The molecular epidemiology of *Salmonella* Typhi and applications for policy

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This thesis is dedicated to my loving, brilliant partner, Bas.

Declaration

I hereby declare that except where specific reference is made to the work of others, the contents of this dissertation are original and have not been submitted in whole or in part for consideration for any other degree or qualification in this, or any other University. This dissertation is the result of my own work and includes nothing which is the outcome of work done in collaboration, except where specifically indicated in the text. This dissertation contains less than 60,000 words excluding appendices, bibliography, footnotes, tables, and equations, and has less than 150 figures.

Megan Carey

October 4, 2022

Some of the results presented in the thesis have been published during this PhD in the following peer-reviewed papers: Carey ME et al (2020) *Clin Inf Dis*, Iqbal J et al (2020) *mSphere*, Carey ME et al (2021) *Clin Inf Dis*, Carey ME et al (2022) *Curr Opin in Infect Dis*.

Abstract

Salmonella Typhi (*S*. Typhi) is a Gram-negative bacterium and the etiologic agent of typhoid fever. High rates of typhoid were historically associated with urban slums in South Asia with poor sanitation, but recent multicentre surveillance studies have demonstrated that typhoid is also a major problem of urban and rural areas in sub-Saharan Africa. Typhoid necessitates antimicrobial therapy; however, antimicrobial resistance (AMR) poses a serious threat to the effective clinical management of typhoid, particularly in South Asia, where resistance to all oral antimicrobials used to treat typhoid has been reported. New typhoid conjugate vaccines (TCVs) are highly efficacious and two have been prequalified by the World Health Organization (WHO); a small number of countries have introduced TCVs into their national immunization programs. However, many countries lack primary surveillance data to inform decision-making for TCV introduction. Here, aiming to aggregate and analyse globally representative whole genome sequencing (WGS) data to inform public health action, I conducted a genomic investigation of the global distribution and transmission dynamics of AMR *S*. Typhi. With colleagues from Pakistan and India, I conducted phylogenetic analyses of the first molecularly confirmed azithromycin-resistant *S*. Typhi isolated in Pakistan and India and put them into context with contemporaneous azithromycin-resistant isolates. I found that single point mutations in *acrB* (efflux pump) were emerging independently in these settings, potentially associated with selective pressure. AMR in *S*. Typhi is associated with the H58 lineage, which arose recently before becoming globally dominant within a relatively short time. Given this lineage's association with AMR, I sought to investigate how, when, and where it emerged. Working with collaborators from the United Kingdom Health Security Agency (UKHSA), we performed phylogenetic and phylodynamic analyses using *S*.

Typhi from returning travellers to the UK between 1980 and 1995. This dataset, which contained the earliest described H58 *S*. Typhi, indicates that the prototype H58 organisms were MDR, and that they emerged spontaneously in India in 1987 and became radially distributed throughout South Asia. These early organisms were associated with a single long branch and possessed mutations associated with increased bile tolerance, suggesting that the first H58 organism was generated during chronic carriage. The subsequent increased use of fluoroquinolones led to several independent mutations in *gyrA*, leading to decreased fluoroquinolone susceptibility. The apparent ability of H58 to acquire and maintain AMR genes continues to pose a threat, suggesting that TCVs should be deployed across South Asia to minimise the potential emergence of new drug-resistant variants. I worked with a broad range of collaborators to establish the Global Typhoid Genomics Consortium (GTGC) to encourage sharing of *S*. Typhi genomic data and standardised metadata, and to enable analysis and visualisation of these data in context to support public health decision-making. Here, I engaged with groups sequencing *S*. Typhi genomes to analyse and draft a global update paper; the final dataset contained >13,000 sequences. This work provides an updated overview of AMR and genotype distribution and illustrates key international transmission events, with the aim of informing TCV introduction decision-making and treatment guidelines. To create a translational link between laboratory scientists, genomics experts, clinicians, and policy makers, I outlined use cases for WGS in surveillance, diagnostic development, clinical management, and informing vaccine introduction as well as typhoid control. I hope to advance this work by fostering broader participation in the GTGC, and by encouraging funding support for the generation and analysis of *S*. Typhi WGS data. I also intend to create greater visibility for, and advocate for the value of, such data in conversations with country decision-makers and other policymakers via the WHO.

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I would like to thank Florian Marks for the opportunity to do a PhD while maintaining a position in global health at the University of Cambridge. I would like to thank Gordon Dougan for his wisdom and guidance. I am extremely grateful to Stephen Baker for his ideas, enthusiasm, and friendship. I'm grateful to Mailis Maes and Ellen Higginson for their friendship and support. I have worked with several wonderful colleagues as part of the Typhoid Conjugate Vaccine in Africa (THECA) Consortium – many thanks to Susan Tonks, Justin Im, and Andrea Haselbeck. I would like to thank Zoe Dyson for always finding time to share insights. I am appreciative of Caroline Zellmer's extraordinary positivity. I am grateful to Malick Gibani for his friendship and encouragement. I would like to thank Kathy Neuzil for her mentorship. I am very grateful to my parents for listening and providing the occasional escape to wine country. I am thankful for my brothers, who always provide much-needed hilarity and perspective. Finally, I am grateful to my partner Bastiaan Haak, who inspires me with his intellect, creativity, and kindness every day.

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Abbreviations

AMR	Antimicrobial resistance
AST	Antimicrobial Susceptibility Testing
BEAST	Bayesian Evolutionary Analysis Sampling Trees
CRT	Cluster-randomized trial
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
GMT	Geometric mean titre
H58	Haplotype 58 (*Salmonella* Typhi)
LMIC(s)	Low-and middle-income countries
MDR	Multi-drug resistant (in the context of *S*. Typhi, resistant to traditional first-line antimicrobials ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole)
MIC	minimum inhibitory concentration
MRCA	Most recent common ancestor
QRDR	Quinolone Resistance Determining Region
RCT	Randomized controlled trial
SAGE	Strategic Advisory Group of Experts
SEAP	Surveillance of Enteric Fever in Asia Project
SEFI	Surveillance of Enteric Fever in India
SETA	Severe Typhoid in Africa project
SNP	Single nucleotide polymorphism
STRATAA	Strategic Typhoid Alliance across Africa and Asia
TCV	Typhoid conjugate vaccine
Ty21a	Orally administered typhoid vaccine based on live-attenuated Ty2 *Salmonella* Typhi strain
TyVAC	Typhoid Vaccine Acceleration Consortium
Vi-CRM_{197}	Vi polysaccharide conjugated to CRM_{197} (genetically detoxified form of diphtheria toxin) carrier protein
Vi-DT	Vi polysaccharide conjugated to diphtheria toxoid carrier protein
Vi-PS	unconjugated Vi polysaccharide vaccine
Vi-rEPA	Vi polysaccharide antigen conjugated to the recombinant exoprotein A of *Pseudomonas aeruginosa* (carrier protein)
Vi-TT	Vi polysaccharide conjugated to tetanus toxoid carrier protein
WASH	Water Sanitation and Hygiene
WGS	Whole Genome Sequencing
WHO	World Health Organization
XDR	Extensively-drug resistant (in the context of *S*. Typhi, MDR as defined above and fully resistant to fluoroquinolones and third generation cephalosporins)

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Chapter 1: Introduction

1.1 *Salmonella* Typhi, Epidemiology and Control

1.1.1 Pathophysiology and clinical disease

Salmonella enterica subspecies enterica serovar Typhi (*S*. Typhi) is a Gram negative, rod-shaped bacterium that causes the clinical syndrome typhoid fever. *S*. Typhi was estimated to be responsible for 10.9 million cases and 116,800 deaths in 2017.[1] *S*. Typhi is human-restricted, and is spread via the faecal-oral route, typically through the ingestion of contaminated food or water.[2] The incubation period is typically 7 to 14 days.[3] Infecting bacteria adhere to the mucosa in the small intestine and invade the epithelial cells. After penetrating the mucosal layer, the bacteria translocate to the intestinal lymphoid follicles and mesenteric lymph nodes, and some may continue to the mononuclear phagocyte system in the spleen and liver. During the bacteraemia phase of infection, the bacteria become widely disseminated. Secondary infection can occur in the spleen, liver, bone marrow, Peyer's patches, or gallbladder. The gallbladder is a major reservoir for chronic infection. In rare instances, typhoid fever may result in intestinal perforation and peritonitis following necrosis of Peyer's patches, or toxic encephalopathy. The pathophysiology of *S*. Typhi infection is illustrated in **Figure 1**.[3]

Symptomatic typhoid patients typically experience fever, headache, malaise, abdominal pain, and nausea. Hepatomegaly, splenomegaly, and a relative brachycardia are also common[3]. Without treatment, patients may experience increasing fever, weakness, weight loss, altered mental state, and other complications including intestinal bleeding, intestinal perforation, and toxic encephalopathy. Prior to the advent of antimicrobials, the estimated

case fatality rate (CFR) associated with typhoid fever was 10-30%.[4] The estimated CFR is presently 1%, but this number has the potential to increase due to an increasing prevalence of antimicrobial resistance (AMR).

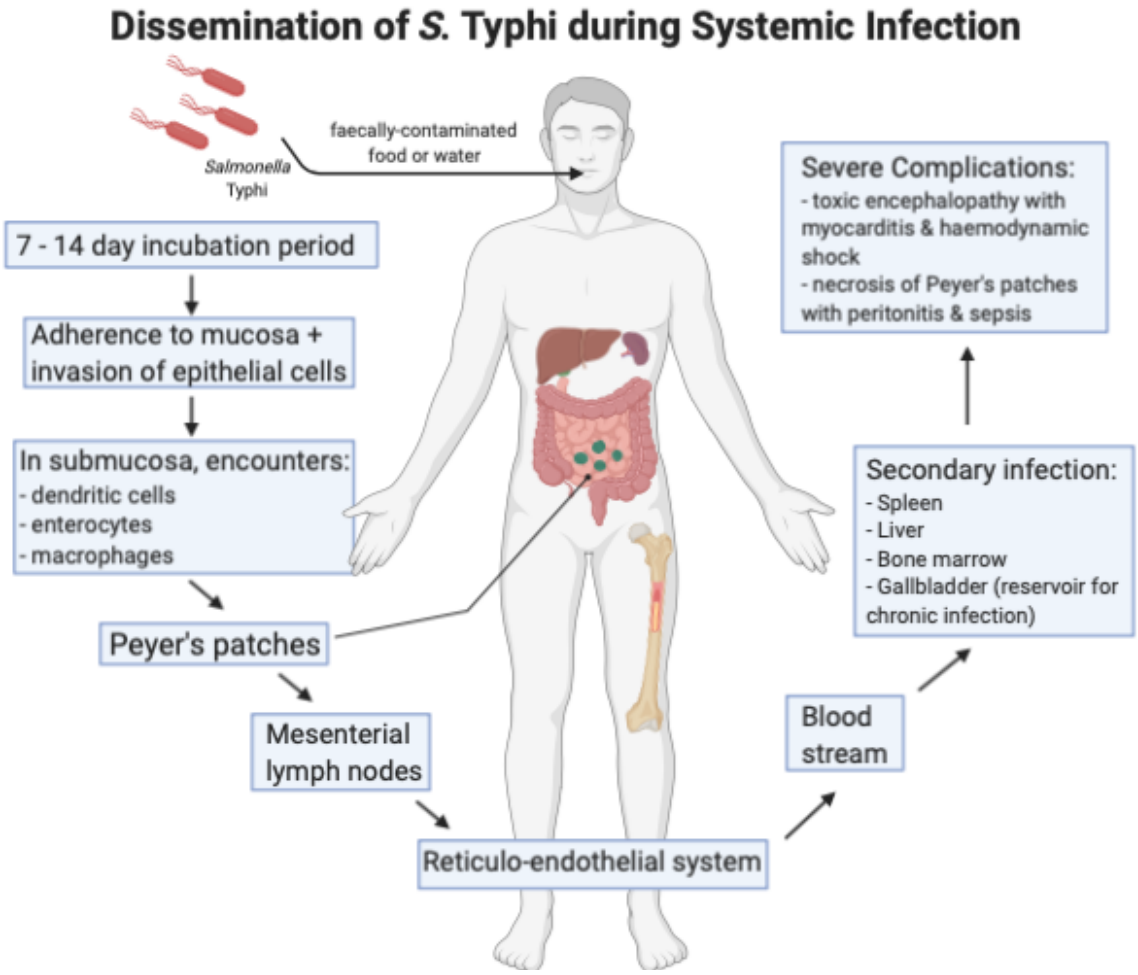


Figure 1. Dissemination of *S*. Typhi during systemic Infection (figure adapted from de Jong *et al* 2012).[3]

S. Typhi is typically transmitted through the consumption of faecally-contaminated food or water and has a 7-14 day incubation period. Bacteria adhere to mucosal cells and invade epithelial cells, then translocate to intestinal lymphoid follicles and mesenteric lymph nodes, and potentially on to the mononuclear phagocyte system. Secondary infections can occur in the spleen, liver, bone marrow, Peyer's patches, or gall bladder, which is also a major niche for chronic carriage.

In addition to causing acute disease, *S*. Typhi can lead to a chronic asymptomatic carriage state, during which time the bacteria can colonize the gallbladder for extended periods of time.[5] Previous reports estimated the prevalence of chronic carriers to be 2-5% in typhoid-endemic regions, but there are few studies from sub-Saharan Africa. Increased risk of chronic carriage is associated with advanced age, and the risk is higher in women than in men.[6]

1.1.2 Typhoid diagnostics

A lack of sufficiently sensitive diagnostic tests, as well as inconsistent usage of existing tests, poses a major impediment to appropriate treatment of typhoid fever, as well as an accurate view of global incidence of disease.[7] Bone marrow culture was once considered the gold standard for diagnosis of typhoid fever, but given the invasive and challenging nature of obtaining bone marrow aspirates, it is now rarely performed.[7] Some treating physicians rely solely on serological tests like the Widal test, which has limited accuracy and utility in endemic settings.[8] Blood culture–based diagnostics are recommended for use in the surveillance of typhoid fever and other invasive *Salmonella* infections by the World Health Organization (WHO),[9] but these tests are often not feasible to perform at scale in low-resource setting; significant infrastructure and trained personnel are required to conduct these tests and interpret results.[7] Where blood culture is available, it is only 40%–60% sensitive, depending in part on the volume of blood collected and prior antimicrobial usage.[10] Furthermore, the concentration of *S*. Typhi in peripheral blood of typhoid patients is low and highly transient, further complicating diagnostic efforts.[2] In addition, due to the

required incubation time, results are not usually available for several days, so they are not useful for rapid clinical decisions to inform empiric therapy.[10]

Febrile patients may not always present to healthcare facilities for diagnostic testing and treatment, particularly during the ongoing COVID-19 pandemic.[11] Potential constraints to healthcare seeking include distance to and accessibility of the closest healthcare facility, or costs associated with treatment and/or hospitalization, combined with ease of access and affordability of antimicrobials in the community. As a result, the true number of invasive *Salmonella* infections may be severely underestimated.[12]

1.1.3 Global burden of disease

1.1.3.1 Systematic reviews and modelled estimates

A 2019 systematic review and meta-analysis conducted by Marchello *et al.* provides an overview of 33 surveillance studies reporting estimated typhoid fever incidence rates published between 1954 and 2018, stratified by method - either population-based or hybrid surveillance, which uses one of several multipliers to account for under-ascertainment of cases at sentinel surveillance sites.[13] High incidence rates were reported in Africa and Asia, but with considerable variation in rates over time, including in the same location over time, and geography. Major regional data gaps were noted. **Figures 2 & 3** show forest plots of incidence rates from population-based studies conducted in Asia and Africa, while **Figure 4** shows incidence rate estimates from multiplier studies from Africa.

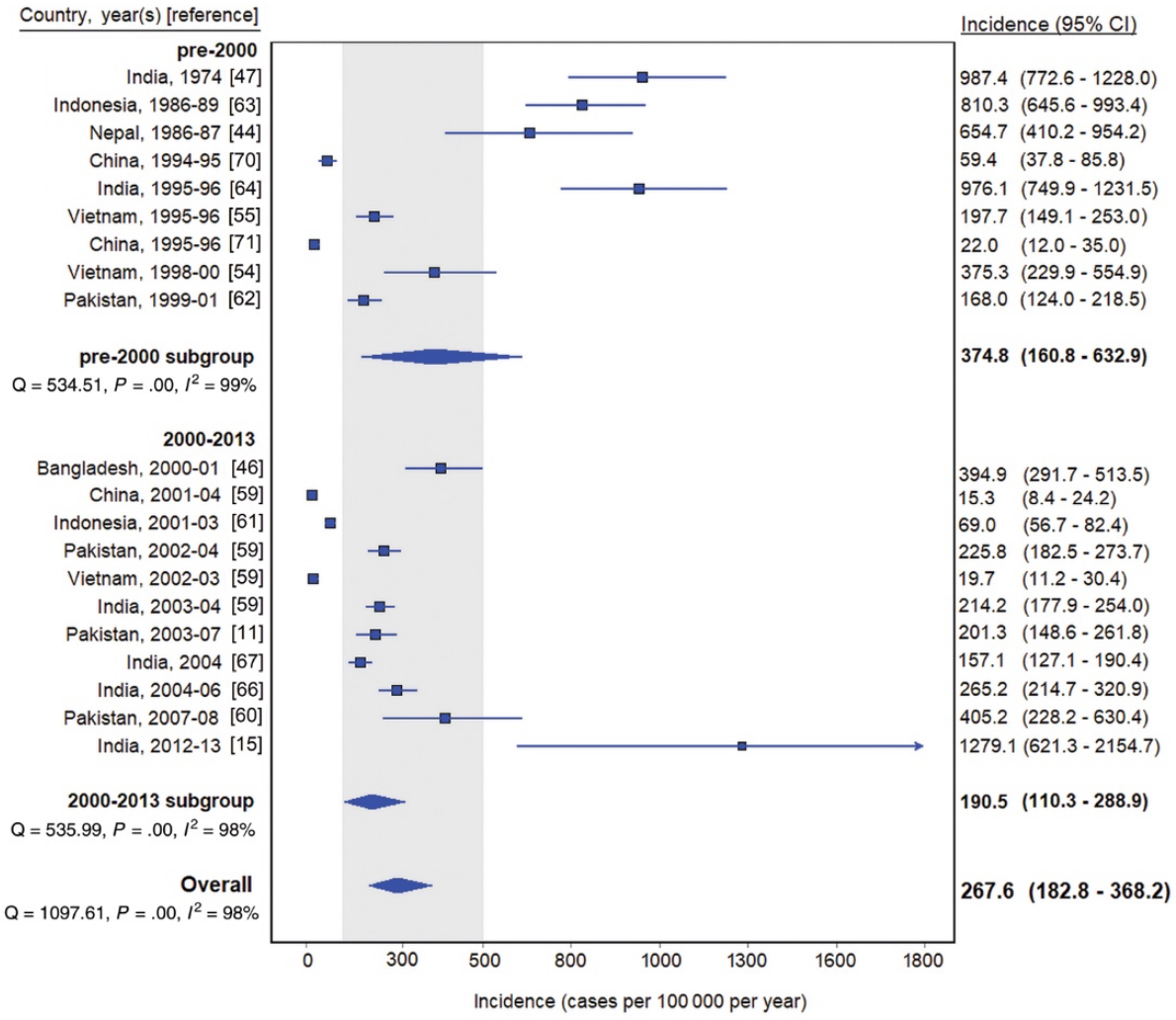


Figure 2. Typhoid fever incidence rate estimates among population-based studies in Asia 1954 -2018 (Marchello *et al* 2019).[13]

Incidence rate estimates are split into two time periods – pre-2000 and 2000-2013. Estimates range from 19.7 cases (Vietnam 2002-2003) to 1,279.1 cases (India 2012 – 2013) per 100,000 person-years. Grey shading indicates a range of 100-500 cases per 100,000 per-year, within which range most of the estimates fall. CI = confidence interval.

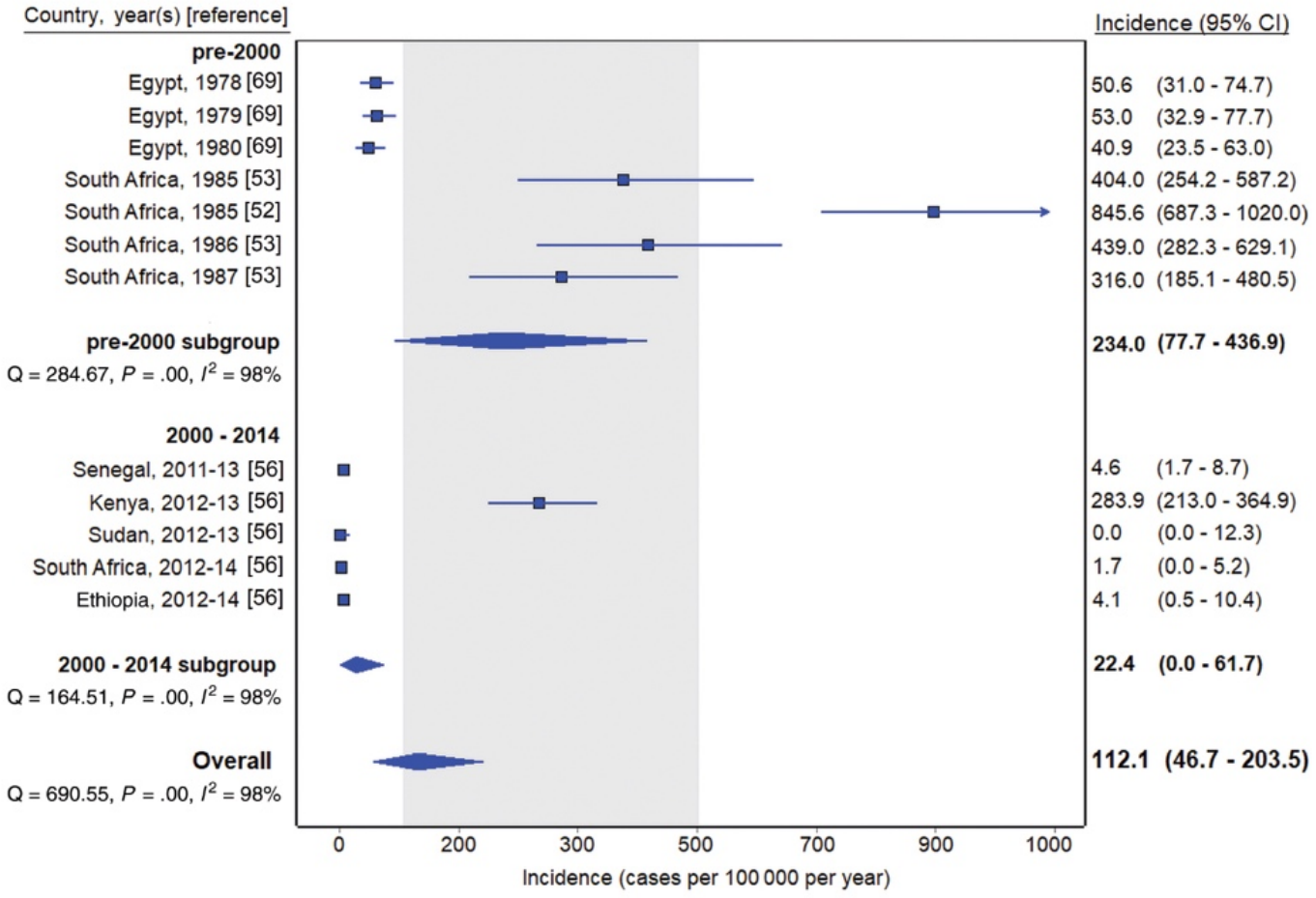


Figure 3. Typhoid fever incidence rate estimates among population-based studies in Africa, 1954 – 2018 (Marchello *et al* 2019).[13]

Incidence rate estimates are split into two time periods – pre-2000 and 2000-2014. Estimates range from 0 cases (Sudan 2012-2013) to 845.6 cases (South Africa 1985) per 100,000 person-years. Grey shading indicates a range of 100-500 cases per 100,000 per-years. CI = confidence interval.

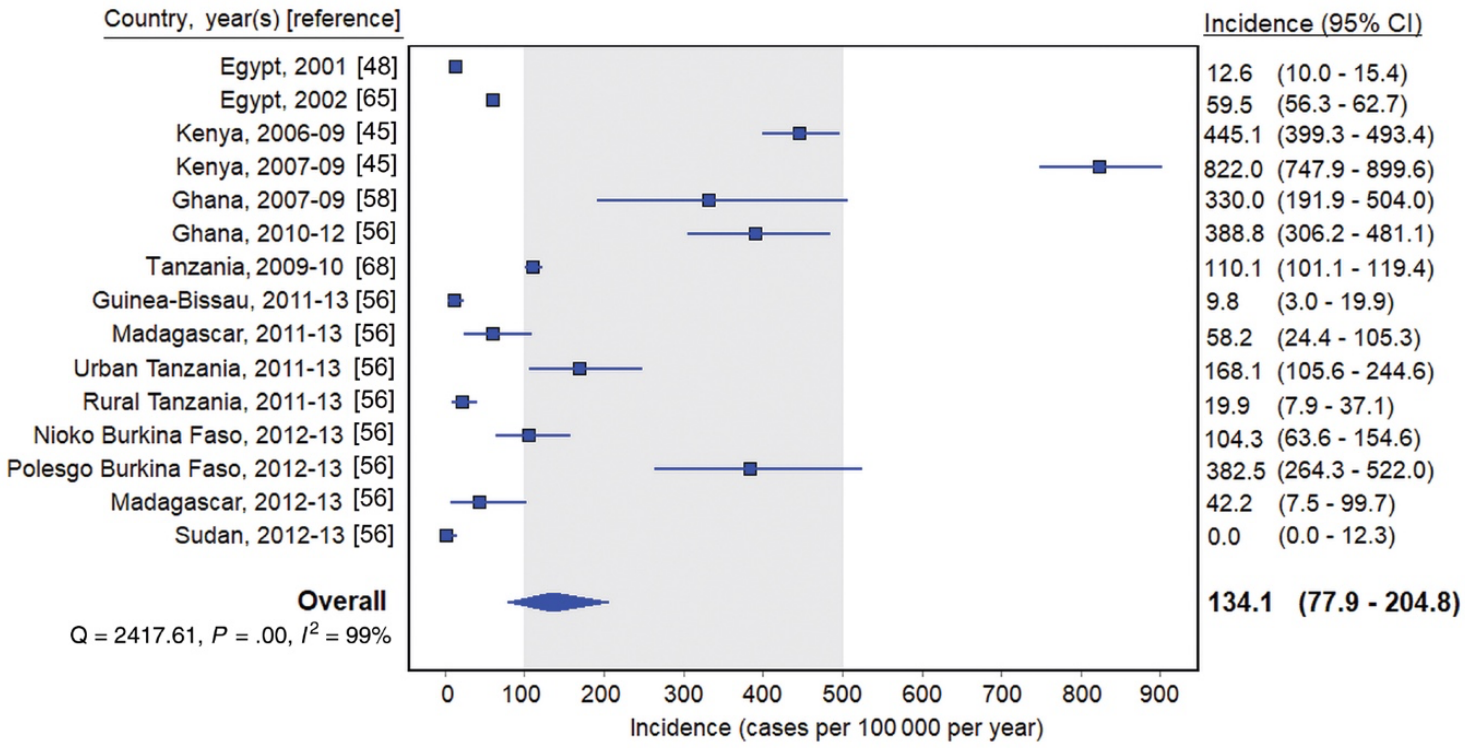


Figure 4. Typhoid fever incidence rate estimates among multiplier studies in Africa, 1954 – 2018. (Marchello *et al* 2019).[13]

Estimates range from 0 cases (Sudan 2012-2013) to 822 cases (Kenya 2007-2009) per 100,000 person-years. Grey shading indicates a range of 100-500 cases per 100,000 per-years. CI = confidence interval.

The Institute for Health Metrics and Evaluation at the University of Washington and collaborators have generated updated modelled estimates of typhoid fever burden as a component of the Global Burden of Disease Study 2019, which are publicly available on the Global Health Data Exchange (GHDx) website (https://ghdx.healthdata.org). Authors estimated that *S*. Typhi was responsible for 9.24 million (95% CI 5.94, 14.13) cases, 8.05 million (95% CI 3.86, 13.93) Disability-Adjusted Life Years (DALYs), and 110,029 (95% CI 52,810, 191,205) deaths in 2019.[14] The estimated age distribution of disease is shown below in **Figure 5**. These estimates suggest that there is low disease incidence during the early, late, and post neonatal periods, but that incidence of disease is 124 cases per 100,000 person-years among 1–4-year-olds, and peaks at 333 cases per 100,00 person-years in 10–14-year-olds. This has important implications for vaccination policy in terms of when to target children in routine immunisation programs, which ages to include in catch-up vaccination campaigns, and potentially when to provide booster vaccinations if these are deemed necessary.

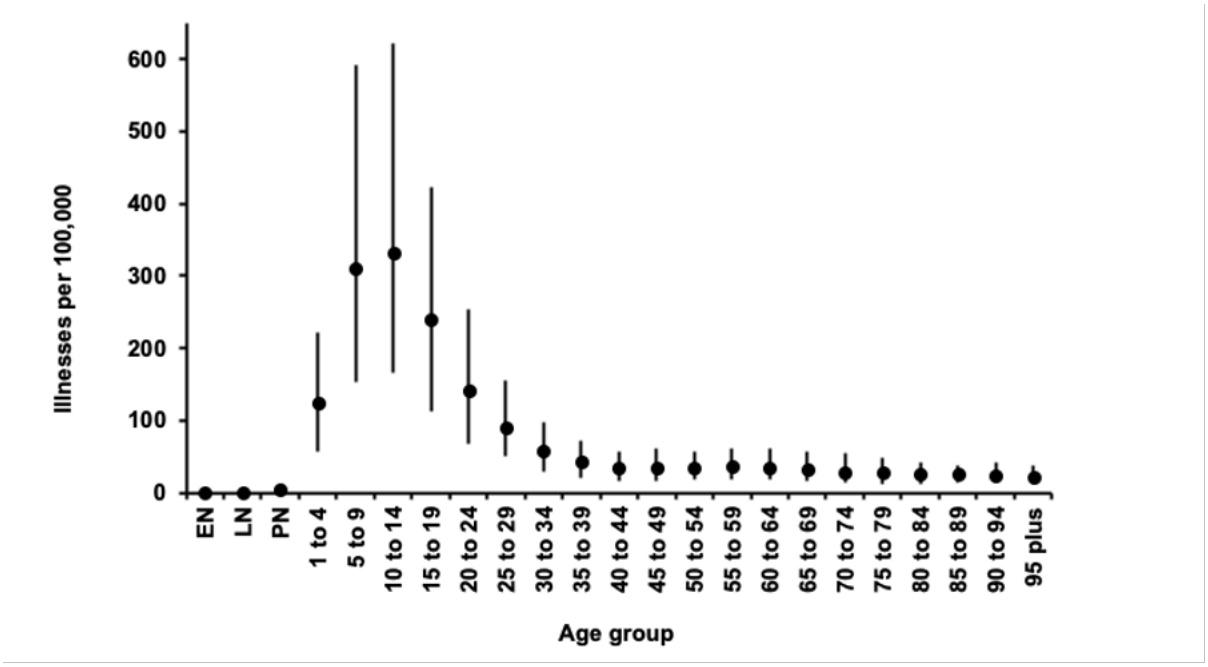


Figure 5. Typhoid fever incidence per 100,000 person-years by age group in 2019 (GHDx - http://vizhub.healthdata.org/gbd-compare/).

This figure shows the age distribution of typhoid fever incidence rate estimates generated by the Institute for Health Metrics and Evaluation (IHME) as part of the Global Burden of Disease 2019 study. Highest age-specific incidence rates are observed among 10–14-year-olds (<300 cases per 100,000 person-years), followed by 5–9-year-olds, and 15–19-year-olds. Incidence is still estimated as high (<100 cases per 100,000 person-years) among 1–4-year-olds as well.

In the previously published GBD 2017 study, authors estimated that highest estimated incidence rate overall was observed in South Asia, followed by Southeast Asia, western sub-Saharan Africa, eastern sub-Saharan Africa, and Oceania.[1] Authors noted that while overall estimated numbers of cases and deaths were similar to those reported in previously published estimates,[15–17] regional estimated differed significantly, particularly for Oceania and central sub-Saharan Africa, from whence data are relatively scarce.

IHME and other modelled estimates suggest that both typhoid fever incidence (**Figure 6**) and mortality (**Figure 7**) are declining from 1990 to 2019, although incidence is declining more sharply than mortality, perhaps owing in part to increasing prevalence of AMR in *S*. Typhi, which renders antimicrobial therapy less effective.

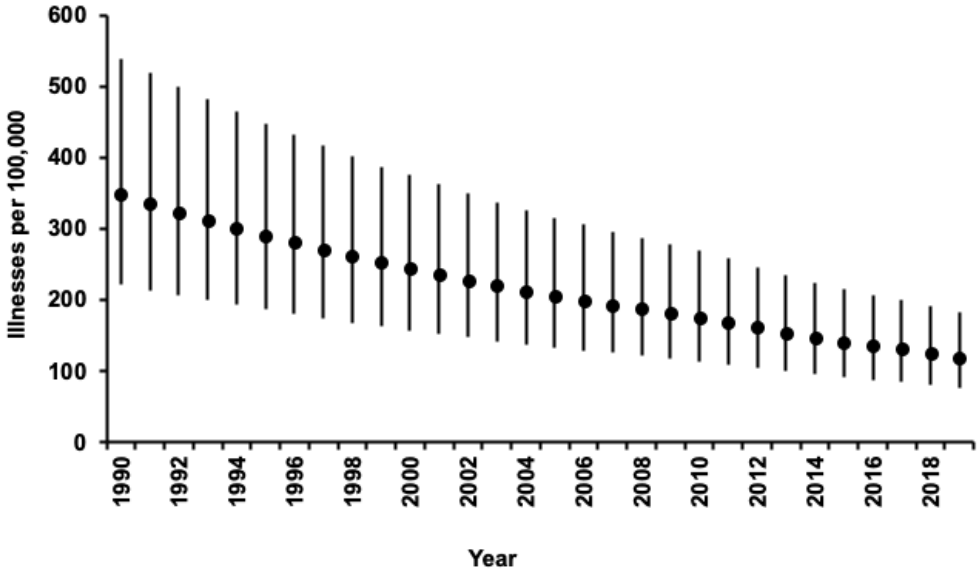


Figure 6. Typhoid fever incidence rates (per 100,000) per year, 1990 – 2019 (GHDx - http://vizhub.healthdata.org/gbd-compare/).

This figure shows the estimated annual incidence rates from 1990 – 2019. The number has declined dramatically over the thirty-year period, from over 300 cases per 100,000 person-years in 1990 to just over 100 cases per 100,000 person-years in 2019.

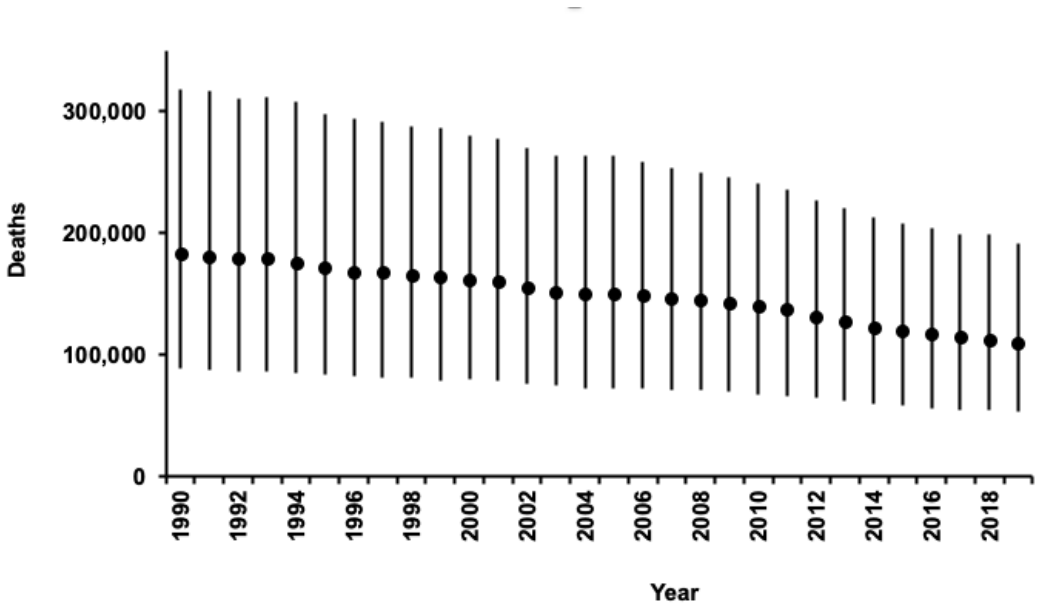


Figure 7. Typhoid fever deaths per year, 1990 – 2019 (GHDx - http://vizhub.healthdata.org/gbd-compare/).

This figure shows the estimated number of deaths attributable to typhoid fever ever year from 1990 – 2019. This number has declined steadily.

1.1.3.2 Background and overview of global population-based surveillance studies and methods

In 2009, the WHO recommended the generation of additional data on the burden of invasive *Salmonella* disease.[18] Existing disease burden estimates were based on the extrapolation of data obtained from a small number of surveillance studies conducted in limited geographical regions, which did not represent the full diversity of epidemiological settings in which typhoid is endemic.[19] A paucity of population-based surveillance studies has also contributed to uncertainty around disease burden, particularly in Africa. Indeed, a systematic review of the global burden of enteric fever conducted in 2004 showed that systematic, population-based surveillance had only been conducted in two African countries between 1954 and 2000 – South Africa and Egypt.[19]

Several standardized, multisite surveillance studies have been established over the past decade, funded primarily by the Bill & Melinda Gates Foundation and the Wellcome Trust, to address these regional data gaps as well as other important research questions. One of these studies was the Typhoid Fever Surveillance in Africa Program (TSAP), which was coordinated by the International Vaccine Institute (IVI) and partners from 2010 to 2014. The TSAP study, which was conducted at thirteen different sites in ten different countries in Africa, demonstrated high overall incidence rates of typhoid fever in several sub-Saharan African (both rural and urban), as well as high incidence rates of invasive non-typhoidal *Salmonella* (iNTS) disease across multiple sites.[20] Following the TSAP study, several additional surveillance studies were initiated to further elucidate burden of disease in diverse epidemiological settings and to interrogate additional questions about clinical

manifestations of enteric fever, such as the clinical spectrum of severity, and prevalence of severe disease and chronic intestinal carriage. These studies included the Severe Typhoid Fever Surveillance in Africa program (SETA, IVI); the Surveillance for Enteric Fever in Asia Project (SEAP, Sabin Vaccine Institute); the Strategic Typhoid Alliance Across Africa and Asia (STRATAA, University of Oxford); and the Surveillance of Enteric Fever in India (SEFI, Christian Medical College, Vellore) study. Preliminary data from these studies informed the WHO Strategic Advisory Group of Experts (SAGE) recommendation for typhoid conjugate vaccine (TCV) use in the control of typhoid fever in endemic settings that was published in 2018,[21] and additional data generated by these studies was discussed in the 2022 SAGE Typhoid session and can be used to inform TCV introduction decision-making and identification of optimal vaccination strategies in the future.

Each of the described studies aimed to address slightly different questions relating to invasive *Salmonella* disease burden in different epidemiological settings, using different approaches to sampling (eligibility criteria, samples collected, sample collection schedule, duration of follow-up) and different adjustment factors to calculate incidence rates. I have compared the methodological similarities and differences between these diverse and complementary studies, which are summarised in **Table 1** below.[12] We also identified early lessons learned and outstanding data gaps and issued recommendations for optimizing sustainable surveillance systems going forward. These key findings included the spatiotemporal heterogeneity of typhoid fever incidence, even in settings that are considered highly endemic, and the difficulty of extrapolation of disease burden estimates within regions or even within countries. Another key insight is that identifying study facilities that serve a large proportion of the population catchment area can help reduce

uncertainty around incidence rate estimates by reducing the magnitude of the healthcare seeking adjustment factor. These difficulties, as well as those detailed in the diagnostics section of this chapter, underscore the need for new, easy-to-use, low-cost diagnostics, both for point of care (to inform appropriate diagnosis and antimicrobial therapy) and for surveillance (to understand regional disease incidence and inform vaccine prioritisation discussions).

Table 1. Comparison of Surveillance Methods (Carey *et al* 2020)[12]

	SETA	SEAP	SEFI	STRATAA
Design	*Prospective passive, facility-based surveillance paired with population-based healthcare utilization surveys; Prospective case-controlled cohort for long-term follow-up*	*Retrospective and prospective passive, facility-based surveillance paired with population-based healthcare utilization surveys.*	*Tier 1: Prospective population-based cohort with active surveillance* *Tier 2: Prospective passive, hospital-based paired with population-based healthcare utilization surveys* *Tier 3: laboratory-based surveillance*	*Prospective population-based cohort with passive surveillance paired with population-based healthcare utilization surveys and seroincidence surveys*
Eligibility criteria	*Primary / Secondary Health Facilities* • *Objective fever of ≥38°C OR* • *Subjective fever ≥ 3 consecutive days in the last week,* • *AND reside in the nested catchment area;* *Referral Hospitals* • *Subjective fever ≥ 3 consecutive days in the last week, OR* • *Clinically suspected typhoid fever* • *AND reside in the catchment area* • *OR pathognomonic gastrointestinal perforations even in the absence of laboratory confirmation and regardless of catchment area (special cases)*	*Outpatient* • *3 days of consecutive fever in the last 7 days,* • *AND reside in the study catchment area* • *AND physician must advise blood culture* *Inpatient* • *Clinical suspicion of enteric fever AND physician must advise blood culture OR* • *Confirmed diagnosis of enteric fever at any time during hospitalization OR* • *Non-traumatic ileal perforations, even in the absence of laboratory confirmation* *Laboratory:* • *Blood culture positive for S. Typhi or Paratyphi A only*	*Tier 1* • *Subjective fever ≥ 3 consecutive days (families given thermometers and diary cards to record)* • *AND reside in census population area* • *AND fever in the last 12 hours before presentation,* *Tier 2* • *All inpatients presenting with fever OR* • *Patient with non-traumatic ileal perforation* • *AND residing in geographic catchment area* *Tier 3* • *Blood culture positive for S. Typhi or Paratyphi A only*	• *Objective fever of ≥38°C OR* • *Subjective fever of ≥ 2 days* • *AND reside in census population area*
Sample collection and follow-up	• *Blood samples taken from enrolled subjects at baseline* • *For blood culture confirmed cases of S.Typhi and iNTS and associated controls, blood, urine,and stool samples and oropharyngeal swabs were taken at predefined timepoints, as well as,* • *Ileal tissue or other surgical samples taken in cases of non-traumatic ileal perforation regardless of blood culture positivity* • *1-year follow-up of blood-culture confirmed S.Typhi and iNTS case and controls)*	• *Blood samples taken from enrolled subjects at baseline* • *Urine samples taken from a sample of enrolled subjects at baseline* • *Ileal tissue samples taken in cases of non-traumatic ileal perforation regardless of blood culture positivity* • *6-week phone call for blood-culture confirmed cases of S. Typhi or Paratyphi A – patients with complications followed up*	• *Blood samples taken from enrolled subjects at baseline* • *Ileal tissue samples taken in cases of non-traumatic ileal perforation regardless of blood culture positivity* • *Tier 1 – weekly follow-up, and in-person follow-up & blood collection at 28 days for enteric fever sub-cohort* • *Tier 2 – phone contact at 14 and 28 days post discharge for cost-of-illness data*	• *Blood, plasma, and stool samples taken from enrolled subjects at baseline* • *Blood, plasma, and stool samples taken from cases and household members of culture-confirmed cases)* • *Day 8, 30, 180 follow-up*

	SETA	SEAP	SEFI	STRATAA
Incidence rate adjustment factors	• *Probability of seeking care at a study facility, based on HCUS* • *Proportion of eligible patients enrolled in study* • *Proportion of eligible patients consenting to participate with a blood culture taken* • *Sensitivity of blood culture (assumed 60%)*	• *Probability of eligible patient seeking care at a study facility, based on HCUS* • *Proportion of eligible patients who consented and received a blood culture* • *Difference in healthcare-seeking according to socio-economic status* • *Sensitivity of blood culture (assumed 59%)*	• *Probability of seeking care at a study facility, based on HCUS* • *Proportion of eligible patients who consented and received a blood culture* • *Sensitivity of blood culture (assumed 59%)*	• *Probability of seeking care at a study facility, based on HCUS; adjusted for the prevalence of previously identified typhoid risk factors* • *Proportion of eligible patients who consented and had blood drawn for culturing; adjusted for age, duration of fever, temperature at presentation and clinical suspicion (Nepal and Bangladesh only)* • *Sensitivity of blood culture; adjusted for volume and reported prior antibiotic usage*
Additional objectives	• *Long-term sequelae, antimicrobial resistance, natural immune response, prevalence of chronic carriage, cost-of-illness, quality of life, long-term socio-economic study*	• *Long-term sequelae, antimicrobial resistance, cost-of-illness*	• *Antimicrobial resistance* • *Cost of illness*	• *Prevalence of chronic carriage, seroincidence, antimicrobial resistance, household transmission*

1.1.3.3 Published data from new population-based surveillance studies

Over the course of my PhD and during the time since the surveillance study methodology paper was published, each of the studies described has been completed, and data from two of the studies (STRATAA and SEAP) have been made publicly available. These published data, which include data from several South Asian sites and one African site, report high overall incidence of typhoid fever, with substantial burden in children less than 4 years of age. These data are summarised at a high level below.

1) Surveillance for Enteric Fever in Asia Project (SEAP) (2016 – 2019)[22]

Standardized prospective blood culture surveillance was conducted at five hospitals in pre-defined catchment areas in Dhaka, Bangladesh; Kathmandu and Kavrepalanchok, Nepal; and Karachi, Pakistan. A hybrid surveillance methodology that combined facility-based blood culture surveillance with healthcare utilization surveys was used to estimate overall and age-specific adjusted incidence rates for *S.* Typhi and *S.* Paratyphi A at age site. High crude and adjusted incidence rates were observed among young children at all sites, particularly in Bangladesh and Pakistan. Overall adjusted incidence rates of *S*. Typhi by site were:

- Dhaka, Bangladesh: 1,110 cases/100,000 per-years (p-y) (95% CI: 949, 1,305)
- Kathmandu, Nepal: 330 cases/100,000 p-y (95% CI: 232, 476)
- Kavrepalanchok, Nepal: 271 cases/100,000 p-y (95% CI:205, 365)
- Karachi, Pakistan: 195 cases/ 100,000 p-y (95% CI:163, 236)

2) Strategic Typhoid Alliance Across Africa and Asia (STRATAA) – Bangladesh, Malawi, Nepal (2016-2018)

A prospective, multi-component passive febrile illness surveillance study was conducted in three densely populated urban sites in Bangladesh, Nepal, and Malawi (each location with a catchment population of ~100,000) that were pre-defined following a demographic census. This surveillance program also included serological surveillance and stool screening to identify chronic carriers. Overall and age-adjusted incidence rates were estimated for each site (see **Table 2** below).[23]

Table 2. The incidence of blood-culture confirmed typhoid fever by site and age (Meiring *et al* 2021).[23]

	Blantyre, Malawi			Kathmandu, Nepal			Dhaka, Bangladesh		
	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)
0–4 years	83 (53–124)	632 (398–965)	7·6	72 (33–136)	764 (307–1921)	10·7	417 (337–511)	2625 (1764–4244)	6·3
5–9 years	146 (103–201)	861 (599–1203)	5·9	341 (250–455)	6713 (3085–18 730)	19·7	554 (456–666)	3228 (2276–4757)	5·8
10–14 years	88 (56–132)	602 (377–915)	6·9	191 (128–275)	3750 (1653–10 559)	19·6	268 (203–348)	1564 (1050–2384)	5·8
15–29 years	32 (20–48)	361 (219–567)	11·4	92 (71–119)	1457 (684–3918)	15·8	98 (76–124)	956 (603–1635)	9·8
≥30 years	21 (10–37)	248 (124–447)	12·0	6 (2–13)	92 (29–301)	15·0	29 (19–42)	279 (157–514)	9·7
All ages	58 (48–70)	444 (347–717)	7·7	74 (62–87)	1062 (683–1839)	14·4	161 (145–179)	1135 (898–1480)	7·0

Rates are per 100 000 person-years of observation. CrI=credible interval. *Adjusted for blood-culture sensitivity, probability of receiving a blood culture diagnostic test, and probability of health-care seeking.

These new data confirm that there is high incidence of typhoid fever in South Asia and in parts of sub-Saharan Africa and that there is substantial disease burden in young children as well as school-aged children, further strengthening the case for typhoid-endemic countries to introduce TCV into routine immunisation programs and to conduct catch-up campaigns where feasible. However, as stated above, large regional data gaps exist, and the difficulties of establishing and maintaining blood culture surveillance in many remote areas mean that these data gaps are likely to persist until such time as newer surveillance diagnostics are developed, validated, and made widely available at a low cost.

1.2 Antimicrobial resistance in *S*. Typhi

AMR has been observed in *S*. Typhi since the advent of use of antimicrobial agents to treat typhoid fever, beginning with the introduction of chloramphenicol in 1948, which was followed closely by reports of chloramphenicol resistance in 1950 and widespread chloramphenicol resistance by the early 1970s (see **Figure 8**).[4] By the late 1980s, multidrug resistance (MDR; resistance to first-line antimicrobials chloramphenicol, trimethoprim–sulfamethoxazole, and ampicillin) had become common, which led to widespread use of fluoroquinolones as first-line therapy in typhoid fever treatment. Decreased fluoroquinolone susceptibility soon emerged and became common, particularly in South and South-East Asia. The rise of fluoroquinolone resistance led to broader use of azithromycin or third generation cephalosporins to treat typhoid fever.

