-5463.12710 PMID: 31369208; PubMed Central PMCID: PMC6768106.

43. Su CC, Rutherford DJ, Yu EW. Characterization of the multidrug efflux regulator AcrR from Escherichia coli. Biochem Biophys Res Commun. 2007; 361(1):85–90. Epub 2007/07/24. https://doi.org/10.1016/j.bbrc.2007.06.175 PMID: 17644067; PubMed Central PMCID: PMC2104644.

44. Neupane DP, Dulal HP, Song J. Enteric Fever Diagnosis: Current Challenges and Future Directions. Pathogens. 2021; 10(4):410. Epub 2021/05/01. https://doi.org/10.3390/pathogens10040410 PMID: 33915749; PubMed Central PMCID: PMC8065732.

45. Lee S, Yang YA, Milano SK, Nguyen T, Ahn C, Sim JH, et al. Salmonella Typhoid Toxin PltB Subunit and Its Non-typhoidal Salmonella Ortholog Confer Differential Host Adaptation and Virulence. Cell Host Microbe. 2020; 27(6):937–49. Epub 2020/05/13. https://doi.org/10.1016/j.chom.2020.04.005 PMID: 32396840.

46. Ahn C, Yang Y-A, Neupane DP, Nguyen T, Richards AF, Sim JH, et al. Mechanisms of typhoid toxin neutralization by antibodies targeting glycan receptor binding and nuclease subunits. iScience. 2021; 24(5):102454. https://doi.org/10.1016/j.isci.2021.102454 PMID: 34113815

47. Taitt CR, Leski TA, Prouty MG, Ford GW, Heang V, House BL, et al. Tracking Antimicrobial Resistance Determinants in Diarrheal Pathogens: A Cross-Institutional Pilot Study. Int J Mol Sci. 2020; 21(16). Epub 2020/08/23. https://doi.org/10.3390/ijms21165928 PMID: 32824772; PubMed Central PMCID: PMC7460656.

48. Poirel L, Potron A, Nordmann P. OXA-48-like carbapenemases: the phantom menace. J Antimicrob Chemother. 2012; 67(7):1597–606. Epub 2012/04/14. https://doi.org/10.1093/jac/dks121 PMID: 22499996.