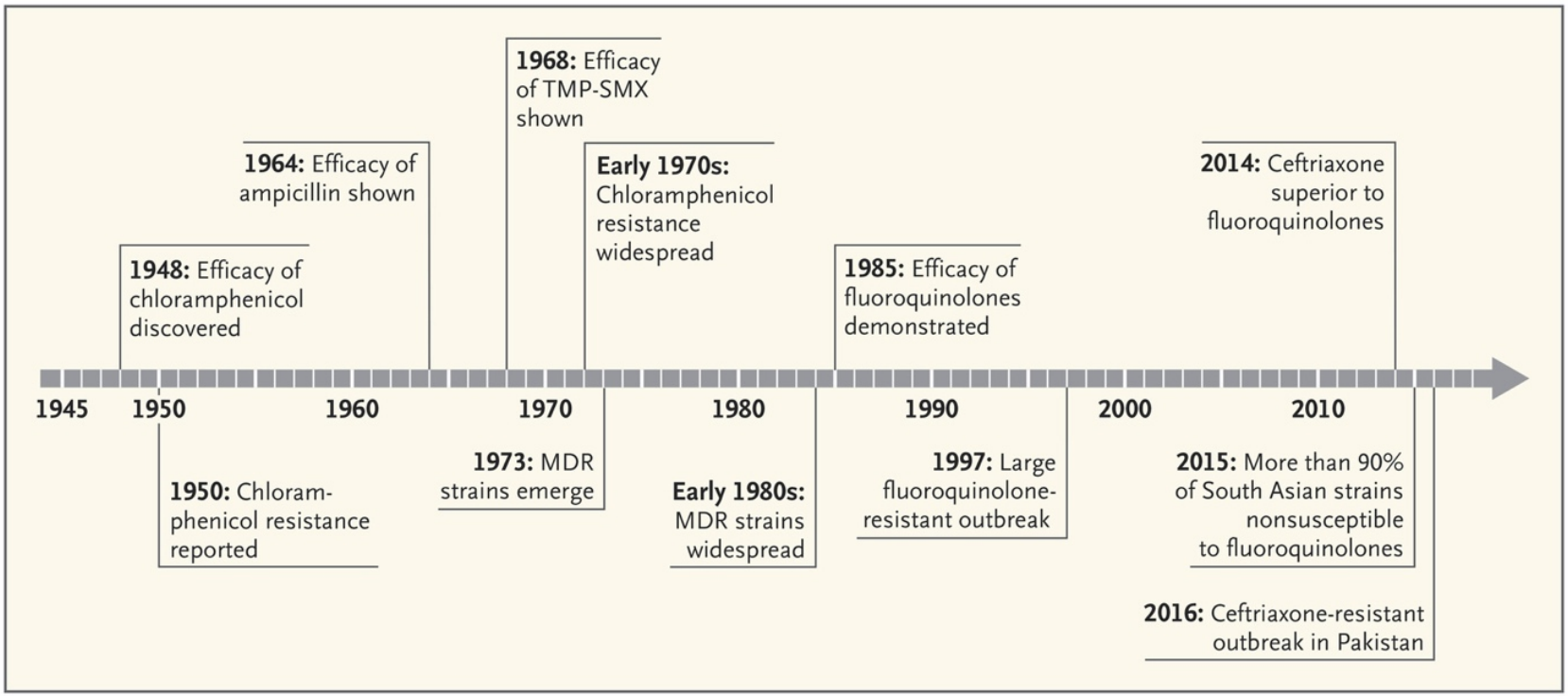


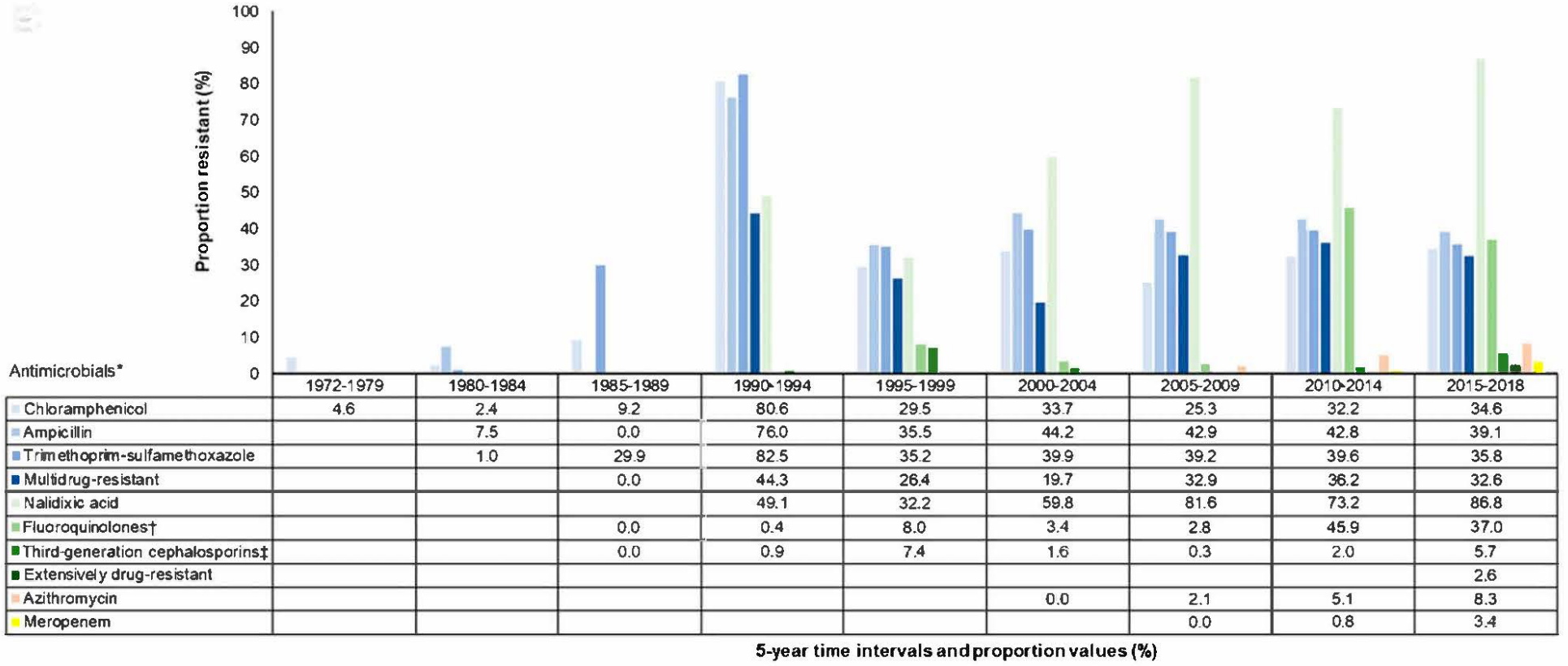
Figure 8. History of Antibiotic Efficacy Studies and the Emergence of AMR in *S.* Typhi (Andrews *et al* 2018).

Following reports of ceftriaxone treatment failure in late 2016 in Hyderabad, Pakistan, investigators from Aga Khan University and the Sanger Wellcome Trust Institute discovered and characterised a new extensively drug-resistant (XDR; defined as MDR and resistant to fluoroquinolones and third-generation cephalosporins) variant of *S*. Typhi (genotype 4.3.1.1.P1), which spread throughout Sindh province and into Punjab.[24,25] Several instances of travel-associated XDR typhoid have been reported in Europe, Australia, and North America.[26–30] In addition, azithromycin-resistance has emerged independently in different lineages of *S.* Typhi in Bangladesh, Pakistan, Nepal, India, Singapore, and Samoa.[31–36] There is an urgent need to track the emergence and spread of drug-resistant *S*. Typhi to inform empirical treatment guidelines and to inform decisions around use of preventative interventions like TCVs.

1.2.1 Phenotypic data

A 2019 systematic review and meta-analysis from Christian Marchello and colleagues provides an overview of antimicrobial susceptibility testing (AST) data collected between 1974 and 2018 found in published literature.[37] These data show increasing prevalence rates of AMR over time, beginning with resistance to traditional first line antimicrobials (MDR), followed by widespread fluoroquinolone non-susceptibility, particularly in South and Southeast Asia, and at lower prevalence levels in Africa (see **Figures 9** and **10**). These summary figures also illustrate the emergence (at low levels of prevalence) of resistance to third generation cephalosporins and azithromycin in Asia (**Figure 10**).[37] This includes XDR *S*. Typhi described above that emerged in Pakistan in late 2016. Resistance to carbapenems has also been reported from one study in Pakistan and one study in Indonesia,[38,39] but these

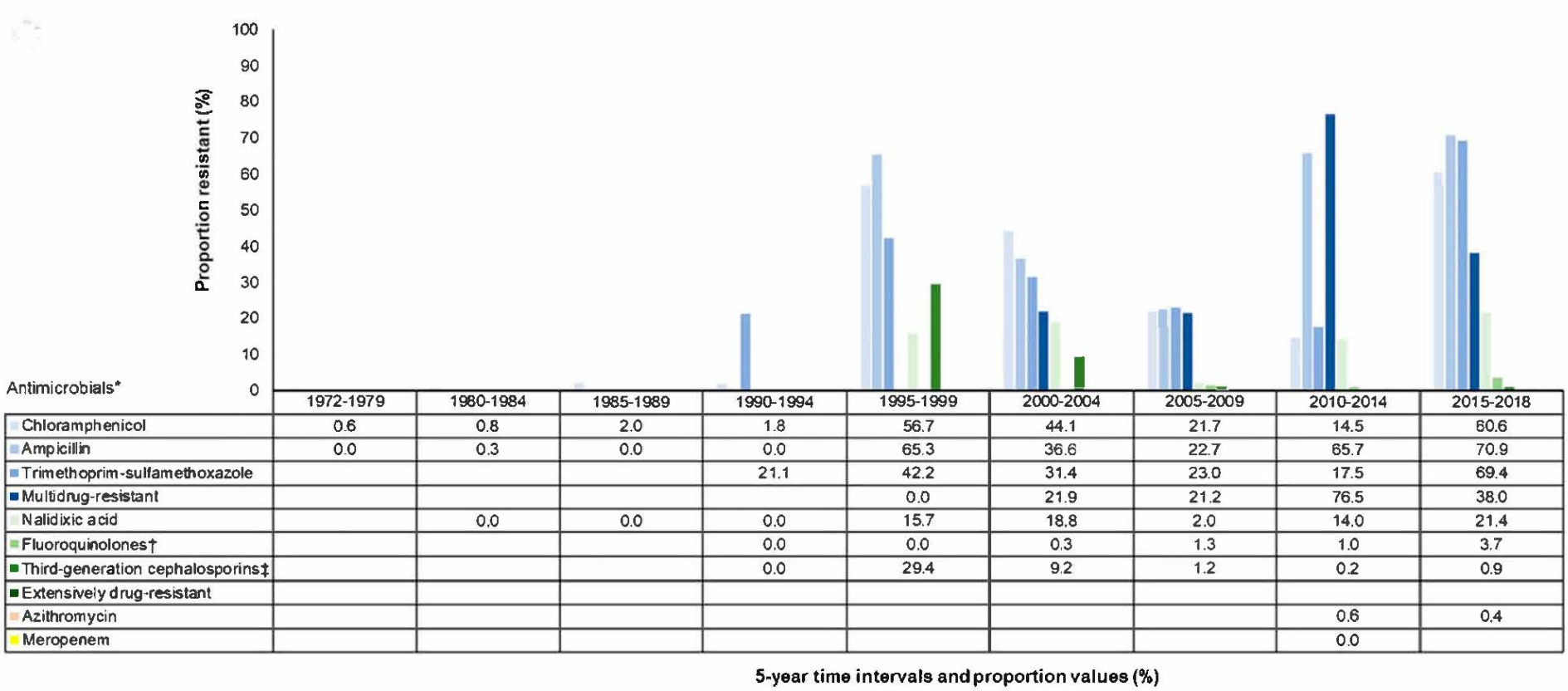
results have not been confirmed with additional testing and attempts to contact the authors for additional information were unsuccessful.



Antimicrobials*	1972-1979	1980-1984	1985-1989	1990-1994	1995-1999	2000-2004	2005-2009	2010-2014	2015-2018
Chloramphenicol	4.6	2.4	9.2	80.6	29.5	33.7	25.3	32.2	34.6
Ampicillin		7.5	0.0	76.0	35.5	44.2	42.9	42.8	39.1
Trimethoprim-sulfamethoxazole		1.0	29.9	82.5	35.2	39.9	39.2	39.6	35.8
Multidrug-resistant			0.0	44.3	26.4	19.7	32.9	36.2	32.6
Nalidixic acid				49.1	32.2	59.8	81.6	73.2	86.8
Fluoroquinolones†			0.0	0.4	8.0	3.4	2.8	45.9	37.0
Third-generation cephalosporins‡			0.0	0.9	7.4	1.6	0.3	2.0	5.7
Extensively drug-resistant									2.6
Azithromycin						0.0	2.1	5.1	8.3
Meropenem							0.0	0.8	3.4

Figure 9. Antimicrobial resistant *S.* Typhi isolates in Asia, 1972–2018 (Marchello *et al* 2020).[37]

This figure shows trends in resistance to antimicrobial agents used to treat typhoid fever in Asia between 1972 and 2018. Prevalence of MDR appears in the early 1990s and remains prevalent. Fluoroquinolone resistance also appears in the 1990s and becomes highly prevalent by the early 2000s. Resistance to third-generation cephalosporins is increasing overall, and XDR typhoid appears during the most recent period (2015-2018).



Antimicrobials*	1972-1979	1980-1984	1985-1989	1990-1994	1995-1999	2000-2004	2005-2009	2010-2014	2015-2018
Chloramphenicol	0.6	0.8	2.0	1.8	56.7	44.1	21.7	14.5	60.6
Ampicillin	0.0	0.3	0.0	0.0	65.3	36.6	22.7	65.7	70.9
Trimethoprim-sulfamethoxazole				21.1	42.2	31.4	23.0	17.5	69.4
Multidrug-resistant					0.0	21.9	21.2	76.5	38.0
Nalidixic acid		0.0	0.0	0.0	15.7	18.8	2.0	14.0	21.4
Fluoroquinolones†				0.0	0.0	0.3	1.3	1.0	3.7
Third-generation cephalosporins‡				0.0	29.4	9.2	1.2	0.2	0.9
Extensively drug-resistant									
Azithromycin								0.6	0.4
Meropenem								0.0	

Figure 10. Antimicrobial resistant *S.* Typhi isolates in Africa, 1972–2018 (Marchello *et al* 2020).[37]

This figure shows trends in resistance to antimicrobial agents used to treat typhoid fever in Africa between 1972 and 2018. MDR appears in the early 2000s and its prevalence increases until the most recent period (2015-2018), and resistance to fluoroquinolones appears in the late 1990s and remains prevalent.

Chris Murray and colleagues from the Global Research on Antimicrobial Resistance (GRAM) Project developed a model to estimate the effect of AMR on disease incidence, mortality, duration of hospital stay, and healthcare costs for 88 pathogen-antimicrobial combinations in 204 countries and territories. The authors estimated that there were 4.95 million deaths (95% CI: 3.62, 6.57) associated with bacterial AMR, including 1.27 million deaths (95% CI: 911,000, 1,710,000) that were attributable to bacterial AMR in 2019.[40] Of the 23 pathogens evaluated, *S*. Typhi was estimated to be the 11th highest in terms of global deaths attributable to and associated with bacterial AMR, with 23,700 deaths attributable to AMR in 2019 (**Figure 11**). Authors only included MDR *S*. Typhi and fluoroquinolone-resistant *S*. Typhi in this analysis, so the overall estimated burden of AMR *S*. Typhi is likely to have been underestimated.

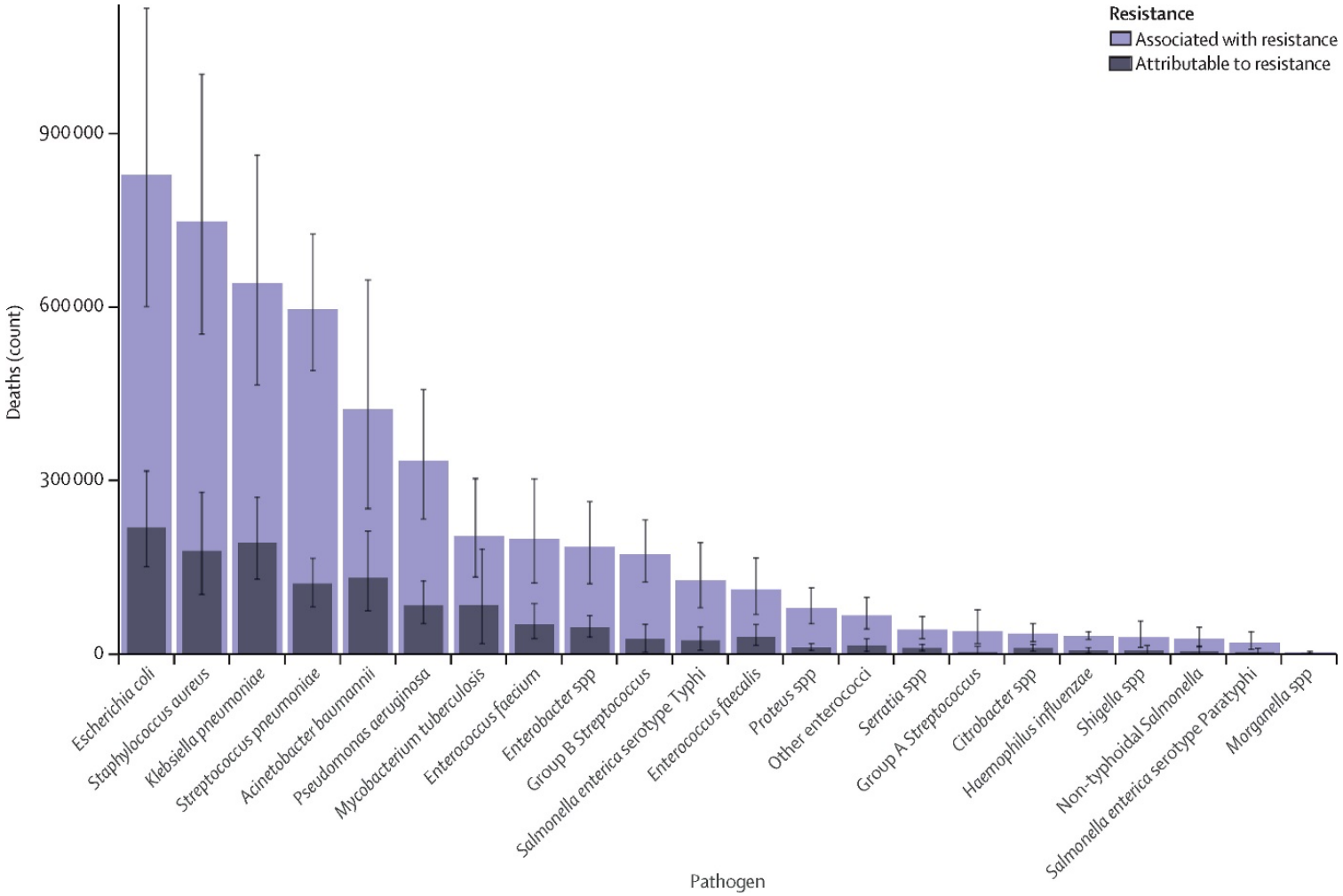


Figure 11. Global death counts attributable to and associated with bacterial AMR by pathogen in 2019 (Murray *et al* 2022). [41]

1.2.2 Genomic data

1.2.2.1 Whole Genome Sequencing and S. Typhi

The first whole genome sequence of *S*. Typhi, CT18, was published in 2001.[42] A second *S*. Typhi genome, Ty2, was published in 2003.[43] Since that time, high-throughput sequencing technologies have been developed and are more broadly available, along with reagents, training, and access to supportive resources to process and store high volumes of data. At time of writing, there are 12,001 *S*. Typhi genomes that are publicly available on Typhi Pathogenwatch (https://pathogen.watch/organisms/styphi) with accompanying metadata from 111 different countries as a result of work described in **Chapter 6**. The availability of additional genomic data, particularly from different parts of the world, will help facilitate monitoring the emergence and spread of new variants, particularly those with AMR phenotypes. The potential value of such surveillance has also been illustrated very clearly and rapidly by widespread generation, sharing, and contextualisation of SARS-CoV2 genomic data.

S. Typhi evolves very slowly (at a rate of 0.5 substitutions per year, as compared with 5 substitutions per year in host-generalist *Salmonella* serovars)[44] and is highly clonal, so most analyses are reliant on the identification of single nucleotide polymorphisms (SNPs), which are then used to generate phylogenies to analyse transmission dynamics and monitor the emergence and spread of drug-resistant lineages.[24,45,46] In order to further facilitate such analyses, a genotyping framework, GenoTyphi, was developed using specific marker SNPs to assign *S*. Typhi genomes to clades and subclades. The first iteration was developed based on approximately 2,000 *S*. Typhi genomes from 63 countries and used 68 marker SNPs to

define 4 primary clades, 15 clades, and 49 subclades.[47] This analysis showed a highly structured bacterial population consisting of subclades that were largely geographically restricted, except for Haplotype 58 or H58[48] (genotype 4.3.1), which was highly associated with drug-resistance and present throughout Asia and eastern and southern Africa. The GenoTyphi framework has expanded to reflect the current bacterial population structure and the emergence of additional AMR sublineages, including XDR typhoid.[49] This framework is available alongside tools to identify AMR determinants and generate phylogenetic trees as part of the online genomic analysis platform Typhi Pathogenwatch. This platform facilitates genomic surveillance and outbreak analysis, allowing users without advanced bioinformatics knowledge to upload their own data and put them into context with additional publicly available global data.[43]

1.2.2.2 Molecular mechanisms of resistance in S. Typhi

Whole Genome Sequencing (WGS) data facilitate investigation of molecular mechanisms of resistance, which in turn enables monitoring of the emergence and spread of drug resistance. The genetic basis for MDR was initially a conjugative plasmid of incompatibility type IncHI1,[50] which was first sequenced in the CT18 genome.[42] This plasmid had genes encoding resistance to all three first-line drugs (*cat*, *dfr*, *sul*, $bla_{\text{TEM-1}}$), mobilised by nested transposons (Tn*6029* in Tn*21*, in Tn*9*).[45,51] The earliest published H58 isolates were MDR, and it has been proposed that MDR drove the emergence and dissemination of H58,[51] which is estimated to have originated in South Asia in the late 1980s[45,52] before spreading throughout Southeastern Asia and into Eastern and Southern Africa.[45,53,54] This hypothesis is also investigated in further detail later in **Chapter 5**. The MDR transposon has subsequently

migrated to the *S*. Typhi chromosome on several independent occasions, allowing for loss of the plasmid and fixation of MDR in various lineages. Other AMR plasmid types do occur in *S*. Typhi but are relatively rare.[43,45,55–57]

The emergence of MDR *S*. Typhi led to widespread use of fluoroquinolones as first-line therapy in typhoid fever treatment and decreased fluoroquinolone susceptibility soon emerged and became common, particularly in South and Southeast Asia.[58,59] The genetic basis for this is primarily substitutions in the quinolone resistance determining region (QRDR) of core chromosomal genes *gyrA* and *parC*, both of which directly impact fluoroquinolone binding. These substitutions have arisen in diverse lineages (estimated >80 independent emergences)[52] but are particularly common in H58 (4.3.1) subtypes.[45,48,52] The most common genetic marker is a single QRDR mutation (typically at *gyrA* codon 83 or 87), which results in a moderate increase in ciprofloxacin MIC to 0.06-0.25 mg/L [60] and is associated with prolonged fever clearance times and increased chance of clinical failure when treating with fluoroquinolones.[61,62] QRDR mutations appear to be synergistic, as the presence of two QRDR mutations leads to intermediate resistance, and an accumulation of three QRDR mutations raises ciprofloxacin MIC to 8–32 mg/L and is associated with high rates of clinical failure.[61] The acquisition of a *qnrS* gene (plasmid-mediated quinolone-resistance gene that protects DNA gyrase and topoisomerase IV against quinolone compounds) in combination with a single QRDR mutation also leads to a fully resistant phenotype. In this thesis, single-QRDR or double-QRDR mutations are defined as CipI (ciprofloxacin intermediate) and triple-QRDR mutants are defined as CipR (ciprofloxacin resistant), to reflect the marked difference in expected MICs and clinical phenotypes between these genotypes. Triple mutants appear to be rare, except for a subclade of 4.3.1.2

bearing GyrA-S83F, GyrA-D87N and ParC-S80I, which emerged in India in the mid-1990s and has since been introduced into Pakistan, Nepal, and Bangladesh.[52,61,63]

The challenge of fluoroquinolone non-susceptibility was met with broader use of azithromycin or third-generation cephalosporins in typhoid fever treatment.[64–66] Reports of ceftriaxone treatment failure in late 2016 in Hyderabad, Pakistan led to the discovery of an extensively drug-resistant (XDR; defined as MDR plus resistance to fluoroquinolones and third-generation cephalosporins) clone of *S*. Typhi (genotype 4.3.1.1.P1, a subtype of H58), which subsequently spread throughout Pakistan.[24,67] This XDR clone harbours a common combination of chromosomal AMR determinants (an integrated MDR transposon plus single QRDR mutation, GyrA-83) but has also acquired a novel IncY-type plasmid carrying resistance genes, including *qnrS* (which, combined with GyrA-83, resulted in a fully ciprofloxacin resistant phenotype) and the extended-spectrum beta-lactamase (ESBL) gene $bla_{\text{CTX-M-15}}$.[24] This ESBL gene has subsequently migrated from plasmid to chromosome in some 4.3.1.1.P1 isolates.[68] Other ESBL-producing, ceftriaxone resistant (CefR) *S*. Typhi have been identified in India,[68–72] via both local 'in-country' surveillance and travel-associated cases. The only oral therapy available to treat XDR *S*. Typhi infection is azithromycin,[73] which although effective, shows prolonged bacteremia and fever clearance times in a controlled human infection model conducted in immunologically-naive adults in Oxford, UK.[74] As will be described in subsequent chapters, azithromycin resistance (AziR) *S*. Typhi, which is mediated by a nonsynonymous point mutation at codon 717 in the *acrB* gene, which codes for an efflux pump, was initially discovered in Bangladesh.[32] These initial *acrB* mutants all had azithromycin MIC values of ≥32 μg/ml. Such mutations have now been reported across South Asia [32,34,75–77]; however, the prevalence so far remains low.[52,75]

Imported cases of XDR 4.3.1.1.P1 have been identified in Europe, Australia and North America;[26–29,68] imported AziR cases are rarer but have been reported in Singapore.[36]

The accumulation of resistance to all therapeutic options means there is an urgent need to track the emergence and spread of AMR *S*. Typhi, to guide empirical therapy to prevent treatment failure, and to direct the deployment of preventative interventions like TCVs. A high degree of concordance (98-99.7%) between antimicrobial susceptibility data and known genetic determinants of AMR has been demonstrated by multiple groups using matched AST and WGS data (Center for Genomic Pathogen Surveillance/Pathogenwatch, United Kingdom Health Security Agency, the Severe Enteric fever in Asia Project [SEAP], Sabin Vaccine Institute),[43,52,78] and these AMR determinants can be called directly from raw reads or assemblies using freely-available online genomic resources like Pathogenwatch.[43] (This and other freely available genomic resources will be described in greater detail in **Chapter 7**). Given the high positive predictive value of molecular determinants of AMR and the ease with which these genetic elements can be identified from sequence data, there is a clear role for WGS in standardised and scalable AMR surveillance.

1.2.2.3 Potential applications of WGS in informing policy

Given the continued emergence and spread of drug resistance, there is an urgent need to establish and expand AMR surveillance to inform vaccine introduction and antimicrobial stewardship policy. Classically, this has been conducted using phenotypic data, but there are several challenges associated with this approach. Phenotypic data are not always widely or consistently reported. Where reported, these data may hard to standardize across different labs as different tests are often used, along with different interpretive criteria, and these criteria change over time. In addition, not all breakpoints have been validated extensively using clinical data. Phenotypic data also cannot provide information about relatedness or transmission dynamics. For these reasons, implementation of genomic surveillance alongside AST testing is advisable.

Genomic surveillance provides several advantages. WGS data yield standardized AMR outputs where molecular mechanisms of resistance are understood and can facilitate investigation of new molecular mechanisms of resistance and pathogen discovery. WGS data can provide information about international transmission and emergence and spread of drug-resistant organisms. In addition, these tests are repeatable. Extrapolation of data from returning travellers can be used to draw inferences about circulating genotypes and AMR profiles in the countries from which travellers are returning.[55] However, there are challenges in generating and analysing genomic data associated with access to technology, reagents, bioinformatic training, high compute clusters and internet bandwidth in many low- and middle-income countries (LMICs).[79] These challenges are not insurmountable but will require a different approach to funding and collaborative work between institutions. In

addition to generation and analysis of genomic data, interpretation and generalisability of these data may also not be straightforward, as data from genomic studies may not always be representative. Often, isolates that are drug-resistant are more likely to be sequenced. Many isolates are sequenced as part of outbreak investigations, and sampling frame may not always be reported. Use cases for WGS in informing typhoid control measures, as well as challenges associated with generating and analysing such data, are described in greater detail in **Chapter 7**.

The Global Typhoid Genomics Consortium (https://www.typhoidgenomics.org) was established in 2021 to address several of these concerns. The consortium's mission is to engage with the wider typhoid research community to aggregate *S*. Typhi genomic data and standardized metadata to facilitate the extraction of relevant insights to inform public health policy through inclusive, repeatable analysis using freely available and accessible pipelines and intuitive data visualization. One of the first activities of the consortium was to generate a global update paper, in which a large, geographically representative dataset of more than thirteen thousand *S*. Typhi genomes is presented. This update paper, which is described in greater detail in **Chapter 6**, provides a contemporary snapshot of the global genetic diversity in *S*. Typhi and its spectrum of AMR determinants. In addition, we have leveraged this rich dataset to identify key international transmission events involving drug-resistant *S*. Typhi. The establishment of the Global Typhoid Genomics Consortium marks twenty years of typhoid genomics and provides a platform for future typhoid genomics activities, which can inform more sophisticated disease control.

1.3 Typhoid vaccines

1.3.1 History of typhoid vaccines and vaccine policy

The first known typhoid vaccine, a heat-phenol-inactivated whole cell parenteral vaccine, was developed and distributed in Europe in 1896. It was primarily used in military populations, and ultimately was deemed too reactogenic to have broader public health utility.[80] Since that time, several additional vaccines have been developed and licensed, including a parenteral capsular polysaccharide vaccine based on *S.* Typhi Vi antigen,[81] and a live-attenuated oral vaccine based on the Ty21a strain of *S*. Typhi.[82] The WHO recommended use of both vaccines for endemic and outbreak control of typhoid in its 2008 position paper, but neither vaccine was implemented more broadly.[83] The lack of obvious demand for these products may be due in part to modest efficacy, limited duration of protection, and inappropriateness for use in children less than two years of age.[84]

At the previous WHO SAGE review for typhoid vaccine policy in October 2017, the most robust evidence for a licensed typhoid conjugate vaccine (TCV) was from immunogenicity data on Typbar-TCV® (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Bharat Biotech India Limited, Hyderabad, India) and efficacy estimates from a Controlled Human Infection Model (CHIM) study in immunologically naïve, healthy adult volunteers in Oxford, England. Vaccine efficacy of Typbar-TCV® in the CHIM was estimated as 87.1% (95% CI 47.2, 96.9%) against a definition of typhoid fever (fever ≥38˚C lasting for >12 hours followed by blood culture-confirmed *S*. Typhi infection), and 54.6% (95% CI 26.8, 71.8) efficacious against blood culture-confirmed *S*. Typhi infection, which was the primary endpoint of the study.[85] Several additional TCVs were in early-stage clinical trials.[86]

While no field efficacy data were available for TCVs with ongoing clinical development programmes, a large-scale Phase III efficacy study evaluating a previous TCV construct (Vi polysaccharide linked to the recombinant exoprotein A of *Pseudomonas aeruginosa*, or Vi-rEPA) developed by the US National Institutes of Health (NIH) was conducted in Vietnam in the early 2000s. In this study, 1,091 Vietnamese children received two doses of Vi-rEPA vaccine, which provided 91.5% (95% CI 77.1, 96.6) protective efficacy over 27 months of follow-up (per protocol analysis). In addition, among 771 children who only received one dose of Vi-rEPA (intention to treat analysis), 87.7% efficacy was observed.[87] 75 children who received a single dose of Vi-rEPA still had anti-Vi IgG antibody levels above an assumed protective threshold eight years later,[88] strengthening the assertion that conjugate vaccines could confer longer duration of protection than previously licensed polysaccharide vaccines (Vi-PS) or Ty21a vaccines. This vaccine was never commercialized.

Following SAGE's review of the geographic and age distribution of typhoid fever, the increasing threat posed by AMR, performance of Vi-PS and Ty21a vaccines as well as TCV, and cost-effectiveness of routine vaccination) and recommendations, WHO issued a revised global policy on typhoid vaccines in October 2017, which was published in March 2018.[21] WHO recommended the introduction of a single dose of TCV for infants and children 6 months of age and over in typhoid-endemic countries, noting that it is likely to be most feasible at existing vaccine visits at 9 months of age or in the second year of life. TCV was recommended over previously recommended typhoid vaccines at all ages in view of its improved immunological properties, suitability for use in younger children and anticipated longer duration of protection. SAGE also suggested that TCV should be prioritized for

countries with the highest burden of disease or a high burden of AMR *S.* Typhi and that catch-up vaccination in children up to 15 years of age should be conducted at the time of introduction routine immunization where feasible for maximal public health impact.

1.3.2 Currently available and pipeline vaccines

There are now two WHO prequalified TCVs, both licensed for use in infants and children from 6 months of age and in adults up to 45 years:

- **Typbar-TCV®** (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Bharat Biotech India Limited, Hyderabad, India, WHO PQ date December 2017), and
- **TYPHIBEV®** (Vi polysaccharide conjugated to CRM_{197} carrier protein, Biological E. Limited, Pune, India, WHO PQ date December 2020).

Typbar-TCV® has been evaluated in three large-scale post-licensure efficacy/effectiveness studies in Bangladesh, Malawi, and Nepal by the Typhoid Vaccine Acceleration Consortium (TyVAC), which is led by the Center for Vaccine Development and Global Health at the University of Maryland School of Medicine, the Oxford Vaccine Group at the University of Oxford, and PATH.[89–91] TyVAC investigators also conducted an immunogenicity and co-administration study in infants and toddlers in Burkina Faso.[92–94] These studies show that a single dose of Typbar-TCV® vaccine is safe, immunogenic, and highly efficacious (81-85%) in preventing symptomatic typhoid fever in children 9 months to 15 years of age over a 18-24 month follow-up period. The results of these studies are described in additional detail in the April 2022 WHO SAGE background document,[95] which is also appended to this thesis (**Appendix 9)**.

In addition, Typbar-TCV® was shown to be 95% effective against blood culture confirmed *S*. Typhi and 97% effective against extensively drug-resistant (XDR) *S*. Typhi in 6 month – 10-year-olds in Hyderabad, Pakistan.[96] New efficacy and effectiveness data for Typbar-TCV® are summarised in **Table 3** below. Additional studies assessing longer-term (≥3 years) effectiveness and potential herd effects of a single dose of Typbar-TCV® are in planning or early execution stages in Ghana, Democratic Republic of Congo, and Fiji, and effectiveness studies using TYPHIBEV® are being planned in India and Madagascar, as well as a post-introduction impact assessment in Malawi. In addition, investigators from Biological E. have recently published data from their Phase II/III licensure study in India, demonstrating immunogenicity non-inferiority of TYPHIBEV as compared to Typbar-TCV® in participants 6 months – 45 years of age.[97] A summary of completed and planned clinical development activities for both Typbar-TCV® and TYPHIBEV® is included in **Table 4**.

Table 3. Protective efficacy/effectiveness of a single TCV dose (Typbar-TCV®) against primary endpoint of blood culture confirmed typhoid fever[95]

Study, Design	Age (# vaccinated)	Control vaccine	Follow up	Vaccine efficacy or effectiveness (95% CI)
TyVAC TRIALS				
Nepal, Individually randomized[91]	9 mths – 16 yrs (20,019)	Group A meningitis	24 months	79.1% (62.0, 88.5)
Malawi, Individually randomized[90]	9 mths – 12 yrs (28,130)	Group A meningitis	18-24 months	83.7% (68.1, 91.6)
Bangladesh, Cluster randomized[89]	9 mths – 16 yrs (67,395)	SA-14-14-2 JE	24 months	85.0% (76, 91)
ADDITIONAL STUDIES				
Navi Mumbai, India (routine immunization), Case-control[98]	9 mths – 14 yrs (540,000)	None	15 months	80.2% (53.2, 91.6)
Karachi, Pakistan (outbreak response campaign) Case control[99]	6 mths – 15 yrs (87,993)	None	4 months	72% (34, 88)
Hyderabad, Pakistan (outbreak response campaign) Cohort study[100]	6 mths – 10 yrs (207,000)	None	18 months	95% (93, 96)

Table 4. Overview of available data for WHO Prequalified TCVs[95]

	Typbar-TCV® (Vi-TT, Bharat Biotech India Ltd., India)	**TYPHIBEV® (Vi-CRM$_{197}$, Biological E Ltd., India)**
Composition	*Salmonella* Typhi Vi polysaccharide conjugated to tetanus toxoid	Vi polysaccharide prepared from *Citrobacter freundii sensu latoa* conjugated to CRM
Phase of Development	WHO PQ 2017, post-licensure studies ongoing	WHO PQ 2020, post-licensure studies being planned
Indication/Target Ages	≥6 months- ≤45 years. ≥6 months-≤65 years DCGI approved, WHO filing pending	≥ 6 months to ≤ 45 years
Safety Data	>500,000 subjects (pre-licensure studies & post-marketing surveillance in India, TyVAC studies, Pakistan impact studies, Navi Mumbai)[101–105]	3000+ subjects (licensure studies in India)
Immunogenicity data (geographic representation, special populations, age range, duration of response)	Ph III (India) • 6 mo – 45 yrs: 3-, 5- & 7-years post immunization[106] TyVAC (2-3 yrs post-vaccination follow-up underway) • Burkina n=250, 9-11 months & 15-23 months[93,94] • Malawi n=600, 9 months – 12 years, HIV-infected & malnourished[107] • Nepal n=1500, 9 months – 16 years[91] • Bangladesh n=1300, 9 months – 16 years[108]	Ph II/III (India, n=622) • Immunogenicity non-inferiority compared to Typbar-TCV, participants ages 6 months - <64 years (not published) • Immunogenicity data 3 years post primary immunization expected July 2022
Efficacy and/or Effectiveness Data?	Efficacy data (TyVAC) • Nepal - 79% (95%CI 61.9, 88.5) efficacy, 24 months follow-up[91] • Malawi - 83.7% (68.1, 91.6) efficacy, 24 months follow-up[90] • Bangladesh - 85% (67, 91) total protection, 18-24 months follow-up[89] Effectiveness • Hyderabad, Pakistan - 95% (93-96%) effective against *S.* Typhi, and 97% (95-98%) against XDR *S*. Typhi, 18 months follow-up[96] • Lyari, Pakistan - 72% (34, 88) effective, 4 months follow-up[99] • Navi Mumbai– 80.2% (53.2, 91.6) effective, 15 months follow-up[98]	Planned evaluations in effectiveness studies in Madagascar (TyCOMA) & India
Coadministration/non-interference data?	• MCV & MMR in India @ 9 & 15 mo (DCGI approved, to be submitted to WHO, publication under review at IJID) • Yellow fever & measles-rubella (9 mo) & MCV-A (15 mo) in Burkina Faso	• MR coadministration study planned to start March 2022 in India
2 dose schedule tested?	No, 1 dose only assessed in primary series	No, 1 dose only assessed in primary series
Booster dose schedules tested?	Comparison of single dose vs booster after 2 yr in 6-23 mo cohort of PhIII 3-, 5- & 7-years post primary immunization in India [106]	Evaluation of booster dose 3 years post primary immunization planned for extended Ph II/III study in India

MCV – measles-containing vaccine. MMR – measles-mumps-rubella vaccine. MCV-A – meningococcal conjugate serogroup A vaccine.

There is a robust vaccine development pipeline. Four companies have programmes with a TCV candidate with Phase III clinical trials ongoing or completed –**BioTCV** (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, PT Biofarma, Indonesia),[109–112] **EuTYPH-C** (Vi polysaccharide conjugated to CRM197 carrier protein, EuBiologics, South Korea),[113] **SKYTyphoid** (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, SK Bioscience, South Korea),[114,115] and **ZYVAC TCV** (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Zydus Cadila, India).[116] SKYTyphoid recently received an export-only license from the Korean regulatory authority,[117] and ZYVAC-TCV was licensed in India in 2017.[118] All four manufacturers are seeking WHO Prequalification. The number and diversity of TCV manufacturers bodes well for future supply security as part of a healthy markets framework.[119] Licensure and prequalification of these vaccines will be based on safety and immunogenicity data (non-inferiority compared to Typbar-TCV®)[120], and manufacturers do not have any current plans to conduct additional studies demonstrating efficacy or effectiveness of their candidate vaccines against clinical disease, which may have an impact on country product preferences. Key data (published and expected) for pipeline vaccines are summarised in **Table 5**.

Table 5. Overview of available and expected data for pipeline TCVs[95]

	Bio-TCV (Vi-DT, PT Bio Farma, Indonesia)	SKYTyphoid (Vi-DT, SK Bioscience, Korea)	ZYVAC TCV (Vi-TT, Zydus Cadila, India)	EuTYPH-C (Vi-CRM_{197}, EuBiologics, Korea)
Phase of Development	Phase III study completed (Indonesia)	Two parallel phase III studies completed (Philippines & Nepal)	Phase III, Phase IV, & active Post-Marketing Surveillance ongoing (India)	Phase II/III study completed (Philippines), Phase III starting (Kenya & Senegal)
Manufacturer's target date for PQ	2025	2023	2023 (submission planned Sept 2022)	2023
Indication/ Target Ages	≥ 6 months to ≤ 60 years	≥ 6 months	≥ 6 months to ≤ 45 years. Extended age indication (to 65 years) to be sought.	≥6 months to ≤45 years
Safety Data	3000+ subjects	3000+ subjects	<3000 subjects, but PMS study (n=3000) fully enrolled	<3000 subjects
Immunogenicity data (geographic representation, special populations, age range, duration of response)	Phase I (Indonesia) n=30, 18-45 years, 6 month follow-up[112] Ph II (Indonesia) n=600, 6 months – 40 years[110,111] • Long-term follow-up (5 years) of subjects aged 6-23 months at initial vaccination ongoing Phase III (Indonesia) • Non-inferiority to Typbar-TCV, n=3071, 6 months – 60 years (completed)	Phase I (Philippines) • n=144, 2-45 years[114] Phase II (Philippines) • n=285, 6-23 months[115,121] • 27.5 month follow-up published,[122] longer-term (5 years) follow-up ongoing Ph III (Philippines) • n=1800, 6 months- 45 years Phase III (Nepal) • Non-inferiority vs Typbar-TCV, n=1800, 6 months–45 years[123]	Phase I (India) • n= 24, 18-45 years Phase II/III (India) • Non-inferiority to Typbar-TCV, n=240 6 mo - 45 years[116] • 3 years post vx data submitted & manuscript under review Phase III (India) • Immunogenicity non-inferiority to Typbar-TCV, n = 238, 45-65 years (completed)	Phase I (Philippines) • Non-inferiority to Typbar-TCV & Vi-PS, n=75, 18 -45 years[113] Phase II/III (Philippines) • Non-inferiority to Typbar-TCV, n=444, 6 months – 45 years (completed) Phase III (Kenya & Senegal) Immunogenicity non-inferiority to Typbar-TCV, 6 months – 45 years, n= 3255
Coadmin non-interference data w/ routine vaccines?	Measles-rubella included in Ph III study	Coadministration with measles-rubella & measles-mumps-rubella included in Ph III in Nepal (complete)	• Coadministration study with measles-rubella vaccine (n=900) in infants (9-10 months) started March 2022	• Coadministration with measles-rubella & yellow fever will be assessed in Ph III (Kenya & Senegal)
2 dose schedule tested?	2 dose schedule (4 weeks apart) assessed in Ph I	2 dose schedule (4 weeks apart) was assessed in Ph I & II	No, 1 dose only in primary series	No, 1 dose only in primary series
Booster dose schedules tested?	Booster 3 years ±6 months post primary immunization tested in extended Ph II study.	Boosters 6 months and 2 years post primary immunization compared in extended Ph II study (out to 5 years post primary immunization)	Booster 3 years post primary immunization tested in extended Ph II/III subset (manuscript under review)	No

1.3.3 Global policy and vaccine introduction

1.3.3.1 Vaccine policy & research agenda

The WHO SAGE met in April 2022 to review new data on typhoid fever epidemiology, AMR, and TCV performance. Some of the key topics included the geographic distribution of typhoid fever, the increasing threat of AMR, duration of protection of a single dose of TCV, and whether a booster dose will be required. I was a consultant with the WHO Immunisations, Vaccinations, and Biologicals group from 2021-2023, during which time, I organized and presented at a TCV Research Agenda Stakeholder meeting and drafted the SAGE background paper for the April 2022 typhoid session, where I presented new data on licensed and pipeline TCVs. The high-level meeting TCV Research Agenda report is included as **Appendix 8**, and the WHO SAGE Typhoid Background Paper is included as **Appendix 9**. The broad conclusions of the WHO SAGE session were as follows:[124]

- Typhoid fever incidence is high in South Asia and parts of Africa, particularly in school-aged children and young children, but major regional data gaps remain
- AMR in *S*. Typhi is a major threat to effective treatment, particularly resistance to ciprofloxacin, azithromycin, and cephalosporins
- New data showing high efficacy & effectiveness of a single dose of TCV in diverse epidemiological settings strengthen the existing 2017 SAGE recommendation for TCV introduction
- No evidence of waning immunity or protection over two years, but questions about longer-term duration of protection and the need for and optimal timing of a booster dose remain. Additional data will be made available in the next 18-24 months that should help settle this question.

Key outstanding questions include:

- What is the overall global disease burden (incidence and severity) - not just in areas where multiple surveillance studies have been established)?
- What is the global burden and distribution of AMR in *S*. Typhi?
- What is the impact of TCV on AMR?
- What is the duration of protection conferred by one dose of TCV?
- Is a booster dose required and, if so, when?

1.3.3.2 Vaccine introduction status and barriers

Typhoid conjugate vaccines have been introduced into routine childhood immunization programmes in six countries to date. The first public sector TCV introduction was conducted at a subnational level in India in the Navi Mumbai Municipal Corporation and included an effectiveness and safety evaluation.[105,125] Pakistan was the first country to initiate a Gavi-supported national introduction in 2019,[126] followed by Liberia (2021),[127] Zimbabwe (2021),[128] and Nepal (2022).[129] Malawi also plans to introduce TCV with Gavi support in 2023. The Samoan government initiated a self-financed TCV introduction in 2021.[130] Several additional countries are considering Gavi applications for TCV introduction support.

There are still some key barriers to decision-making around TCV introduction as well as identification of optimal vaccination strategy. Early introduction countries like Pakistan, Malawi, Nepal, and Zimbabwe have had access to reliable, laboratory-confirmed *S*. Typhi data and information about AMR profiles, but many countries lack the requisite laboratory infrastructure to generate these data. Other key drivers that support decision-making include cost-effectiveness and availability of vaccine performance data. Ample data exist demonstrating the short-term (1-2 year) efficacy and effectiveness of Typbar-TCV® but no

such data exist for other TCVs, which may have an impact on product selection in countries. This has been observed for other vaccination programs, like rotavirus, where countries have chosen to delay introduction until their preferred product is available.[131] Operational challenges with recent introductions have included COVID-19-related delays and setbacks relating to misinformation around COVID-19 vaccination, school closures disrupting planned mass vaccinations in schools, difficulties integrating a new vaccination into already-crowded routine immunisation schedules, and human resource limitations. Additional operational research for how best to integrate TCV introduction into existing programmes should also be considered.

1.4 Aims of the thesis

Enormous progress has been made in the typhoid field in recent years. Our understanding of disease incidence and AMR have increased, and TCVs have been evaluated and deployed in typhoid-endemic regions. Some of the key challenges to effective typhoid control that remain include: 1) a lack of standardized, geographically representative incidence rate data, 2) the rapid emergence and spread of AMR and the deficit of new antimicrobials in development, and 3) the need for widespread deployment of TCVs and sustained investments in improved water and sanitation interventions. Due to financial, technical, and logistic limitations, it is unlikely that standardized blood culture surveillance will be implemented broadly, but it may be feasible and cost-effective to establish and implement sentinel genomic surveillance for *S*. Typhi and other viral and bacterial pathogens in key geographies to improve our understanding of transmission dynamics and AMR patterns. Over the course of my PhD, I aimed to expand our global understanding of the molecular epidemiology of *S*. Typhi and explore how to link this to effective policy interventions. By making information on the emergence and spread of drug-resistant strains more accessible to a lay audience, these data are more likely to inform typhoid control policy, at both global and regional levels.

Aims of the PhD:

1. To track the emergence and possible spread of azithromycin-resistant *S*. Typhi with international collaborators in AMR hotspots (South Asia) using genomic data and metadata, and to ensure that these data are made widely available and understandable to both academic and policy audiences.

2. To generate an updated overview of global genotype and AMR distribution for *S*. Typhi and to describe how it varies by time and geography by working with international collaborators to aggregate and analyse genomic data and metadata from key geographies.

3. To create a research consortium comprised of a broad network of typhoid researchers to encourage *S*. Typhi WGS data sharing, and to facilitate visualisation and analysis of data in a transparent, reproducible manner, to inform public health action.

Chapter 2: Materials and Methods

2.1 Overview

The high-level workflows and methods used to support the analysis in Chapters 3-6 are similar and will be described at a conceptual level in this section. However, as each chapter describes a discrete analysis of a different genomic dataset with different research questions, the specific methods used in each chapter will be described individually for each data chapter in subsequent sections as well.

2.1.1 Bacterial identification and antimicrobial susceptibility testing

In the studies described in this dissertation, bacterial isolates (isolated almost exclusively from blood of febrile patients who were consented and enrolled into a blood culture surveillance study) were identified as *S*. Typhi using conventional biochemical methods. Serologically confirmed *S*. Typhi isolates were subjected to antimicrobial susceptibility testing against azithromycin, ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, and additional cephalosporins and/or fluoroquinolones in some instances using disk diffusion. Results were interpreted according to Clinical Laboratory Standards Institute (CLSI) Guidelines.[132] Minimum inhibitory concentration (MIC) testing was conducted on all organisms showing resistance to any antimicrobials by gradient diffusion strips using E-tests (bioMerieux, France).

2.1.2 DNA Extraction and Whole Genome Sequencing

Isolated *S*. Typhi were subcultured, and total genomic DNA was extracted in all studies using commercial genomic DNA extraction kits, and then library preps were performed. In brief, quality control was conducted to ensure that ample high-quality genomic DNA was available for sequencing, samples were diluted to a prespecified concentration, DNA tagmentation was performed, tagmented DNA were amplified, the sample was purified using a PCR clean-up step, and a final assessment of DNA concentration was performed before libraries were normalised and pooled. Pooled samples were submitted to whole genome sequencing using one of several Illumina machines to generate paired end reads of a prespecified base pair length. Quality control assessments were conducted to look at indicators including phred scores (distribution of per base quality), GC content (content of nucleotides guanine and cytosine, as well as adenine and thymine, should be roughly equal, assuming high quality, accurate, minimally contaminated reads), and presence of adaptors. Low-quality reads (typically phred score <20) were trimmed before generating alignments.

2.1.3 Phylogenetic and SNP analysis

Paired-end reads were assembled and mapped to a reference genome using one of several standardized mapping pipelines. Plasmids and recombinant regions were filtered out of alignments, and read alignments were used to call genotypes in accordance with the GenoTyphi typing scheme.[133,134] AMR phenotypes are also called from assemblies using standard pipelines. SNP alignments were generated with all read alignments in each dataset, as well as additional genomes for context. These larger SNP alignments were used to infer maximum likelihood (ML) phylogenetic trees using RAxML.[135]

2.2 Methods used for Chapter 3

2.2.1 Overview of Severe Enteric Fever in Asia Project (SEAP)

Collaborators from the Aga Khan University conducted standardized prospective facility and laboratory-based blood culture surveillance in outpatient and inpatient wards at Aga Khan University Hospital and Kharadar General Hospital from September 2016 and September 2019.[136] These hospitals serve a catchment area of 30 million people in Karachi, including densely populated informal urban areas. Subjects presenting to outpatient clinics who lived in predefined catchment areas and who reported three consecutive days of fever were considered eligible for participation in the study, and those for whom a study clinician recommended a blood culture were enrolled. Inpatients with clinical suspicion of typhoid or with nontraumatic ileal perforation were also enrolled in the study. The major objectives of the study were to establish crude and adjusted incidence rate estimates for *S*. Typhi and Paratyphi A at sites in Bangladesh, Nepal, and Pakistan, as well as to estimate the prevalence of AMR and characterise severe enteric fever in these settings.[136]

2.2.2 Laboratory Samples and Analysis

Trained phlebotomists collected whole blood samples from enrolled participants prior to antimicrobial administration. Samples were incubated for up to five days at 37°C using either the BACTEC blood culture system (Becton Dickinson, Franklin Lakes, NJ, USA) or the BacTAlert 3D automated blood culture system (BioMérieux, Marcy-l'Étoile, France). Positive samples were subcultured onto chocolate agar, sheep blood agar, MacConkey agar, or a combination of the three, and species identification was confirmed using biochemical testing and O and H antisera (MAST ASSURE, Mast Group, Liverpool, UK) when available.

Antimicrobial susceptibility testing by disc diffusion was conducted according to Clinical and Laboratory Standard Institute (CLSI) Guidelines – M100-ED-29, 2019.[132] MDR, XDR, and azithromycin non-susceptibility were reported.

2.2.3. Whole Genome Sequencing and Phylogenetic Analysis

We aimed to investigate the genetic basis of any organism with potential azithromycin resistance through whole-genome sequencing (WGS). Genomic DNA was extracted and subjected to WGS on a Hiseq2500 (Illumina, San Diego, CA) to generate 125-bp paired-end reads. The resulting sequence data were mapped against the CT18 reference sequence (accession number AL513382) using the RedDog mapping pipeline to identify SNPs, and to determine the genotype in accordance with the GenoTyphi scheme.[24,47,56,57,75,137–142] (https://github.com/katholt/genotyphi). The single high azithromycin MIC organism was categorized as genotype 4.3.1.1, so we aimed to put it into phylogenetic context with contemporaneous *S*. Typhi organisms from South Asia belonging to genotype 4.3.1.1 (n=663). After removing repetitive sequences and recombination regions,[143] we generated a final alignment 7,661 chromosomal SNPs for 664 isolates (**Table S1**). Maximum-likelihood phylogenetic trees were inferred from the chromosomal SNP alignments with RAxML (v8.2.9)[144] and visualized in Microreact[145] (https://microreact.org/project/8FjPCdisk) and the ggtree package in R.[146] SRST2[147] was used with ARGannot[148] and PlasmidFinder[149] to identify AMR genes and plasmid replicons, respectively. Mutations in *gyrA*, and *parC*, as well as the R717Q mutation in *acrB*, were detected using GenoTyphi (https://github.com/katholt/genotyphi).

2.3 Methods used for Chapter 3

2.3.1 Ethical approvals

Ethical clearance was granted by the Institutional Ethics Committee of the Government Multi-specialty Hospital, Sector 16, Chandigarh (letter no GMSH/2018/8763 dated 26.7.2018) and the Postgraduate Institute of Medical Education & Research (PGIMER) Institutional Ethics Committee (IEC-08/2018-285 dated 24-9-2018). Administrative approval to conduct this study was provided by the Chandigarh Health Department (CHMM-2017/2991 dated 28.8.2017). The Collaborative Research Committee of PGIMER (no 79/227-Edu-18/4997 dated 12/12/2018) also provided ethical approval. Provision of informed consent was a requirement for participation in the study.

2.3.2 Study design

Febrile patients who presented to Civil Hospital Manimajra in Chandigarh (CHMM) between September 2016 and December 2017 were approached for inclusion in this study, informed consent was administered, and blood cultures were taken following enrollment. CHMM is a 100-bed secondary health care facility located in a peri-urban neighbourhood in Chandigarh. CHMM serves a catchment area of approximately 200,000 people, including patients who are referred for advanced care from one of four local primary health care centers. Passive community-based blood culture surveillance has been ongoing at this facility since November 2013. Study investigators recorded clinical history, laboratory test results, and risk factor data for each patient residing in Manimajra with a blood culture-confirmed case of enteric fever. This analysis focused exclusively on *S*. Typhi isolated over the course of this enteric fever study.

2.3.3 Identification and Antimicrobial Susceptibility Testing

Bacterial isolates were identified as *S*. Typhi using conventional biochemical tests (Motility agar, Hugh–Leifson Oxidative-Fermentation [OF] test, the Triple Sugar Iron [TSI] test, citrate test, urease test, phenyl pyruvic acid [PPA] test, and indole test). Isolate identification was confirmed using antisera from Central Research Institute (CRI), Kasauli. Antimicrobial susceptibility was determined for the following antimicrobials by disc diffusion: ampicillin (10µg), chloramphenicol (30µg), trimethoprim/sulfamethoxazole (1.25/23.75µg), ceftriaxone (30µg), azithromycin (15µg), ciprofloxacin (5µg), and pefloxacin (5 µg). Zone diameters were measured and interpreted in accordance with Clinical Laboratory Standards Institute (CLSI) guidelines.[132] MIC testing was also conducted on all organisms showing resistance to any of the above antimicrobials by gradient diffusion strips using E-tests (bioMerieux, France).

2.3.4 Whole-Genome Sequencing and Phylogenetic Analysis

All *S*. Typhi samples were shipped to PGIMER Chandigarh. Isolates from patients residing outside of Manimajra, as were those for which there were inadequate clinical metadata, or the DNA yield was below the amount required for WGS. Total genomic DNA was extracted from the *S.* Typhi using the Wizard genomic DNA extraction Kit (Promega, Wisconsin, USA). and genomic DNA was subjected to WGS using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) to generate 250-bp paired-end reads. We then aimed to put these sequences into global and regional context. These reads were mapped against the CT18 reference sequence (accession no. AL513382) using the RedDog mapping pipeline (available at: https://github.com/katholt/RedDog) to identify SNPs.[47,137,150] After removing prophages and recombinant[143] and repetitive sequences, we generated a final alignment of 25,832

chromosomal SNPs for 3472 isolates. SRST2[147] was used with ARGannot[148] and PlasmidFinder[149] to identify AMR genes and plasmid replicons, respectively. Mutations in *gyrA*, and *parC*, as well as the R717Q mutation in *acrB,* were detected using GenoTyphi (https://github.com/katholt/genotyphi). Maximum likelihood phylogenetic trees were inferred from the chromosomal SNP alignments using RAxML (v8.2.9)[135], and then visualized using Microreact[145] (https://microreact.org/project/nniNzBL2uq3XZXYDKgG374) and the Interactive Tree of Life (ITOL).[151] Raw read data were deposited in the European Nucleotide under accession ERP124488 (**Table S2**).

2.4 Methods used for Chapter 5

2.4.1 Study design and bacterial isolates

To better understand the global population structure during the time that H58 was thought to have emerged, we collaborated with the United Kingdom Health Security Agency (UKHSA, formerly Public Health England) to aggregate *S.* Typhi organisms that were collected from returning travellers between 1980 and 1995. The database was queried and organisms were selected using a random number generator from the following three categories: i) 126 *S*. Typhi with the E1 Phage type (which is considered to be associated with H58)[51] originating from South Asia (India, Nepal, Pakistan, and Bangladesh), ii) 159 *S*. Typhi organisms with a variety of non-E1 phage types originating from South Asia, and iii) 184 *S*. Typhi organisms with a variety of phage types (both E1 and non-E1) originating from locations outside of South Asia. A total of 470 *S*. Typhi organisms meeting these criteria were randomly selected, revived, subjected to DNA extraction and whole genome sequenced. Ultimately, our dataset was composed of 463 novel sequences generated as a component of this study and 305 existing sequences[47,56,152] known to belong to the H58 lineage and its nearest neighbours, yielding a total of 768 whole genome sequences on which to structure subsequent analysis.

2.4.2 DNA extraction and Whole Genome Sequencing

Genomic DNA was extracted from *S*. Typhi isolates using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA), following standardized manufacturer's protocol. Two ng of genomic DNA from each sample was fragmented and tagged for multiplexing with Nextera DNA Sample Preparation Kits, followed by paired-end sequencing on an Illumina HiSeq2000 Platform to produce 101 bp paired end reads (Illumina, Cambridge, UK). Raw

reads were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB15284 (**Table S3**).

2.4.3 Read alignment and SNP analysis

FastQC and FASTX-Toolkit bioinformatics pipelines were used to check the quality of raw reads.[153,154] Six samples were excluded from the analysis, one was determined to not be *Salmonella*, one appeared to be comprised of multiple genotypes, and four samples were on a long branch length and were determined to be contaminated. Paired end reads for the remaining 464 samples were mapped to the *S*. Typhi CT18 reference genome (accession number: AL513382)[155] using the RedDog mapping pipeline (v1beta.10b, available at http://githib.com/katholt/reddog). RedDog uses Bowtie2 v2.2.9[141] to map all raw reads to the CT18 reference genome and then uses SAMtools v1.3.1[156156] to identify high quality SNP calls. SNPs that did not meet predefined criteria (a minimal phred quality score of 30 and depth coverage of 5 were filtered out).[57] A failed mapping sequence was defined as when <50% of total reads mapped to the reference genome. 2 isolates were excluded from additional analysis after mapping failed, due to depth coverage of less than 10 (as per the RedDog pipeline default). A concatenation of core SNPs that were present in >95% of all genomes was generated and filtered to exclude all SNPs from phage regions or repetitive sequences in the genome reference CT18 as defined previously (**Table S3**).[60] Gubbins (v2.3.2)[143] was used to filter out SNPs in recombination regions. Finally, the alignment of 17,325 SNPs from mapping of the remaining 462 isolates was utilized for phylogenetic analysis. Resultant BAM files for all isolates from RedDog mapping were used to determine previously defined genotypes according to an extended genotyping framework using the GenoTyphi pipeline[49] (available: https://github.com/katholt/genotyphi).

Based on the results of our mapping, we also undertook a detailed genetic analysis to look for non-synonymous SNPs that were unique to the early (isolated between 1980 and 1995) H58 (genotype 4.3.1) isolates in our dataset, as well as SNPs that were unique to early H58 isolates that were MDR, to investigate why this lineage was so globally successful, and how it maintained its MDR IncH1 plasmid.

2.4.3 Phylogenetic analysis

RAxML (v8.2.9)[26] was used to infer maximum likelihood (ML) phylogenetic trees from the final chromosomal SNP alignment, with a generalized time-reversible model, a gamma distribution to model site-specific rate variation (the GTR+ Γ substitution model; GTRGAMMA in RAxML), and 100 bootstrap pseudo-replicates to assess branch support. *Salmonella* Paratyphi A AKU1_12601 (accession no: FM200053)[157] was used as an outgroup. The resultant trees were visualized using Interactive Tree of Life (iTOL)[151] and the ggtree package in R.[158] An interactive visualisation of this phylogeny and associated metadata can be found in Microreact (https://microreact.org/project/hzELvWqY3UCvsyAw892fnd-origins-of-h58-s-typhi).[145]

2.4.4 Characterisation of AMR associated genes and mobile elements

SRST2 (v0.2.0) [147] was used to detect AMR genes and plasmid replicons using the ARGannot[148] and PlasmidFinder[149] databases, respectively. Mutations in the *gyrA* and *parC* genes, as well as the R717Q mutation in *acrB*, were detected using Mykrobe v0.10.0.[159]

2.4.5 Bayesian phylogenetic analysis of H58 and nearest neighbours

To infer where and when the first H58 (genotype 4.3.1) organism emerged, we conducted Bayesian phylogenetic analyses on a subset (n=345) of H58 (genotype 4.3.1) from our dataset and from published literature isolated between 1980 and 2000.[45,56,152] This analysis of 345 *S*. Typhi isolates was conducted in the BEAST v1.8.4.[160] The temporal signal of the data was checked initially. The maximum likelihood tree, constructed using the GTR+ Γ substitution model and GTRGAMMA, was subjected to TempEst v1.5 to test the best fit of

linear regression between sampling dates and their root-to-tip genetic distances, using default TempEst parameters.[161] To further test temporal signal, the TipDatingBeast R package was used to randomly reassign the sampling dates of sequences 20 times to create date-randomized data sets. BEAST analyses were conducted for these randomized data sets and the mean rates were compared between runs. The data had sufficient temporal signal if the 95% credible interval of mean rates of the date-randomized datasets did not overlap with that of the original sampling dataset.[162,163]

An automatic model selection program (ModelFinder)[164] was implemented through IQ-TREE[165] and run on the non-recombinant SNP alignment (724 variable sites) to select the best-fit sequence evolution model for BEAST analysis. ModelFinder showed that GTR had the lowest Bayesian Information Criteria (BIC) score and thus it was chosen as the best-fit substitution model.

As part of the BEAST analysis, six different model combinations were run for six combinations, and the final analysis was conducted using the best fitting model The path sampling and stepping-stone sampling approaches were applied to compare the log marginal likelihoods of the different runs.[166,167] The GTR+Γ4 with strict clock and Bayesian skyline was identified as the best-fit model for running BEAST. Finally, BEAST was run three independent times using the best-fit model, using a Bayesian Markov chain Monte Carlo (MCMC) parameter-fitting approach (generated 10^7 chains and sampled every 1000 iterations). The log files after three runs were combined using LogCombiner v1.8.3[168] with a burn-in rate of 10%. The effective sample size (ESS) of all parameters was assessed by Tracer v1.8.3.[169] If the ESS of any parameters was less than 200, we increased the MCMC chain

length by 50% and reduced the sampling frequency accordingly.[166] The trees were combined and summarized using LogCombiner v1.8.3 and TreeAnnotator v1.8.3.[160]

2.5 Methods used for Chapter 6

2.5.1 Ethics

Each contributing study or surveillance program obtained local ethical and governance approvals, as reported in the primary publication for each dataset. For the present study, inclusion of data that were not yet public domain by August 2021 was approved by the Observational / Interventions Research Ethics Committee of the London School of Hygiene and Tropical Medicine (ref #26408), based on details provided on the local ethical approvals for sample and data collection.

2.5.2 Sequence data aggregation and processing

Attempts were made to include all *S*. Typhi sequence data generated in the 21 years since the first genome was sequenced, through August 2021. Genome data and the corresponding data owners were identified from literature searches and sequence database searches [European Nucleotide Archive (ENA); NCBI Short Read Archive (SRA) and GenBank; Enterobase]. Unpublished data, including data from ongoing burden studies and routine public health laboratory sequencing, were identified through professional networks, published study protocols,[12] and an open call for participation in the Global Typhoid Genomics Consortium. All data generators identified through the above means were invited to join the Global Typhoid Genomics Consortium and to provide or verify corresponding source information, with year and location being required fields (see below). Nearly all those contacted responded and will be included as Consortium authors on this study. The exceptions were one genome reported from Malaysia[170] and n=133 draft genomes reported from India,[171] for which reads were not available in NCBI and authors did not respond to email inquiries. A further n=850 genomes sequenced by United States Centers for Disease

Control (CDC) were excluded from analysis as they are likely travel-associated but lacked sufficient epidemiological information to determine country of origin.

Whole genome sequence data, in the form of Illumina fastq files, were sourced from the European Nucleotide Archive (ENA) or Short Read Archive (SRA) using the run accessions provided in **Table S4** or were provided directly by the data contributors in the case of data that was unpublished in August 2021. Primary sequence analysis was conducted at the Wellcome Sanger Institute.

Illumina reads were assembled using the Centre for Genomic Pathogen Surveillance (CGPS) assembly pipeline v2.1.0 (https://gitlab.com/cgps/ghru/pipelines/dsl2/pipelines/assembly/),[172] which utilises the SPAdes assembler (v3.12.0).[173] Assemblies were uploaded to Pathogenwatch to confirm species and identify AMR determinants and plasmid replicons.[72] Assemblies of >5.5 Mbp or <4.5 Mbp in size were excluded from further analysis. Reads were mapped, using the CGPS mapping pipeline v1.2.2 (https://gitlab.com/cgps/ghru/pipelines/snp_phylogeny/), to a concatenation of the *S*. Typhi CT18 chromosome and plasmid (pHCM1, pHCM2) reference sequences (accessions AL513382-AL513384). The pipeline uses bwa-mem v0.7.17[174–176] to map reads and samtools v1.16[156] to call SNPs, and we used the default filtering options to obtain high-quality SNP calls as follows: '%QUAL<25 || FORMAT/DP<10 || MAX(FORMAT/ADF)<5 || MAX(FORMAT/ADR)<5 || MAX(FORMAT/AD)/SUM(FORMAT/DP)<0.9 || MQ<30 || MQ0F>0.1'. SNPs in known phage or repetitive regions (coordinates available at: https://bit.ly/33kmypF) were also filtered out *a priori* using remove_blocks_from_aln.py (available at: https://github.com/sanger-

pathogens/remove_blocks_from_aln) as these are unsuitable for phylogenetic analysis. Genotypes, as defined under the GenoTyphi scheme,[47,133] were called directly from Illumina reads using Mykrobe v0.10.0 and collated using the Python code available at https://github.com/katholt/genotyphi.

2.5.3 Quality control of sequence data

Of the 13,031 readsets collated, one was excluded as it failed assembly and eight were excluded as they were suspected to be non-*S*. Typhi based on excessive divergence from the *S*. Typhi reference sequence [49,543-112,951 high-quality chromosomal SNPs after filtering, vs median 376 (range 59-804) for the rest of the data set]. Pathogenwatch analysis of the corresponding genome assemblies identified these eight as other *S. enterica* serovars (2 Paratyphi B, 2 Enteritidis, 1 Montevideo, 1 Newport, 1 Durban) or other species (1 *K. pneumoniae*).

A further 103 readsets were identified as likely mixtures of *S*. Typhi strains, based on excessive high-quality heterozygous SNP calls (>2.7% of total SNPs remaining after filtering) that could not be explained by recombination or HGT (i.e. SNPs were not spatially clustered, as would be expected if they result from imported DNA). Code used for this quality control step, to analyse the VCF files resulting from the mapping analysis, is available at https://github.com/zadyson/SNP_Stats_GeneratorXX. These 103 'mixed' Typhi genomes were included in descriptive statistical analyses, as the genotype and AMR calls were considered to represent those of the dominant strain in the mixture, but they were excluded from phylogenetic analyses as these rely on alignments of SNP alleles that are assumed to represent single haplotypes.

2.5.4 Metadata curation and variable definitions

Owners of the contributing studies were asked to provide or update source information relating to their genome data, using a standardised template (http://bit.ly/typhiMeta). Repeat isolates were defined as those that represent the same case of typhoid infection (acute disease or asymptomatic carriage) as one that is already included in the data set. In such cases, data owners were asked to indicate the 'primary' isolate (either the first, or the best quality, genome for each unique case) to use in the analysis. Repeat isolates were then excluded from the data set entirely.

Data provided on the source of isolates (specimen type and patient health status) are shown in **Table S5**. This information was used to identify isolates that represent cases of acute typhoid illness. 'Confirmed acute illness' genomes were defined as those recorded as isolated from blood of symptomatic individuals (n=6,349). A further n=119 were recorded as asymptomatic carriers. The remaining genomes had no health status recorded (i.e. symptomatic vs asymptomatic carrier); of these, the majority were from blood isolates (n=3,375) or the specimen type was not recorded (n=2,529). Since most studies and surveillance programs are set up to capture acute cases rather than asymptomatic carriers, we defined 'Assumed acute illness' genomes as all those not recorded explicitly as asymptomatic carriers (n=119) or coming from gall bladder (n=1) or environmental (n=14) samples; this resulted in a total of 12,888 genomes assumed to represent acute illness.
We defined 'country of origin' as the country of isolation; or for travel-associated infections, the country recorded as the most likely origin of infection based on travel history.[55,177–179] Countries were assigned to geographical regions using the United Nations Statistics Division

standard M49 (see https://unstats.un.org/unsd/methodology/m49/overview/); we used the intermediate region label where assigned and subregion otherwise. To identify isolate collections that were suitably representative of local pathogen populations, for the purpose of calculating genotype and AMR prevalence rates for a given setting, data owners were asked to indicate the purpose of sampling for each study or dataset. Options available were either 'Non-Targeted' (surveillance study, routine diagnostics, reference lab, other; n=11,130), 'Targeted' (cluster investigation, AMR focused, other; n=1,878) or 'Not Provided' (n=14).

2.5.5 AMR determinants and definitions

AMR determinants identified in the genome assemblies using Pathogenwatch were used to define drug resistance variables as follows. Multidrug resistance (MDR): resistance determinants for chloramphenicol (*catA1* or *cmlA*), ampicillin (*bla*TEM-1D, *bla*OXA-7), and co-trimoxazole (at least one *dfrA* gene and at least one *sul* gene). Ciprofloxacin non-susceptible (CipI/R): one or more of the quinolone resistance determining region (QRDR) mutations at *gyrA*-83, *gyrA*-87, *parC*-80, *parC*-84, *gyrB*-464 or presence of a plasmid-mediated quinolone resistance (PMQR) gene (*qnrB*, *qnrD*, *qnrS*). Ciprofloxacin resistant (CipR): QRDR triple mutant (*gyrA*-83 and *gyrA*-87, together with either *parC*-80 or *parC*-84), or plasmid-mediated quinolone resistance (PMQR) together with *gyrA*-83, *gyrA*-87 and/or *gyrB*-464. Third-generation cephalosporin resistance (3GCR): presence of an extended-spectrum beta-lactamase (ESBL) (*bla*CTX-M-12, *bla*CTX-M-15, *bla*CTX-M-23, *bla*CTX-M-55, *bla*SHV-12) or *ampC* gene. Extensive drug resistance (XDR): MDR plus CipR plus 3GCR. Azithromycin resistance (AziR): mutation at *acrB*-717 or presence of *ereA*. The above lists all those AMR determinants that were found here in ≥1 genome and used to define AMR

profiles and prevalence rates; additional AMR genes sought by Typhi Pathogenwatch but not detected are listed in Supplementary Table 2 of Argimon et al, 2021.[43]

2.5.6 Prevalence estimates

Genotype and AMR frequencies were calculated at the level of country and UN world region (based on 'country of origin'). Inclusion criteria for these estimates were: known 'country of origin', known year of isolation, non-targeted sampling, assumed acute illness. A total of 10,768 genomes met these criteria, the subset of 9,467 isolated from 2010 onwards are the focus of the majority of analyses and visualisations. The prevalence estimates reported in text and figures are simple proportions. Robustness of prevalence estimates was assessed informally, by comparing point estimates and 95% confidence intervals for national prevalence rates derived from data contributed by different laboratories (for genomes isolated from 2010, and laboratories with N≥20 genomes meeting the inclusion criteria during this period.

Chapter 3: Azithromycin-resistant *S*. Typhi in Pakistan

3.1 Abstract

This chapter describes the identification and molecular investigation of an azithromycin-resistant *S*. Typhi isolate in Pakistan in the context of the Severe Enteric fever in Asia (SEAP) Project. This is the first published instance of an *S*. Typhi isolate from Pakistan with a single point mutation in the *acrB* gene, which has recently been identified as a molecular mechanism for resistance to azithromycin. Our investigation showed that this isolate was not related to azithromycin-resistant *acrB* mutants identified in Bangladesh,[75] but that this mutation had arisen independently. These findings are particularly worrisome considering the ongoing XDR typhoid outbreak in Pakistan. It is hoped that the national introduction of typhoid conjugate vaccine (TCV) will slow the spread of XDR typhoid and prevent infections caused by XDR and other drug-resistant *S*. Typhi strains. These data were published in *mBio* in 2020 (Iqbal *et al.*). I outlined the research questions and led the drafting of the paper described in this chapter. Colleagues in Pakistan collected blood samples from febrile patients. DNA extraction and WGS were conducted by colleagues from OUCRU Nepal, and another colleague at London School of Hygiene and Tropical Medicine (LSHTM) ran the phylogenetic analysis and generated the phylogenetic tree (**Figure 12**).

3.1.1 Typhoid fever and AMR in Pakistan

Typhoid is a notifiable disease in Pakistan, and Pakistan has a long, well-studied history of high incidence rates of typhoid fever.[180–182] Investigators from the SEAP study (described in **Chapter 1.1.3.3**) estimated all-age adjusted incidence rates between 103 (85-126) and 176 (144-216) cases per 100,000 person-years in Karachi, with highest age-specific adjusted

incidence rate estimates in 2-4 year olds (371 [265-534] and 910 [662-1269] cases per 100,000 person-years at Aga Khan University Hospital and Kharadar General Hospital, respectively). AMR, particularly MDR and fluoroquinolone resistance, has also been prevalent in *S*. Typhi in Pakistan for decades, with 91.7% of *S*. Typhi isolated by the Aga Khan University Hospital laboratory (with 190 collection sites across the country) exhibiting fluoroquinolone resistance by 2011.[183] A small number of *S*. Typhi organisms that were resistant to third generation cephalosporins were also reported in Pakistan between 2009 and 2011.[183] The emergence and spread of an extensively drug-resistant (XDR) *S*. Typhi variant in Hyderabad, Pakistan,[24,25] which is resistant to chloramphenicol, ampicillin, co-trimoxazole, streptomycin, fluoroquinolones, and third generation cephalosporins, was a major cause for concern and led to a reactive TCV vaccination campaign in this area, followed by national introduction of TCV into routine immunisation.[100] XDR typhoid has since spread throughout other parts of the country and become prevalent,[22,184,185] leaving azithromycin as the only effective oral option for typhoid treatment in many parts of the country.

3.1.2 Azithromycin resistance in *S*. Typhi

In 2019, researchers from Child Health Research Foundation in Dhaka, Bangladesh identified a new molecular mechanism of resistance in *S*. Typhi – a single non-synonymous point mutation at position 717 in the gene coding for AcrAB, which is an efflux pump that removes small molecules from bacterial cells, which they identified in 12 *S*. Typhi strains and one *Salmonella* Paratyphi A strain from their study setting.[75] This mutation resulted in the conversion of an arginine (R) to a glutamine (Q). To investigate the potential role this mutation might play in mediating azithromycin resistance, colleagues cloned *acrB* from

azithromycin-sensitive and resistant strains of *S*. Typhi, expressed them in *E. coli*, *S*. Typhi, and Paratyphi A strains, and tested their MICs. The presence of *acrB*-R717Q resulted in 11-fold and 3-folder higher MICs in *E. coli* and *S*. Typhi, respectively. The single azithromycin resistant Paratyphi A strain contained a different single point mutation at position 7171 in *acrB*, leading to the conversion of an arginine (R) to a leucine (L). The presence of this R717L mutation in *E. coli* and Paratyphi A led to 7-fold and 3-fold increases in MIC, respectively. This established the potential of R717 mutations to mediate azithromycin resistance.

This first report of molecularly confirmed azithromycin-resistant *S*. Typhi in Bangladesh highlighted the dangers associated with overreliance on azithromycin to treat enteric fever. When collaborators from the Aga Kha University (AKU) and the broader Surveillance for Enteric fever in Asia Project (SEAP) identified potential azithromycin resistance among *S*. Typhi organisms isolated during the SEAP study, we established a collaboration to sequence these strains and to investigate whether this molecular mechanism of resistance to azithromycin was present in these organisms.

3.2 Results

3.2.1 Sample collection and laboratory analysis

Between the specified dates, 10,080 patients were enrolled in the SEAP study at the two sites in Karachi; 2,104 had a positive blood culture for *S*. Typhi, and 139 had a positive blood culture for *S*. Paratyphi A. Following antimicrobial susceptibility testing by disc diffusion, six *S*. Typhi isolates exhibited potential azithromycin resistance (diameter >12 mm). MIC testing was conducted on these six isolates. One failed to revive, four isolates had azithromycin MICs ranging between 1 and 2g/ml and one *S*. Typhi isolate had an MIC of 12g/ml. This

places this isolate at the upper range of the wild-type azithromycin susceptibility distribution (the current CLSI susceptibility breakpoint for azithromycin resistance is 16g/ml). This isolate also exhibited resistance to chloramphenicol, fluoroquinolones, and co-trimoxazole, but it was susceptible to third generation cephalosporins. The use of a consistent case definition and inclusion and exclusion criteria make this sample set more likely to be representative and unbiased, although there may be a bias towards more severe disease and/or illness caused by a drug-resistant pathogen, as less serious illness caused by a drug-susceptible pathogen might resolve more quickly and/or be treated in the community, given the widespread availability of antimicrobials. Given that this is one isolate out of a relatively large sample set, we did not attempt to make any prevalence calculations.

3.2.2 Phylogenetic interpretation

Following WGS and phylogenetic analysis, this higher azithromycin MIC *S*. Typhi isolate was classified as genotype 4.3.1.1 (H58 lineage I), placing it in the same sublineage at that of the XDR clade circulating at high prevalence in Pakistan. The organism also had a single mutation in *gyrA* (S83F), resulting in reduced fluoroquinolone susceptibility. The putative mechanism of higher MIC against azithromycin was the same R717Q mutation in the gene encoding *acrB* as that identified in *S*. Typhi belonging to genotype 4.3.1.1 in Bangladesh.[75] The identification of this mutation in *S*. Typhi in Pakistan raised an important question - was this a *de novo* mutation in the Pakistan-specific 4.3.1.1 cluster or was this organism part of larger, internationally disseminating, azithromycin-resistant clone?

The phylogenetic tree shown in **Figure 12** indicated that this was a spontaneous mutation that emerged independently in Pakistan, since it was distantly related (relative within H58

lineage I) to the organisms with *acrB* mutations in Bangladesh, and independent from the proximal XDR sublineage.

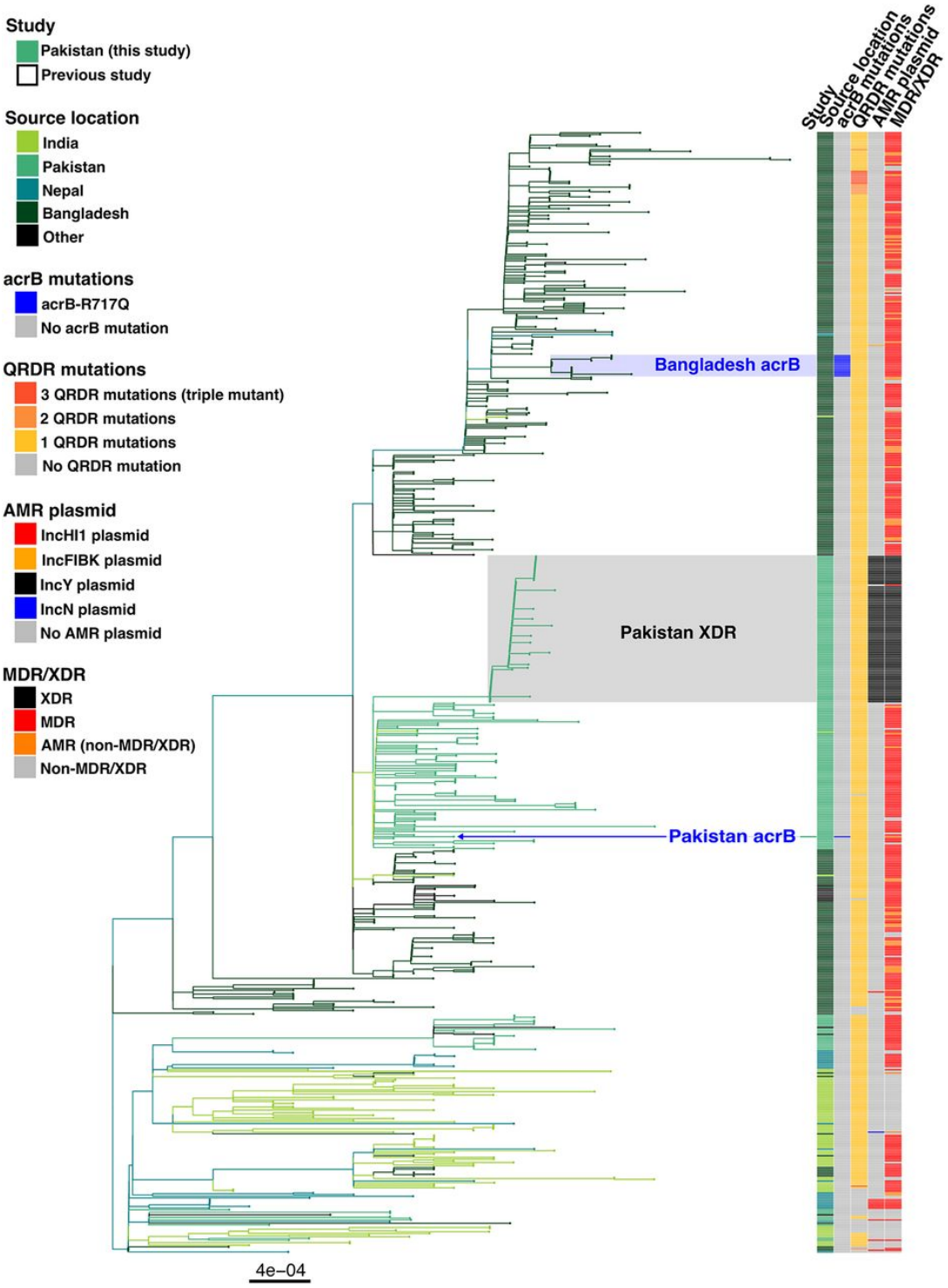


Figure 12. South Asian H58 lineage I (genotype 4.3.1.1) phylogenetic tree (Iqbal *et al* 2020).[32]

Phylogenetic tree showing genotype 4.3.1.1 isolates from South Asia (n=664). Branches are coloured by source country according to the inset legend and first colour bar. The second colour bar indicates genomes containing the *acrB*-R717Q mutation. The third colour bar indicates mutations in the quinolone resistance deter-mining region (QRDR) of genes *gyrA*, and *parC*. The final colour bar indicates MDR and XDR sequences.

3.3 Discussion

Typically, the isolation of a single *S*. Typhi organism exhibiting resistance to an antimicrobial agent being used as the primary therapy for typhoid fever would be notable, if not a grave public health issue. However, considering the emergence of XDR typhoid in Pakistan and the subsequent reliance of azithromycin for the treatment of typhoid and other bacterial infections, this first report of azithromycin resistant *S*. Typhi in Pakistan is a major cause for concern. When one considers the rapidity with which single point in the quinolone resistance determining region (QRDR; mutations in this region confer reduced fluoroquinolone susceptibility) emerged independently in settings where fluoroquinolones were also be used widely, we can reasonably anticipate the emergence of similar homoplasies elsewhere. It is too early to predict how these organisms may spread, and it is encouraging that these mutations have not yet been reported in any XDR *S*. Typhi in Pakistan or elsewhere. However, given the spontaneous nature of these mutations, it is conceivable that one could arise in an XDR *S*. Typhi organism, and/or that the IncY plasmid found in XDR organisms could be mobilised into an azithromycin-resistant *S*. Typhi lineage.

There is reason to be hopeful. Driven in part by the emergence and spread of XDR typhoid, Pakistan became the first country to initiate a Gavi-supported nationwide typhoid conjugate vaccine (TCV) introduction, which started with a mass vaccination campaign in Sindh province in November 2019.[126,186] In addition, a recently published evaluation of the reactive TCV campaign that was conducted in Hyderabad, Pakistan showed that TCV was highly effective against both blood culture-confirmed *S*. Typhi (95%; 93-96) as well as XDR *S*. Typhi (97%; 95-98).[100] This bodes well for the potential of widespread TCV use to lead to reduced incidence of typhoid fever and XDR typhoid fever in Pakistan. However, drug-

resistant *S*. Typhi, particularly azithromycin resistant *S*. Typhi, have been observed in many other countries where TCV has not yet been introduced.

Now, there is a race against time to prevent the inevitable emergence and spread of untreatable typhoid fever. There are two TCVs that have been prequalified by the World Health Organization, but uptake of TCVs has been slower than expected. Effective treatment is still paramount for typhoid control, as are water and sanitation interventions. It will be important progress with additional intervention strategies and to continue to track AMR in *S*. Typhi, both to inform best practices for antimicrobial treatment guidelines and to inform where and how best to deploy preventative interventions like TCVs.

Chapter 4: Emergence of azithromycin-resistant *S*. Typhi in independent lineages in Chandigarh, India

4.1 Abstract

This chapter describes a phylogenetic analysis of *S*. Typhi isolated as part of a blood culture surveillance study conducted in a typhoid-endemic state in northern India. Sixty-six *S*. Typhi were isolated over a fifteen-month period, seven of which were azithromycin resistant because of the same point mutation in the *acrB* gene that had been observed in Bangladesh and Pakistan. We aimed to investigate the relatedness of these organisms and found that within this relatively short time and in a small catchment area, *acrB* mutations appeared independently in two separate genetic lineages. We also put these sequences into phylogenetic context with other azithromycin resistant organisms from Bangladesh, Pakistan, and Nepal and demonstrating that these *acrB* mutations have been occurring spontaneously and independently in multiple locations in South Asia where there is intense selection pressure, warranting urgent use of preventative interventions like typhoid conjugate vaccines, as well as careful monitoring of azithromycin use throughout the region to prevent additional emergence of resistance. The results of this analysis were published in *Clinical Infectious Diseases* in 2021 (Carey*et al*). I outlined the research questions, conducted the data analysis, generated visualisations of typhoid fever seasonality (**Figure 13**) and the phylogenetic trees of our data (**Figure 14**) and our data in context with a globally representative collection of *S*. Typhi isolates (**Figure 15**), and wrote the manuscript. Colleagues in Chandigarh collected blood samples, colleagues from OUCRU Nepal conducted DNA extractions and WGS, and a colleague at LSHTM ran the bioinformatic analyses.

4.2 Introduction

The majority of typhoid fever disease burden is believed to be concentrated in South Asia, which has a modelled overall incidence rate of 592 cases per 100,000 person-years.[1] The burden of disease in India has not been well described in published literature, although a pooled incidence rate estimate of 377 cases per 100,000 person-years has been calculated using limited population-based data; significant geographical heterogeneity was observed.[187] The Surveillance for Enteric Fever in India (SEFI) study was established to generate geographically representative, age-specific incidence data for typhoid fever, as well as additional information regarding cost of illness, range of clinical severity, and AMR.[188] These data will undoubtedly provide a more comprehensive understanding of typhoid fever incidence rates and drug resistance profiles across the Indian sub-continent, and ultimately, should inform decision-making around typhoid conjugate vaccine (TCV) introduction and identification of optimal introduction strategy in India.[189]

As indicated in previous chapters, there is an escalating problem caused by drug resistant *S*. Typhi in South Asia. This is likely due in part to empirical treatment of febrile patients and widespread community availability of antimicrobials. While patterns of resistance to first-line antimicrobials and fluoroquinolones have been well established and described, there are open questions about the global and regional distribution of azithromycin resistance, which appears to have emerged more recently, and the potential for the emergence of a specific sub-lineage of *S*. Typhi with this phenotype. In this study, we sought to characterise the molecular basis of drug-resistant *S*. Typhi isolated during a cross-sectional enteric fever surveillance study conducted in Chandigarh in Northern India. Using WGS and phylogenetic analysis, we described the distribution of a collection of azithromycin resistant *S*. Typhi and

demonstrated that these organisms arose independently from those reported in Pakistan and Bangladesh through the development of an identical mutation in *arcB*. Our data support the prioritization of TCV introduction in India to prevent the continued emergence and spread of drug-resistant *S*. Typhi infections in South Asia.

4.2 Results

4.2.1 Epidemiological observations

Typhoid fever is a major public health concern among children and young adults in this region of Northern India. It is thought that many cases in this area, a hub for the states of Punjab, Haryana, and Himachal Pradesh, are associated with the mixing of large populations of seasonal workers from the adjoining states. These workers generally live in informal dwellings with poor sanitation and limited access to safe water. Approximately 1,500 suspected enteric fever patients present to the CHMM facility annually and receive blood cultures, of which ~10% are positive for *S*. Typhi. There is a seasonal peak of typhoid fever in this facility during the monsoon months from May to September (**Figure 13**).

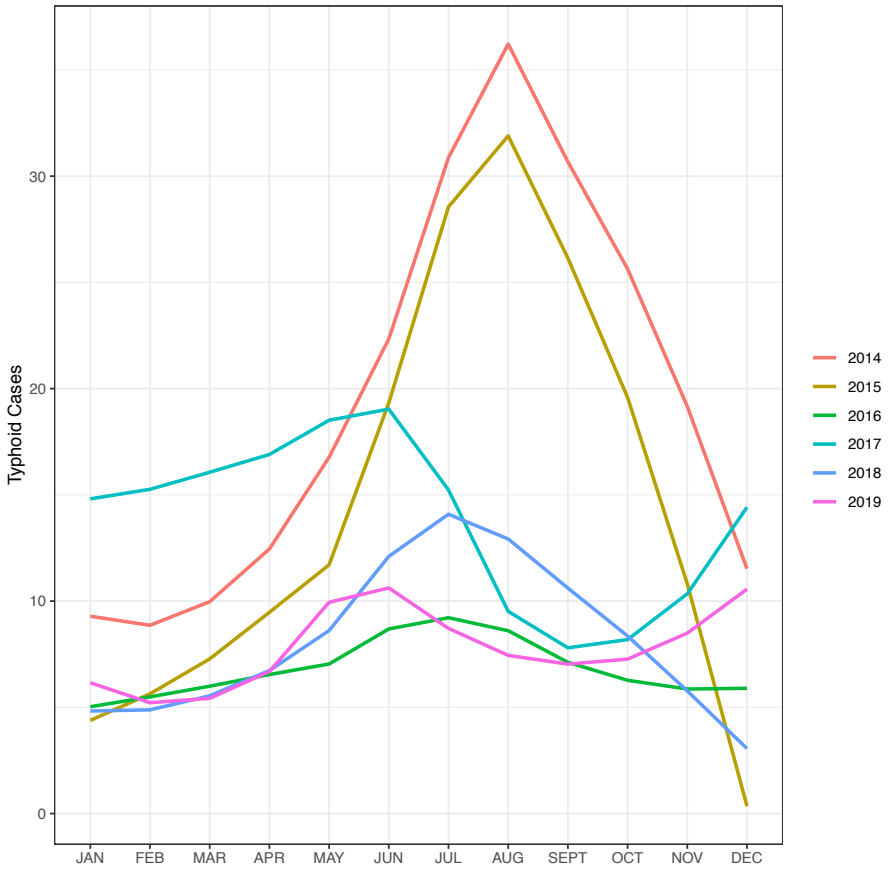


Figure 13. The annual seasonality of typhoid fever in Manimajra, Chandigarh (Carey *et al* 2021).

Plots showing the number of typhoid cases recorded the civil hospital in Manimajra from 2014 to 2019. The observed annual peak in typhoid cases corresponds with the monsoon season in Northern India (May to September).

The *S*. Typhi organisms interrogated here by WGS were isolated between September 2016 and December 2017 and all originated from blood cultures taken from febrile patients attending CHMM using standardized enrollment criteria. This reduces the likelihood of obtaining a biased sample, although one can reasonably assume that patients presenting to a healthcare facility might be more likely to have severe disease, possibly caused by a drug-resistant pathogen, as less severe illnesses and/or those caused by sensitive pathogens might resolve without treatment or be treated successfully in the community. All patients with a positive blood culture for *S*. Typhi resided within 12.9 km of the healthcare facility and were in an area of ~28 km^2. *S.* Typhi was isolated throughout the specified months, again with higher number of cases observed between May and September. The median age of typhoid patients included in this analysis was seven years of age. The standard of care antimicrobials at this facility for patients with suspected enteric fever in outpatient settings are cotrimoxazole, cefixime, and/or azithromycin, and ceftriaxone for inpatients.

4.2.2 The local phylogenetic structure of *S*. Typhi

Following data quality control, we generated and analysed 66 *S*. Typhi genome sequences from typhoid fever patients in Chandigarh. We observed that the population structure of *S*. Typhi around Chandigarh exhibited a high level of genetic diversity, with eight co-circulating genotypes, which was suggestive of population mixing and sustained introduction of organisms from a variety of locations across India (**Figure 14**). However, as has been observed in several other locations in Asia and East Africa, most organisms (80%; 53/66) belonged to lineage 4.3.1 (H58), with the majority of those (66%; 35/53) belonging to genotype 4.3.1.2. In total, 24% (16/66) of isolates were subclade 4.3.1, and 3% (2/66) were H58 sublineage II (genotype 4.3.1.1). Additional genotypes included clade 3.3 (7.5%, 5/66), clade 2.5 (7.5%, 5/66), subclade 3.3.1 (1.5%, 1/66), clade 4.1 (1.5%, 1/66) and major lineage 2 (genotype 2; 1.5%, 1/66).

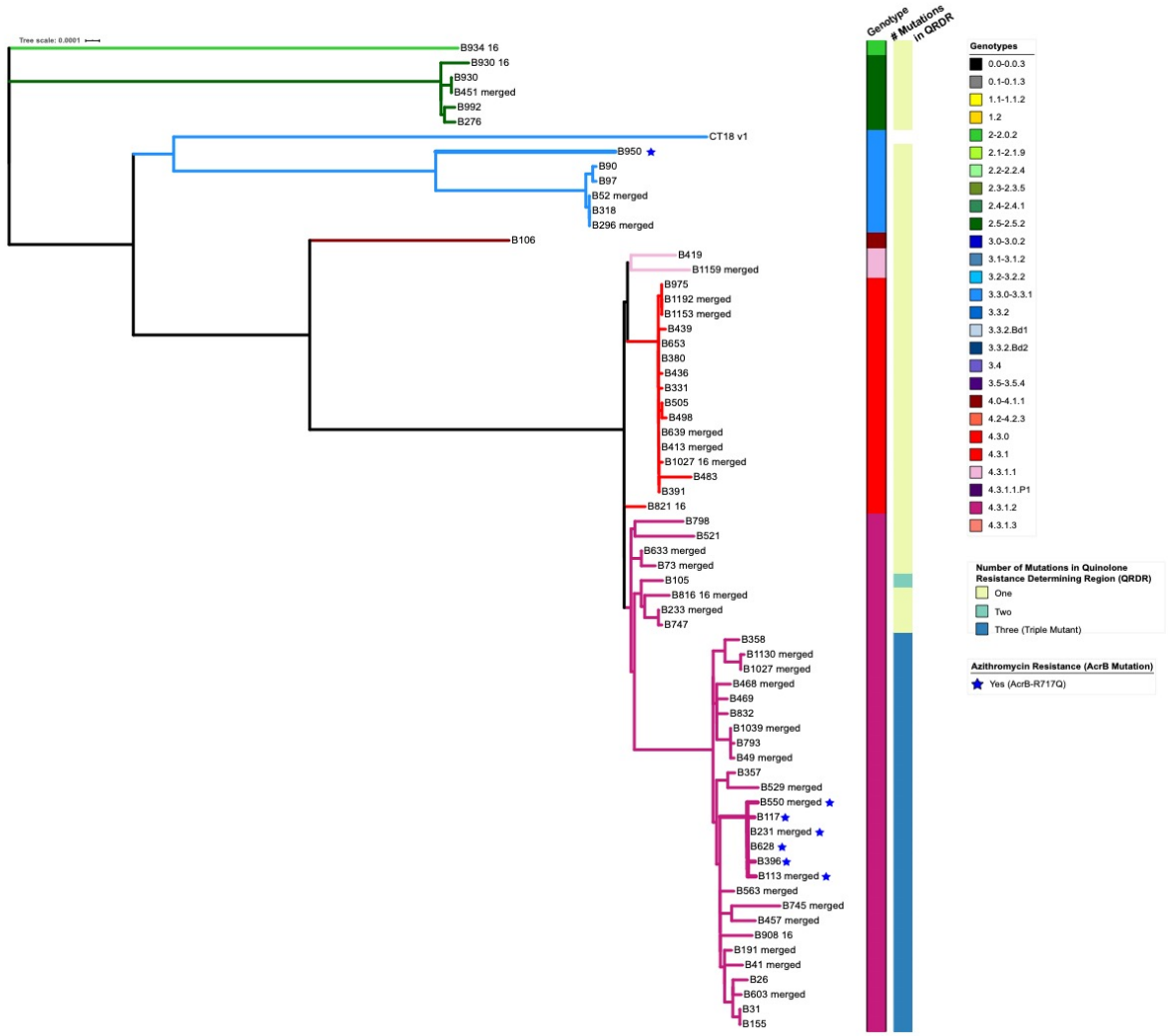


Figure 14. The phylogenetic distribution of *S*. Typhi isolated at the civil hospital in Manimajra, Chandigarh (Carey *et al* 2021).

Phylogenetic tree generated in RaxML of the 66 *S*. Typhi isolates. This collection shows considerable genetic diversity, with eight genotypes represented (indicated by colours on branches and in first column). Mutations in the Quinolone Resistance Determining Region (QRDR), and presence of the *acrB*-R717Q mutation are shown for each organism. There are two distinct clusters of organisms with the *acrB* mutation that confers azithromycin resistance; each of these individual organisms is indicated with a star.

4.2.3 Fluoroquinolone resistance

All (66/66) *S*. Typhi genome sequences, regardless of the genotype, possessed mutations in the *gyrA* gene, conferring reduced susceptibility to fluoroquinolones. Notably, given that these mutations were observed in a range of genotypes, these had occurred independently, likely because of sustained antimicrobial pressure from widespread fluoroquinolone use in the region. We also observed multiple *gyrA* mutation profiles in 4.3.1 organisms conferring intermediate resistance against fluoroquinolones (0.12µg/ml < ciprofloxacin MIC < 1µg/ml). These mutations included S83Y (29.1%; 16/55), S83F (16.4%; 9/55), and D87N (1.8%, 1/55). Additionally, we identified a subclade of organisms that represented 49.1% (27/55) of the H58 isolates, all of which were genotype 4.3.1.2, that contained the classical triple mutations associated with full fluoroquinolone resistance (S83F and D87N in *gyrA* and S80I in *parC*)[137]. These organisms exhibited high-level fluoroquinolone resistance (ciprofloxacin MIC >24µg/ml). Our observations with respect to ubiquitous fluoroquinolone resistance were concerning; however, none of the *S*. Typhi isolates were MDR, which may be associated with a reduced reliance on older classes of antimicrobials.

4.2.4 Azithromycin resistance

We identified that 7/66 (10.6%) of the sequenced isolates contained a mutation in *acrB*, a gene encoding a component of the AcrAB efflux pump.[190] Mutations in *acrB* have been previously observed to be associated with resistance to azithromycin[75]. Here, the *acrB* mutation was non-synonymous (R717Q) and identified in six genotype 4.3.1.2 organisms and in one genotype 3.3.1 organism. These data are indicative of convergent mutation in different lineages, highlighting a potential increasing reliance on azithromycin; this selective

pressure is further accentuated by the small clonal expansion in genotype 4.3.1.2 (**Figure 14**).

As discussed in **Chapter 3**, the R717Q mutation in *acrB* has been linked to high azithromycin MICs in genotype 4.3.1.1 *S*. Typhi isolates from Bangladesh[24] and in a genotype 4.3.1.1 *S*. Typhi organism isolated in Pakistan.[75] The contemporary Indian *S*. Typhi isolates identified here with the same R717Q mutation in *acrB* showed resistance to azithromycin with an MIC >16µg/ml (range: 16 ->256µg/ml). Our data suggested that these R7171Q mutations in the *acrB* gene have arisen spontaneously in India. To test this hypothesis, we constructed an expanded phylogenetic tree comprising a global *S.* Typhi collection, including organisms from across South Asia and the recently described azithromycin-resistant organisms from Bangladesh, Pakistan, and Nepal. We found that the azithromycin-resistant *S*. Typhi from India were phylogenetically distinct from those reported from Bangladesh, Pakistan, and Nepal (Figure 16). Additionally, we found that the azithromycin-resistant organisms associated with *acrB* mutations were dispersed around the tree and appear to have arisen on at least five different occasions, with a different *acrB* point mutation in organisms from Nepal (R717L), which was also associated with azithromycin resistance (MIC >256 mg/L).[191]

The azithromycin-resistant isolates from India described here were isolated in 2017, meaning that they are contemporaneous with those reported from Bangladesh and Pakistan, and arose independently in phylogenetically distinct lineages. Six of the seven Indian isolates with the R717Q mutation in *arcB* (all 4.3.1.2) were also within the group of organisms with the triple mutation associated with high level fluoroquinolone resistance,

making these organisms highly resistant to these two key oral antimicrobials used to treat typhoid fever and other bloodstream infections in the region.

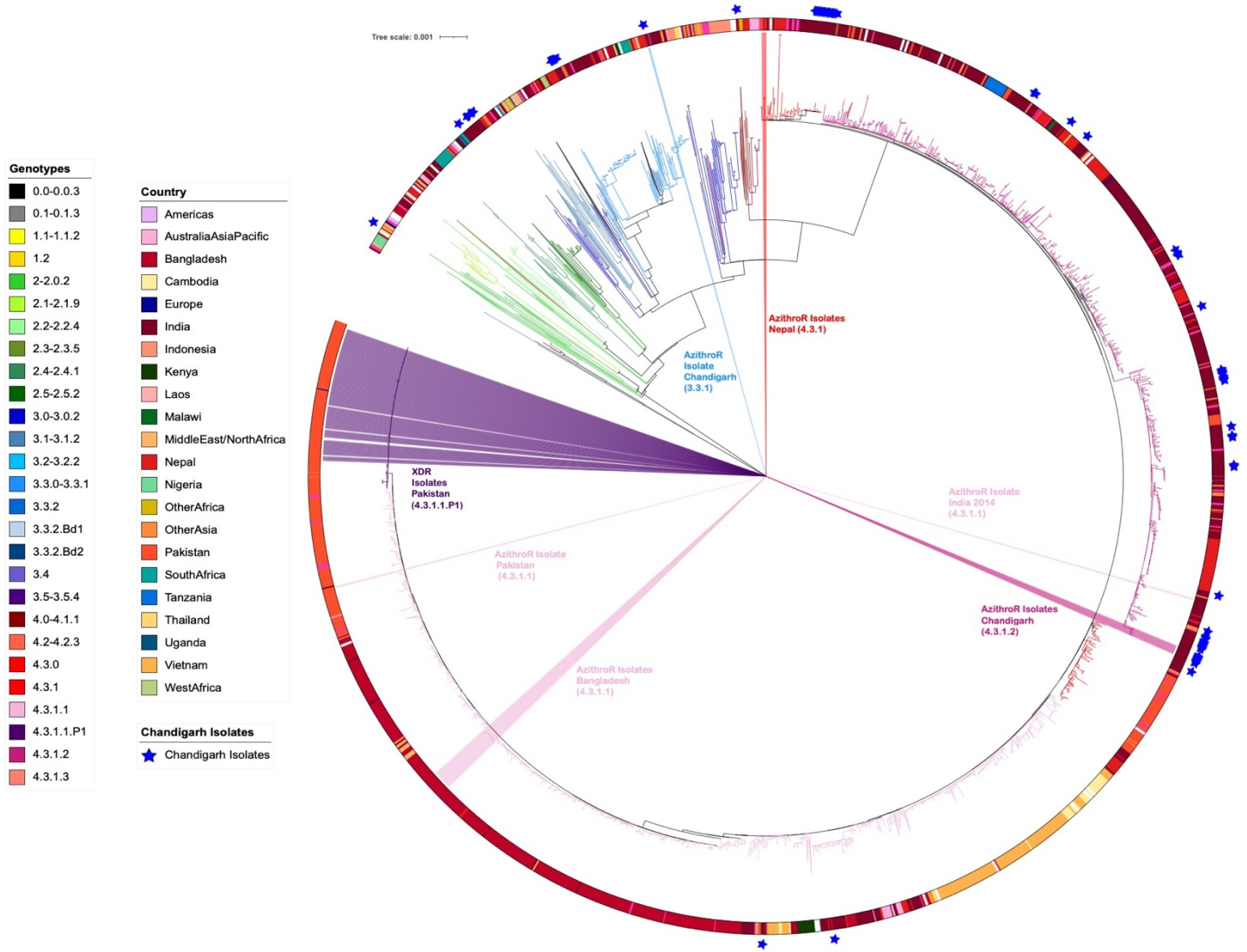


Figure 15. Azithromycin resistant *S*. Typhi from Chandigarh in global context.
Diagram depicts a maximum likelihood rooted phylogenetic tree with a final alignment of 25,832 chromosomal SNPs for 3,472 globally representative *S*. Typhi isolates, including all publicly available isolates from India. The colour of the internal branches represents the genotype, the coloured ring around the tree indicates the country or region of origin for each isolate, and the blue stars indicate which isolates were originate from this study. Additionally, the tree contains each known *S*. Typhi isolate with an *acrB* mutation in public databases, these originate from India, Nepal, Bangladesh, and Pakistan. The location of the XDR isolates from Pakistan are added for context.

4.3 Discussion

In this study, we aimed to describe the genomic aspects of *S*. Typhi causing febrile illness in a typhoid-endemic region in Northern India. We investigated antimicrobial susceptibility patterns using phenotypic testing and WGS data and then placed these data into a regional and global context using published genomic data. Notably, we identified seven azithromycin resistant *S*. Typhi isolates. These organisms belonged to two different lineages and were genetically distinct from azithromycin resistant isolates recently reported from Bangladesh, Pakistan, and Nepal. Our observations suggest that azithromycin resistance mutations at codon 717 in *arcB* are occurring independently in locations where there is substantial selective pressure induced by widespread azithromycin use. An increased reliance on azithromycin for treatment of typhoid fever and other invasive bacterial infections in South Asia, along with ongoing clinical trials measuring the impact of prophylactic administration of azithromycin on growth and mortality of infants and young children in Pakistan, Bangladesh, and India,[192] signal the inevitability of what Hooda and colleagues have termed pan-oral drug-resistant (PoDR) Typhi.[192] This scenario would necessitate inpatient intravenous drug administration for effective treatment of typhoid fever in the region at enormous cost to patients and to healthcare systems. Where intravenous drug administration is not an option, typhoid fever could once again become an extremely deadly disease, as it was in the pre-antimicrobial era.

While the catchment area of this study is not representative of the entire Indian sub-continent, the phenomenon described herein is unlikely to be restricted to Chandigarh. India is currently the largest consumer of antimicrobials of all low- and middle-income countries (LMICs), consuming a reported 6.5 billion Defined Daily Doses (DDDs) in the year

2015, or 13.6 DDDs per 1,000 inhabitants per day.[193,194] With such widespread availability and use of antimicrobials nationally, selective pressure on circulating pathogens is likely to be substantial. Recently published genomic data from the SEFI study suggest that fluoroquinolone non-susceptibility is increasingly prevalent across multiple, geographically representative sites in India (18 facilities across 16 cities).[46] Additional forthcoming data from the SEFI study will soon further elucidate AMR patterns across the country. Ideally, these data will inform local antimicrobial stewardship practices and may be used as a basis for prioritization of future interventions.

How then to address this evolutionary arms race? Drug development efforts cannot keep pace with bacterial evolution. Therefore, there is an urgent need for preventative interventions, namely water, sanitation, and hygiene (WASH) interventions and TCV introduction, in India and across South Asia. Pakistan became the first country to introduce TCV into its national immunization programme in late 2019,[126] followed by Liberia,[127] Zimbabwe,[128] and Nepal,[129] but TCV introduction is urgently warranted in India and Bangladesh as well. There is also a need for enhanced typhoid surveillance, in South Asia and globally, specifically to monitor the emergence and spread of this and other resistance phenotypes. Historic genomic data show us that drug-resistant *S*. Typhi lineages emerge in South Asia and then spread to East Africa and even Latin America.[195] With the widespread prophylactic deployment of azithromycin through clinical studies and public health programs in West Africa and South Asia,[192,196] it will be critical to monitor global AMR patterns to mitigate a public health catastrophe.

This emerging problem additionally represents an opportunity for use of genomics to inform policy. WGS data provides clear information regarding AMR in organisms where molecular mechanisms of resistance are understood. Genomic surveillance also enables the identification and characterization of new resistance phenotypes, as was the case for XDR typhoid[24] and the azithromycin-resistant organisms identified in Bangladesh and described further here.[75] The outputs of antimicrobial susceptibility testing are not always straightforward, particularly in cases where susceptibility breakpoints have not been validated extensively using clinical data, as is the case for azithromycin.[197] Genomic AMR data can inform prioritization of TCV introduction, as well as implementation of WASH interventions. Genomic surveillance should also be an important component of long-term monitoring of the impact of widespread TCV deployment. Not only can genomic surveillance provide additional information on the impact of TCV on AMR, but it can also demonstrate the impact of vaccine on bacterial population structures and facilitate the identification of any vaccine escape mutants. Such information is vital to understanding the long-term impact of vaccine, and to critically assess the feasibility global typhoid elimination.

Chapter 5: The origins of haplotype 58 (H58) *S*. Typhi

5.1 Abstract

AMR in *S*. Typhi is associated with the H58 lineage, which arose comparatively recently before becoming globally disseminated. To better understand when and how this lineage emerged and became dominant, we performed detailed phylogenetic and phylodynamic analyses on contemporary genome sequences from *S*. Typhi isolated in the period spanning the emergence. Our dataset, which contains the earliest described H58 *S*. Typhi, indicates that the prototype H58 organisms were MDR. These organisms emerged spontaneously in India in 1987 and became radially distributed throughout South Asia and then globally in the ensuing years. These early organisms were associated with a single long branch, possessing mutations associated with increased bile tolerance, suggesting that the first H58 organism was generated during chronic carriage. The subsequent use of fluoroquinolones led to several independent mutations in *gyrA*. The ability of H58 to acquire and maintain AMR genes continues to pose a threat, as XDR variants have emerged recently in this lineage. Understanding where and how H58 *S*. Typhi originated and became successful is key to understand how AMR drives successful lineages of bacterial pathogens. Additionally, these data can inform optimal targeting of TCVs for reducing the potential for emergence and the impact of new drug-resistant variants. Emphasis should also be placed upon the prospective identification and treatment of chronic carriers to prevent the emergence of new drug resistant variants with the ability to spread efficiently. [45,52] These results have been preprinted on *bioRxiv* (Carey *et al* 2022)[198] and submitted for publication. I identified the research questions, generated the visualisations for and annotated the maximum-likelihood phylogenetic trees and the BEAST tree, and drafted the paper. Colleagues from UKHSA

located and revived bacterial samples, a colleague from OUCRU Nepal conducted DNA extraction and WGS, and a colleague at LSHTM ran the phylogenetic analyses.

5.2 Introduction

Given the importance of antimicrobials for the management and control of typhoid, AMR in *S*. Typhi has the potential to be a major public health issue. Indeed, the problem of AMR in *S*. Typhi first appeared in the 1950s with the emergence of resistance against the most widely used drug, chloramphenicol.[199] MDR *S.* Typhi, which was first identified in the 1970s and became common in the early 1990s,[200,201] is frequently conferred by self-transmissible IncH1 plasmids carrying a suite of resistance genes, include resistance determinants for chloramphenicol (*catA1* or *cmlA*), ampicillin (*bla*TEM-1D, *bla*OXA-7), and co-trimoxazole (at least one *dfr*A gene and at least one *sul* gene).[51] Lower efficacy of first-line antimicrobials led to the increased use of fluoroquinolones, but decreased fluoroquinolone susceptibility became apparent in the mid-1990s, and was widespread in South and Southeast Asia in the early 2000s.[45,202] Inevitably, as treatment options have become limited, third-generation cephalosporins and azithromycin have been used more widely for effective treatment of typhoid fever.[64–66] However, newly circulating XDR *S*. Typhi has left azithromycin as the only feasible oral antimicrobial for the treatment of typhoid fever across South Asia.[24] We are arguably at a tipping point, as azithromycin-resistant *S*. Typhi has since been reported in Bangladesh, Pakistan, Nepal, and India, thereby threatening efficacy of common oral antimicrobials for effective typhoid treatment.[32–34,75] If an XDR organism were to acquire azithromycin resistance (single base pair mutation), this would lead to what Hooda and colleagues have referred to as pan-oral drug-resistant (PoDR) *S.* Typhi, which would require inpatient intravenous treatment.[192] This would come at substantial additional cost to

patients and their families, and place additional strain on already overburdened health systems. [203–205]

In contrast to many other Gram-negative bacteria, *S*. Typhi is human restricted with limited genetic diversity that can be described by a comparatively straightforward phylogenetic structure.[206] Therefore, the phylogeny and evolution of *S*. Typhi provide a model for how AMR emerges, spreads, and becomes maintained in a human pathogen. AMR phenotypes in *S*. Typhi are typically dominated by a single lineage; H58 (genotype 4.3.1 and consequent sublineages), which was the 58th *S*. Typhi haplotype to be described in the original genome wide typing system.[48] This highly successful lineage is commonly associated with MDR phenotypes and decreased fluoroquinolone susceptibility.[45] Previous phylogeographic analysis suggested that H58 emerged initially in Asia between 1985 and 1992 and then disseminated rapidly to become the dominant clade in Asia and subsequently in East Africa.[45] H58 is currently subdivided into three distinct lineages – lineage I (4.3.1.1) and lineage II (4.3.1.2), which were first identified in a paediatric study conducted in Kathmandu,[207] and lineage III (4.3.1.3), which was identified in Dhaka, Bangladesh.[56] A recent study of acute typhoid fever patients and asymptomatic carriers in Kenya demonstrated the co-circulation of genotypes 4.3.1.1 and 4.3.1.2 in this setting, and closer analysis showed that these East African sequences had distinct AMR profiles and were the result of several introduction events.[133,208] These events led to the designation of three additional genotypes: H58 lineage I sublineage East Africa I (4.3.1.1.EA1), H58 lineage II sublineage East Africa II (4.3.1.2.EA2), and H58 lineage II sublineage East Africa III (4.3.1.2.EA3).[133] In addition, the XDR *S*. Typhi clone, which was caused by a monophyletic

outbreak of genotype 4.3.1.1 organisms, was designated genotype 4.3.1.1.P1 to facilitate monitoring of its spread.

It is apparent from investigating the phylogeny of *S*. Typhi that H58 is atypical in comparison to other lineages. This lineage became dominant in under a decade, and first appeared on a long basal branch length, indicative of a larger number of single base pair mutations separating it from its nearest neighbour (**Figure 16a**). These observations suggest that there is something 'unique' about the evolution of this lineage, but we have limited understanding of how H58 emerged, what enabled its rapid spread, and when it initially appeared. Here, by collating new genome sequences of *S*. Typhi that were associated with travel to South Asia in the late 1980s and early 1990s and comparing them to a global population over the same period, we explore an expanded early phylogenetic dataset to resolve the origins and rapid success of this important and successful AMR clone.

5.3 Results and discussion

5.3.1 Sampling

The main questions that we aimed to address with this study were: i) when and where did H58 *S*. Typhi first emerge; ii) can we better resolve the evolutionary events that lead to the long branch length observed for H58 *S*. Typhi; and iii) how quickly did this lineage spread and why? Therefore, to investigate the origins of *S*. Typhi H58, data from United Kingdom Health Security Agency (UKHSA, formerly Public Health England) containing information on stored *S*. Typhi organisms isolated between 1980 and 1995 from travellers returning to the UK from overseas and receiving a blood culture were analysed.

The database was queried and organisms were selected from the following three categories: i) 126 *S*. Typhi with the E1 Phage type (which is considered to be associated with H58)[51] originating from South Asia (India, Nepal, Pakistan, and Bangladesh), ii) 159 *S*. Typhi organisms with a variety of non-E1 phage types originating from South Asia, and iii) 184 *S*. Typhi organisms with a variety of phage types (both E1 and non-E1) originating from locations outside of South Asia. A total of 470 *S*. Typhi organisms meeting these criteria were randomly selected, revived, subjected to DNA extraction and whole genome sequenced. Ultimately, our dataset was composed of 463 novel sequences generated as a component of this study and 305 existing sequences[47,56,152] known to belong to the H58 lineage and its nearest neighbours, yielding a total of 768 whole genome sequences on which to structure subsequent analysis. It is worth noting that our dataset was enriched for samples of E1 phage type and samples isolated from travellers returning from South Asia, so is not globally representative. This was done in order to provide the highest possibility of identifying early H58 isolates. Subsequent work conducted by UKHSA and other colleagues has suggested that *S*. Typhi WGS data isolated from returning travellers is often representative of the circulating *S*. Typhi populations in the countries of origin,[55] and that routine surveillance of *S*. Typhi isolated from returning travellers could be used as informal sentinel surveillance for countries of travel. However, there may also be some bias associated with data obtained from returning travellers (e.g. differences in health-seeking behaviours, which may bias towards greater severity and likelihood of AMR), as well as differences in patterns of travel, that are worth considering in interpreting the representativeness of this dataset.

5.3.2 Population structure, genotype distribution, and AMR profiles of historical *S*. Typhi

We inferred a maximum likelihood phylogenetic tree from the sequencing data to examine the population structure of this historic (1980 – 1995) collection of global *S*. Typhi sequences (**Figure 16a**). Notably, unlike the extent global population of *S*. Typhi, which is largely dominated by a single lineage,[45,52] this historic population exhibited considerable genetic diversity, with 37 genotypes represented (Figure 1a). The majority of isolates belonged to primary clade 2 (194/463), of which clade 2.0 was most common (36%, 69/194) followed by subclades 2.3.3 (14%, 28/194) and 2.2.2 (13%, 25/194). An additional 23% of isolates belonged to primary clade 3 (108/463) and 3% of isolates were classified as major lineage 1 (12/463). Ultimately, 29% of isolates belonged to major lineage 4 (134/463), of which 63% (84/134) were H58. Among these, H58 lineage I (genotype 4.3.1.1) was most common (67%, 56/84), followed by genotype 4.3.1 (H58 not differentiated into any sublineage; 24%, 20/84). The earliest H58 isolates in our dataset are illustrated in higher resolution in **Figure 16b**.

Although we enriched our historical dataset for samples isolated from travellers returning from South Asia, our final dataset generated substantial geographic coverage, with 39 different countries represented. Therefore, these data are likely to be reasonably representative of the circulating *S.* Typhi in this pivotal period. Notably, the earliest H58 organism in our dataset that was classified as H58 according to the GenoTyphi scheme was isolated in 1983 from an individual entering the UK from India, followed by two additional Indian isolates (1988) that were also classified as H58. These organisms differed from the larger cluster of H58 organisms by 61-64 SNPs (**Figure 16a**). All H58 *S*. Typhi in this dataset were isolated from travellers returning from South Asia, with the majority (51/84; 61%)

originating from Pakistan and the remainder from India and Bangladesh; 31/84 (37%) and 2/84 (2%), respectively.

Given that this dataset included isolates from the early MDR era and then following the emergence of reduced fluoroquinolone susceptibility, we analysed the data for genes associated with MDR and mutations in the DNA gyrase gene, *gyrA*. Overall, 7% (34/463) of the organisms in this historical dataset were genetically defined as being MDR; significantly, all were H58 (genotypes 4.3.1, 4.3.1.1, 4.3.1.2, and 4.3.1.3) and isolated between 1991 and 1995, and 97% (33/34) possessed an IncH1 plasmid (**Figure 16b**). Thirteen H58 organisms contained an IncH1 plasmid carrying AMR genes, but did not possess the genes conferring resistance to all three first-line antimicrobials, and thus were not genetically defined as MDR (**Figure 16b**). The first mutation in the quinolone resistance determining region (QRDR) in our dataset was a *gyrA*-D87Y substitutions identified in a genotype 2.5 organism originating in South Africa in 1986 (**Figure 16a**). This was clearly a spontaneous mutation that evolved *de novo* and did not appear to become fixed in the population. Similarly, two single QRDR mutations (*gyrA*-S83F and *gyrA*-S83Y) occurred independently in H58 organisms (genotype 4.3.1) in India in 1995 (**Figure 16b**) and were not observed in descendant populations. No mutations in *gyrB* or *parC* were observed in this dataset.

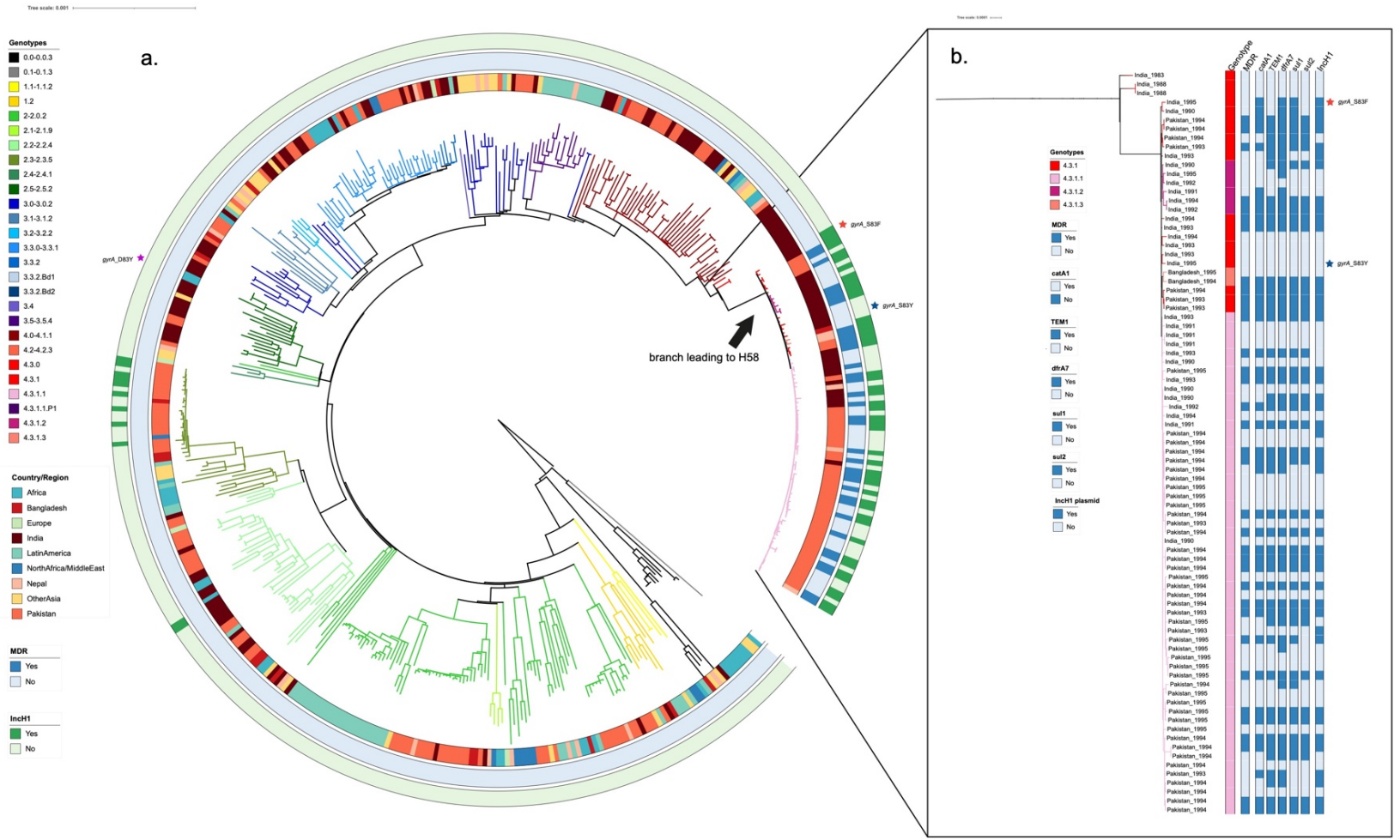


Figure 16. The phylogenetic structure of historical (1980 – 1995) *S*. Typhi isolates.
Maximum likelihood outgroup rooted phylogenetic tree depicting the genomic sequences of 463 *S*. Typhi isolated from returning travellers to the United Kingdom. Branch colour indicates genotype, and rings outside of the tree indicate country of origin, MDR, and presence of IncH1 plasmid, coloured as per the inset legend. QRDR mutations indicated by stars outside the rings. b) A zoomed-in view of H58 *S*. Typhi isolates from historical collection. Genotype, country of origin, presence of MDR, AMR mutations, and presence of IncH1 plasmids are indicated by bars to the right of the tree and coloured as per the inset legend. QRDR mutations indicated by stars to the right of the bars.

In order to contextualise these isolates to understand the evolutionary events leading to this clone, we selected H58 and nearest neighbours (from genotypes 4.1 and 4.2) *S*. Typhi organisms (n=305) that were already available in the public domain from previous studies[47,56,152] (**Table S3**) and generated a phylogenetic tree combining these isolates with early H58 and nearest neighbour isolates from our unpublished dataset (n=117). In our H58 and nearest neighbour dataset (n= 422, **Figure 17**), which included both published data as well as our contemporary data, 17 countries were represented.[47,56,152] Of the non-H58 isolates (nearest neighbours), 42% were genotype 4.1 (32/76), 13% (10/76) were genotype 4.2, 28% (21/76) were 4.2.1, and 16% (12/76) were 4.2.3. These non-H58 nearest neighbour organisms were isolated between 1981 and 2000. None of them were MDR, and none carried an IncH1 plasmid. Of our H58 isolates (defined as having informative SNPs indicative of lineage 4.3.1), the earliest organism was isolated in 1983 in India, followed by two additional Indian isolates (1988) that were also classified as H58. However, we can observe that there were no more recent isolates from this founder group (**Figure 17**), implying that this lineage became extinct; these isolates did not contain IncH1 plasmids and were non-MDR. Within the H58 lineage, most of the organisms belonged to H58 sublineage I (4.3.1.1; 84%, 290/346), followed by sublineage II (4.3.1.2; 7%, 25/346), genotype 4.3.1 (6%, 21/346), and sublineage III (4.3.1.3; 3%, 10/346). Overall, 63% (219/346) of these H58 organisms were MDR, and 87% (191/219) of these MDR H58 organisms carried an IncH1 plasmid. All of the MDR H58 organisms lacking an IncH1 plasmid were genotype 4.3.1.1, the earliest of which was isolated in India in 1991. Within this group, the first single point mutation in the QRDR occurred comparatively early in an organism isolated in India in 1991.[152]

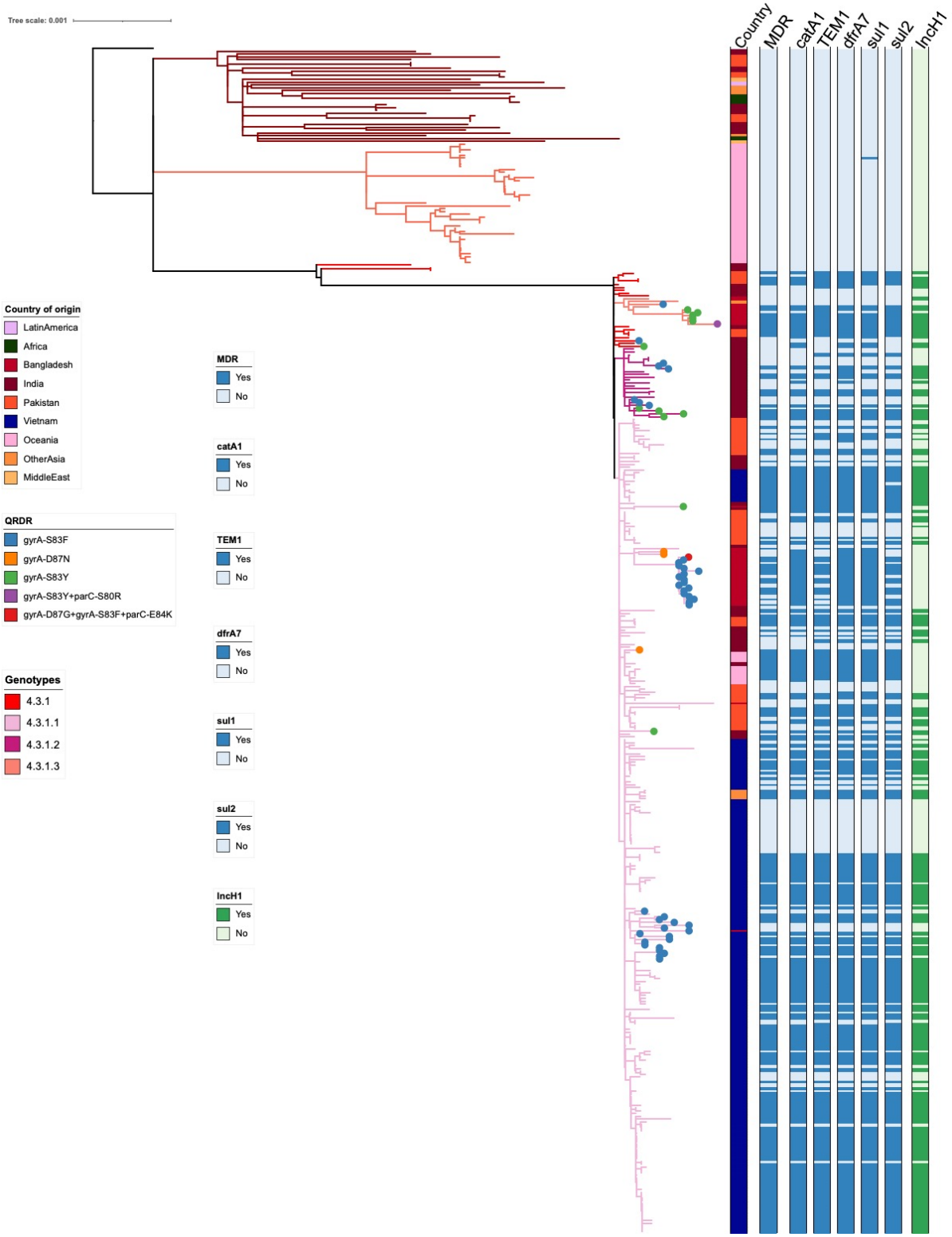


Figure 17. The phylogenetic structure of early H58 organisms and nearest neighbours.

Maximum likelihood rooted phylogenetic tree showing *S*. Typhi organisms (genotype 4.3.1) and nearest neighbours (genotypes 4.1 and 4.2) from our historical collection and from published literature (n=422 total). Genotype is indicated by branch colour, presence of QRDR mutation(s) are indicated by coloured circles at the end of the branches, and country of origin, presence of MDR, AMR mutations, and presence of incH1 plasmid are indicated by bars to the right of the tree and coloured as per the inset legend.

5.3.3 Evolutionary history of H58 *S.* Typhi

Our estimation of the temporal signal of our H58 and nearest neighbours data exhibited a strong correlation between the sampling dates and the root-to-tip distances, with a positive value for the slope and an R^2 value of 0.4743 (**Figure 18**).

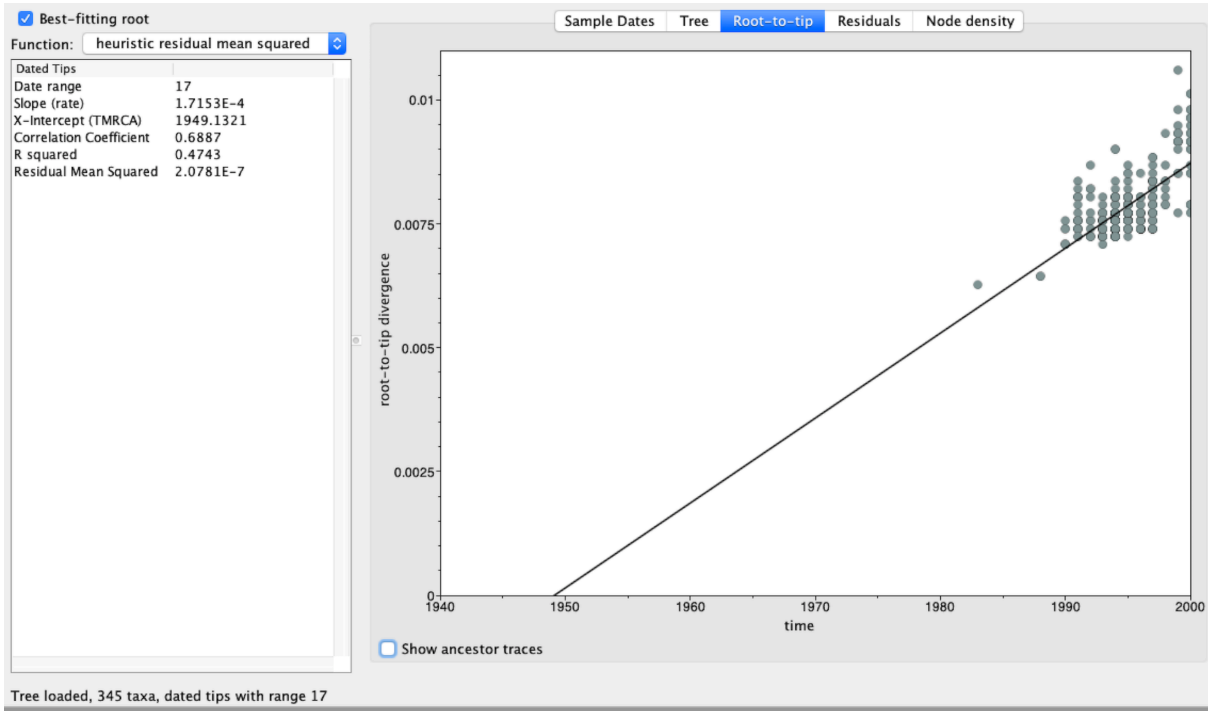


Figure 18. An estimation of temporal signal in H58 *S.* Typhi data.

Path-O-Gen/TempEst results (analysis included n=345 isolates using an alignment of 724 non-recombinant SNPs), demonstrating strong correlation between sampling dates and root-to-tip distance (R^2 = 0.4743).

Additionally, the randomly reassigned sampling time of sequences 20 times to generate the mean rates indicated that there was no overlap between the 95% credible intervals of the mean rate of the real data set and that of the date randomization data (**Figure 19**), thus warranting BEAST analysis.

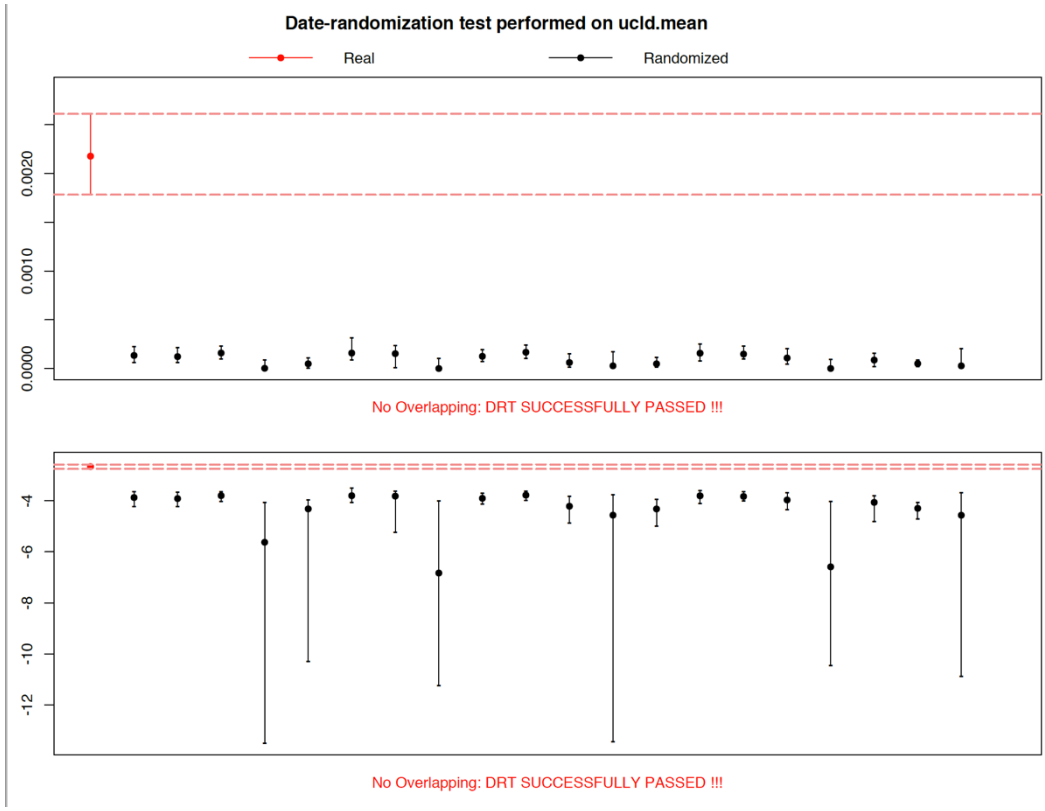


Figure 19. A TipDatingBeast estimation of temporal signal in H58 *S*. Typhi data.

TipDatingBeast results (analysis incorporated n=345 isolates and an alignment of 724 non-recombinant SNPs), demonstrating no overlap between the original mean rates of mutation and mean rates of date randomization.

Using BEAST analysis, we determined that the median substitution rate of the H58 was 2.79 × 10^{-7} substitutions $base^{-1}$ $year^{-1}$ [95% highest posterior density (HPD): 2.40 × 10^{-7} - 3.24 × 10^{-7}), which is comparable to that observed in previous studies.[166] We found that the most recent common ancestor (MRCA) of the H58 was estimated to have emerged in late 1987 (95% HPD: 1986 – 1988). Two H58 sublineages (4.3.1.1 and 4.3.1.2, **Figure 20**) then emerged almost simultaneously in India in 1987 and 1988. The time-inferred phylogeny shows a clonal expansion of H58 that originated from South Asia, specifically in India, and then disseminated globally. As noted above, 63% (219/346) of isolates were MDR, and 87% (191/219) of those isolates contained an IncH1 plasmid known to carry AMR genes. Detailed genetic analysis of the IncH1 plasmids observed in most of these MDR isolates revealed high genetic similarity, with an average of 1.3755 SNPs difference between them (**Figure 21**). These data suggest that the ancestral H58 organism that was the basis for the major clonal expansion was already MDR before undergoing clonal expansion and subsequent global dissemination; some ensuing H58 organisms then lost the MDR plasmid in certain settings, presumably because of decreased antimicrobial selection pressure with first-line antimicrobials, with a corresponding impact on fitness. The fact the three early precursor organisms that were not MDR appear to have become extinct supports our hypothesis that the presence of an MDR phenotype was a selective event.

It is also stark that QRDR mutations appeared in H58 organisms quickly and frequently. Within this H58 dataset, organisms with one or more QRDR mutations appeared within six independent lineages of H58, as illustrated in **Figures 17 and 20**. The earliest H58 lineage (lineage II) to develop QRDR mutations was observed in 1991 in India, with a single S83Y mutation in the *gyrA* gene, and the first isolate containing two QRDR mutations (*gyr*AS83Y;

*par*C-S80R) appeared in a genotype 4.3.1.3 organism in Bangladesh in 1999 (**Figure 20**). The first “triple mutant” (mutations in *gyr*A-D87G, *gyr*A-S83F, and *parC*-E84K) was found in a 4.3.1.1 Bangladeshi isolate in 1999 (**Figure 20**).[169]

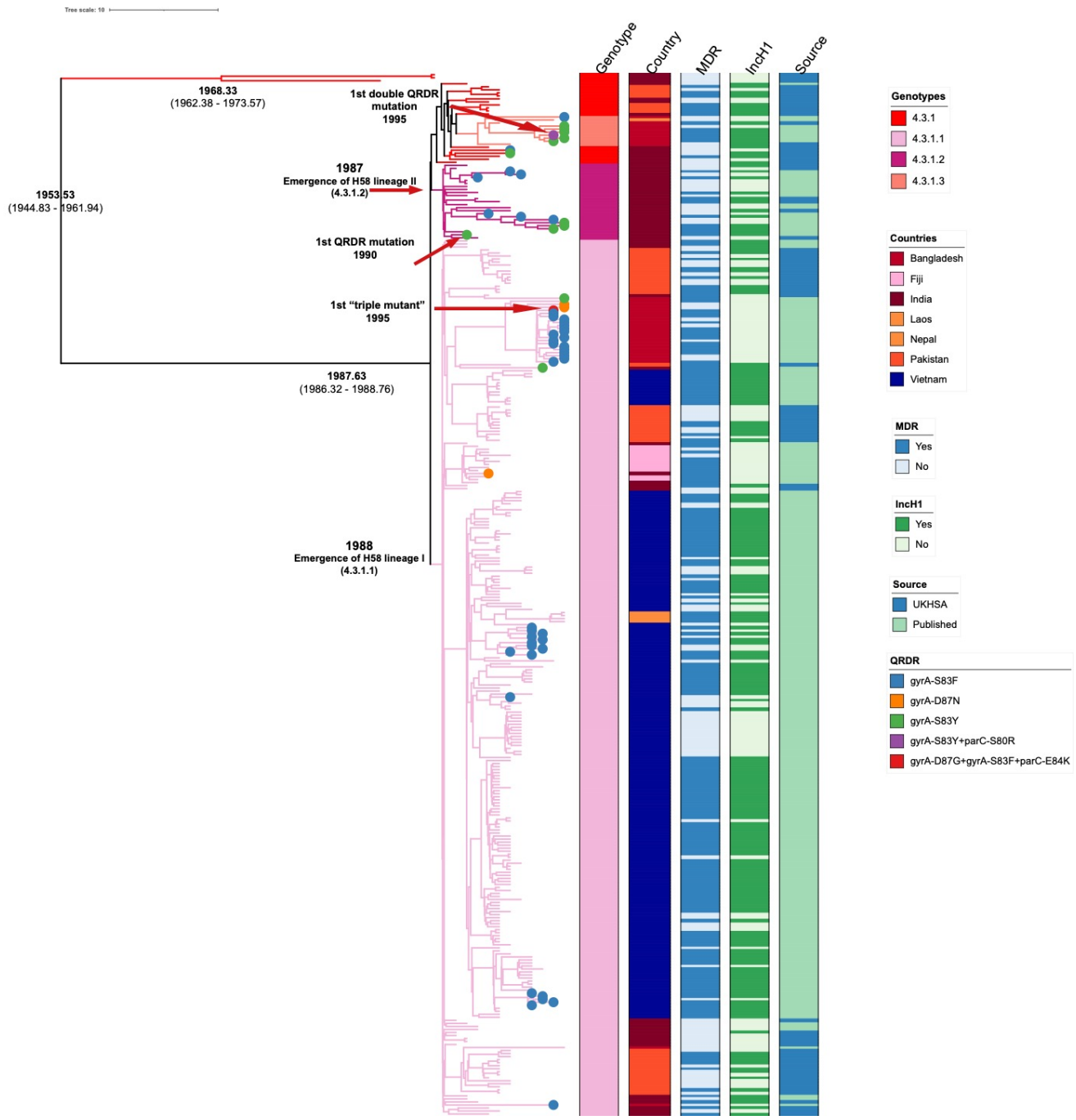


Figure 20. A dated phylogenetic structure of historical H58 *S*. Typhi isolates.

BEAST-generated dated phylogeny of H58 *S*. Typhi isolates from UKHSA collection and published literature (n=345). Tip colours indicate presence of specific mutation(s) in the QRDR as per inset legend. Branch colour and the first column to the right of the tree indicate genotype, the second column indicates country of origin, the third represents presence of MDR, and the final column indicates presence of an incH1 plasmid. Our analysis suggests that the Most Recent Common Ancestor (MCRA) of H58 appeared in 1987, and that two sublineages (I and II) emerged almost simultaneously in India in 1987 and 1988. The first single point mutation in the QRDR was observed in 1990, and the first "triple mutant" was observed in Bangladesh in 1999.

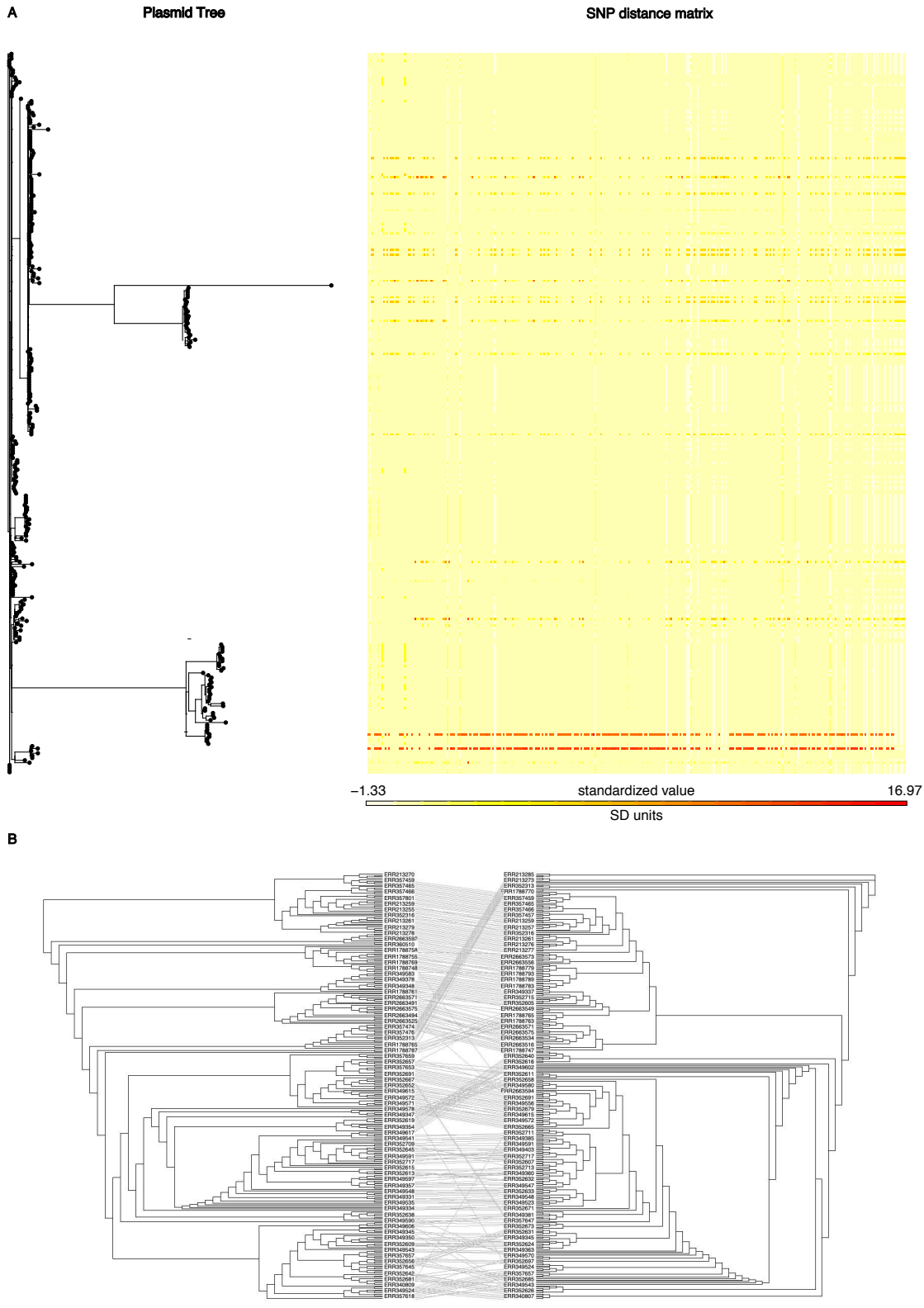


Figure 21. A genetic and phylogenetic evaluation of IncH1 plasmid content of early H58 *S.* Typhi isolates.
A. Heatmap of SNP differences between Inch1 plasmids of early H58 isolates shown alongside ML phylogenetic plasmid tree. B. Tanglegram depicting topological relationship of ML phylogenetic trees for early H58 organisms and their respective plasmids.

5.3.4 Genetic variation associated with H58 *S.* Typhi

Our data support the hypothesis that H58 *S*. Typhi was successful specifically because of the acquisition and maintenance of an MDR plasmid. This selection meant that later *gyrA* mutations were more likely to occur in this lineage given its dominance (and therefore, higher rates of replication leading to additional opportunities for mutations to occur), as well as assumed frequent fluoroquinolone exposure, given its existing MDR phenotype. Based on the results of our mapping, we undertook further genetic analysis to identify non-synonymous SNPs unique to the early H58 isolates, as well as SNPs that were unique to early H58 isolates that were MDR. The motivation was to explain the origins of the long branch length illustrated on **Figure 16a**, to infer why this lineage was so globally successful, and identify genetic elements that may stabilize an MDR IncH1 plasmid.

We identified 16 unique non-synonymous SNPs that were exclusive to the early H58 isolates as compared to precursor 4.1 and 4.2 organisms, the majority of which were present in genes associated with central metabolism and outer membrane structures; one of which was associated with pathogenicity (**Table 6**). Within the early H58 isolates that were also MDR, we identified an additional 23 unique non-synonymous SNPs, most of which were found in genes encoding proteins predicted to regulate metabolism, degrade small molecules, membrane/surface structures, as well as regulators, pathogenicity adaptation, and information transfer (**Table 7**). We additionally identified mutations in a gene (t2518/STY0376) encoding a hypothetical protein with an EAL (diguanylate phosphodiesterase) domain with a non-synonymous mutation, which has previously been identified as being associated with H58 organisms.[37] The homologous gene (STM0343) in *Salmonella* Typhimurium has been described as regulating motility and invasion, which

suggests that SNPs in this gene might also contribute to virulence.[38,39] In addition, we identified SNPs in genes that have previously been associated with tolerance to bile in *S*. Typhi (*sirA*, *recB*, *wecF*, *dsdA*, and *yjjV*) among the early MDR H58 isolates.[40,41]

We also aimed to compare the gene content of the early H58 plasmids, in part to investigate our hypothesis that the original H58 organism contained an MDR IncH1 plasmid and that this plasmid co-evolved with H58 *S*. Typhi before becoming chromosomally integrated or lost in subsequent years, as compared to multiple independent acquisitions of an MDR plasmid within the H58 lineage. We assessed genetic similarity of the plasmids by comparing alignments for the plasmid sequences of our early H58 organisms using snp-dists (https://github.com/tseemann/snp-dists) (see **Figure 21b**). As mentioned above, these plasmids are virtually identical, with a mean pairwise SNP distance of 1.3755 SNPs and median distance of 0 SNPs. We generated a tanglegram using the Dendroscope program (https://uni-tuebingen.de/en/fakultaeten/mathematisch-naturwissenschaftliche-fakultaet/fachbereiche/informatik/lehrstuehle/algorithms-in-bioinformatics/software/dendroscope/) to compare the topologies of maximum likelihood phylogenetic trees for our early H58 S. Typhi organisms (n=305) and their respective plasmids (**Figure 21a**). The tanglegram illustrates that there is a high degree of concordance between the host (chromosomal) and plasmid trees, which is suggestive of coevolution of the plasmid with *S*. Typhi rather than several distinct plasmid acquisitions.

Table 6. Non-synonymous SNPs unique to early H58 *S*. Typhi.

Gene ID	SNP Position in CT18	Gene	Reference nucleotide	4.3.1 nucleotide	Ancestral codon	Derived codon	Ancestral amino acid	Derived amino acid	Functional Category	Product
STY0376	387595		C	T	ACC	ATC	T	I	Central/intermediary metabolism	putative rtn protein; diguanylate cyclase/ phosphodiesterase domain-containing protein
STY0452	461438	*yajI*	G	A	CGT	TGT	R	C	Membrane/surface structures	putative lipoprotein
STY0522	529155	*kefA/aefA*	A	G	AAA	GAA	K	E	Membrane/surface structures	integral membrane protein AefA
STY0698	693560	*rlpB*	C	T	ATG	ATA	M	I	Central/intermediary metabolism	rare lipoprotein B precursor
STY1458	1408039		C	T	CAG	TAG	Q	*		putative lipoprotein
STY1703	1629304	*ssaP*	G	A	GCG	GTG	A	V	Pathogenicity/adaptation/chaperones	putative type III secretion protein
STY2371	2202853		C	T	CGT	TGT	R	C	Membrane/surface structures	putative nucleoside permease
STY2513	2348633	*glpA*	G	A	GGC	AGC	G	S	Central/intermediary metabolism	anaerobic glycerol-3-phosphate dehydrogenase subunit A
STY2553	2388057	*nuoG*	G	A	ACT	ATT	T	I	Information transfer	NADH dehydrogenase I chain G
STY2564	2401233	*yfbT*	G	A	CGC	TGC	R	C	Central/intermediary metabolism	putative phosphatase
STY3518	3360344	*nanE2*	T	C	ATA	GTA	I	V	Pseudogenes	conserved hypothetical protein (pseudogene)
STY3795	3659647	*Ydey*	C	T	GGG	GAG	G	E	Membrane/surface structures	Putative ABC transporter protein, AI-2 transport system permease
STY4405	4273783	*metH*	C	A	CGT	AGT	R	S	Central/intermediary metabolism	B12-dependent homocysteine-N5-methyltetrahydrofolate transmethylase
STY4451	4321540		A	G	AAG	AGG	K	R		single-strand DNA-binding protein
STY4890	4754034		C	T	TGG	TAG	W	*		probable carbon starvation protein
STY4890	4754035		A	G	TGG	CGG	W	R		probable carbon starvation protein

Table 7. Non-synonymous SNPs unique to early MDR H58 *S*. Typhi

Gene ID	SNP Position in CT18	Gene	Reference nucleotide	4.3.1 nucleotide	Ancestral codon	Derived codon	Ancestral amino acid	Derived amino acid	Functional Category	Product
STY0042	40159	*betC*	G	A	GGG	GAG	G	E	Central/intermediary metabolism	putative secreted sulfatase
STY0085	89102	*etfB/ fixA*	A	G	AAA	AGA	K	R	Degradation of small molecules	FixA protein
STY0376	387082		G	A	CGA	CAA	R	Q	Central/intermediary metabolism	putative rtn protein; diguanylate cyclase/phosphodiesterase domain-containing protein
STY0886	880083	*ybiK*	A	G	ACC	GCC	T	A	Central/intermediary metabolism	putative L-asparaginase
STY1310	1270888		G	A	CGA	CAA	R	Q	Membrane/surface structures	Voltage-gated potassium channel
STY1328	1286044	*trpE*	T	C	GAC	GGC	D	G	Central/intermediary metabolism	Anthranilate synthase component
STY1410	1360939	*dbpA*	C	T	CAG	TAG	Q	*	Pseudogenes	ATP-dependent RNA helicase (pseudogene)
STY1917	1810914	*hyaE*	G	A	GCT	ACT	A	T	Information transfer	hydrogenase-1 operon protein HyaE
STY2155	2002943	*uvrY*	G	A	CTT	TTT	L	F	Regulators	invasion response-regulator
STY2875	2750755		G	A	GCG	ACG	A	T	Pathogenicity adaptation/chaperones	large repetitive protein
STY3001	2875160	*sptP*	G	A	CAA	TAA	Q	*	Pathogenicity adaptation/chaperones	Pathogenicity island 1 tyrosine phosphatase (associated with virulence)
STY3132	3004181	*recB*	T	C	GAA	GGA	E	G	Degradation of macromolecules	exonuclease V subunit
STY3297	3144053	*ordL*	A	C	GTG	GGG	V	G	Central/intermediary metabolism	Putative gamma-glutamylputrescine oxidoreductase
STY3555	3398551	*yhdA*	G	A	GCT	GTT	A	V	Membrane/surface structures	Putative lipoprotein
STY3628	3484294	*wecF*	C	T	GTA	ATA	V	I	Conserved hypothetical proteins	Putative 4-alpha-L-fucosyl transferase
STY3955	3824631	*torC*	T	G	TCA	GCA	S	A	Pseudogenes	Cytochrome c-type protein
STY3977	3843665	*dsdA*	C	T	GGC	GAC	G	D	Degradation of small molecules	D-serine dehydratase
STY4161	4020211	*yhjY*	C	T	ACG	ATG	T	M	Membrane/surface structures	putative membrane protein

STY4314	4192687	*gph*	C	T	GCG	GTG	A	V	Degradation of small molecules	phosphoglycolate phosphatase
STY4318	4196909	*bigA*	G	A	CCA	TCA	P	S	Pseudogenes	putative surface-exposed virulence protein (pseudogene)
STY4392	4253640	*dprA*	G	A	GCT	ACT	A	T	Conserved hypothetical proteins	Putative DNA protecting protein
STY4805	4665891		G	A	GCG	GTG	A	V	Regulators	arginine deiminase
STY4915	4775254	*yjjV*	C	A	CCG	CAG	P	Q	Conserved hypothetical proteins	TatD DNase family protein

These analyses suggest that there was a cascade of events that corresponded with the genesis of the first H58 *S.* Typhi. The cumulative mutations signified by the observed long branch length is uncommon in *S*. Typhi and has two feasible explanations. The first is that the progenitor organism was a hyper mutator, and that a key mutation in *mutS* was responsible for generating a large amount of genetic diversity in a short time frame.[209] However, no such informative SNPs were observed in the early H58 isolates, although any such mutations may have reverted. The second, and more likely explanation, is that the organism was in an environment that created an atypical selective pressure to induce mutations that facilitated its ability become exposed to, and then accept, an MDR plasmid. Previous data on *S*. Typhi carriage in the gallbladder determined that this environment creates an atypical selective pressure and stimulates mutations in metabolism and outer membrane structures.[208,210] This genetic variation was associated with organisms being located on signature long branches; observations here are comparable. Our data suggest that H58 *S.* Typhi became successful due to its early ability to accept and stabilise a large MDR plasmid, which may have occurred whilst in the gallbladder; this one-off event and onward transmission then created this successful lineage. Therefore, we speculate that gallbladder carriage acts as a niche for generation of new variants with both modest (single SNPs) and large (plasmid acquisition) events capable of generating new lineages of *S*. Typhi with a selective advantage.

5.4. Implications of these findings

Our phylodynamic analysis of the origins of H58 *S*. Typhi has important consequences for how we understand the emergence and spread of new drug-resistant variants and can help inform optimal use of typhoid conjugate vaccines (TCVs). Our data suggest that whilst rare, these events can happen given the specific selective pressures, allowing MDR organisms to arise and spread rapidly. This observation comes at a critical time in global typhoid control. Two TCVs have been prequalified by the World Health Organization, with additional candidates in late-stage clinical development, and promising clinical efficacy and effectiveness data.[95,124] The continued spread of drug-resistant H58 genotypes is clearly a major argument for the use of TCV, particularly as resistance to all oral antimicrobials has been reported in *S*. Typhi in South Asia.[32–34,75] We continue to observe a pattern in which drug-resistant variants emerge in South Asia and spread radially, with H58 being the only major genotype to do so to date. [45,52–54,58,63] H58 *S*. Typhi was first isolated in Kenya shortly after its estimated emergence in 1988, further illustrating the potential for rapid spread of this lineage.[54] This suggests that prioritization of widespread use of TCVs in South Asia can not only prevent significant morbidity in the region but should limit the continued emergence and spread of drug-resistant organisms elsewhere, which could expand the useful lifespan of existing therapeutic options in some parts of the world until TCVs become more widely available.

Our data strongly suggest that H58 *S.* Typhi, which is highly associated with being MDR and decreased fluoroquinolone susceptibility, is highly adept at acquiring and maintaining drug resistance determinants, which is likely facilitated by unique mutations that occurred in the earliest H58 organism. The rapid international dissemination of the H58 lineage, starting in

South Asia and spreading throughout Southeast Asia,[45,152] into Africa[45,53–55,208,211,212] and more recently, Latin America,[213] suggest that expanded genomic surveillance is warranted to monitor its continued global spread. Such information can also help inform the development of transmission dynamics models that predict the spread of newer drug-resistant variants, like XDR, which can also inform TCV introduction decision-making.

We hypothesise that H58 *S*. Typhi may have emerged in India from a chronic carrier, which is supported by the indicative long branch length between early H58 organisms and its nearest non-H58 neighbours. This deduction is consistent with observations from previous studies conducted in Nepal and Kenya, in which higher mean branch lengths were observed in carriage isolates as compared to isolates from symptomatic patients;[208,210] this phenomenon is to be expected, assuming that chronic carriers will have had a longer time from acquisition of infection to shedding and sampling. Notably, considering the structure of the phylogenetic tree and the loss of the early non-MDR H58 organisms from the population, it is likely that the MDR phenotype was the main catalysing factor for the success of this lineage. Additionally, we observed non-synonymous mutations in genes associated with outer membrane structures, metabolism and virulence, which we have been observed previously in organisms isolated directly from the gallbladder.[208,210] Our analysis also supports previous transcriptomic analysis showing that H58 *S*. Typhi has higher bile tolerance relative to other laboratory strains (Ty2 and CT18) and show increased virulence in the presence of bile, thereby increasing the potential of H58 organisms to colonize and persist in the gallbladder. These observations suggest that gallbladder is the ideal location for the generation of variants and highlights the potential for chronic carriage to lead to the emergence of novel *S*. Typhi (and other invasive *Salmonella*) that are

genetically predisposed to express new phenotypes, which may include drug resistance. Therefore, emphasis should be placed upon the prospective identification and treatment of chronic carriers to prevent the emergence of new variants with the ability to spread. Contemporary data from returning travellers to the UK suggest that 1.4% of those infected with *S*. Typhi are chronic carriers, and 0.7% are carrying MDR *S*. Typhi.[78] A comparable frequency of carriage (1.1%) has been observed among children aged 16 years and younger in Mukuru, an informal settlement close to Nairobi, Kenya.[208] This prevalence rate is likely to be higher in older age groups,[6,214,215] and among people living in settings where typhoid is hyperendemic, and thus, may present a more substantial risk in terms of sustained transmission of drug-resistant *S*. Typhi and the potential emergence of additional drug-resistant variants. Scalable, low-cost assays to detect carriers will become vital if we aim to eliminate typhoid and prevent future resurgence.

We suggest that H58 *S*. Typhi likely emerged from a chronic carrier in India in 1987. The prototype organism of the successful clonal expansion was already MDR and became highly successful across South Asia in over a period of <10 years. Ultimately, sustained use of, and exposure to, fluoroquinolones led to selective mutations in *gyrA* on many independent occasions. The dominance of this organism and its ability to maintain AMR genes has latterly meant it has become resistant to additional antimicrobials. Our work represents a blueprint of how such organisms can arise and become dominant, but also provides the justification and evidence for the introduction of new interventions for disease control; if we reduce disease burden by vaccination, we will additionally reduce the likelihood of comparable events occurring in other *S*. Typhi organisms and other pathogens. Widespread vaccine deployment, as well as screen and treat programs, can not only impact AMR directly

through the prevention of drug-resistant infections, but also indirectly, as reduced transmission leads to decreased selection pressure on account of lower bacterial replication, and potentially decreased antimicrobial use following the prevention of clinical disease warranting treatment.

Chapter 6: Twenty-one Years of Typhoid Genomics – a global genomic update paper

6.1 Abstract

Working closely with collaborators from the LSHTM, the University of Melbourne, Oxford Big Data Institute, and the University of Cambridge, I established a Global Typhoid Genomics Consortium, which was established to promote sharing of genomic data and standardised metadata, and to facilitate its analysis for maximal public health impact and is described in greater detail in Chapter 8. One of the first major undertakings of the consortium was to generate a global genomic update paper to provide an updated view of global genome diversity and AMR patterns over time. This chapter provides an overview of that work and includes analysis of the largest *S*. Typhi genomic dataset to date. This work has been conducted in close collaboration with other consortium coordinators from the above-listed institutions as well as many international collaborators who have contributed data and given feedback on data visualisations and analyses. In addition to establishing the consortium, which now has almost 200 members, I identified members of each group to have sequenced an *S*. Typhi genome and invited them to join the consortium, developed a memorandum of understanding and code of ethics to establish principles of data use and authorship, created a standardised metadata template (bit.ly/typhiMeta) and asked data contributors to populate it, generated several summary analyses and visualisations using R, and drafted most of the global update paper described in this chapter. Other global collaborators generated the WGS data and provided standardised metadata, and colleagues from LSHTM generated phylogenetic trees and generated the R markdown file from which all analyses may be repeated and uploaded figures, tables, and code to github (https://github.com/typhoidgenomics/TyphoidGenomicsConsortiumWG1). These results

have been preprinted in *medRxiv* (Carey et al 2022) and the manuscript is under full review at eLife.

6.2 Introduction

We are at a pivotal stage in the history of typhoid control, and there is potential for typhoid endemic LMICs to deploy preventative interventions, leading to large reductions in disease incidence. Wider access to clean water and improved sanitation have led to a major reduction in global incidence of typhoid fever, which has also been reflected in declining incidence of many enteric diseases. This process needs to continue but will require sustained investment from national and local governments and thus remains a long-term objective. In the short to medium term, widespread use of TCVs can help to further reduce global incidence of typhoid fever.[80] The WHO has recommended their use in routine immunization and catch-up campaigns in endemic countries, specifically highlighting their utility in settings where a high prevalence of AMR typhoid fever has been reported.[21] Notably in Pakistan and Zimbabwe, both reactive TCV campaigns and national vaccine introduction were motivated by drug-resistant *S*. Typhi outbreaks in major urban centres, highlighting that the case for prevention can be stronger when treatment options are limited.[67,216] Additional support is likely required to inform TCV decision-making in other typhoid endemic countries, particularly where burden and AMR data are scarce and other competing immunization priorities must be considered. Given the lack of geographically representative blood culture surveillance data and the difficulties associated with establishing and sustaining such surveillance, it is important to fully leverage available surveillance data. Pathogen sequencing is a key tool that facilitates extracting data on the

emergence and spread of AMR variants, which can be informative in decision-making around TCV introduction.

In 2001, the first completed whole genome sequence of *S*. Typhi was published.[42] This multi-drug resistant organisms, CT18, was isolated from a typhoid fever patient in the Mekong Delta region of Viet Nam in 1993, and generating this data required two years' of work assembling plasmid-cloned paired-end sequence reads generated by Sanger capillary sequencing. Together with other early bacterial pathogen genomes, including a second Typhi genome (Ty2) that was published two years later in 2003,[217] the CT18 genome constituted a major turning point in the potential for disease control, treatment, and diagnostics, forming the basis for new insights into comparative and functional genomics,[218,219] and facilitating early genotyping efforts.[48,220] When high-throughput sequencing technologies 454 and Solexa (subsequently Illumina) emerged, *S*. Typhi was an obvious first target for in-depth characterisation of a single pathogen population,[221] and genomics has been increasingly exploited to describe the population structure and global expansion of this highly clonal pathogen.[45] Now, WGS is becoming a more routine component of typhoid surveillance. *Salmonella* were amongst the first pathogens to transition to routine sequencing by public health laboratories in high-income countries,[78] and these systems often capture *S*. Typhi isolated from travel-associated typhoid cases, providing an informal mechanism for sentinel genomic surveillance of pathogen populations in typhoid endemic countries.[55] More recently, WGS has been adopted for typhoid surveillance by national reference laboratories in endemic countries including the Philippines,[222,223] and PulseNet International is gradually transitioning to WGS.[224] Following the first global genomic snapshot study, which included nearly 2000 genomes of *S*. Typhi

isolated from numerous typhoid burden studies across Asia and Africa,[45] WGS has become the standard tool for characterising clinical isolates. Given the very high concordance between susceptibility to clinically relevant drugs and known genetic determinants of AMR in *S*. Typhi,[43,52,78] WGS is also increasingly relied on to infer resistance patterns.

As described in previous chapters, the increasing prevalence of AMR poses a major threat to effective typhoid fever control. Given the wealth of existing and emerging WGS data for *S*. Typhi, we aimed to create a system to enhance visibility and accessibility of genomic data to inform current and future disease control strategies, including controlling the emergence and spread of AMR, identifying where empirical therapy may need review, and monitoring the impact of vaccines on AMR and potential vaccine escape. In establishing the Global Typhoid Genomics Consortium (https://www.typhoidgenomics.org/), we aimed to engage with the wider typhoid research community to aggregate *S*. Typhi genomic data and standardized metadata to facilitate the extraction of relevant insights to inform public health policy through inclusive, repeatable analysis using freely available and accessible pipelines and intuitive data visualization. Here, I present a large, geographically representative dataset of thirteen thousand *S*. Typhi genomes and provide a contemporary snapshot of the global genetic diversity in *S*. Typhi and its spectrum of AMR determinants. The establishment of the Global Typhoid Genomics Consortium marks twenty-one years of typhoid genomics and provides a platform for future typhoid genomics activities, which we hope will inform more sophisticated disease control. It has also provided a basis for the new AMRnet project (http://www.amrnet.org), which I am collaborating on as a postdoctoral research policy fellow at LSHTM.

6.3 Results

6.3.1 Overview of available data

A total of 13,000 high quality *S*. Typhi genomes were collated, from 65 studies and five unpublished datasets provided by public health laboratories. The distribution of samples by region is shown in **Table 8**, with country breakdown in **Figure 22**. The majority originated from Southern Asia (n=8,231), specifically India (n=2,705), Bangladesh (n=2,268), Pakistan (n=1,810) and Nepal (n=1,436). A total of n=1,140 originated from South-eastern Asia, with >100 each from Cambodia (n=279), Vietnam (n=224), the Philippines (n=209), Indonesia (n=145), and Laos (n=139). Overall, 1,106 genomes originated from Eastern Africa, including >100 each from Malawi (n=569), Kenya (n=254), Zimbabwe (n=110). Other regions of Africa were less well represented, with n=384 from Western Africa, n=317 from Southern Africa, n=59 from Middle Africa (so-named in the M49 region definitions, although more commonly referred to as Central Africa), and n=41 from Northern Africa

Table 8. Regional distribution of genomes included in this study.[1]

UN World Region	Total genomes	[1]Untargeted illness sample, since 2010	[2]Travel (%) amongst untargeted illness samples, since 2010
Australia and New Zealand	57	57	0 (0%)
Caribbean	20	20	20 (100%)
Central America	103	100	100 (100%)
Eastern Africa	1,143	854	49 (5.7%)
Eastern Asia	12	3	3 (100%)
Eastern Europe	3	1	1 (100%)
Melanesia	232	37	30 (81.1%)
Micronesia	4	1	1 (100%)
Middle Africa	59	21	6 (28.6%)
Northern Africa	41	6	6 (100%)
Northern America	167	140	2 (1.4%)
Northern Europe	109	105	0 (0%)
Polynesia	324	262	45 (17.2%)
South America	367	105	5 (4.8%)
South-eastern Asia	1,140	584	72 (12.3%)
Southern Africa	317	286	2 (0.7%)
Southern Asia	8,231	6,623	1,878 (28.4%)
Southern Europe	10	6	6 (100%)
Western Africa	384	267	34 (12.7%)
Western Asia	47	21	21 (100%)
Western Europe	7	3	3 (100%)
Unknown	225	0	0
Total	12,965	9,478	2,284 (24.1%)

[1] Genomes associated with assumed acute typhoid cases, isolated from 2010 onwards from non-targeted sampling frames; this is the subset of data used to generate genotype prevalence distributions shown in **Figures 23 & 24**. Genomes recorded as travel-associated and with known travel to a specific country in this region, associated with assumed acute typhoid isolated from 2010 onwards from non-targeted sampling frames. Countries were assigned to world regions based on the United Nations (UN) Statistics Division standard M49.

Figure 23. Total number of genome sequences per country and year.

Heatmap shows number of genomes per country per year incorporated into this analysis. Coloured squares indicate the number of genome sequences per country (y) per annum (x) as per the inset legend.

In total, there were 36 countries with ≥20 genomes (total n=12,409 genomes, 95.7%) and 20 countries with ≥100 genomes (n=11,761 genomes, 90.7%). Countries with the most genomes available (n≥100 each) were mainly those where local burden studies have utilised WGS for isolate characterisation (India, Bangladesh, Nepal, Pakistan, Cambodia, Laos, Kenya, Malawi, Zimbabwe, Ghana, Nigeria, Chile, Samoa); plus, South Africa, the Philippines, United Kingdom and United States, where S. Typhi isolates are sequenced as part of national surveillance programmes. [34,35,52,53,55,68,72,78,208,212,213,216,225–228]

The genome collection includes n=3,381 isolates recorded as travel-associated, contributed mainly by public health reference laboratories in England (n=1,740), USA (n=749), Australia (n=490), New Zealand (n=144), France (n=116) and Japan (n=104). The most common countries of origin for travel-associated isolates were India (n=1,241), Pakistan (n=783), Bangladesh (n=264), Fiji (n=102), Samoa (n=87), Mexico (n=60), Chile (n=49), Papua New Guinea (n=45), Nigeria (n=42) and Nepal (n=39). For some typhoid-endemic countries, the majority of genome data originated from travel-associated cases captured in other countries; those in this category with total n≥10 genomes are Guatemala (n=22/22), El Salvador (n=19/19), Mexico (n=60/61), Peru (n=14/14), Haiti (n=12/12), Morocco (n=12/13), Iraq (n=19/19), Malaysia (n=35/35), Fiji (n=102/144) and Papua New Guinea (n=45/86).

In total, n=10,726 genomes were assumed to represent acute typhoid fever and recorded as derived from ‘non-targeted’ sampling frames, i.e., local surveillance studies or reference laboratory-based national surveillance programs that could be considered representative of a given time (year of isolation) and geography (country and region of origin) (see **Chapter**

2.5.5 for definitions). The majority of these isolates (9,478, 88.4%) originate from 2010 onwards, hence genotype and AMR prevalence rates are estimated for this period.

6.3.2 Geographic distribution of genotypes

The breakdown of genotype prevalence rates by world region, for genomes isolated from 2010 onwards, is shown in **Figure 23a** and annual breakdown of genotype prevalence by region is given in **Figure 23b**. (Regional denominators may be found in **Table 8**). Notably, while our data confirm that H58 genotypes dominate in Asia and Eastern and Southern Africa, they were virtually absent from other parts of Africa, from South and Central America, and from Polynesia and Melanesia. Instead, each of these regions was dominated by their own local genotypes. Typhoid fever is no longer endemic in Northern America, Europe, or Australia/New Zealand. The genotype distributions shown for these regions were estimated from *S*. Typhi that were isolated locally but not recorded as being travel-associated; nevertheless, these genomes can be assumed to result from limited local transmission of travel-associated infections, and thus to reflect the diversity of travel destinations for individuals living in those regions. Annual national genotype prevalence rates for well-sampled countries (with N≥50 representative genomes each) with endemic typhoid are shown in **Figure 23b**, and annual national genotype prevalence rates for less well sampled countries (with N<50 genomes) are shown in **Figure 24**. Below, we summarise notable features of the global genotype distribution, by region.

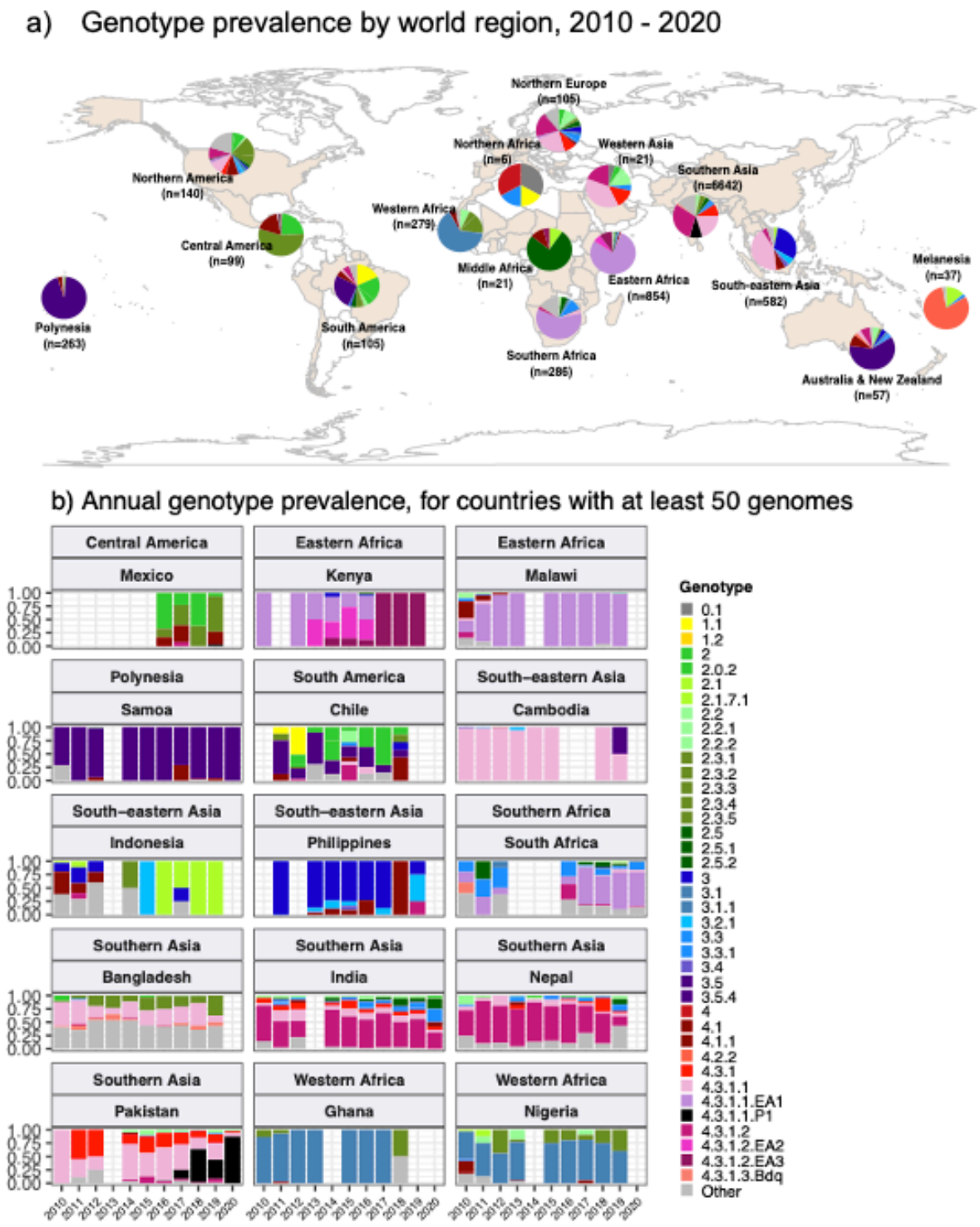


Figure 23. Annual breakdown of genotypes per world region, 2010-2020.

Annual genotype distribution by world region for regions with ≥20 representative genomes. Bars show genotypes observed per annum, coloured as per inset legend. Genotypes present at ≥20% frequency in any country are indicated separately, rare genotypes are aggregated as 'Other'.

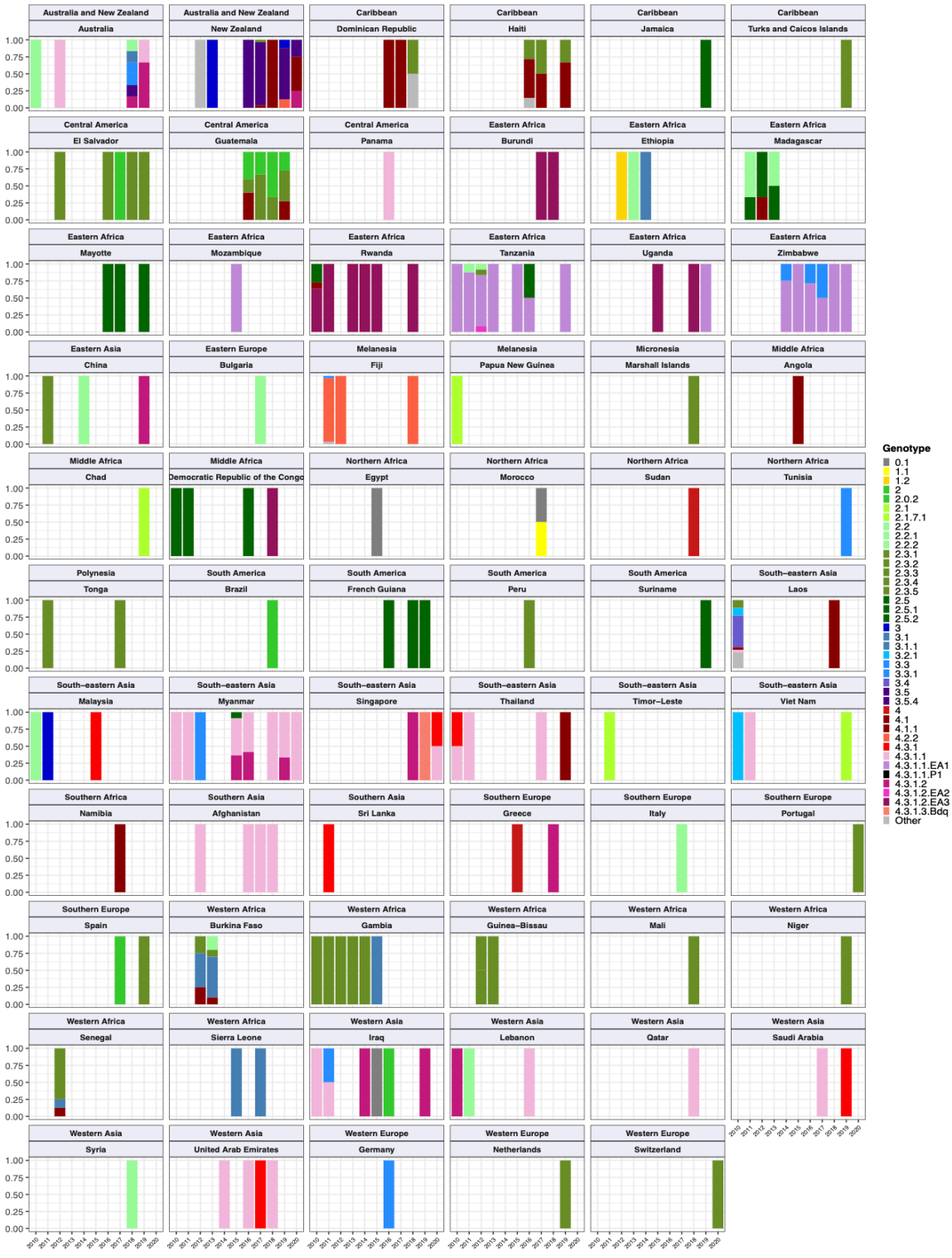


Figure 24. Annual breakdown of genotypes per country.

Annual genotype distribution for countries with <50 representative genomes between 2010-2020 (plots for countries with ≥50 genomes are shown in Figure 23b). Bars show genotypes observed per annum, coloured as per inset legend. Genotypes present at ≥20% frequency in any country are indicated separately, rare genotypes are aggregated as 'Other'

6.3.2.1 Southern Asia

Southern Asia was the most represented region, with 6,623 genomes suitable for prevalence analysis. The genotype distribution confirms the widely reported finding that the H58 lineage (4.3.1 and derived genotypes) is the dominant form of *S*. Typhi in Southern Asia, where it is thought to have originated[45,52] (overall prevalence, 70.4%; see **Figure 23a**). Notably though, the distribution of H58 genotypes was different between countries in the region (see **Figure 23b**), and in Bangladesh it was associated with a minority of genomes (42% [n=670/1,591], compared with 73% in India [n=1,655/2,267], 74% in Nepal [n=941/1,275], and 94% in Pakistan [n=1,390/1,484]). India and Nepal were dominated by sublineage 2 (genotype 4.3.1.2 and derived genotypes; 54% [n=1,214/2,267] and 57% [n=736/1,275], respectively), which was rare in Bangladesh (0.6%; n=9/1,591) and Pakistan (3.2%; n=47/1,484). In India, H58 lineage 1 (4.3.1.1) was also present at appreciable frequency (12%; n=268/2,267) as was 4.3.1 (i.e. H58 that does not belong to any of the defined sublineages 4.3.1.1-3; 7.4% [n=168/2,267]). In Nepal, 4.3.1 was present at 12% frequency (n=152/1,275) and 4.3.1.1 at just 4.9% (n=63/1,275).

In Pakistan, lineage 1 (genotype 4.3.1.1 and derived genotypes) was most common (73%; 1,089/1.484), with the XDR sublineage (genotype 4.3.1.1.P1) appearing in 2016[24,67] and rapidly rising to dominance (87% in 2020 [n=27/31]; see **Figure 23b**). Pakistan also had prevalent 4.3.1 (17%; [n=254/1,484]). H58 lineage 1 (4.3.1.1) was the single most common genotype in Bangladesh; however, it constituted only 34% of the total pathogen population (n=546/1,591). Bangladesh has its own H58 lineage 3 (4.3.1.3), which had a total prevalence of 8%; only two isolates of 4.3.1 and nine isolates of 4.3.1.2 were detected. Non-H58 genotypes were also evident in South Asia, with the greatest diversity evident in Bangladesh (see **Figure 23b**). Those exceeding 5% in any one country were: 3.3.2 (5.8% in Bangladesh

[n=93/1,591], 12.9% in Nepal [n=164/1,275]), 2.5 in India (8.4%; n=190/2,267), 3.3 in India (6.6%; n=150/2,267), 2.3.3 in Bangladesh (17.2%; n=274/1,591), and 3.2.2 in Bangladesh (6.6%; n=264/1,591).Annual prevalence estimates were stable over the past decade, except for the 4.3.1.1.P1 in Pakistan, which emerged in 2016 and became dominant shortly thereafter (see **Figure 23b**).

6.3.2.2 South-eastern and Western Asia

In Southeast Asia, H58 accounted for 47.3% (95% CI, 43.2-51.3%) in aggregate (mostly lineage 1, 43.0% of total genomes). However, the picture was quite mixed (see **Figures 23b and 24**), with H58 accounting for nearly all isolates in Cambodia (98%; n=216/221; all lineage 1), Myanmar (94%; n=46/49; mixed lineages) and Singapore (n=4/4, mixed lineages), but largely absent from Indonesia (3%; n=2/65), Laos (n=1/27, 4%) and the Philippines (n=1/205, 0.5%). These latter countries showed distinct populations with multiple genotypes exceeding 5% frequency: 4.1 (26%), 3 (18%), 2.1 (15%) and 3.1.2 (12%) in Indonesia; 3.4 (44%), 3.5.2 (15%), 2.3.4 (11%), 3.2.1 (11%) and 4.1 (7%) in Laos; 3 (79%), 3.2.1 (11%) and 4.1 (7%) in the Philippines.[229]

Data from Western Asia was limited to a small number of travel-associated cases (total n=21, from Iraq, Lebanon, Qatar, Saudi Arabia, Syria, and the United Arab Emirates) but was mostly H58 (n=71%; n=15/21) with 38% lineage 1 (n=8/21) and 19% lineage 2 (n=4/21).

6.3.2.3 Africa

Only 1,410 (15%) of the 9,478 genomes from untargeted sampling frames in 2010-2020 were isolated from residents in or travellers to Africa. There is significant

underrepresentation from this continent with high endemicity and varying epidemiology across subregions. Our aggregated data confirmed that H58 was the dominant cause of typhoid in Eastern Africa during the study period (93.3% H58 [95% CI, 91.5-95.0%] 774/830; see **Figure 23a**). It was recently shown that H58 in Kenya derive from three separate introductions of H58 into the region, which are now assigned their own genotypes (4.3.1.1.EA1, 4.3.1.2.EA2, 4.3.1.2.EA3).[208] At the regional level, 4.3.1.1.EA1 dominated (**Figure 23b**). However, there were country-level differences, with 4.3.1.1.EA1 dominating in Malawi (94%; n=524/558), Tanzania (83%; n=15/18), Zimbabwe (80%; n=20/25) and earlier years in Kenya (59%, n=86/145 in 2012-2016), and 4.3.1.2.EA3 dominating in Rwanda (85%, n=20/25) and Uganda (97%, n=35/36) (**Figure 23b, 24**). Although the specific periods of sampling differ for these countries, the rate of H58 was consistently high across the available time frames for all countries, with no change in dominant genotypes except for in Kenya (see **Figure 23b, 24**; note the apparent shift to 4.3.1.2.EA3 in Kenya is based on n=4 isolates only so requires confirmation).

The Southern African region was represented by South Africa, where the majority of data came from 2017-2020 (92%; n=262/285), via routine sequencing at the National Institute of Communicable Diseases reference laboratory. H58 prevalence in South Africa was high (69.5%, [95%CI, 63.9-75.1%]) during this period (mostly 4.3.1.1.EA1, 64.1%), but was much lower (25% [95% CI, 4-46%]) among the smaller sampling of earlier years (n=4/16 for 2010-2012) (see **Figure 23b**).

In Western Africa, the most common genotypes were 3.1.1 (64.4%, [95% CI, 58.7-70.2%]; n=172/266) and 2.3.2 (13.9%, [95% CI, 9.7-18.0%] n=37/266) (**Figure 23a**). Most of these

data come from the Typhoid Fever Surveillance in Africa Programme (TSAP) genomics report[212] and a study of typhoid in Abuja and Kano in Nigeria,[230] which showed that in the period 2010-2013, 3.1.1 dominated in Nigeria and nearby Ghana and Burkina Faso whereas 2.3.2 dominated in The Gambia and neighbouring Senegal and Guinea Bissau.[212] Here, we found that additional data from travel cases and recent Nigerian national surveillance suggest that these patterns reflect long-established and persisting populations (see **Figure 24**): 3.1.1 was detected from Benin (2002-2009; n=4/4), Burkina Faso (2006-2013; n=11/17), Cote d'Ivoire (2006-2008; n=4/4), The Gambia (2015; n=2/28), Ghana (2007-2017; n=93/109), Guinea (2009; n=1/2), Mali (2008; n=1/5), Mauritania (2009; n=1/2), Nigeria (2008-2019; n=122/192), Sierra Leone (2015-2017; n=2/2) and Togo (2004-2006; n=2/3); and 2.3.2 from Burkina Faso (2012-2013; n=2/17), The Gambia (2008-2014; n=25/28), Ghana (2010-2018; n=9/109), Guinea Bissau (2012-2013; n=2/3), Mali (1999-2018; n=3/5), Niger (1990-1999; n=2/4), Nigeria (1984-2002; n=4/192), Senegal (2012; n=6/10), and Togo (2001; n=1/3).

Limited genome data (n=19) was available for the Middle Africa region (also known as Central Africa) (**Table 8**). Genomes from Democratic Republic of the Congo (DRC) comprised n=17 genotype 2.5.1 (16 isolated locally, plus one from USA CDC) and a single 4.3.1.2.EA3 isolate (from the UK reference lab). Two genomes each were available from Angola (both 4.1.1, via UK) and Chad (both 2.1, via France). Northern Africa was similarly poorly represented, with one isolate from Egypt (0.1, via UK), two from Morocco (0.1, via UK and 1.1, via USA), two from Sudan (genotype 4, via UK) and one from Tunisia (3.3, from UK).

6.3.2.4 The Americas

Notably, Central American isolates were dominated by 2.3.2 (55%, [95% CI, 45.2-64.8%] n= 55/100), which was also common in Western Africa (13.9%, [95% CI, 9.7-18.0%]; n=37/266) (**Figure 23a**). Little has been reported about *S*. Typhi populations from this region previously, and the genomes collated here were almost exclusively novel and contributed via the US CDC and isolated between 2016 and 2019. They mainly originated from El Salvador (n=19, 2012-2019, 89% 2.3.2), Guatemala (n=22, 2016-2019, 41% 2.3.2) and Mexico (n=58, 2011-2019, 50% 2.3.2). Prior to 2010, genotype 2.3.2 was also identified in isolates from Mexico referred to the French reference lab in 1972 (representing a large national outbreak) and 1998. The maximum-likelihood phylogeny for 2.3.2 included several discrete clades from different geographical regions in West Africa and the Americas (see **Figure 25**), consistent with occasional continental transfers between these regions followed by local clonal expansions. Three clades were dominated by West African isolates (one with isolates from West Coast countries, and two smaller clades from Nigeria and neighbouring countries); two clades of South American isolates (from Chile, Argentina, and Peru); one small clade of Caribbean (mainly Haiti) and USA isolates; and one large clade of Central American isolates (from Mexico, Guatemala and El Salvador) (see **Figure 25).** Other common genotypes identified in Central America were 2.0.2 (overall prevalence 23%; 32% in Guatemala, 25% in Mexico, 11% in El Salvador,) and 4.1 (17%; 23% in Guatemala, 21% in Mexico, not detected from El Salvador) (see **Figure 24**).

Genomes in neighbouring South America (n=105) were more diverse, with no dominant genotype accounting for a majority of cases in the 2010-2020 period (**Figure 23a**). This mainly reflects the diversity of genotypes present in Chile (see **Figure 23b**), as recently

reported in a large study from Santiago (n=97).[227] However, this diversity of genotypes was also reflected in the smaller datasets from Argentina, and traveller isolates from Peru. Genotypes with ≥5% prevalence in the region were 3.5 (27%; n=28/105), 1.1 (18%; n=19/105), 2 (18%; n=19/105), 1.2.1 (5.7%; n=6/105) and 2.0.2 (5.7%; n=6/105). WGS data recently reported by Colombia's Instituto Nacional de Salud[231] were not included in the regional prevalence estimates as they covered only a subset (5%) of surveillance isolates that were selected to maximise diversity, rather than to be representative. However, only four genotypes were detected in the Colombia study (1.1, 2, 2.5, 3.5), and two-thirds of isolates sequenced were genotype 2.5 (67%; n=51/77); 3.5 was also common, at 25% (n=20/77). Similarly, all five isolates from French Guiana (sequenced via the French reference laboratory) were genotype 2.5, consistent with limited diversity and a preponderance of this genotype in the north of the continent.

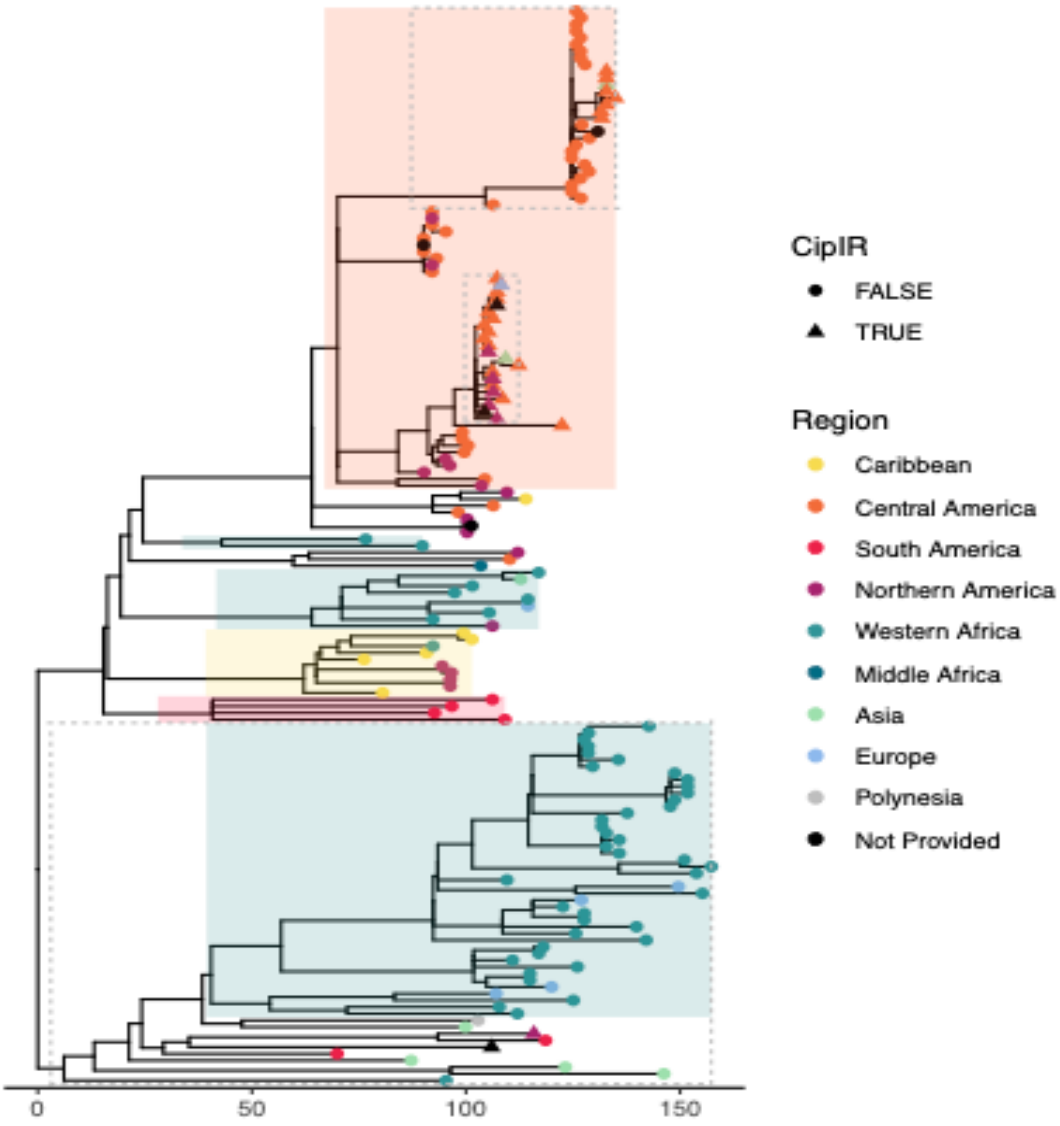


Figure 25. Maximum likelihood phylogeny for genotype 2.3.2.

Maximum likelihood phylogenetic tree showing relationships amongst all available genotype 2.3.2 genomes (n=161), inferred using Gubbins and RaxML. The tree was outgroup rooted using representative genomes of the other defined genotypes; scale bar is presented at the bottom, units are substitutions. Tips are coloured by world region, according to inset legend; triangles indicate genomes harbouring QRDR mutations resulting in predicted nonsusceptibility to ciprofloxacin (CipIR). Clades representing local clonal expansions are shaded; subtrees subjected to dating analyses are highlighted with dashed boxes.

6.3.2.5 Pacific Islands

In Melanesia and Polynesia, each island has their own dominant genotype (Figure 24a): 4.2 and its derivatives in Fiji (n=31/32, 97% of all Fiji genomes), 2.1.7 in Papua New Guinea (n=5/5 in post-2010 genomes, 100%, consistent with the longer-term trend),[232] and 3.5.4 in Samoa (n=249/259, 96%).[35]

6.3.3 Global distribution of AMR

We estimated the regional (**Figure 26**) and national (**Figure 27**) prevalence of clinically relevant AMR profiles for the period 2010-2020, inferred from WGS data from non-targeted sampling frames for which country of origin and year of isolation could be determined (see **Chapter 2.5.6**). To understand the potential implications of these AMR prevalence rates for local empirical therapy, we categorised them according to a traffic light-style system, whereby amber colours signal emerging resistance of potential concern (<10%), and red colours signal levels of resistance that may warrant reconsideration of empirical antimicrobial use (>10%; see **Figure 26, 27**). The regional view highlights that CipNS is widespread, whereas CipR, AziR, and XDR are mostly restricted to Southern Asia, and MDR is most prevalent in African regions and to a lesser degree in Asia (**Figure 26**). National estimates for countries with sufficient data (≥50 representative genomes available for the period 2010-2020, see **Figure 27**) indicate that MDR remains a very significant problem across all well-sampled African countries ((39% in Nigeria, 61% in South Africa, 66% in Ghana, 78% in Kenya, 93% in Malawi), but is much more variable in Asia (3% in India [n=67/2,267] and Nepal [n=36/1,275], 25% in Bangladesh [n=393/1,591], 68% in Pakistan [n=1,004/1,484], 76% in Cambodia [n=167/221]) and essentially absent from Indonesia (n=0), the Philippines (n=0), Samoa (n=0), Mexico (n=1, 1.7%) and Chile (n=0). The

underlying genotypes are shown in **Figure 29** and highlight that MDR in Asian and Eastern African countries is mostly associated with H58 (i.e., 4.3.1 and derived genotypes) but in West African is associated with the dominant genotype in the region, 3.1.1. In contrast, ciprofloxacin non-susceptibility was associated with more diverse *S*. Typhi genotypes in each country, including essentially all common genotypes in Southern Asian countries (**Figure 29**). National annual prevalence data suggest AMR profiles were mostly quite stable over the last decade (with the notable exception of the emergence and rapid spread of XDR *S*. Typhi in Pakistan) but reveal some interesting differences between settings in terms of AMR trends and the underlying genotypes (**Figures 27 – 29**).

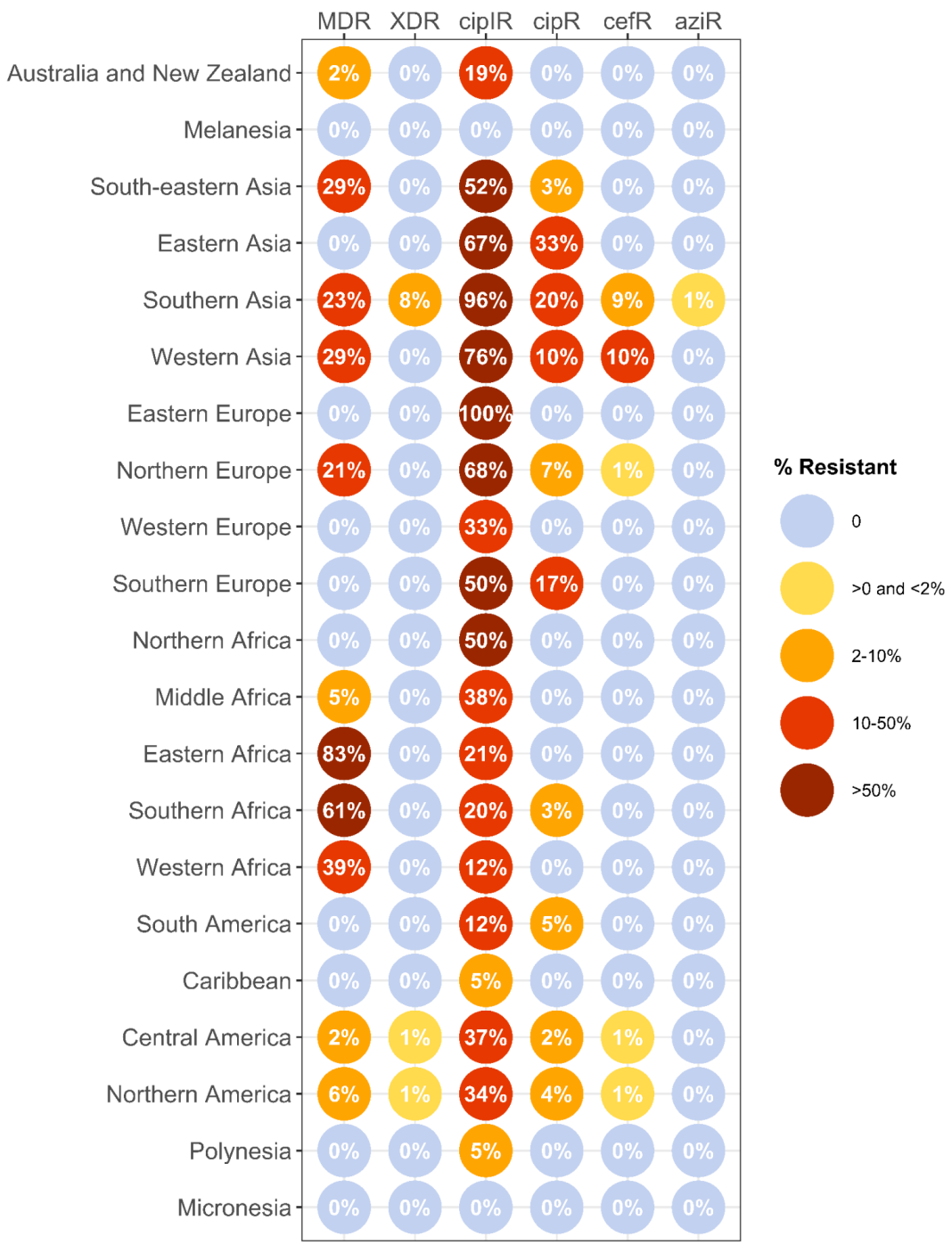


Figure 26. Prevalence of key AMR genotype profiles by world region (2010-2020).

Prevalence of key AMR profiles by world region, based on representative genomes, i.e. untargeted sampling, assumed acute cases, 2010-2020. Percentage resistance values are printed for each region/drug combination and are coloured by categorical ranges to reflect escalating levels of concern for empirical antimicrobial use: (i) 0: no resistance detected; (ii) >0 and ≤2%: resistance present but rare; (iii) 2-10%: emerging resistance; (iv) 10-50%: resistance common; (v) >50%: established resistance.

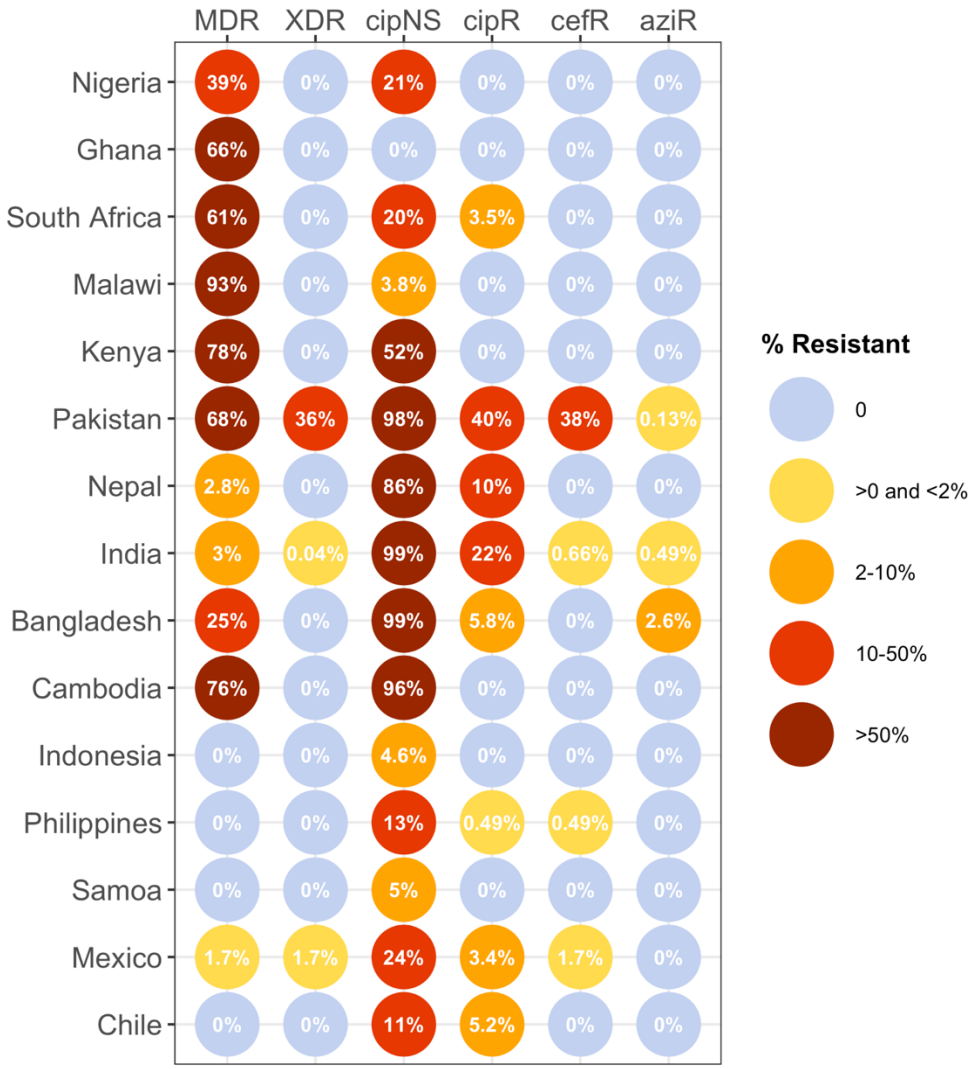


Figure 27. Prevalence of key AMR genotype profiles by country, (2010-2020).

Prevalence of key AMR genotype profiles for all countries with at ≥50 representative genomes (untargeted, assumed acute cases) from 2010-2020. Percentage resistance values are printed for each country/drug combination and are coloured by categorical ranges to reflect escalating levels of concern for empirical antimicrobial use: (i) 0: no resistance detected; (ii) >0 and ≤2%: resistance present but rare; (iii) 2-10%: emerging resistance; (iv) 10-50%: resistance common; (v) >50%: established resistance. Annual rates underlying these summary rates are shown in Figure 29

6.3.3.1 Ciprofloxacin non-susceptibility (CipNS)

In Bangladesh and India, ciprofloxacin non-susceptibility (CipNS) has been near-universal since at least 2005 (91-100% prevalence in all years with N≥10 samples since 2005, see **Figure 28**). This was associated mainly with GyrA-S83F (79% prevalence in Bangladesh, 70% in India) and GyrA-S83Y mutations (9.2% prevalence in Bangladesh, 26% in India), which were detected across diverse genotype backgrounds (see **Figure 30**); in total, CipNS variants were present in 30 genotype backgrounds in India (out of n=34 genotypes, 88%) and 17 in Bangladesh (out of n=21 genotypes, 81%). In neighbouring Nepal, CipNS prevalence has stabilised in the 85-95% range since 2011 (70% GyrA-S83F, 12% GyrA-S83Y; CipNS in 12 genotype backgrounds). The persistence of ciprofloxacin-susceptible Typhi in Nepal was largely associated with genotype 3.3.2, which maintained annual prevalence of 3-10% (mean 5.8%) throughout 2010-2018, rising to 39% in 2019. In Pakistan, CipNS has exceeded 95% since 2012 (**Figure 28**), across n=14/17 genotypes (**Figure 29**). Sustained high prevalence of CipNS was also evident in Cambodia (4.3.1.1 with GyrA-S83F). In contrast, CipNS has been relatively rare in African countries, but has been increasing in recent years, especially in Kenya (from 20% in 2012 to 65% in 2016, $p=3x10^{-9}$ using proportion trend test) and Nigeria (from 8% in 2013 to 80% in 2019, $p=7x10^{-6}$; see **Figure 28**). CipNS in these settings was associated with QRDR mutations in the locally dominant genotypes, specifically GyrA-S83F (15% of 4.3.1.1.EA1), GyrA-S83Y (100% of 4.3.1.2.EA3) and GyrA-S464F in Kenya (100% of 4.3.1.2.EA2), and GyrA-S83Y (27% of 3.1.1) in Nigeria (see **Figure 30**).

6.3.3.2 Ciprofloxacin resistance (CipR)

Ciprofloxacin resistance emerges in a stepwise manner in *S*. Typhi, through acquisition of additional QRDR mutations and/or PMQR genes in strains already carrying a QRDR mutation. CipR genomes were common (≥10%) in Pakistan, India, and Nepal, and emerging (3-6%) in Bangladesh, South Africa, Chile and Mexico (**Figure 27**). A total of 26 distinct CipR genotypes (comprising unique combinations of *S*. Typhi genotype, QRDR mutations and/or PMQR genes) were identified, of which five were found in appreciable numbers (>5 genomes each, see **Figure 30**). The XDR strain 4.3.1.1.P1 (carrying GyrA-S83F + qnrS) was first identified in Pakistan in 2016,[24,233] and here accounted for 75% of *S*. Typhi genomes from Pakistan in 2020 and a dramatic rise in CipR prevalence (**Figure 28**). This genotype was only detected three times without a known origin in Pakistan (one isolate each in India, Mexico, and USA). The CipR strain 4.3.1.3.Bdq (carrying GyrA-S83F and *qnrS*) emerged in Bangladesh in ~1989[52] and here accounted for 95% of CipR genomes in this country. 4.3.1.3.Bdq genomes were also detected in India (n=4), Singapore (n=1) and South Africa (n=1). The other major CipR genotypes were the QRDR triple-mutant 4.3.1.2.1, its derivative 4.3.1.2.1.1 (which also carries plasmid-borne *qnrB*), and a QRDR triple-mutant sublineage of 3.3. These three CipR variants were most common in India, where we estimated consistently high CipR prevalence (19-27% per year) from 2014 onwards (**Figure 28**), associated with 15 unique CipR genotypes (**Figure 29**). Most Indian CipR genomes belong to 4.3.1.2.1 (92.3%). CipR 4.3.1.2.1 was also found in 12 other countries, most notably Nepal (accounting for 95% of CipR genomes), where it has been shown to have been introduced from India and result in treatment failure;[61] Pakistan (accounting for 6.6% of CipR genomes); Myanmar (accounting for n=17/17 CipR genomes); and Chile (accounting for n=5/5 CipR

genomes) (see **Figure 29**). The 3.3 QRDR triple-mutant accounted for 3.8% of CipR genomes in India and was also found in neighbouring Nepal (n=4, 3% of CipR). CipR genomes were identified from Zimbabwe (4.3.1.1.EA1 with gyrA S83F + *qnrS*, associated with recent CipR outbreaks)[211] and South Africa (five different genotypes, totaling 3.5%; see **Figure 30**), but were otherwise absent from African *S*. Typhi genomes.

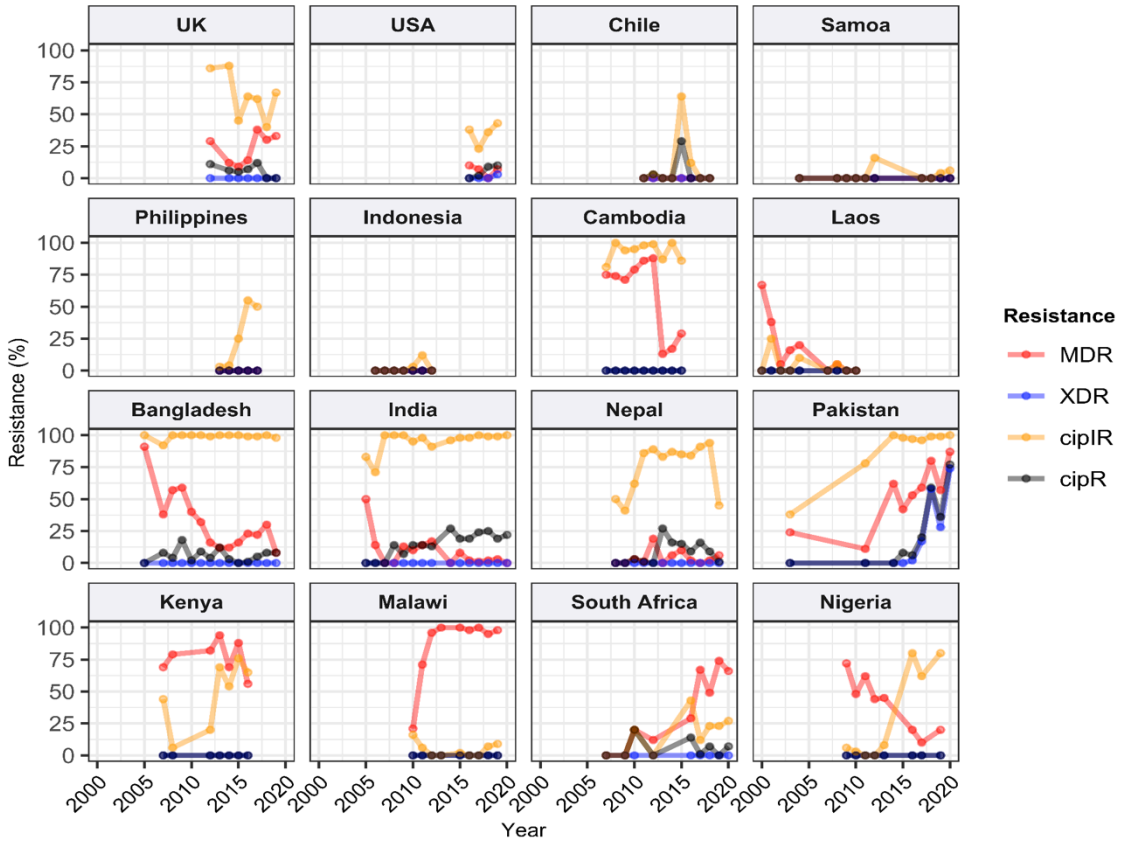


Figure 28. Frequency of key AMR profiles by country, 2000-2020.

Frequency of key AMR profiles inferred from WGS data, for countries with ≥3 years with ≥10 representative genomes (untargeted, assumed acute cases) from 2000-2020. Data is shown only for country/year combinations with N≥5 isolates. MDR, multidrug resistant; XDR, extensively drug resistant; cipIR, ciprofloxacin non-susceptible; cipR, ciprofloxacin resistant.

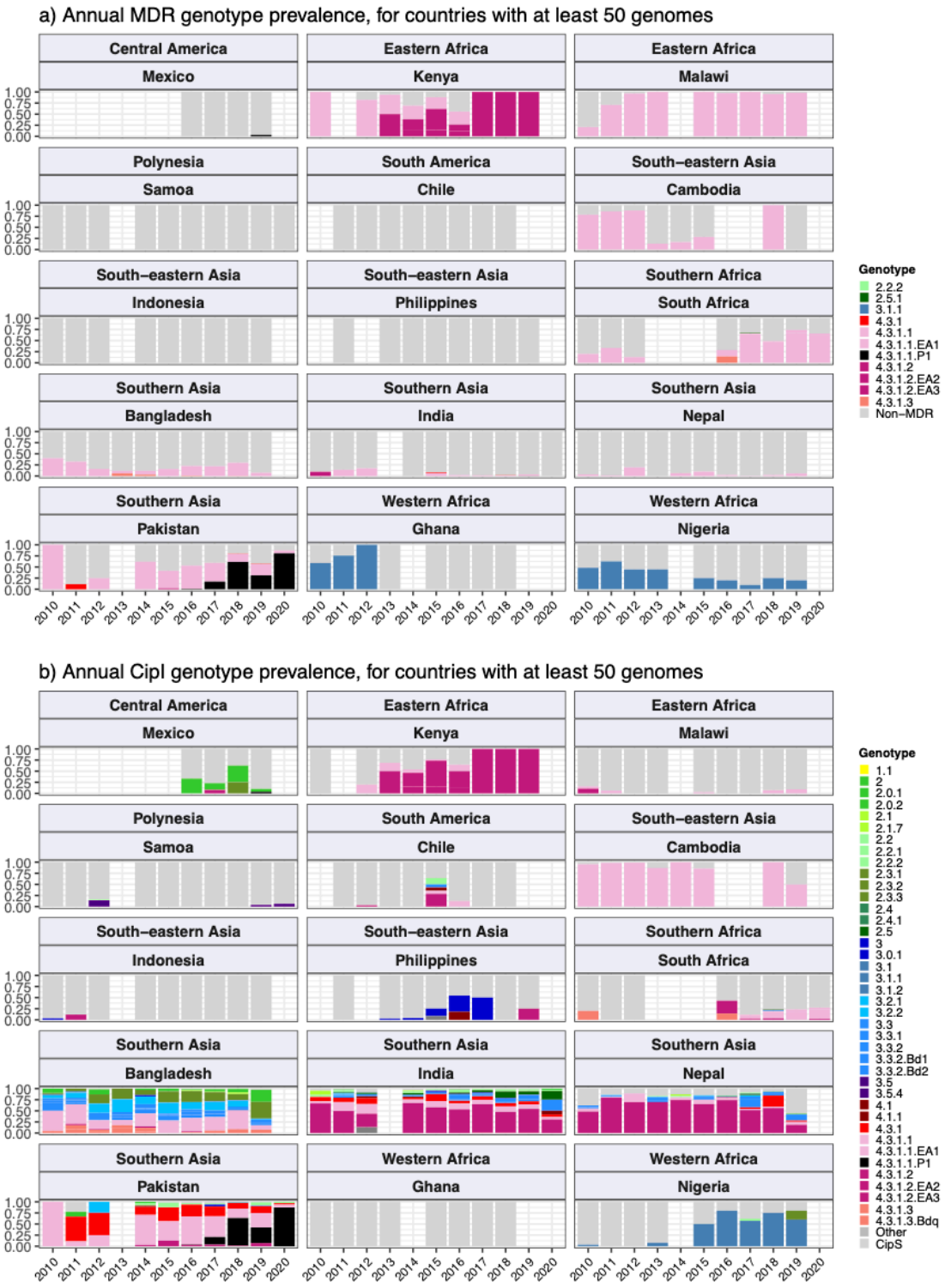


Figure 29. Annual genotype prevalence amongst MDR and CipNS/R genomes, 2010-2020.

Annual genotype prevalence amongst (a) MDR and (b) CipNS/R genomes, for countries with ≥50 representative genomes between 2010-2020. Genotypes for (a) MDR and (b) CipNS/R genomes are coloured according to the inset legends; sensitive genomes of all genotypes are aggregated and coloured grey.

Figure 30. Distribution of fluoroquinolone resistance determinants by genotype by country.

Distribution of fluoroquinolone resistance determinants by genotype for selected countries discussed in text. Node size indicates total number of isolates for a given combination of genotype (row) and determinant (column); nodes are coloured to indicate the frequency of the determinant within that genotype. Wt = wildtype; i.e. no quinolone resistance determining mutations were detected in gyrA or parC and no plasmid-borne quinolone resistance (qnr) genes were detected.

6.3.3.3 Multidrug resistant (MDR)

Prevalence of MDR (co-resistance to ampicillin, chloramphenicol and co-trimoxazole) has declined in India ($p=2x10^{-9}$ using proportion trend test) to 2% (0-3% per year, 2016-2020), and is similarly rare in Nepal (mean 5% in 2011-2019) (see **Figure 27**). MDR prevalence has also declined in Bangladesh (p=2x10-4 using proportion trend test) but remains high enough to discourage deployment of older first-line drugs, with prevalence exceeding 20% in most years (see **Figure 28**). In Pakistan, the emergence of the XDR strain 4.3.1.1.P1 has driven up MDR prevalence dramatically ($p=4x10^{-11}$ using proportion trend test), to 87% in 2020. MDR prevalence has remained high in Kenya and Malawi since the arrival of MDR H58 strains (estimated in the late 1990s),[208] but has declined steadily in Nigeria, from 72% in 2010 to 10% in 2017 (p=0.0003 using proportion trend test; see **Figure 29**). We hypothesised that this difference in MDR trends may be linked to the fitness cost imposed by carriage of the large IncHI1 MDR plasmid[234] (present in all MDR isolates in Nigeria, which were genotype 3.1.1), a burden not associated with chromosomally integrated MDR (100% of MDR in Malawi, 19% in Kenya, all H58 genotypes). To explore this, we examined the relationship between the proportion of MDR isolates that carry the IncHI1 plasmid vs trends in MDR prevalence, across countries with appreciable MDR levels (total 7 countries with ≥5% MDR prevalence; see **Figure 31**). Two countries showed a significant rise in MDR prevalence (Pakistan, $p=3x10^{-11}$; South Africa, $p=9x10^{-8}$); in both cases, this rise coincided with loss of IncHI1 plasmids (see **Figure 31**) and presumably migration of MDR to the chromosome (as has been clearly shown in XDR 4.3.1.1.P1 strains in Pakistan).[24] Cambodia followed a similar pattern to Nigeria, whereby all MDR organisms belonged to the same genotype (4.3.1.1 in Cambodia, 3.1.1 in Nigeria) and carried the IncHI1 plasmid, and MDR

prevalence has declined over time in each case (see **Figure 31**). As noted above, MDR was maintained at high levels in Kenya and Malawi, where the IncHI1 plasmid frequency was either in decline (Kenya) or entirely absent (Malawi; see **Figure 31**). Notably, Bangladesh showed a significant decline in total MDR prevalence (p=0.0002), and in MDR prevalence within the dominant genotype 4.3.1.1 (p=0.049), despite the majority of MDR (and all MDR within 4.3.1.1) being chromosomal rather than plasmid associated.[57]

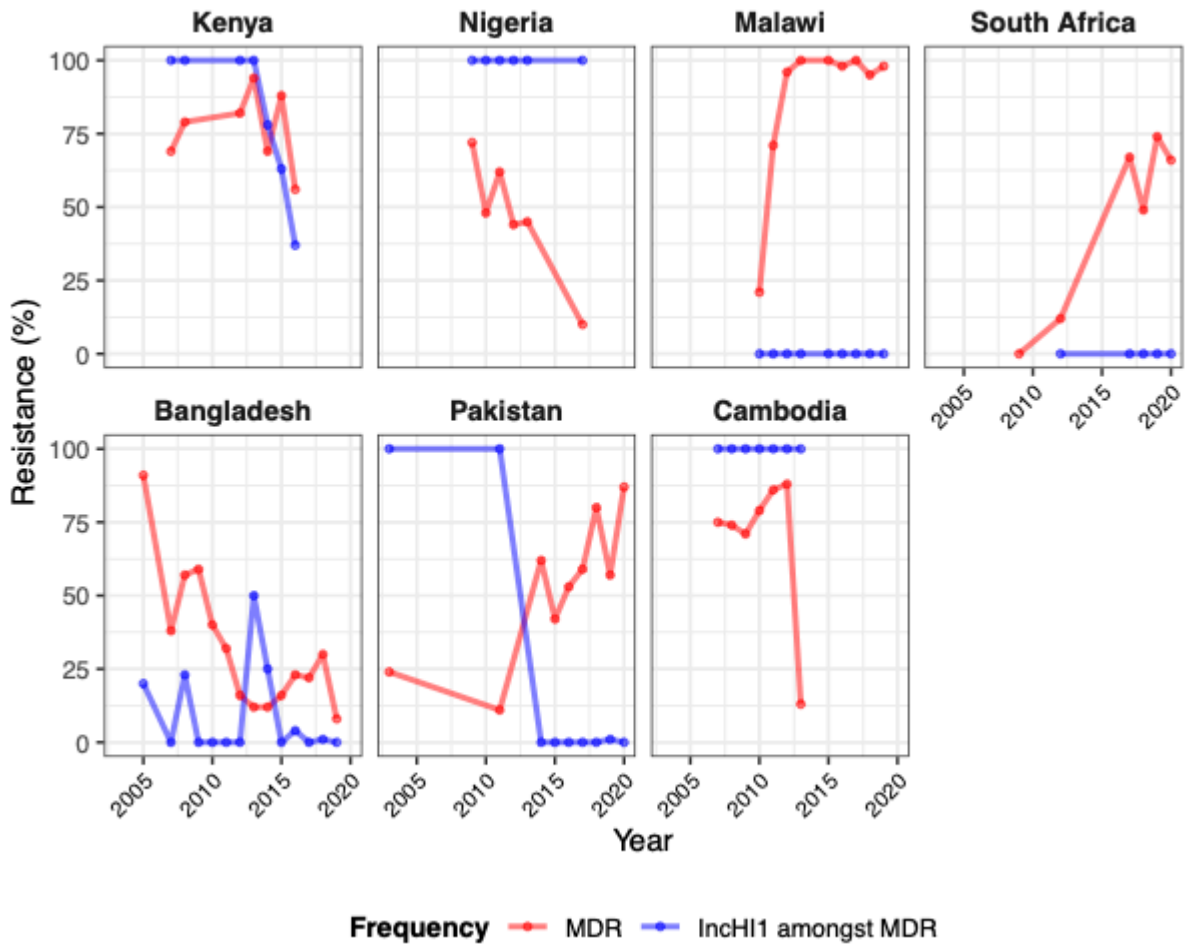


Figure 31. Trends in annual MDR frequency and proportion of MDR explained by prevalence of IncHI1 plasmids, 2000-2020.

Trends in annual MDR frequency (red) and proportion of MDR explained by prevalence of IncH1 plasmids (blue). In Pakistan and South Africa, MDR prevalence is increasing dramatically, which coincides with a decline of prevalence of IncH1 plasmids among MDR isolates, likely due to integration of MDR genes into the chromosome. MDR prevalence remained high in Kenya and Malawi, where IncH1 prevalence was either in decline or absent.

6.3.3.4 Extensively drug-resistant (XDR)

The XDR 4.3.1.1.P1 sublineage (that is, MDR with additional resistance to fluoroquinolones and third-generation cephalosporins including ceftriaxone) was recognised as emerging in late 2016 in Sindh Province, where it caused an outbreak of XDR typhoid that has since spread throughout Pakistan.[24,68,233] Here, we identified the genome of strain Rwp1-PK1 (assembly accession NIFP01000000), isolated from Rawalpindi in July 2015, as genotype 4.3.1.1.P1. Rwp1-PK1 was isolated from a 17-year-old male with symptomatic typhoid whose infection did not resolve following ceftriaxone treatment and was found to be phenotypically XDR (resistant to ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, ceftriaxone).[235] The isolate was later sequenced and reported as carrying *bla*CTX-M-15, *bla*TEM-1, *qnrS1* and GyrA-S83F,[236] but was not genotyped nor included in comparative genomics analyses investigating the emergence of XDR in Pakistan, so has not previously been recognised as belonging to the 4.3.1.1.P1 XDR sublineage. We found that the Rwp1-PK1 genome carries the 4.3.1.1.P1 marker SNV, clusters with the 4.3.1.1.P1 sublineage in a core-genome tree (**Figure 32**) and shares the full set of AMR determinants typical of 4.3.1.1.P1, indicating that this XDR strain was present in northern Pakistan for at least a full year before it was reported as causing outbreaks in the southern province of Sindh.

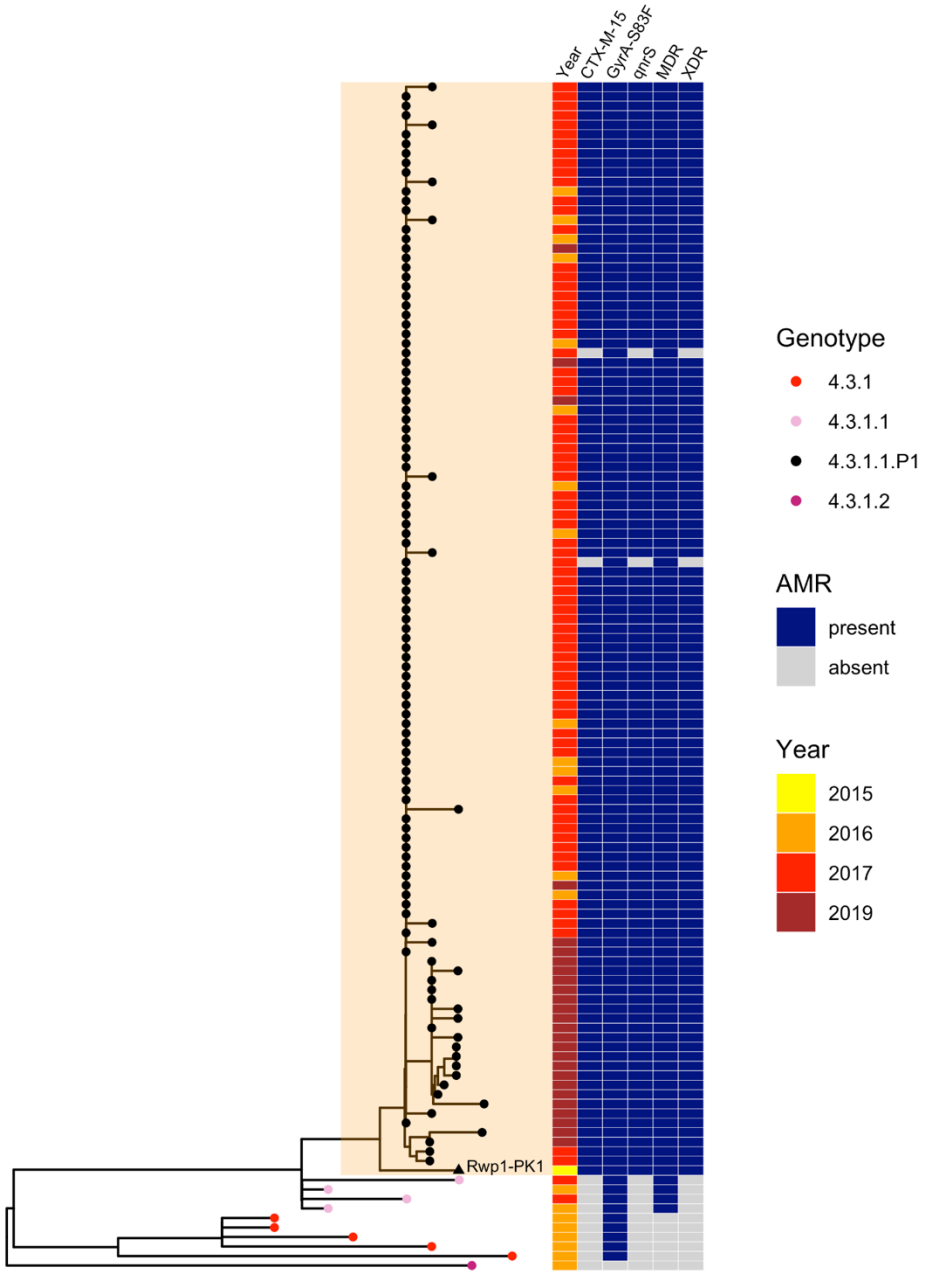


Figure 32. Phylogenetic tree showing position of 2015 Rwalpindi isolate, Rwp1-PK1, in context with other genomes from Pakistan.

Core-genome distance-based neighbour-joining tree generated in Pathogenwatch, using all genomes from (Klemm et al., 2018) (the first genomic characterisation of the XDR outbreak clade, including outbreak strains and local context strains from Sindh Province in 2016-2017) and (Rasheed et al., 2020) (genomics report of XDR outbreak strains from Lahore in 2019). Tree tips are coloured by genotype, according to inset legend; the 2015 strain Rwp1-PK1 is labelled in the tree and indicated with a triangle. Year of isolation and presence of AMR determinants are indicated in the heatmap, according to inset legend.

6.3.3.5 Ceftriaxone resistant (CefR)

There was no evidence for establishment of the lineage 4.3.1.1.P1 or other XDR lineages outside of Pakistan; however, ESBL genes were identified in an additional 33 genomes, belonging to 8 other genotypes (**Table 9**). Several of these carried a *bla*$_{CTX\text{-}M\text{-}15}$ IncY plasmid similar to that of 4.3.1.1.P1. These include instances with no other acquired AMR genes in plasmid or chromosome (genotype 3 in Philippines; genotype 4.3.1.2 in Iraq); one instance with chromosomally integrated AMR genes plus IncY plasmid-borne *bla*$_{CTX\text{-}M\text{-}15}$ (genotype 2.5.1 in DRC); and instances with a 4.3.1.1.P1-like profile carrying *qnrS* in the IncY plasmid and the MDR locus in the chromosome (n=4 4.3.1, India and Pakistan; n=1 4.3.1.1, Pakistan; see **Table 9**). Notably *bla*$_{CTX\text{-}M\text{-}15}$ IncY plasmids were rare (n=1 to 4 genomes) in all strain backgrounds except 4.3.1.1.P1 (total n=654), suggesting that the IncY *bla*$_{CTX\text{-}M\text{-}15}$ plasmid has not been stably maintained in other *S*. Typhi lineages (see **Table 9**). IncY plasmids were also identified in a single genotype 2.3.3 isolated in the UK in 1989 associated with travel to Pakistan (carrying *catA1, tetA(B)*); and in a sublineage of IncHI1-negative 3.1.1 genomes from Nigeria carrying *bla*$_{TEM\text{-}1D}$, *dfrA14*, *sul2*, *tetA(A)*, as has been recently reported elsewhere.[225] Other examples of ESBL carriage appear to represent isolated events (1 or 2 genomes per ESBL/plasmid or ESBL/genotype combination, see **Table 9**), except for a sublineage of 4.3.1.2 from India carrying *bla*$_{SHV\text{-}12}$ in a IncX3 plasmid backbone. Concerningly, the plasmid also carries *qnrB* and is present in a QRDR triple-mutant strain background (*gyrA*-S83F, *gyrA*-D87N, *parC*-S80I), resulting in a combination of ciprofloxacin resistance and third-generation cephalosporin resistance. This group comprised 15 isolates from Mumbai (2015-2018), plus three additional isolates from travellers returning to England, Australia and the USA from India (2018-2020). [71,72,78,140] This strain therefore

appears to have originated in Mumbai and persisted there since at least 2015 for at least six years, but our data do not suggest onward spread out of Maharashtra or India.

Table 9. Extended spectrum beta-lactamase (ESBL) genes detected in *S*. Typhi genomes.[2]

Genotype	ESBL	3GCR	Country of Origin	n	Years	Other plasmid / AMR markers
2.5.1	CTX-M-15	Y	DRC[237]	1	2015	**IncY**[*]; *bla*$_{TEM-1}$, *dfrA7*, *sul1* (*gyrA*-S83F)
3	CTX-M-15	Y	Philippines[229]	1	2013	-
	SHV-12	Y	Philippines[238]	2	2007	**IncHI2A**[$]; *bla*$_{TEM-1}$, *dfrA18, tetA*(D)
3.3	CTX-M-15	Y	UK[68]	1	2012	(*gyrA*-S83F)
3.3.2	CTX-M-15	Y	Bangladesh[56,239]	2	2000	**IncI1**[$]; *bla*$_{TEM-1}$
3.5	CTX-M-12	Y	Colombia[231]	1	2012	**IncL, IncFIB(pHCM2)**; *bla*$_{TEM-1}$, *sul1*
4.3.1	CTX-M-15	Y	India[69]	1	2019	**IncY**[$]; *qnrS, bla*$_{TEM-1}$, *dfrA14, sul2,* (*gyrA*-S83Y)
		Y/N	Pakistan[24,52]	3	2017-18	**IncY**; *qnrS, bla*$_{TEM-1}$, *sul2, catA1, dfrA7, sul1, tetA*(A) (*gyrA*-S83F)
4.3.1.1	CTX-M-15	N	Pakistan[24]	1	2016	**IncY**; *qnrS, bla*$_{TEM-1}$, *sul2, catA1, dfrA7, sul1, tetA*(A) (*gyrA*-S83F)
4.3.1.1.P1	CTX-M-15	Y[24]	India[68]	1	2019	**IncY** # *qnrS*, *bla*$_{TEM-1}$, *sul2, catA1*, *dfrA7*, *sul1* (*gyrA*-S83F)
		?	Mexico	1	2019	
		Y	Pakistan	654	2016-20	
		?	USA *§	1	2019	
	CTX-M-55	Y	Pakistan[236,240]*§	2	2015-18	
4.3.1.2	CTX-M-15	Y	Iraq[27,78]	2	2019	**IncY**; (*gyrA*-S83F)
	SHV-12	Y	India[71,72,78,179] §	18	2015-20	**IncX3**[$]; *qnrB* (*gyrA*-S83F, *gyrA*-D87N, *parC*-S80I)

[2] 'Other plasmid/AMR markers' column includes: (i) plasmid replicons (Inc types) identified in the genome (in bold); (ii) other acquired AMR genes; (iii) chromosomal AMR mutations (in brackets). $ indicates this plasmid is the reported location of the ESBL gene in the genome assembly; # n=31 4.3.1.1.P1 isolates from Pakistan lacked plasmid replicons.

6.3.3.6 Azithromycin resistance (AziR)

AziR associated mutations in *acrB* were identified in 74 genomes. The majority of *acrB* mutants were from Bangladesh (n=55, 73%), followed by India (n=11, 15%) (see **Figure 33a**), although the overall frequency of resistance was very low even in these locations (2.6% in Bangladesh, 0.5% in India). Thirteen distinct combinations of genotype and *acrB* SNP were identified, implying at least thirteen distinct events of AziR emergence; six were singleton isolates, and four were represented by 2–3 isolates each (**Figure 33b**). The three more common AziR variants all carried R717Q, in H58 lineage 1 or genotype 4.3.1.1 (n=38, mainly from Bangladesh), 3.2.2 (n=12, from Bangladesh) or H58 lineage 2 (n=7, from India). Notably half (n=7/13) of all *acrB*/genotype combinations were identified in Bangladesh (see **Figure 33b**). All *acrB* mutants also carried QRDR mutations, and eight were cipR: n=6 belong to the CipR 4.3.1.2.1 lineage in India (all carried R717Q and were isolated in 2017 in Chandigarh) and n=2 belong to the CipR 4.3.1.3.Bdq lineage (both carried R717L and were isolated in 2019, one in Singapore and one in Bangladesh).

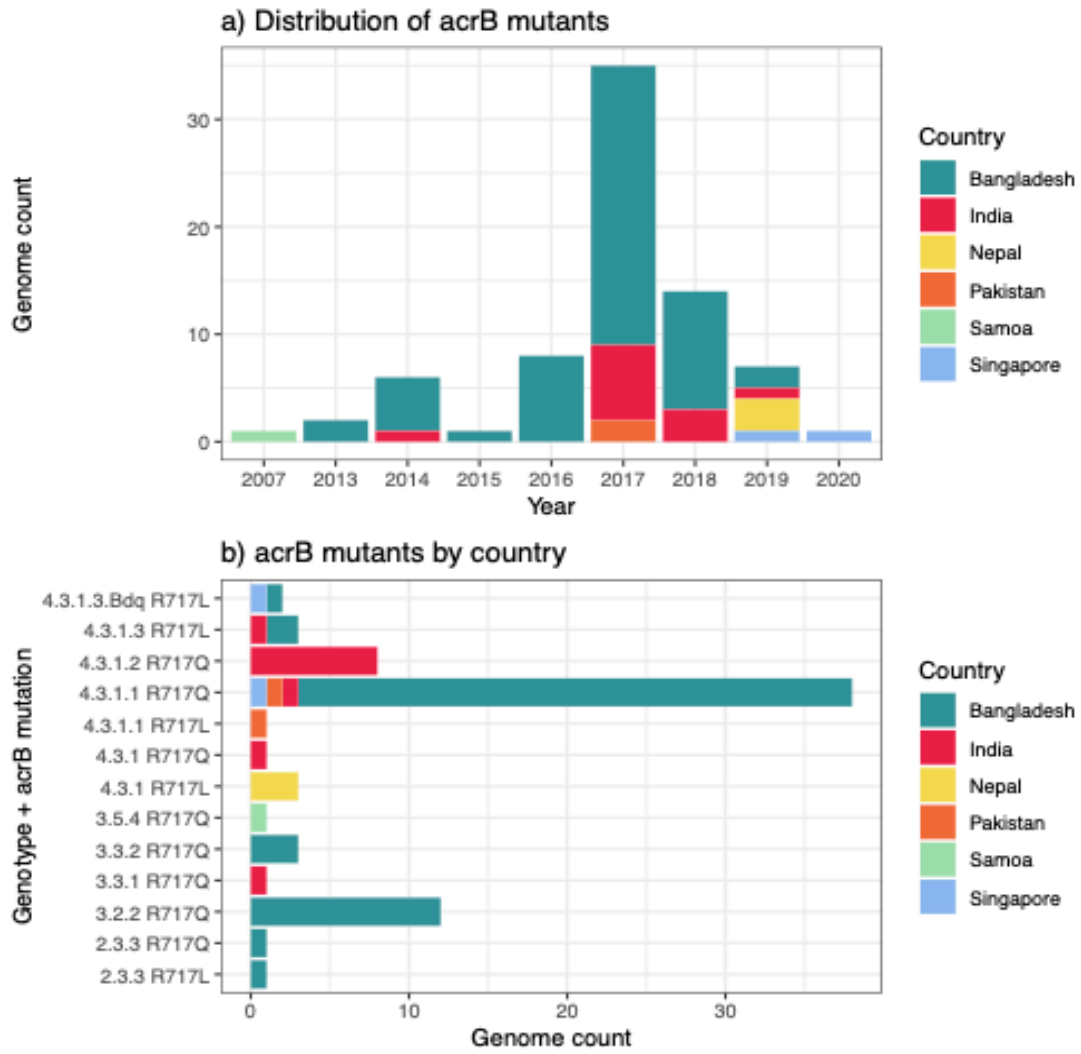


Figure 33. Distribution of azithromycin resistance-associated *acrB* mutations detected in *S*. Typhi genomes.

Distribution of *acrB* mutants by (a) year of isolation and (b) country of isolation. The first *acrB* mutant appeared in Samoa in 2007. Other mutants have appeared independently across a range of genetic backgrounds, largely in South Asian countries. Country of origin is coloured as per inset label.

6.3.4 Robustness of national estimates across studies

The estimates of genotype and AMR prevalence represented here reflect *post hoc* analyses of data that were generated for a variety of different primary purposes, by different groups, including in-country surveillance and travel-associated cases captured in other countries. To explore the robustness of these national-level estimates, we compared prevalence rates estimated from different studies/sources from the same country, where sufficient data existed to do so. South Asian countries were each represented by 2 to 4 in-country data sources plus travel-associated data collected in 3 to 4 other countries. **Figure 34** shows genotype prevalence estimates derived from these different sources (with ≥20 isolates each) and **Figure 35a** shows the annual genotype frequency distributions (for years with ≥20 isolates). In most cases (67% of genotype-source combinations), genotype prevalence rates estimated from individual source laboratories yielded 95% confidence intervals (CIs) that overlapped with those of the pooled national estimates (see **Figure 34**). The main exception was for 4.3.1.2 in India, where all data sources confirmed 4.3.1.2 was the most prevalent genotype, but the point estimates ranged from 40% to 82%, compared with the pooled estimate of 53.4% (95% CI, 50.6–56.2%),[52] and 95% CIs were frequently non-overlapping (see **Figure 34**). High prevalence of 4.3.1.2 was estimated from contributing laboratories in urban Vellore (82% [95% CI, 78-87%]), Chennai (67% [56-77%]), Bengaluru (70% [62-78%]), and Mumbai (two laboratories, estimates 74% [65-83%] and 63% [46-79%]); with lower prevalence in northern India, New Delhi (three laboratories, estimates 48% [28-68%], 40% [31-49%], 39% [22-56%) and Chandigarh (39% [33-45%]). Two Indian laboratories were clear outliers, with little or no 4.3.1.2 but very high prevalence of a different genotype: 4.3.1.1 in rural Bathalapalli (81% [67-95%]) and 2.5 in the northern city of Ludhiana (77% [66-88]).

The relative prevalence of 4.3.1.1.P1 (XDR lineage) in Pakistan vs its parent lineage 4.3.1.1 also varied between sources, however, this was clearly driven by differences in the time frames and locations relative to the emergence of 4.3.1.1.P1, which first arose in Karachi and later spread to cause outbreaks elsewhere including Lahore (sampled in 2019) (see **Figure 35**). AMR prevalence estimates were also highly concordant across data sources (see **Figure 36**) and showed strikingly similar temporal trends (**Figure 35b**).

Figure 34. Genotype prevalence estimated from different data sources, for South Asian countries.

Genotype prevalence estimates from different labs within the same country for Bangladesh, India, Pakistan, and Nepal. Data is shown only for source labs with N≥20 isolates from which to estimate prevalence. Lines show 95% confidence interval for each proportion (prevalence) estimate; solid circles highlight the pooled point estimate for national prevalence in each country. Lines are coloured by country as per the inset legend.

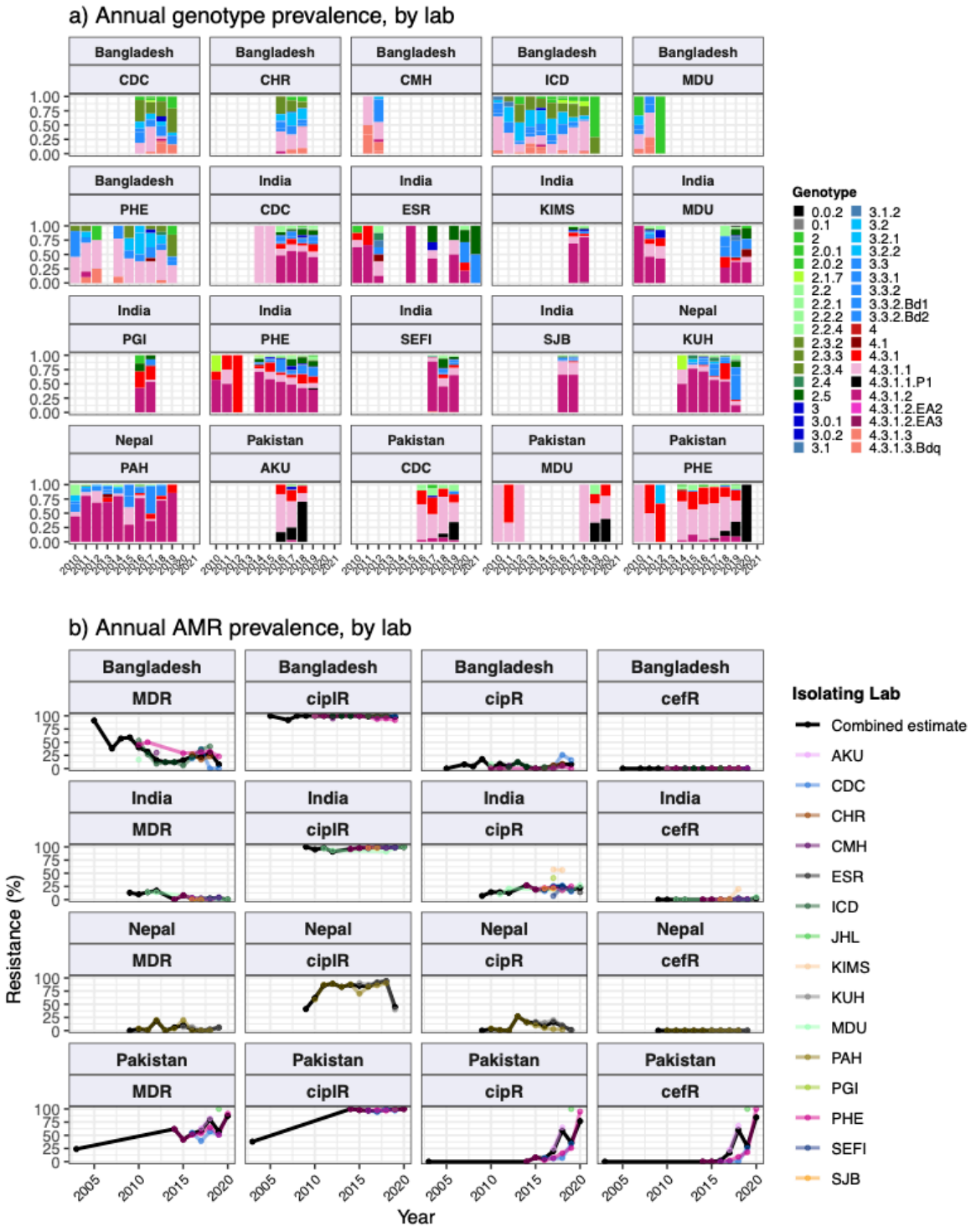


Figure 35. Annual genotype and AMR frequencies by isolating lab, for South Asian countries with multiple data sources, 2000-2020.

Annual genotype **(a)** and AMR **(b)** frequencies by isolating lab for South Asian countries. Labs shown are those with ≥20 isolates; and years shown for each lab are those with N≥5 isolates from that site. **(a)** Bars are coloured to indicate annual genotype prevalence, as per inset legend. **(b)** Lines indicate annual frequencies of key AMR profiles (MDR, cipIR, cipR, cefR), coloured by isolating laboratory as per inset legend.

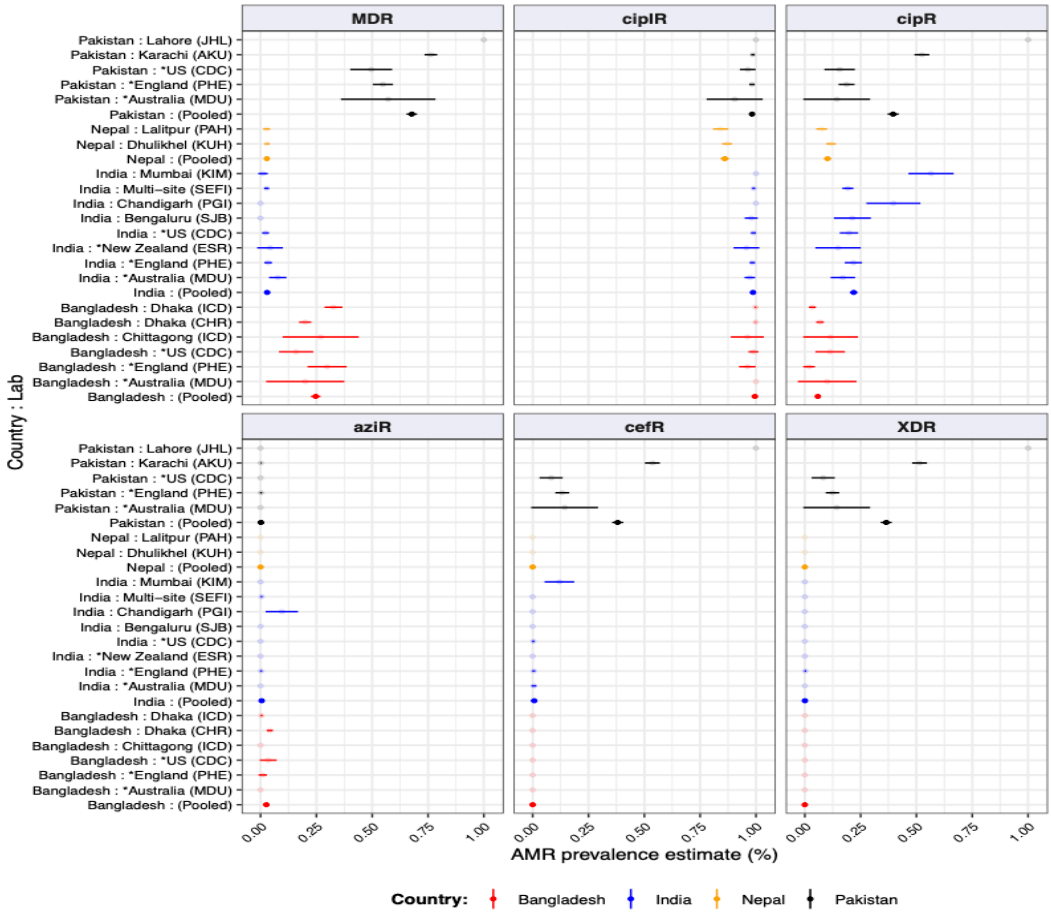


Figure 36. AMR prevalence estimated from different data sources, for South Asian countries.

AMR prevalence from different data sources within Bangladesh, Nepal, India, and Pakistan. Data is shown only for source labs with N≥20 isolates from which to estimate prevalence. Lines show 95% confidence interval for each proportion (prevalence) estimate; solid circles highlight the pooled point estimate for national prevalence in each country. Lines are coloured by country as per the inset legend.

The only other country represented by ≥10 sequenced isolates each from multiple laboratories was Nigeria; these were from Abuja (Zankli Medical Center, n=105, 2010-2013) and Ibadan (University of Ibadan, n=14, 2017-2018), and reference laboratories in England (n=15, 2015-2019) and the USA (n=10, 2016-2019) (see **Figure 37**). Genotype prevalence estimates were concordant across different sources, with single-laboratory 95% CIs overlapping with one another and with the pooled point estimate, for all five common genotypes (see **Figure 37a**). The exception was that genotype 3.1.1 accounted for all n=14/14 isolates sequenced from Ibadan but ranged from 53-70% prevalence at other laboratories and yielded a pooled national prevalence estimate of 67% [95% CI, 60-75%) (see **Figure 37a, c**). AMR prevalence estimates were more variable for Nigeria across laboratories (see **Figure 37b**), but this could be explained by their non-overlapping sampling times: Abuja data from early years (2010–2013) showed high MDR (49%) and low CipNS (4%) prevalence rates; whereas Ibadan data from later years (2017-2018) showed comparatively lower MDR (21%) and higher CipNS (79%) prevalence rates, which is consistent with contemporaneous travel data (12% MDR, 60% CipNS, from total n=25 isolated 2015-2019) (**Figure 37b**).

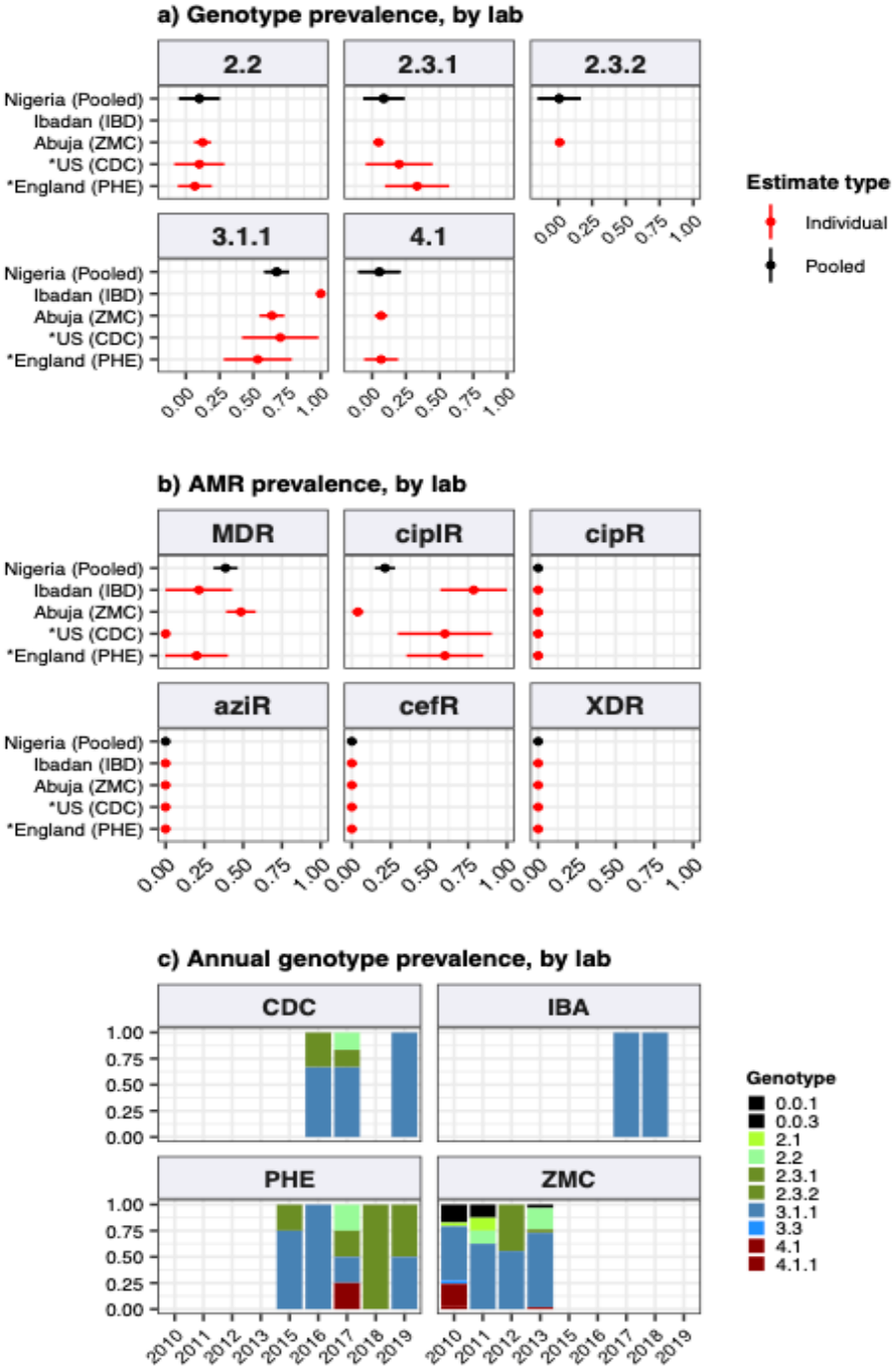


Figure 37. Genotype and AMR prevalence rate estimates for Nigeria from different data sources, 2010-2020.

Data are shown only from source labs with N≥10 isolates from which to estimate prevalence. **(a)** Genotype prevalence and **(b)** AMR prevalence, using all available isolates per lab, 2010-2020. Lines show 95% CI for each proportion (prevalence) estimate. Red indicates estimates based on data from individual labs, black indicates pooled estimates (i.e. from all labs), as per inset legend. **(c)** Annual genotype frequencies. Bars are coloured by genotype as per inset legend. Lab abbreviations are shown in y-axis labels for panels **(a-b)**.

6.4 Discussion

6.4.1 Data availability

This study presents the most comprehensive genomic snapshot of Typhi to date, with 12,965 high quality genomes originating from 111 countries in 21 world regions. The consortium model provides improved consistency and completeness of source data aggregated from 77 laboratories and 66 unique studies. Our dataset also includes 1,290 novel genomes sequenced by public health laboratories that would not otherwise have been published, including travel data from countries not previously represented in published Typhi genomics studies (e.g. El Salvador, Guatemala, Haiti, Mexico and Peru). However, it is a *post hoc* analysis of isolates that were cultured in different contexts (including routine diagnostics, as well as study settings where culture would not normally be undertaken) and sequenced for different reasons (including retrospective studies, outbreak investigations, and routine surveillance). The study therefore has important limitations, most notably the scarcity of genomic data from many countries and world regions where typhoid is believed to be endemic,[1] including Northern and Middle Africa, Western Asia, as well as Central and South America (**Figure 38**). These genomic data gaps reflect an underlying lack of routine blood culture or sustained blood-culture surveillance, and limited resources and expertise in many settings.[241] In addition, public health authorities may be disincentivized to generate, analyse, and publish genomic data; we hope that this analysis strengthens the case for data generation and sharing for public good. Substantial investments have been made in recent years to improve and expand microbiological surveillance capacity in some low- and middle-income countries, but major regional surveillance gaps remain. It is therefore important to maximise information recovery from available data sources, especially WGS, which provides data on the emergence and spread

of AMR variants. While the inference of AMR phenotype from WGS is currently highly reproducible and accurate for *S*. Typhi,[43,78] continued phenotypic antimicrobial susceptibility testing remains crucial to monitor for emerging mechanisms and to guide changes in empiric therapy.

For now, routine sequencing of travel-associated *S*. Typhi infections diagnosed in high-income countries helps to fill some molecular surveillance gaps for some regions, assuming that accurate travel history information is available and the sequence and metadata (including country of origin) are shared.[55] For example, our study included >3,000 genomes shared by public health reference laboratories in England, Australia, New Zealand, France, Japan, and the USA. These infections mostly originate in other countries, and can in principle provide informative, if informal, sentinel surveillance for pathogen populations in countries with strong travel and/or immigration links to those with routine sequencing.[140] Indeed, for some countries and regions, travel data represented most or all the available genome data (see **Table 8**). In this study, where multiple data sources were available for the same country, we found that national genotype and AMR prevalence estimates for the period 2010-2020 were largely concordant between local surveillance studies and travel-associated cases captured elsewhere (**Figures 35 & 37**) particularly when comparing contemporaneous annual prevalence estimates. This shows clearly that travel-associated *S*. Typhi isolated in low burden countries can be informative for surveillance of some high burden countries, which should serve as incentive for public health reference laboratories to share their data to the fullest extent they are able to under local regulations.

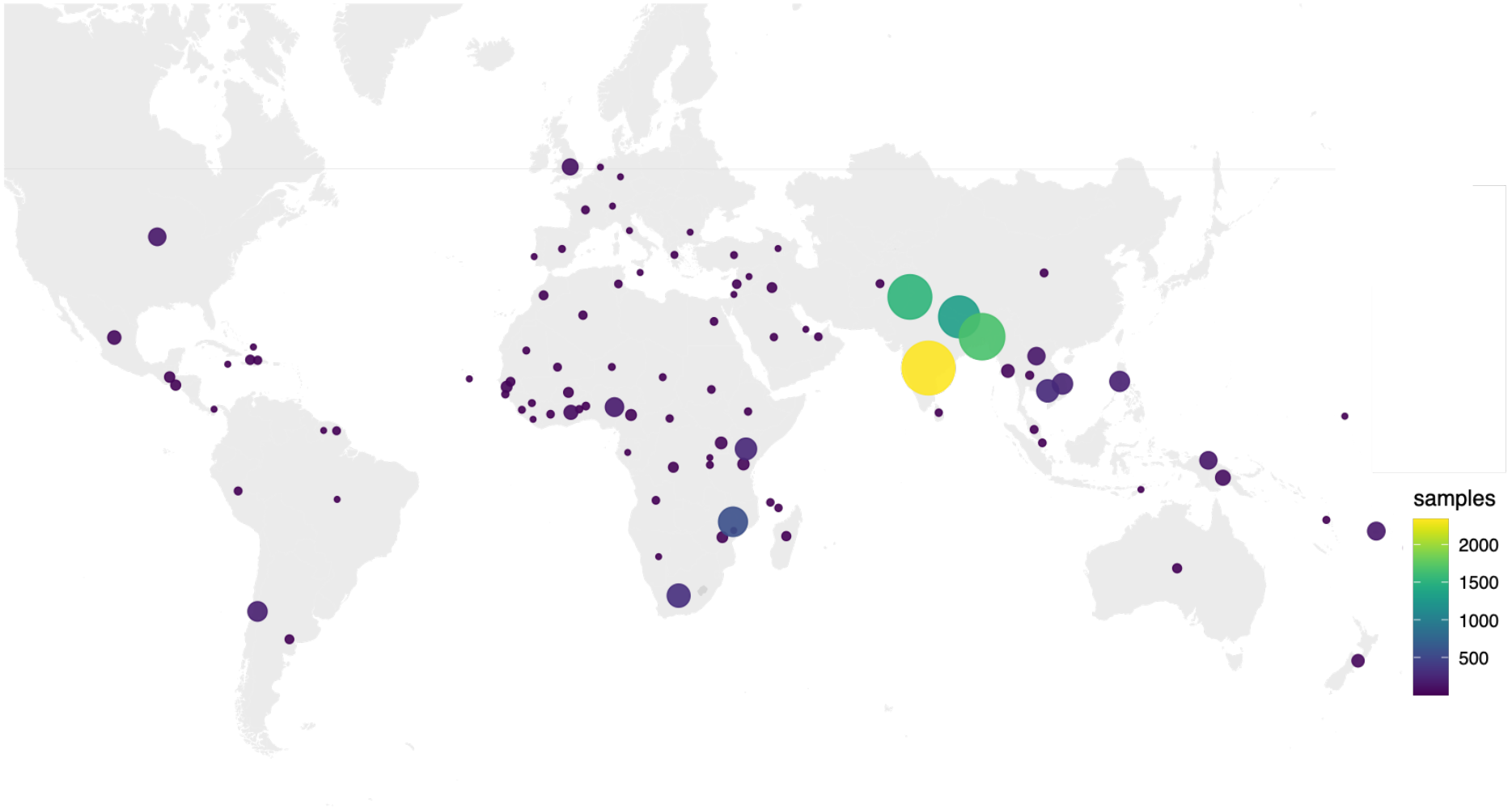


Figure 38. Geographic distribution of *S*. Typhi genomic data over duration of study period (n=13,022)

Another key limitation stemming from the post hoc nature of this study is that it is hard to assess how representative the prevalence estimates are for a given region/country and timeframe. The GTGC has developed new source/metadata standards for *S*. Typhi (see bit.ly/typhiMeta), that include information on the purpose of sampling, which were completed by the original owners of each dataset. Such 'purpose-of-sampling' fields are currently lacking from metadata templates used for submission of bacterial genomes to the public sequencing archives (e.g. NCBI, ENA), and our approach was modelled on that established for sharing of SARS-CoV-2 sequence data, designed by the PHA4GE consortium.[242] In this study, the purpose-of-sampling information was used to identify the subset of genome data that could be reasonably considered to be representative of national annual trends in genotype and AMR prevalence for public health surveillance purposes (n=9,478 genomes post 2010; **Figs 1-3**). These originate mainly from local typhoid surveillance studies (59%), or routine diagnostics/surveillance capturing locally acquired (19%) and travel-associated (24%) infections. The comparisons of estimates for a given country based on different sources of genomes (**Figures 34-37**) are reassuring that the general scale and trends of AMR prevalence are reliable. The genome-based estimates are also in broad agreement with available phenotypic prevalence data on AMR in *S*. Typhi,[243] although systematic aggregation of susceptibility data is limited. Notably, the genome data adds an additional layer of information on resistance mechanisms and the emergence and spread of lineages or variants. Importantly, our study shows clearly that, whilst much attention has been given to the emergence and spread of drug-resistant H58 *S*. Typhi, other clones predominate outside of Southern Asia and Eastern Africa (**Figure 23**) and can be associated with non-susceptibility to ciprofloxacin (**Figure 30**), azithromycin (**Figure 33**) or

ceftriaxone (**Table 9**), which are included in the World Health Organization Essential Medicines List as first choice treatment for enteric fever.[244]

6.4.2 AMR

Our data demonstrate that CipNS is emerging or established in all regions except Melanesia (here represented by n=35 genomes from Fiji and Papua New Guinea, mainly from 2010, although more recent reports support a lack of CipNS in Fiji [245,246] (see **Figure 29**). For countries with sufficient data to assess (≥50 genomes), CipNS was emerging or established in all countries except Ghana (**Figure 27**), with no evidence of declining prevalence (**Figure 29**). A diverse range of genotypes and QRDR mutations are involved (**Figures 29, 30**), likely reflecting the lack of fitness cost associated with these mutations.[247] That QRDR mutations are so widespread is highly concerning, as infections with CipNS strains can take longer to resolve, and full clinical resistance can emerge relatively easily against this background, through acquisition of either a mobile *qnr* gene (as occurred in 4.3.1.1.P1 in Pakistan) or additional QRDR mutations (as occurred in 4.3.1.2.1 in India). Notably, the data suggest CipR typhoid is now a well-established problem across Southern Asia and is emergent in Chile, Mexico and South Africa (**Figures 27, 28**). A recent study estimating national annual antibiotic consumption highlighted differences in rates of fluoroquinolone usage between regions and countries, which could potentially drive these differences in resistance prevalence.[248] The highest rates of fluoroquinolone consumption were estimated in South Asian countries, rising from 1.67 defined daily doses (DDD) per 1,000 per day in 2000 to 2.81 DDD/1,000/day in 2010 and 2.94 DDD/1,000/day in 2018 (see https://www.tropicalmedicine.ox.ac.uk/research/oxford/microbe/gram-project/antibiotic-usage-an d-consumption). Fluoroquinolone consumption was also estimated to increase

substantially in Latin America, rising from 0.64 DDD/1,000/day in 2000 to 1.85 DDD/1,000/day in 2010 and 2.26 DDD/1,000/day in 2018. Our data show the highest incidence of CipR burden is associated with four main variants (**Figure 29**). In Pakistan, India and Bangladesh, it is associated with locally emerged variants; however, the relatively high burden in Nepal is associated with variants acquired from India.[61,202] In other regions, CipR burden is low and so far linked mainly to the spread of 4.3.1.2.1 out of India,[52,63] plus occasional *de novo* emergence of resistant variants, which show no evidence of geographical spread. However, the high rates of CipNS in Kenya (53%) and Nigeria (40%) are concerning, especially given the increasing usage of fluoroquinolones in these countries (estimated 2.1 DDD/1,000/day in 2018 in Kenya and 2.76 DD/1,000/day in Nigeria),[248] which could potentially drive local emergence and spread of CipR.

While resistance to azithromycin and ceftriaxone have been detected (**Table 9, Figures 26-28 & 33**), their prevalence remains low and, except for XDR 4.3.1.1.P1, clonal expansion of resistant variants has not been observed. To our knowledge, there are no data reported on the fitness cost of *acrB* mutations or CefR plasmids in *S*. Typhi; however, the genomic evidence suggests a higher fitness cost compared with QRDR mutations, providing further support for the use of ceftriaxone or azithromycin over ciprofloxacin as we work to introduce preventative measures. Most instances of ESBL-gene carriage in *S*. Typhi (conferring CefR phenotype) have been short-lived (**Table 9**), suggesting selection against the acquisition of new ESBL genes or plasmids. The expansion and dominance of the XDR 4.3.1.1.P1 genotype in Pakistan is obviously concerning (**Figures 27, 28, 32**); however, despite circulating at high prevalence in Pakistan for more than five years, the strain remains azithromycin-susceptible. There is also limited evidence of local transmission of

4.3.1.1.P1 in other countries; however, most countries near Pakistan have limited data available. A short local outbreak of XDR 4.3.1.1.P1 was reported in China, linked to contamination of an apartment block's water[249] and non-travel associated cases have been reported in the USA.[250] Notably, a CefR+CipR lineage of 4.3.1.2.1 that appears to be well-established in Mumbai, India, has been isolated only occasionally since 2015[71,72,78,179] (**Table 9**); however, this is the only example of persistence of a CefR strain besides 4.3.1.1.P1, and there is no evidence it has yet spread outside Mumbai. We hypothesise that the lack of widespread dissemination of 4.3.1.1.P1 and ESBL-positive 4.3.1.2.1 so far may be due to the fitness cost imposed by the associated plasmids (~85 Kbp IncY plasmid in 4.3.1.1.P1;[24] ~43 Kbp IncX3 plasmid in 4.3.1.2.1)[72]. The temporal trend data on MDR prevalence and IncHI1 plasmids (**Figure 31**) suggest that migration of the MDR locus from the plasmid to the chromosome may have mitigated the fitness cost associated with plasmid-borne MDR. The same may be true for ESBL genes, that is, the movement of the ESBL locus from the plasmid to the chromosome (as has recently been reported in 4.3.1.1.P1[68] may result in a fitter CefR or XDR variant that can spread more easily. Our data show *acrB* mutations are occurring spontaneously and independently in multiple locations across a variety of genetic backgrounds (**Figure 33**). While they are still not prevalent, increased use of azithromycin through public health programmes (e.g. trachoma elimination) as well as widespread misuse of azithromycin to treat SARS-CoV-2 infections and use of azithromycin as first-line therapy for typhoid-like illness may lead to increased selection pressure. It will therefore be important to maintain and expand genomic surveillance, particularly in typhoid endemic countries where azithromycin is used widely. It is also notable that, while they are rare overall, *acrB* mutations have already arisen in two of the most common CipR lineages (4.3.1.2 and 4.3.1.3.Bdq); this relatively frequent co-occurrence warrants continued

monitoring and investigation. While we did not detect the mobile azithromycin resistance gene *mphA,* it is circulating in other *S. enterica* serovars[251,252] and other enteric bacteria that share plasmids with *S*. Typhi (including the human-specific *Shigella*),[253] providing another potential mechanism for emergence of azithromycin resistance in *S*. Typhi.

6.4.3 Applications of genomic surveillance for typhoid fever control

We are at a pivotal stage in the history of typhoid control. Wider access to clean water and improved sanitation have led to a major reduction in global incidence of typhoid fever, which has also been reflected in declining incidence of other enteric diseases.[80] This should continue but will require sustained investment from national and local governments and thus remains a long-term objective. In the short to medium term, widespread use of typhoid conjugate vaccines (TCVs) can help to further reduce global incidence of typhoid fever. The WHO has prequalified two TCVs and recommended their use in endemic countries, as well as settings where a high prevalence of AMR *S*. Typhi has been reported.[21] Gavi, the Vaccine Alliance, has committed funds to support the procurement and distribution of TCVs in typhoid endemic countries.[254] Four countries have undertaken Gavi-supported national introductions (Pakistan, Liberia, Zimbabwe, Nepal) and one country has self-financed a national introduction (Samoa).[255] In Pakistan and Zimbabwe, TCV introduction was stimulated by the occurrence of AMR *S*. Typhi outbreaks in major urban centres, highlighting that the case for prevention can be stronger when curative therapy is less available. Additional support is likely required to inform TCV decision-making in other typhoid endemic countries, particularly where burden and AMR data are scarce.

With increasingly limited treatment options, vaccines are an even more important tool to mitigate the public health burden of AMR *S*. Typhi, both through the prevention of drug-resistant infections and through broader, indirect effects, like reduction of empiric antimicrobial use leading to reduced selection pressure. While TCVs have been shown to be highly effective against drug-resistant *S*. Typhi,[25,99] public health policymakers have to weigh

the value of TCVs against other competing immunisation priorities. While TCV introduction is scaled up globally, antimicrobial stewardship should also be prioritised. Aggregated, representative data showing distribution and temporal trends in AMR can inform local treatment guidelines to extend the useful lifespan of antimicrobials licensed to treat typhoid fever, potentially including reverting to former last-line drugs in some settings. The traffic light system presented in this analysis (see **Figures 27 & 28**) provides a framework for monitoring trends in AMR and adjusting empiric therapy guidelines accordingly. Genomic surveillance has a particularly important role to play in monitoring for changes in clinically important resistance in *S*. Typhi, as a shift in resistance mechanism or early evidence of clonal spread, which can only be identified definitively using WGS, could provide early warning of a likely increase in prevalence. This study provides an analytical framework for *S*. Typhi genomic analysis, based on an open, robust, reproducible data flow and analysis framework leveraging open-access online data analysis platforms (Typhi Mykrobe for read-based genotyping;[256] the GHRU pipeline for genome assembly[172] and Typhi Pathogenwatch for assembly-based genotyping and tree-building).[43] We have made available all data processing and statistical analysis code, and underlying sequence and metadata, via GitHub and FigShare. Together, these provide: (i) a comprehensive data and code resource for the research and public health communities interested in typhoid surveillance data; (ii) a model for the inclusion of WGS in project-based or routine surveillance studies of typhoid that can be readily replicated and adapted; and (iii) a sustainable model for aggregated analysis of typhoid genomic surveillance data that can readily incorporate new data and extract features (genotypes, AMR determinants, plasmid replicons) of importance to clinical and public health audiences. Notably, this Consortium-driven effort shows that new insights can be gained from aggregated analysis of published data, which were not evident from the

individual contributing studies, for example (i) the XDR strain 4.3.1.1.P1 existed in Pakistan in 2015, a year earlier than previously reported (**Figure 32**); (ii) the CefR+CipR strain reported in Mumbai[71,72] has persisted between at least 2015-2020 and is now more easily identified as 4.3.1.2.1 with *bla*$_{SHV-12}$; (iii) persistence of MDR in certain settings is correlated with migration of MDR from plasmid to chromosome (**Figure 31**), which has implications for the future persistence and potentially spread of ESBL strains.

This dataset provides clear, actionable information about the distribution and temporal trends in AMR across multiple countries and regions. Where data gaps exist, the potential of travel-associated data to serve as "sentinel" surveillance has been demonstrated previously by Ingle et al[24,140] and supported by additional data included in this analysis. These data can and should inform prioritisation of TCV introduction and improvements to water, sanitation, and hygiene (WASH) infrastructure. Sustaining and expanding genomic surveillance can also facilitate measuring the impact of TCV introduction on local bacterial populations, as has been done for previous vaccines like pneumococcal conjugate vaccines. In addition, monitoring for potential "strain replacement" with other *Salmonella* serovars following TCV introduction can and should inform the prioritisation of the development and deployment of future combination *Salmonella* vaccines.

The SARS-CoV-2 pandemic illustrated the power of open, continuous data sharing and crowdsourced analysis, and the importance of ensuring that genomic surveillance leads to local benefits. The scale of this analysis, which was made possible through the efforts of an

extensive network of collaborators, enables the extraction of key insights of public health relevance. The authors hope that this Consortium effort serves as a starting point for continued data generation and sharing and collective analysis, with additional participation from an expanded group of stakeholders. In particular, we hope that researchers and public health authorities from areas with little publicly available data see the value of reporting and sharing genomic data for collective public health benefit. In addition, we hope that the current momentum for donor and government support of molecular surveillance is sustained, so that additional groups are able to generate their own data and fill regional data gaps to inform local public health action.

6.4.4. Next Steps

This chapter is part of a larger ongoing body of work that is being undertaken by the Global Typhoid Genomics Consortium. Phylodynamic analyses (dating analyses for key genotypes and ancestral state reconstruction) are ongoing and thus have not been included in this section but will ultimately be included in the manuscript. The analysis described in this chapter has been submitted as a manuscript to eLife and is under review. For maximal transparency and reproducibility, all underlying code developed for data analysis is freely available at https://github.com/katholt/TyphoidConsortium20yrs. We are also developing a commentary with members of our Advisory Group (largely composed of public health and infectious disease epidemiology experts) to outline key insights of public health relevance and to highlight the value of continued data sharing, as well as the potential to analyse and visualise data using Typhi Pathogenwatch and TyphiNET. The functionality and public health utility of these two platforms will be described in greater detail in **Chapter 7**

Chapter 7: Applications of genomic data to inform typhoid fever control and next steps

7.1 Abstract

This chapter establishes the global scale of the problem of AMR and outlines three major use cases for the use of WGS in informing typhoid fever control, each of which is described in detail below. We also present freely available resources that can facilitate the generation, analysis, and visualisation of *S*. Typhi genomic data. This information was also presented at the 12th International Conference on Typhoid & Other Invasive Salmonelloses on December 7, 2021 and has been written up in an accepted as part of a supplement in *Open Forum in Infectious Diseases*. Finally, this chapter outlines future directions and next steps for all of the work presented as part of this thesis. Colleagues from the GTGC, Pathogenwatch, and Africa CDC provided overviews of freely available resources for the generation, analysis, and visualisation of genomic data. I developed the use cases, drafted most of the manuscript, and have identified future research directions and applications independently.

7.2 Background – WGS and AMR

AMR is one of the largest threats to global public health, and bacterial AMR was estimated to be associated with 4.95 million (95% 3.62, 6.57) deaths in 2019, including 1.27 million (0.911, 1.71) deaths that were attributable to bacterial AMR.[41] The pathogens associated with the highest attributable mortality in this analysis were *Escherichia coli*, *Staphylococcus aureus*, *Klebsiella pneumoniae*, *Streptococcus pneumoniae*, *Acinetobacter baumannii*, and *Pseudomonas aeruginosa*, which were estimated to be responsible for 929,000 (660,000 - 1,270,000) deaths attributable to AMR and 3.57 million deaths (2.62 - 4.78 million) associated with AMR in 2019.[40] An estimated 23,700 deaths were considered attributable to

drug-resistant *S*. Typhi. However, this analysis only considered two "bug-drug" combinations – MDR *S*. Typhi and fluoroquinolone-resistant *S*. Typhi, so is likely to be an underestimate of associated and attributable mortality caused by drug-resistant typhoid fever.

The WHO published a list of antibiotic-resistant "priority pathogens" that posed the greatest threat to human health based on prevalence and severity of drug resistance for which new antimicrobials are needed in 2017 (**Figure 39**).[257] The list is divided into three categories in accordance with the urgency of need for new antimicrobials based on mortality, requirement for long impatient stays, prevalence of resistance, transmissibility, number of treatment options, and whether or not new antimicrobials to treat a given disease are already in research and development. Critical bacteria include multidrug resistant bacteria that disproportionately affect patients in hospitals, nursing homes, and who rely on ventilators or other devices for care. They can cause serious or even deadly infections. The medium and high priority lists include other increasingly drug-resistant bacteria.

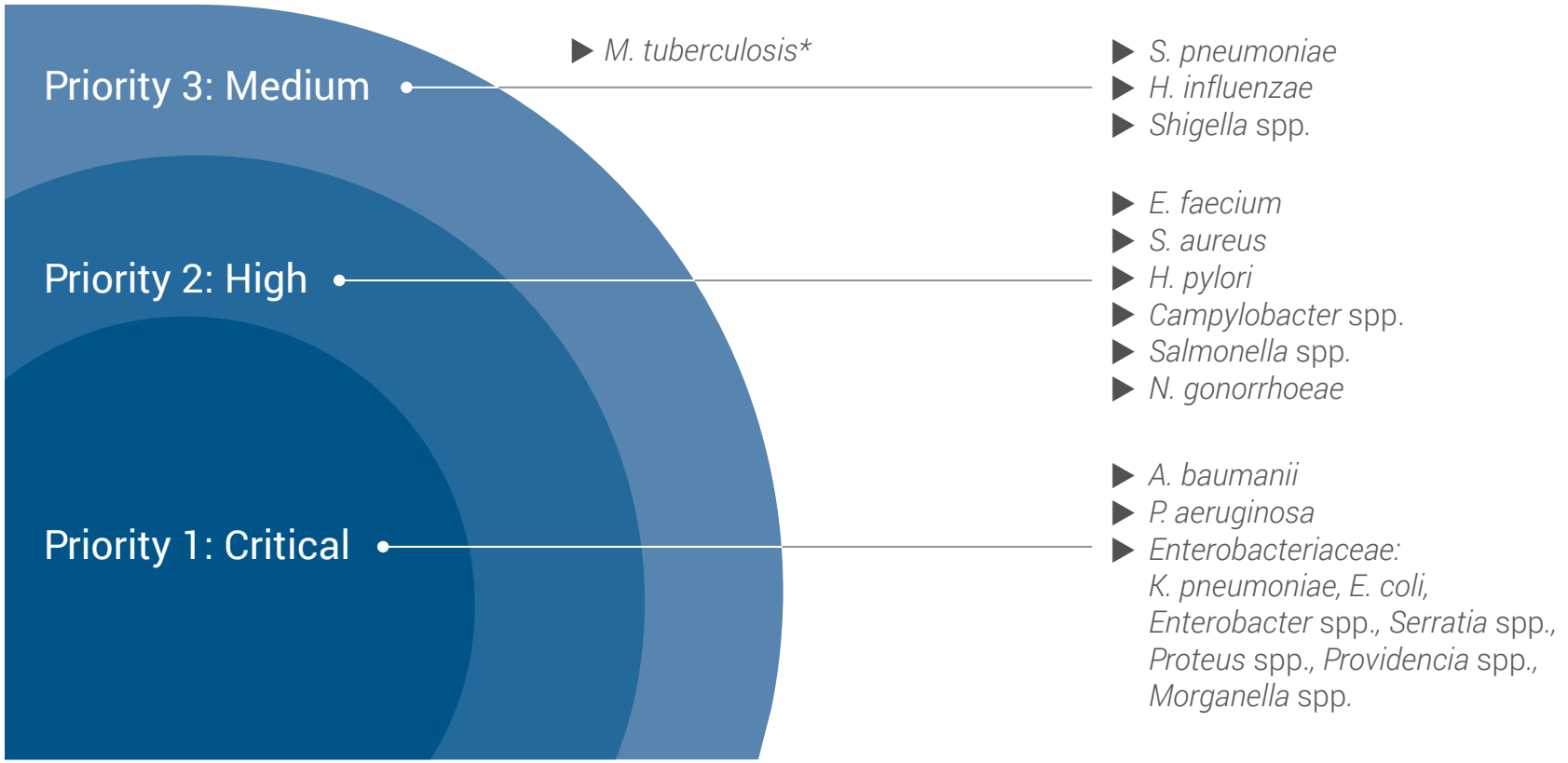


Figure 39. Global priority list of antibiotic-resistant bacteria to guide research, discovery, and development of new antimicrobials (Tacconelli *et al*). [238]

The detection of AMR has classically been conducted using culture-based antimicrobial susceptibility testing (AST), which continues to be standard practice in clinical microbiology. While AST provides useful visual information about how bacteria behave in the presence of antimicrobials at prespecified concentrations, there are limits to the utility of AST data in patient management as well as higher-level disease control. It can be difficult to compare antimicrobial susceptibility testing (AST) data across different labs, as different testing methods may be used, including broth dilution tests, antimicrobial gradient methods including E-tests, disk diffusion tests, and automated instrument systems.[258] Individual laboratory technicians may interpret results differently (e.g. variable measurement of disk diameter in the case of disk diffusion tests), and different clinical breakpoints may be used, from the Clinical Laboratory Standards Institute (CLSI) in the United States or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[259] In addition, these breakpoints are updated periodically, so if precise measurements are not recorded, results may be difficult to compare even within one laboratory over a certain time period. In addition, most AST methods requires culturing of patient samples and subculturing of bacterial colonies identified before any susceptibility testing can be conducted, meaning that patients are often given broad-spectrum antimicrobials well in advance of identification of a causative pathogen, much less any antimicrobial susceptibility results.

In addition to these limitations, AST data do not provide information about molecular mechanisms of resistance, making it impossible to draw any inferences about transmission dynamics, whether it be across different hospital wards or continents, which complicates decision-making around outbreak response. In addition, AST data do not provide

information between relatedness of bacteria, making it difficult to ascertain the source of an outbreak. Genetic-based methods for bacterial typing like multi-locus sequence typing (MLST) provide additional granularity but are limited as they rely on a small fraction of the total genome. WGS data provide genome-wide data (down to single-nucleotide variant or SNP level) that can be used to identify and confirm presence of molecular mechanisms of AMR, confirm pathogen identity, and provide information about pathogen ancestry and virulence.[260–262] Next-generation sequencing (NGS) has facilitated relatively rapid, accurate, and cost-effective determination of entire pathogen genomes, and comparative phylogenetic analysis enables estimation of relatedness of different isolates, which can be combined with clinical and other epidemiological data to describe transmission dynamics and the emergence and spread of AMR.[223] WGS is being used more frequently to address AMR in multiple disease areas, through routine surveillance, outbreak investigation, and informing the development of improved diagnostics and therapeutics.

7.3 Uses cases for WGS to inform typhoid control policy

WGS is a powerful tool that can help to improve our understanding of typhoid epidemiology and the burden of AMR. WGS data can also provide valuable information about transmission pathways, on local, national, and global levels, that can inform better targeting of interventions to achieve typhoid control goals. WGS has facilitated investigations of the molecular mechanisms of new resistance phenotypes, including XDR typhoid and the recent emergence of azithromycin resistant *S*. Typhi in Bangladesh, Pakistan, and India. By providing clear, standardized information about drug resistance profiles of circulating bacterial strains, WGS has the potential to direct local treatment guidelines to extend the useful lifespan of existing antimicrobials that are licensed to treat typhoid fever. Genomic data can also inform the prioritization of vaccine introduction, and phylogenetic analysis can be used to measure the impact of vaccines on bacterial communities. Understanding the genomic impact of TCV introduction and whether vaccines work equally well in preventing disease from different lineages can inform whether typhoid elimination is a feasible goal. In addition, monitoring for potential "strain replacement" with other typhoidal *Salmonella* serovars following TCV introduction can and should inform the prioritization of the development and deployment of future combination *Salmonella* vaccines.

There is an important role for WGS in advancing typhoid control goals. More widespread generation and targeted use of sequence data could help to progress the three following objectives: 1) generating data about typhoid transmission and AMR patterns, 2) informing the prioritization of interventions and monitoring their impact, and 3) extending the lifespan of existing antimicrobials.

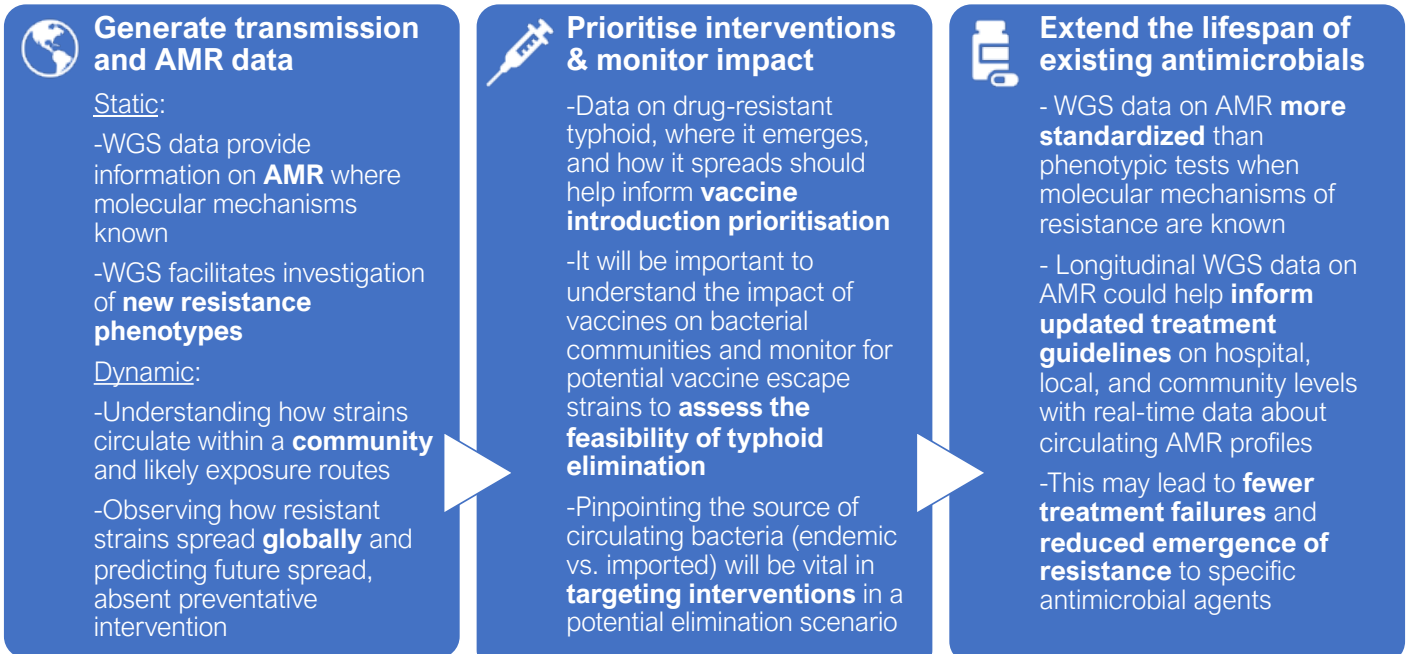


Figure 40. Use cases for WGS to inform typhoid control policy.

WGS data can provide valuable information about AMR patterns and transmission dynamics, which can inform prioritisation of interventions and measurement of their impact. WGS data can also inform more targeted use (and less unnecessary use) of antimicrobial therapy, which can slow the emergence of AMR.

7.3.1 Generating data about typhoid transmission and AMR patterns

AST has long been a mainstay of individual-level patient management as well as higher-level (national, regional) surveillance, and is unlikely to be fully replaced by WGS, but AST has several key limitations. It can be difficult to compare AST data across different labs. Different testing methods may be used: broth dilution tests, antimicrobial gradient methods including E-tests, disk diffusion tests, and automated instrument systems.[258] Individual laboratory technicians may interpret results differently (e.g. variable measurement of disk diameter in the case of disk diffusion tests), and different clinical breakpoints may be used, from the Clinical Laboratory Standards Institute (CLSI) in the United States or the European Committee on Antimicrobial Susceptibility Testing (EUCAST).[259] In addition, these breakpoints are updated periodically, so if precise measurements are not recorded, results may be difficult to compare even within one laboratory over a certain time period. Not all breakpoints have been validated extensively with clinical data, as is the case for azithromycin breakpoints for *S*. Typhi,[197] so categorization of species as susceptible, intermediate, or resistant based on some breakpoints may give a sense of false precision. In addition, most AST methods requires culturing of patient samples and subculturing of bacterial colonies identified before any susceptibility testing can be conducted, meaning that patients are often given broad-spectrum antimicrobials well in advance of identification of a causative pathogen, much less any antimicrobial susceptibility results. Repeat testing may be difficult, as banked isolates may fail to revive.

While WGS may not address the issue of time required for results, WGS data provide valuable, actionable outputs, both statically and longitudinally, that are easily comparable across different laboratories. Where molecular mechanisms of AMR are known, WGS data

provide clear, definitive information about the resistance profile of a given isolate. In addition, phylogenetic analysis of WGS data enables high-throughput comparison of multiple different isolates in terms of AMR profile and molecular mechanism(s) of resistance, and even facilitates inference about relatedness of strains and transmission dynamics. WGS facilitates the investigation and discovery of new molecular mechanisms of resistance, which can then be identified retrospectively in existing sequence data much more easily than reviving banked specimens and then repeating culture and AST. WGS can also facilitate the development of targeted molecular testing to monitor the spread of a specific resistance gene, or lineage, for use in either clinical specimens or environmental specimens.

WGS data from a specific, non-targeted sampling frame – be it a hospital, a regional reference laboratory, or even a national public health laboratory – can provide a valuable snapshot of circulating genotypes and AMR profiles. In addition, sequence data can facilitate the investigation of new AMR phenotypes to determine molecular mechanisms of resistance, enabling heightened surveillance for these new resistant strains as well as investigation into their emergence and spread. One important example of this was the recent investigation of the XDR typhoid outbreak in Pakistan. Following alarming reports of ceftriaxone-resistant typhoid in Hyderabad in November 2016,[67] genomic investigation revealed that a new strain had emerged that was not only resistant to first-line antimicrobials chloramphenicol, ampicillin, and trimethoprim–sulfamethoxazole, but also fluoroquinolones and third generation cephalosporins. This strain, which belongs to the H58 clade, acquired a “promiscuous” plasmid encoding additional resistance elements, including $bla_{\text{CTX-M-15}}$ extended-spectrum β-lactamase, and the *qnrS* fluoroquinolone resistance gene,

that had previously been identified in other enteric bacteria.[24] The discovery of this new strain and subsequent genomic investigation led to intensified nationally representative surveillance, which allowed for the discovery that this novel strain had spread from Hyderabad, Pakistan to Karachi and throughout Sindh province, ultimately spreading to Punjab and beyond.

This public health program follows many years of effort to characterise the global epidemiology of typhoid fever, in which WGS has played a significant role. As previously described, the emergence and spread of AMR have posed an increasing threat to typhoid control, particularly in South Asia, where resistance to each class of oral antimicrobials used to treat typhoid fever has emerged.[34] As new resistance phenotypes have emerged, WGS has facilitated the further investigation of the underlying molecular mechanisms. WGS-based investigation of an outbreak of ceftriaxone-resistant typhoid in Hyderabad, Pakistan revealed the emergence of a new, extensively drug-resistant (XDR) variant that was not only MDR, but also resistant fluoroquinolones and third generation cephalosporins.[67] This variant had acquired a plasmid encoding additional resistance elements, including a $bla_{\text{CTX-M-15}}$ and a *qnrS* fluoroquinolone resistance gene.[24] Determining the molecular mechanisms of XDR typhoid enabled researchers and public health professionals to understand that this was a new strain that had likely emerged in Pakistan (rather than being introduced from elsewhere), and facilitated intensified surveillance of XDR typhoid in Pakistan to understand where it was spreading and how quickly.

In addition to elucidating the molecular mechanisms of AMR and providing well-defined, standardised AMR data where mechanisms of resistance are known, WGS data provide a

more dynamic view of typhoid fever epidemiology by illustrating how and where drug-resistant strains emerge and illustrating where these strains spread and over what timeframe. Tracking the spread of resistant strains and predicting the subsequent spread of new resistant strains can help target interventions more effectively. Understanding whether isolated *S*. Typhi is imported, or endemic will have implications for the urgency, type, and scale of public health response. The 2018 WHO Position Paper on typhoid vaccines indicated that TCV introduction should be prioritised "in countries with the highest burden of typhoid disease or a high burden of antimicrobial-resistant *S*. Typhi".[21] Given the ubiquitous nature of drug-resistant typhoid, the substantial anticipated country demand for TCVs, and the presence of only two WHO prequalified manufacturers of TCV, additional selection criteria may be required. Globally representative WGS data show that drug-resistant typhoid typically emerges and spreads from South Asia;[45,52] therefore, prioritising TCV introduction in this region may reduce the burden of AMR in the region and prevent the spread of new resistance phenotypes globally, as has been observed previously with the spread of H58 typhoid to east and sub-Saharan Africa.[45] Phylogenetic and phylodynamic data can inform optimal vaccine introduction strategies for other key highly drug-resistant pathogens through the identification of drug-resistance "hot spots", as and when new vaccines become available. This will not only prevent infections and save lives in the region, but also limit the continued emergence and spread of resistant *S*. Typhi to other regions.

Of course, access to sequencing is not ubiquitous, and there are many parts of the world from which *S*. Typhi genomic data are not publicly available. However, work undertaken by the colleagues from the United Kingdom Health Security Agency and the University of Melbourne has demonstrated that the genomic profile of *S*. Typhi isolated from returning

travellers to the UK is likely to be representative of the *S*. Typhi population structure in the country of travel. Thus, analysis of returning traveller data can provide some insight into the presence and drug-resistance profile of *S*. Typhi in countries where genomic surveillance is not routinely conducted, acting as a form of sentinel surveillance.[55]

7.3.2 Inform prioritization of interventions and measure their impact

By generating clearer static and dynamic insights into local bacterial populations, genomics can help inform prioritization and targeting of interventions like TCVs, WSH, and health education. Understanding where *S*. Typhi is circulating, what the AMR patterns are, and where the *S*. Typhi have come from (e.g. local, endemic transmission as compared to imported cases) can help policymakers to decide whether or not to introduce TCVs and which strategy to use (which age groups and geographic locations to target). Knowing that there is local transmission of drug-resistant *S*. Typhi should encourage policymakers to introduce TCVs more broadly, and potentially to implement WASH interventions, particularly if *S*. Typhi can be isolated from shared water or sewage samples.[263] If the isolated *S*. Typhi are determined to be travel-associated based on WGS data and associated metadata, policymakers may choose instead to intensify surveillance rather than implementing a national control program.

The example of the discovery and subsequent targeted monitoring of XDR *S*. Typhi transmission in Pakistan provides an excellent example of the potential value of WGS data in informing the deployment of preventative interventions like TCVs. Following initial reports of XDR typhoid in Hyderabad in November 2016, intensified surveillance was undertaken to monitor its spread throughout the country.[264] Reactive vaccination campaigns were initiated

in Hyderabad and Karachi, where the burden was initially greatest, and vaccine safety and effectiveness data were generated.[99,104] These efforts informed the decision of the Federal Expanded Program on Immunization (EPI) to introduce typhoid conjugate vaccine (TCV) into their national immunisation programme, making Pakistan the first country to do so.[126] The phased introduction began with a vaccination campaign targeting ~ 10 million children in the urban areas of Sindh province in November 2019, where the burden of XDR typhoid was highest, and a subsequent campaign was conducted in Islamabad and Punjab province, where XDR had spread, that covered >13 million children.[265] In this instance, WGS and phenotypic AMR data had a direct impact on the decision to introduce TCV and on the vaccine introduction strategy itself.

Crucially, genomics can also help measure the impact of interventions like vaccines on bacterial populations, including identifying potential vaccine escape strains. This targeting and monitoring become particularly critical if the typhoid research and funding community decides to expand current typhoid control goals and targets elimination. To critically assess the feasibility of elimination, it will be important to interrogate the following questions:

1) Do existing conjugate vaccines protect against all lineages of *S*. Typhi? if not, how do we target variants with other measures?

2) What is the role of chronic carriers in sustaining transmission? Do we need to implement screen and treat programs in areas where carriage is thought to be present?

3) Are circulating organisms endemic and/or imported? This will inform additional targeting of interventions, which will require coordinated, networked approach.

To answer some of these questions, it will be essential to establish clinical and genomic surveillance for *S*. Typhi in countries that are introducing TCV into their national immunization programs and implementing large-scale catch-up campaigns to understand the local bacterial population structure before TCV introduction and how if at all that changes following TCV introduction. Ideally, this would be broad-reaching blood culture surveillance in which all *Salmonella* isolates were submitted to WGS, coupled with population screening for chronic carriers. Understanding the age distribution of disease, as well as the distribution of genotypes and AMR types, should inform optimal targeting of TCV introduction. Understanding the impact of TCV on disease incidence, age distribution, and genotypic distribution of *S*. Typhi will help inform better targeting of TCV introduction and potentially address the question of, how much typhoid control can be achieved by widespread TCV deployment? The genomic component of this surveillance should include other *Salmonellae* to ensure any potential strain replacement (either different *S*. Typhi lineages, or "replacement" of *S*. Typhi with *S.* Paratyphi A, for example) could be detected following vaccine introduction. WGS data will also yield important information about the likely origins of any isolated *S*. Typhi, meaning that it will be easier to determine how much local transmission can be reduced following TCV introduction, and how much continued introduction is driven by local transmission as compared to external introduction. In a late-stage elimination scenario, this information will be important in terms of understanding which lever(s) to further reduce transmission, including vaccination entry requirements to prevent additional travel-associated cases, and/or additional local TCV catch-up campaigns to reduce local transmission.

7.3.3 Extending the lifespan of existing antimicrobials

In addition to introducing preventative tools, it is important to maintain viable treatment options when typhoid fever cases are detected. There are a few ways that WGS can be deployed to slow the continued emergence and spread of drug resistance, thereby extending the useful lifespan of existing antimicrobials.

1) Use of WGS as a point of care diagnostic to inform appropriate therapy

At a patient level, phenotypic tests yield uncertain results, as breakpoints are not always validated with clinical data. Presence of established molecular mechanisms of yields unambiguous results about the antimicrobial sensitivity profile of a given isolate. Where possible, using sequencing to directly inform individual patient management would likely slow development of resistance by reducing unnecessary use of antimicrobials, or informing more targeted therapy. This might lead to delays between a patient seeking care and receiving treatment, assuming the treating physician would wait for the AMR readout from sequencing data, and thus might not be practical or advisable in cases of acute illness. In addition, given some of the resource, supply chain, and training constraints detailed in Chapter 7, conducting WGS on all patient samples from patients presenting with non-differentiated febrile illness might not be possible at this time. However, in this case, performing WGS on a subset of samples within large referral hospitals might also yield useful data about local trends in AMR that could still inform better antimicrobial stewardship practices.

2) Use of WGS as a population surveillance diagnostic to track AMR and inform antimicrobial use

Antimicrobial stewardship is limited by availability of timely, geographically relevant AMR data to inform treatment guidelines, and guidelines may be slow to be updated. Ideally, genomic surveillance would be integrated into national AMR surveillance to inform "real time" treatment guidelines. If a stratified randomised (non-targeted) subset of pathogens isolated from relevant patient samples (in this case, *S*. Typhi from blood) from tertiary care hospitals and/or large diagnostic facilities were submitted to WGS and sequence data and AMR information were shared at national, regional, or even hospital levels, this could inform more regular updating of antimicrobial prescribing recommendations based on circulating resistance profiles, thereby "saving" last-line treatments, such as carbapenems until they are absolutely needed.

3) Use of WGS to develop new diagnostic assays for identification of AMR

Efforts have been made previously to develop *S*. Typhi-specific polymerase chain reaction (PCR) diagnostic assays using *S*. Typhi-specific genes, which are identified through analysis of WGS data.[266] Such methods can be used to test environmental samples like shared water supplies or sewage, both to indicate presence or absence of bacteria, as well as a change in detectable levels following the deployment of a large-scale intervention, and can partially mitigate some of the challenges associated with culturing *S.* Typhi from water and other environmental samples. The utility of this approach is somewhat limited by the fact that molecular tests may indicate presence of specific *S*. Typhi DNA, but not of the presence of viable bacteria,[267] but can still provide a good indication that there is, or has recently been,

S. Typhi circulating within a community. This approach can be extended to detect the presence of specific AMR genes for surveillance purposes. This has been performed by colleagues from Child Health Research Foundation in Bangladesh, who used genomic analysis of 56 azithromycin-resistant *S*. Typhi and Paratyphi A isolates with a an AcrB-R717Q/L mutation to develop a simple, low-cost PCR assay to monitor the spread of azithromycin resistance.[77] Such a tool could provide a quicker indication than AST about appropriate therapy at point of care to prevent treatment failures, and could be a useful surveillance tool by providing readouts from multiple patient samples or even pooled environmental samples and facilitating monitoring of trends over time.

7.4 Freely available resources for the generation and analysis of *S*. Typhi genomic data

7.4.1 Overview

In addition to outlining use cases for WGS in informing improved typhoid fever prevention and control, I sought to highlight freely available resources to support the generation, analysis, and visualisation of *S*. Typhi WGS data to inform policy, which we presented in a workshop at the most recent Coalition Against Typhoid international meeting in December 2021. These are described in detail below.

7.4.2 SEQAFRICA

The cost of sequencing a genome continues to drop, but at the time of writing, many laboratories and public health institutes that could benefit from sequence-derived information cannot afford to generate it. In addition to the monetary cost of generating high-quality genomic DNA, preparing and analysing sequencing libraries, generating

sequence reads, performing quality assurance and analysis, the dearth of expertise required to perform these tasks poses a problem in many settings on the African continent.[268] Pathogen sequencing is most easily justified by showcasing data from sequencing.[241] This creates a ratchet effect where those that require sequencing cannot justify the need and therefore cannot access the monetary and training resources to be able to generate or analyse their own sequence data.

WGS is a key tool in understanding mechanisms and transmission dynamics of AMR. Recognizing this, the Fleming Fund, which has provided support for AMR surveillance in over twenty LMICs, has funded SEQAFRICA. The goals of SEAFRICA are to integrate WGS into AMR on the African continent and to sustainably build capacity for generating and understanding sequence data for surveillance. The SEQAFRICA consortium is comprised of regional sequencing centers in Nigeria, Tanzania and South Africa, a national sequence center in Ghana and a South Africa COVID-19 response center (**Figure 41**). The consortium is coordinated by the Danish Technical University in Denmark.

Figure 41. Overview of SEQAFRICA sites.

SEQAFRICA's objectives are to bring whole genome sequencing into AMR surveillance in Africa and to build capacity sustainably through training. The consortium has three regional sequencing centers: Nigeria (University of Ibadan, UI), Tanzania (Kilimanjaro Clinical Research Institute, KCRI) and South Africa (National Institute for Communicable Diseases, NICD); a national center: Ghana (Noguchi Memorial Institute for Medical Research, NMIMR) and a COVID-19 response center: South Africa (NICD Center for Respiratory Diseases and Meningitis (NICD-CRDM))

SEQAFRICA receives and reviews sequencing proposals from across the continent and provides sequencing support for projects that have high priority to inform the epidemiology of AMR, at no cost to participating investigators. This *modus operandi* provides access to sequencing for high priority initiatives that lack adequate resources and facilitates sequencing of small isolate collections of rarer organisms that can fill importance surveillance gaps. Thus far, sequenced isolates have come from a range of public health institutes, African Fleming Fund Fellows leading surveillance in their respective countries, and researchers in 15 African countries. Completed genomes are placed in the public domain within three months of completion, so SEQAFRICA is rapidly filling critical surveillance and research gaps with bacterial genome data from Africa.

Established in 2018, SEQAFRICA is now more than three quarters of the way towards achieving its goal of sequencing 16,000 genomes. *Salmonella* species, including *S*. Typhi, have been priorities from the start. However, SEQAFRICA has received few *S.* Typhi sequencing requests by the time of writing. This reflects overall low isolation rates of *S.* Typhi at various sites on the continent, which in turn is due to poor access to blood culture, as well as initial limited awareness of the program, and the benefits that *S.* Typhi genome sequencing can yield. Additionally, most *S.* Typhi isolates are obtained as part of externally funded term-limited research and surveillance projects, which often have their own arrangements for sequencing.[269] Such initiatives generate a wealth of data, but these tend to come from a smaller range of locales than routine, nationally-owned sequencing, like that conducted by the National Institute for Communicable Diseases (NICD, South Africa).[270,271] For countries setting up surveillance, AMR and *S.* Typhi surveillance are mutually enhancing. For example, Nigeria has found that boosting AMR surveillance in a system that includes a

genomic surveillance component is one way to extend the number and diversity of *S.* Typhi genomes.[272] For countries that do not have their own genomic surveillance for AMR, SEQAFRICA represents an excellent option for health facilities or national surveillance systems to generate *S.* Typhi genomic information and is therefore synergistic with Fleming Fund Country grant projects that focus on constructing the architecture of national surveillance systems, including building clinical microbiology at sentinel and national levels.

Awareness of the value and potential of sequencing, as well as the understanding of how to implement it, will grow with training programs, including those offered by SEQAFRICA. The consortium has taught online introductory courses on WGS in antimicrobial resistance surveillance; the WGS workflow: Isolate to Analysis; SARS-CoV-2 WGS and Basic Bioinformatics using the Command Line. The consortium has also scheduled in-person courses for a subset of >100 online trainees in Ghana that completed these online courses on WGS sequencing and Advanced Bioinformatics using the Command line in September/October 2022 https://antimicrobialresistance.dk/seqafrica/seqafrica-courses.aspx. In addition to the open courses, coordinated sequencing at SEQAFRICA nodes has also allowed staff working at the centers, who received in-person training at DTU at the start of the project, to gain very high-level proficiency. Many of these people work at public health institutes that acquired Illumina and/or Nanopore sequencing facilities during the COVID-19 pandemic[273] and can now apply these tools to bacterial genome sequencing. Thus, the potential for SEQAFRICA and complementary initiatives, like the Africa Pathogen Genomics Initiative, to promote *S.* Typhi surveillance beyond the lifespan of the regional grant is very high.

7.4.3 Typhi Pathogenwatch

Typhi Pathogenwatch[43] is a web application that was developed to support genomic epidemiology and public health surveillance of *S.* Typhi. It enables genome analytics, such as genotyping according to established nomenclature, detecting the presence of AMR determinants and plasmid replicon genes, and contextualization with public genomic data.

Typhi Pathogenwatch uses genome assemblies to perform three essential tasks for surveillance and epidemiological investigations, i.e., (i) placing isolates into lineages or clonal groups based on their genetic distance, (ii) identifying their closest relatives and linking to their geographic distribution, and (iii) detecting the presence of genes and mutations associated with AMR. In addition, Typhi Pathogenwatch provides compatibility with typing information for MLST, cgMLST, *in silico* serotyping (SISTR), the GenoTyphi genotyping scheme, and plasmid replicon sequences (PlasmidFinder).

The application can be accessed at https://pathogen.watch/styphi, where users can create an account to upload and analyse their genomes. User data remains private and stored in their personal account. Public genomes (n=4,909 at the time of writing) available in Pathogenwatch with linked metadata are curated by the Global Typhoid Genomics Consortium. Metadata include, when available, country, date and source of isolation, travel information, patient age, and purpose of sampling. Users can browse and create custom collections of private and/or public genomes available in the application (https://pathogen.watch/genomes/all?organismId=90370) via a set of filters including country, date, MLST, genotype, and resistance (**Figure 42**).

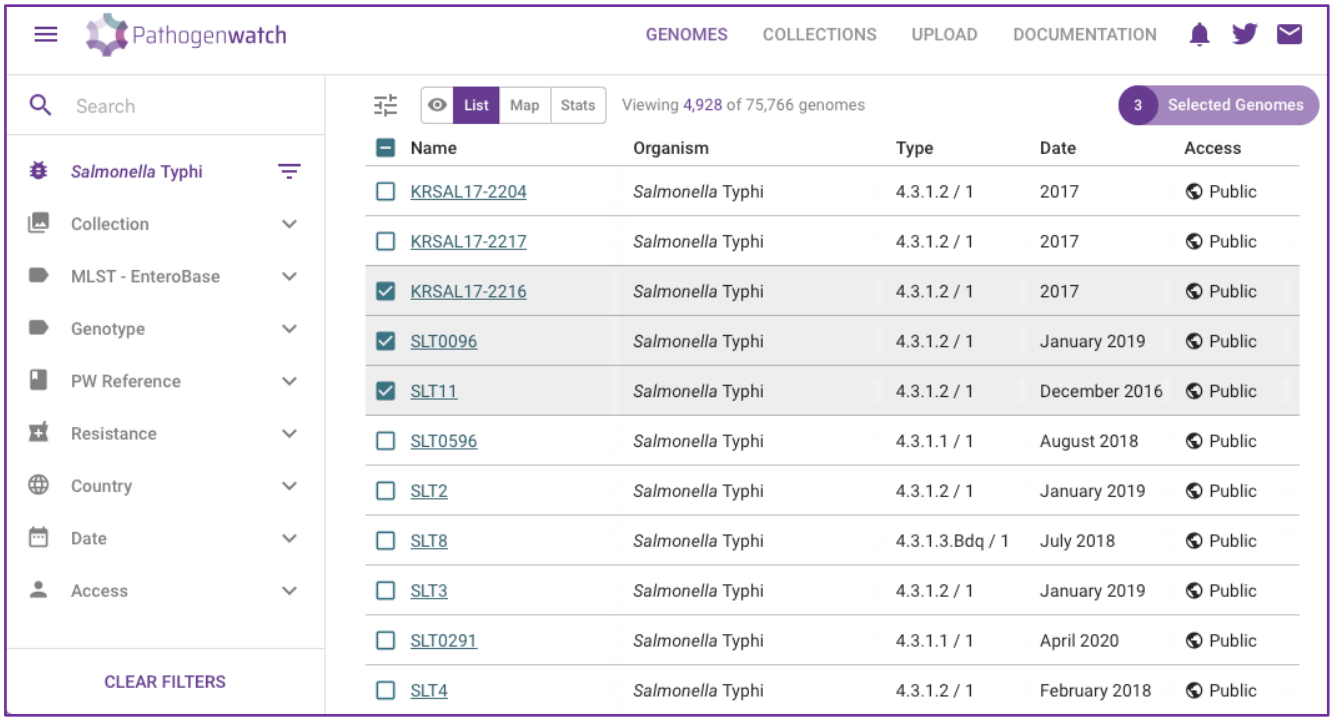


Figure 42. Screen capture of Pathogenwatch data selection page.

The Genomes page in Pathogenwatch allows the user to select a custom dataset of genomes based on available filters (left) or by clicking on individual genomes (ticked boxes). A collection can be created from selected genomes and all analytics can be downloaded.

The results for a single genome are displayed in a genome report that can be downloaded as a PDF. The results for a collection of genomes can be viewed online (**Figure 43**) and downloaded as trees and tables of genotypes, AMR predictions, assembly metrics, and typing information. Results can also be accessed later and shared via a collection ID embedded in a unique weblink, thus facilitating collaborative surveillance. The collection in Figure 3 shows that resistance to broad-spectrum cephalosporins in a subset of genomes from India could be explained by the presence of extended-spectrum beta-lactamase genes, either $bla_{\text{SHV-12}}$ in an IncX3 plasmid (red), or $bla_{\text{CTX-M-15}}$ in an IncY plasmid (white). Genomes with $bla_{\text{SHV-12}}$ show a narrow geographic distribution (map) but wider temporal distribution (timeline), while genomes with $bla_{\text{CTX-M-15}}$ show a wider geographic distribution but narrower temporal distribution.

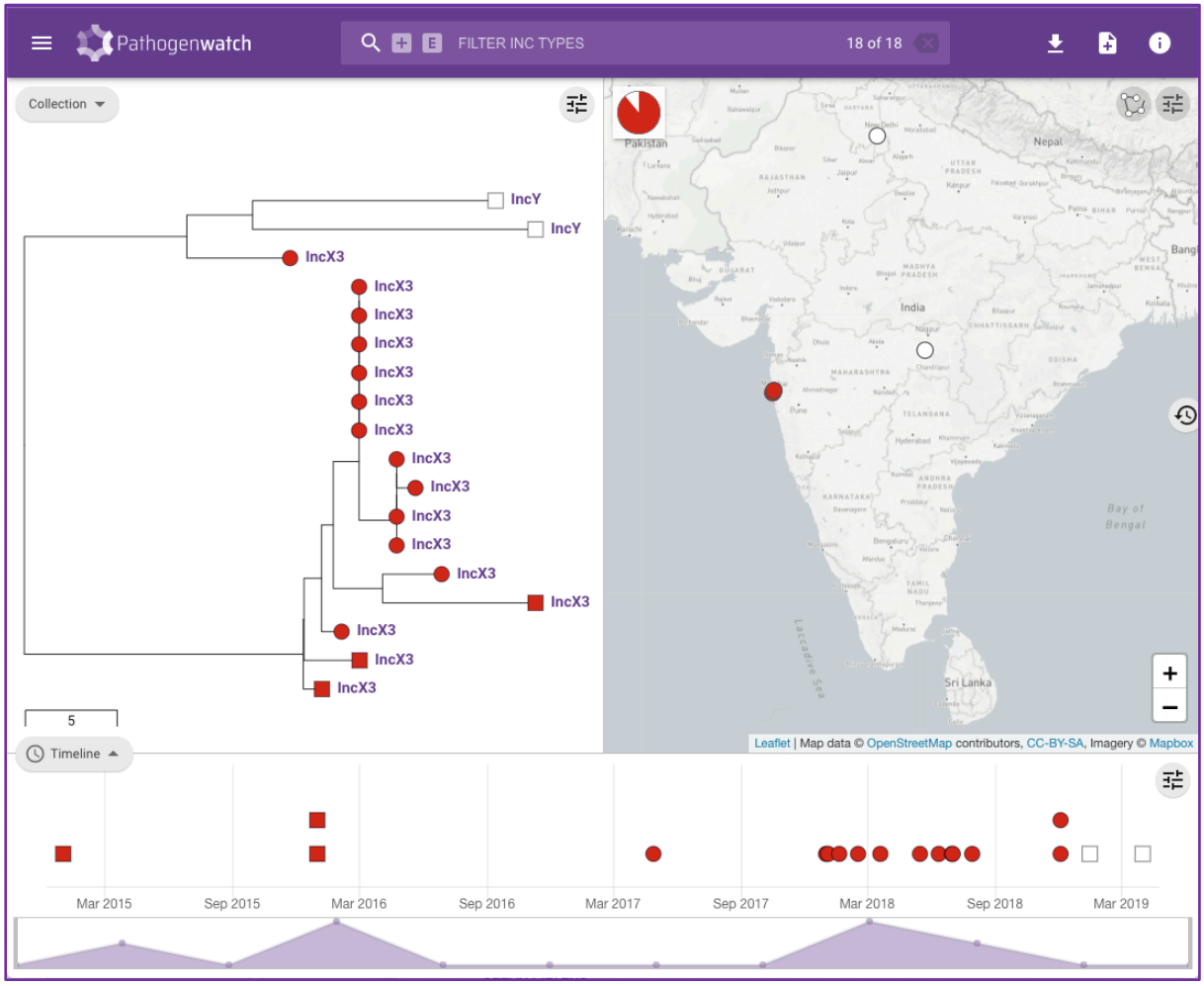


Figure 43. Screen capture of phylogenetic tree, and geographic and temporal distribution of user data using Pathogenwatch.

A collection of 18 genomes uploaded by the user (circles) or public (squares) selected from the Genomes page as resistant to broad-spectrum cephalosporins and from India, highlight the link between the *bla*$_{SHV-12}$ gene (red) and the IncX3 plasmid replicon sequence, and the *bla*$_{CTX-M-15}$ gene (white) and the IncY plasmid replicon.

Typhi Pathogenwatch combines accurate genomic predictions of AMR with broad geographic and population context within an easy-to-use interface accessible to users of all bioinformatics skills levels. Global genotype and AMR data generated by Typhi Pathogenwatch are utilized and aggregated by the TyphiNET dashboard (see next section). Our approach allows the rapid and incremental addition of new data and can be used to underpin surveillance of typhoid and public health decision making at the local, national, or international scales.

7.4.4 TyphiNET: An online AMR surveillance dashboard for global genomic surveillance of *S.* Typhi

TyphiNET is a newly developed online resource that aims to provide easy access to genome-derived data on the global distribution of *S.* Typhi genotypes and AMR determinants (available at: http://typhi.net).[274] TyphiNET empowers users to explore global trends in genome-derived metrics of public health utility, including AMR and genotype frequencies summarised down to national annual prevalence levels, without specialist computing technologies or bioinformatics expertise. Genotype and AMR data are imported from Typhi Pathogenwatch and filtered to include only genome collections that represent non-targeted sampling, suitable for estimating national annual prevalence data. Input data can be further filtered to include only specified time periods, or to exclude data derived from returning travellers. Users can generate 'up-to-date' downloadable reports and data visualisations of typhoid populations at a global- and/or country-level via any standard web browser.

Data can be viewed at global or country levels. Global patterns of national genotype and AMR frequencies can be visualised on the world map (**Figure 44**), with countries coloured to

indicate prevalence ranges (estimated from genome data) for clinically relevant drug resistance phenotypes. Categories of AMR that can be visualised in this way are MDR, aziR, CipI/R, XDR, and sensitive to all relevant antimicrobials. Users can also generate maps showing prevalence of genotype 4.3.1 (H58; commonly associated with AMR), the dominant genotype per country, and the number of genomes available for each country

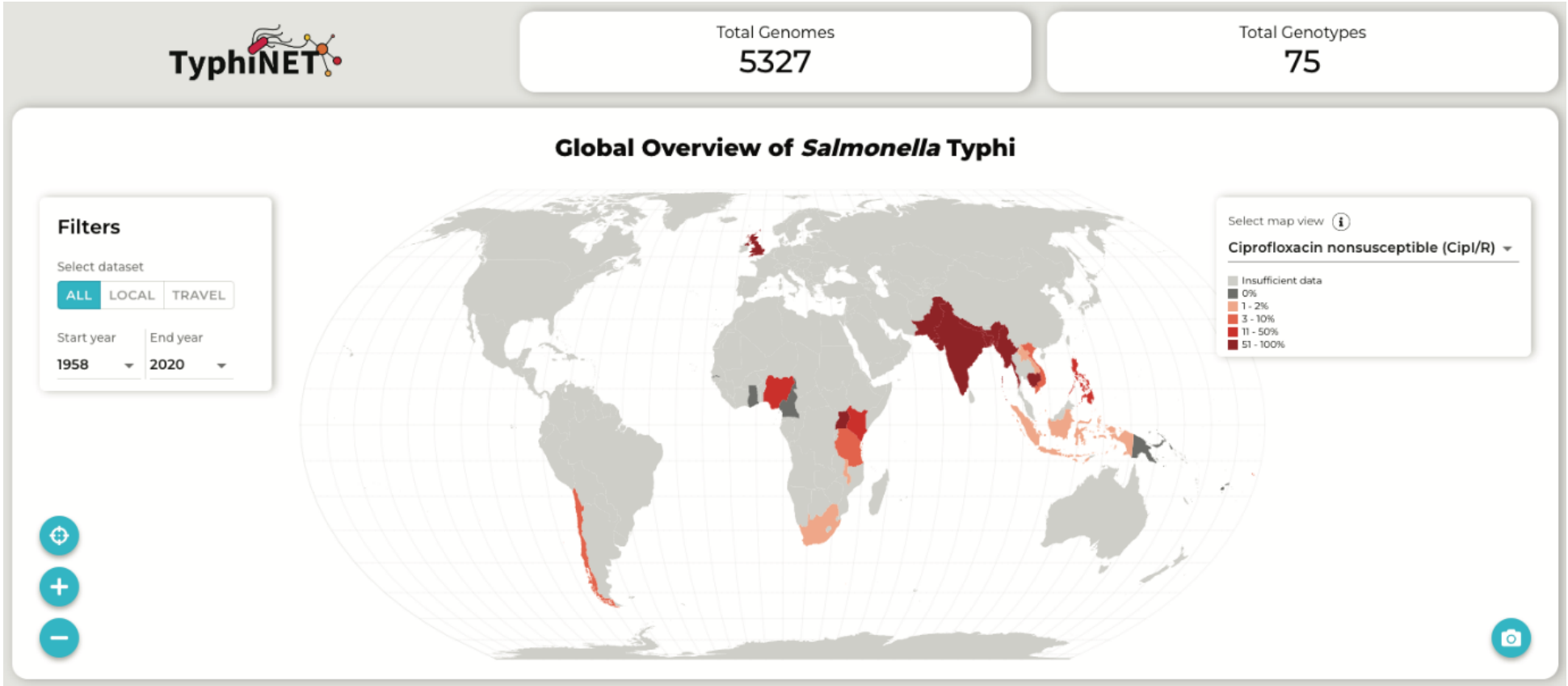


Figure 44. TyphiNET user generated global overview of ciprofloxacin non-susceptibility frequencies.

Top panels indicate the number of sequences and genotypes present in the TyphiNET database as of May 2022. Left panel indicates controls for filtering the data visualised by data source (all data, locally collected, or travel-associated cases) and time period (by providing start and end years for the period). Countries on the map are coloured by the frequency of ciprofloxacin non-susceptibility as per the inset legend (top right of map). Data for each country are shown when n≥20 sequences are available. Controls to zoom in and out of the map, and to centre the map are available at the bottom left of the map. Hovering the mouse over any of the countries on the map provides summary statistics. Clicking on individual countries triggers county-level summaries (shown in **Figure 45**). Camera button allows users to download visualisations.

The following plots can be viewed for either global data or a selected country (**Figure 45**): (i) drug resistance frequencies over time; (ii) trends in genotype frequency over time; (iii) the frequency of resistance to different drug classes among pathogen genotypes; and (iv) the molecular drivers of AMR within pathogen genotypes. Country-level visualisations for Pakistan, using all available genomes from 2003-2020 including returning travellers, are shown in **Figure 45** and illustrate the previously identified emergence and expansion of genotype 4.3.1.1.P1 responsible for ongoing outbreaks of XDR typhoid in Pakistan,[24,233] typified by resistance to former first-line drugs, ciprofloxacin, and third generation cephalosporins (3GC). TyphiNET visualisations show an increase in the proportion of *S.* Typhi that are resistant to classical first-line drugs, ciprofloxacin, and 3GC from around 2017 (**Fig 45A**), coinciding with an increase in genotype 4.3.1.1.P1 (black in **Fig 45B**). Examination of resistance frequencies within genotypes demonstrates that 3GC resistance is associated only with genotype 4.3.1.1.P1 (purple in **Fig 45C**) and mediated by the acquisition of a *blaCTX-M-15* gene (blue in **Fig 45D**).

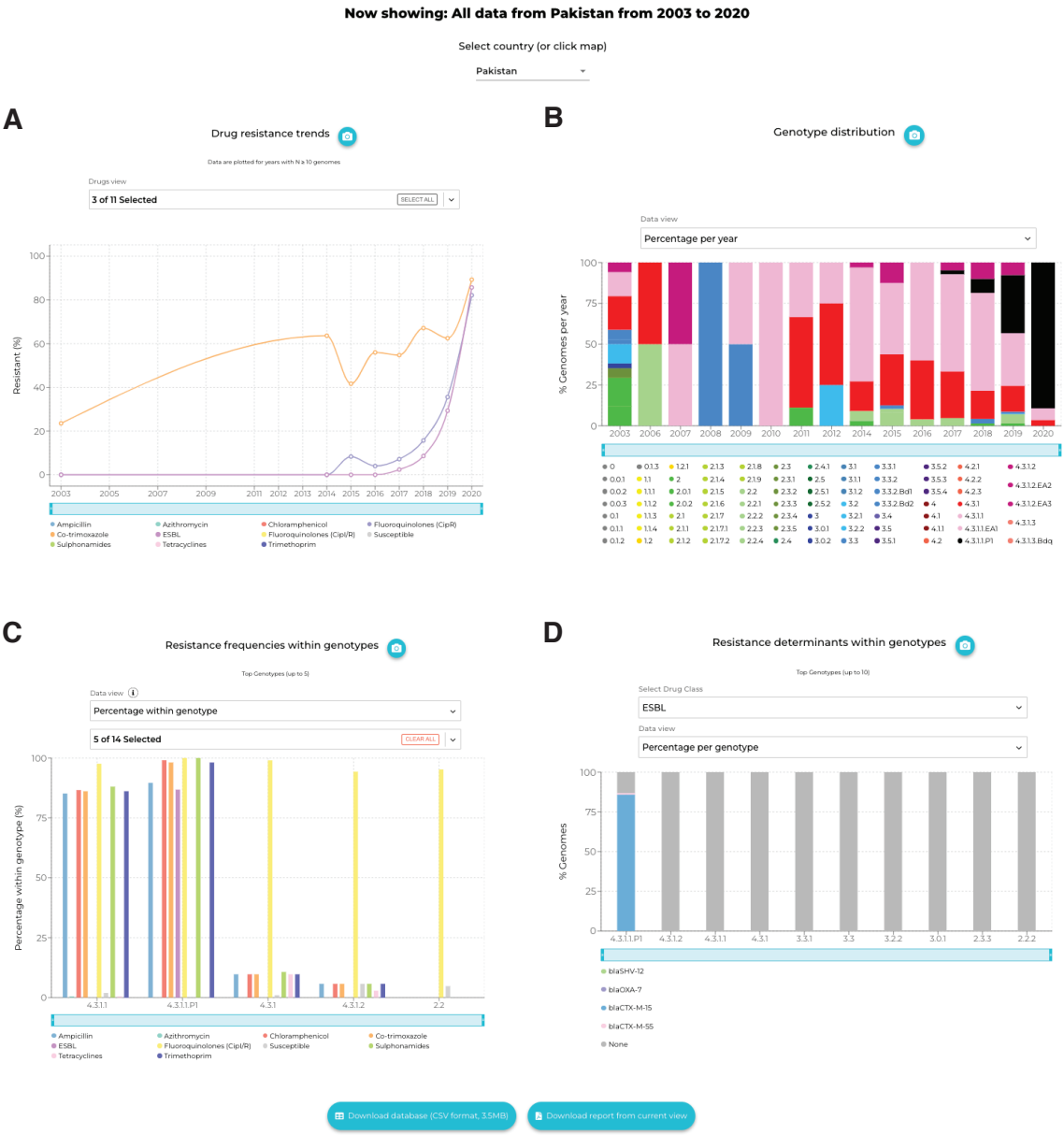


Figure 45. TyphiNET country-level overviews of *S*. Typhi populations in Pakistan.

(A) Drug resistance trends over time plot show resistance frequencies to selected drug classes per year where n≥10 sequences are available. Lines are coloured as per the inset legend, here we have selected 'ESBL' (i.e. 3GC resistance, purple), ciprofloxacin resistance (blue/grey) and co-trimoxazole (orange) to simplify the view. (B) Annual genotype distribution plot reveals the frequencies of pathogen genotypes per year. Genotypes are coloured as per the inset legend. (C) Resistance frequencies within genotypes plot shows frequencies of resistance to different drug classes, within common genotypes. Bars are coloured according to the inset legend. The 5 most resistant genotypes are shown by default. (D) Resistance determinants within genotypes plot shows the distribution of specific genes and mutations mediating resistance to a selected drug class, within common pathogen genotypes.

TyphiNET provides easy access to aggregated genome-derived resistance frequencies for clinically relevant antimicrobials utilised in controlling typhoid, making this data accessible for the first time to a broad range of users without genomics expertise. It is anticipated that these data will be of public health utility as they have the potential to inform national and regional treatment guidelines and control strategies. For example, overviews of resistance frequencies could assist in guiding empirical treatment of typhoid in LMIC settings where the disease is likely endemic, but surveillance data are lacking. In high-income countries where most infections are travel-associated, individual treatment could be informed by resistance frequencies from the country or countries visited. High resistance frequencies to multiple antimicrobials may be informative for targeting other intervention strategies such as the programmatic use of typhoid conjugate vaccines in specific regions. The inclusion of data from travel-associated cases provides informal sentinel surveillance for resistance in countries which *S*. Typhi surveillance data are not available.[55]

TyphiNET was developed as an open source MERN (MongoDB, Express, React, Node.js) stack JavaScript application (code available at: https://github.com/zadyson/TyphiNET). Source, genotypes,[47,133,134] and AMR data from 'non-targeted' sampling frames sequences are imported regularly from Typhi Pathogenwatch[43] and curated by contributors to the Global Typhoid Genomics Consortium.

7.4.5 Global Typhoid Genomics Consortium

The Global Typhoid Genomics Consortium was established in April 2021, to provide a mechanism for the global typhoid research community to engage collaboratively in the aggregation of *S*. Typhi genomic data to facilitate monitoring the emergence and spread of

AMR and to inform targeted public health action. The specific goals of the consortium are to (1) encourage prompt sharing of typhoid genome data for public health benefit; (2) facilitate the extraction and reporting of key data of public health relevance; and (3) promote and facilitate the dissemination and use of information derived from typhoid genomic data to monitor AMR and post-vaccination impact. One of the first major initiatives of the consortium was the generation of a global update paper, which is described **Chapter 6**.

The key activities of the Consortium are to encourage and coordinate sharing and release of typhoid genomics data in a manner that maximise its potential to inform public health. The Consortium does not seek to generate or claim ownership of any genome data; rather the model is to encourage data generators to deposit raw genome data into public databases (ENA or SRA), and share source information using a standardised metadata template (available at https://bit.ly/typhiMeta). This model facilitates harmonization of source information across aggregated *S*. Typhi data – which is crucial to allow downstream integration of the data for public health benefit – while ensuring that Consortium members retain full control over their data and when they choose to make it public. Typhi Pathogenwatch (described above) is used as the central analysis platform to generate inferred genotypes and AMR determinants from raw genomes, and to maintain a publicly available and searchable database of genome assemblies and an interactive global phylogeny. Genotyping is done using the GenoTyphi framework,[47,133] whose ongoing curation will be managed by a working group of the Consortium. A key field in the Consortium metadata template is 'purpose of sampling', which seeks to identify sets of genomes that are derived from 'non-targeted' sampling frames that are suitable for

estimation of national annual prevalence rates of AMR and genotypes (e.g. in the TyphiNET dashboard, described above, and other reports).

Consortium membership is free and open to all (see https://www.typhoidgenomics.org/); the intention is to include all those with an academic or public health interest in using WGS to investigate, monitor and/or understand typhoid epidemiology. Current membership (as at mid-2022) numbers over 150 individuals from 39 countries. A majority of members are from countries in Africa and Asia where typhoid is endemic; however, there is also considerable participation from countries where typhoid is considered a travel-associated disease and subject to routine WGS (which provide useful 'sentinel surveillance' data for common travel destinations).[55] Consortium activities are overseen by a multidisciplinary steering committee of international experts in typhoid surveillance and epidemiology, and an advisory board of stakeholders from the global public health community has been engaged to help identify ways to promote typhoid genomic surveillance – and particularly the use of pathogen WGS data – for public health benefit.

7.4.6 Conclusions

Efforts to "democratize" or broaden access to WGS technologies, reagents, and trainings are well under way, and have been accelerated in some instances as access to sequencing technologies and training have increased during the COVID-19 pandemic.[275] However, there is still a need for additional funding and support for the purchase and maintenance of sequencing machines, reagents, and training. The COVID-19 response clearly illustrated the power of researchers, public health professionals, donors, governments, and bioinformaticians working together with the common goal of data sharing and collaborative

analysis, even if stakeholders in LMICs were disincentivized to be transparent, as in the case of travel bans following the initial reports of the Omicron variant from Southern Africa.[276] There is more work to be done to increase access to sequencing and analysis capacity, and more work to be done to convince governments and donors that supporting these activities is key to future global health security, but the power of data sharing and collaboration through consortia like the COVID-19 Genomics UK (COG-UK) consortium,[277] as well as the utility of freely available data sharing, visualization, and analysis tools like Nextstrain[278] have been demonstrated very clearly.

7.5 Discussion and Next Steps

Genomics and typhoid epidemiology have rapidly become more sophisticated, and the field is still moving forward apace, as epidemiological data gaps are being filled and newly licensed vaccines are being introduced into national immunization programs. However, there are still major regional data gaps, and questions remain in the minds of policymakers about how to locate and analyse existing data as they evaluate the public health value of a TCV programme, particularly when set against the backdrop of multiple competing immunization priorities. The continued emergence and spread of drug-resistant *S*. Typhi heightens the urgency for the widespread deployment of TCVs, and generating, sharing, and visualising WGS data could provide much needed support to decision-makers in typhoid endemic countries.

SARS-CoV-2 has demonstrated the value of global genomic surveillance and data sharing, and one of its legacies will be broader access to and use of sequencing and phylogenetic analysis. Such capacity could be expanded to include surveillance of *S*. Typhi and other priority pathogens if this is not already being done. Generating additional, more geographically representative *S*. Typhi WGS data and standardised metadata and sharing these data more broadly can facilitate a better understanding of where *S*. Typhi exists, what drug-resistance profiles exist, and how and where drug-resistance emerges and spreads. By making this information available and comprehensible to policymakers through efforts like the Global Typhoid Genomics Consortium and platforms like Typhi Pathogenwatch and TyphiNET, we can ensure that this information informs the introduction of TCVs, the selection of vaccination strategies, including timing, location, and target age range for catch-up campaigns. In addition, this information can inform better antimicrobial stewardship

practices to extend the useful lifespan of licensed antimicrobial agents. In addition, WGS and genomic-informed analyses can help us to develop better diagnostic tools, and to understand the impact of TCVs on bacterial population structures. Improved diagnostics and better genomic surveillance can help us to understand global disease incidence, target interventions, measure their impact, and assess the feasibility of typhoid elimination by demonstrating how far large-scale TCV deployment goes towards disease control.

Work presented in this thesis clearly demonstrates the role that WGS can play in identifying molecular mechanisms of resistance, tracking the emergence and spread of drug-resistant *S*. Typhi, and informing decisions to introduce TCV. Discussion in later chapters outlined additional ways that WGS can be used to advance typhoid fever control goals. There are definite next steps for each body of work presented in this thesis, and as well as for some additional typhoid control initiatives.

7.5.1 Azithromycin resistance in South Asia

Work presented in this thesis demonstrating the independent emergence of azithromycin resistant *S*. Typhi in multiple locations in South Asia has been presented at multiple academic conferences and WHO meetings (see **Appendix 1**). As mentioned previously, **Pakistan** has introduced TCVs into their national immunization programme. We are collaborating with the Aga Khan University as part of the TyVAC Consortium to develop sequencing and bioinformatic analysis capacity to ensure that they can generate and analyse their own WGS data to track the spread of XDR and potentially azithromycin-resistant *S*. Typhi, as well as to measure of the genomic impact of TCV introduction in Pakistan. I am also organizing a stakeholder convening through the TyVAC consortium to

develop an agreed-upon framework for measuring the genomic impact of TCV, as this concept is often discussed, but in nebulous terms. This guidance or framework would be incorporated into the World Health Organization's work to develop methodologies on how to measure the impact of vaccines on AMR using TCV as an example, which I am leading as a consultant (**Appendix 7**). This approach could also be leveraged in **Nepal** and **Zimbabwe**, where national TCV introduction is also ongoing. We are working with collaborators in Zimbabwe to establish the "baseline" *S*. Typhi population structure in Harare before a large TCV campaign and will look to support a post-vaccination analysis as well.

At time of writing, neither Bangladesh nor India has publicly made the decision to introduce TCV into their respective national immunisation programmes, but I intend to continue to work with key stakeholders in these settings (and more broadly) to ensure that WGS data are being generated and shared to monitor any additional emergence of drug-resistant *S*. Typhi, both to inform TCV introduction as well as to inform treatment practices.

7.5.2 Origins of H58

The phylogenetic and phylodynamic analysis conducted as part of the origins of H58 *S*. Typhi work will used to inform a transmission dynamic model that has been developed by Virginia Pitzer and colleagues at Yale School of Public health to predict the future spread of XDR typhoid. We hope that this modelling work will encourage countries to consider the implementation of typhoid conjugate vaccines and WSH interventions to guard against the possibility of the XDR typhoid to their respective settings, as well as other enteric pathogens. While the hypothesis that the original H58 isolate came from the gallbladder of a chronic carrier may require additional investigation, this work and other work

investigating chronic carriers in Vietnam, Chile, and Kenya[208,210,227] also strengthens the concept that chronic carriers can play a large role in sustaining typhoid transmission, and a proportionately higher role in formerly endemic settings, which suggests that more work should be done to develop and validate a sensitive, low-cost screening assay for chronic carriers so that they may be identified and treated.

7.5.3 Global Typhoid Genomics Consortium

The results of this analysis have been preprinted and are still under review at eLife. Upon acceptance of the manuscript (hopefully) and submission of an accompanying commentary highlighting key points of public health relevance for submission to a global health journal, I intend to continue to build on the momentum that we have generated to promote additional data sharing and awareness of the consortium activities and the TyphiNET data visualisation platform in support of TCV introduction decision-making and antimicrobial stewardship. This includes promoting awareness and understanding of the potential role of returning traveller data to serve as sentinel surveillance from areas where scant data exist. I plan to do this through my engagement with the TyVAC consortium, as well as my ongoing Enteric Fever Burden of Disease consulting work with the WHO's Immunisations, Vaccines, and Biologicals group. I also intend to work with other academic research groups and public health laboratories to promote generation, sharing, and analysis of genomic data and standardised metadata using the consortium metadata template, with a focus on areas from where few data are publicly available.

References

1. Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis*. 2019;19(4):369-381. doi:10.1016/S1473-3099(18)30685-6
2. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive *Salmonella* infections. *Clin Microbiol Rev*. 2015;28(4):901-937. doi:10.1128/CMR.00002-15
3. de Jong HK, Parry CM, van der Poll T, Wiersinga WJ. Host-Pathogen Interaction in Invasive *Salmonellosis*. *PLoS Pathog*. 2012;8(10):1-9. doi:10.1371/journal.ppat.1002933
4. Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively Drug-Resistant Typhoid — Are Conjugate Vaccines Arriving Just in Time? *New England Journal of Medicine*. 2018;379(16):1491-1493. doi:10.1056/nejmp1805927
5. Dougan G, Baker S. *Salmonella enterica* serovar Typhi and the pathogenesis of typhoid fever. *Annu Rev Microbiol*. 2014;68:317-336. doi:10.1146/annurev-micro-091313-103739
6. Levine MM, Black RE, Lanata C, Committee CT. Precise Estimation of the Numbers of Chronic Carriers of *Salmonella* Typhi in Santiago, Chile, an Endemic Area. *Journal of Infectious Diseases*. 1982;146(6). http://jid.oxfordjournals.org/
7. Parry CM, Wijedoru L, Arjyal A, Baker S. The utility of diagnostic tests for enteric fever in endemic locations. *Expert Rev Anti Infect Ther*. 2011;9(6):711-725. doi:10.1586/eri.11.47
8. Levine MM, Grados O, Gilman RH, Woodward WE, Solis-Plaza R, Waldman W. Diagnostic Value of the Widal Test in Areas Endemic for Typhoid Fever. *American Journal of Tropical Medicine and Hygiene*. 1978;27(7):785-800. doi:10.1093/infdis/jiy494
9. World Health Organisation. Typhoid and other invasive Salmonellosis. Vaccine Preventable Diseases Surveillance Standards. Published online 2018.
10. Antillon M, Saad NJ, Baker S, Pollard AJ, Pitzer VE. The Relationship between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever: A Systematic Review and Meta-Analysis. *Journal of Infectious Diseases*. 2018;218(Suppl 4):S255-S267. doi:10.1093/infdis/jiy471
11. Moynihan R, Sanders S, Michaleff ZA, et al. Impact of COVID-19 pandemic on utilisation of healthcare services: A systematic review. *BMJ Open*. 2021;11(3). doi:10.1136/BMJOPEN-2020-045343
12. Carey ME, Macwright WR, Im J, et al. The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences. *Clinical Infectious Diseases*. 2020;71(Suppl 2). doi:10.1093/cid/ciaa367
13. Marchello CS, Hong CY, Crump JA. Global typhoid fever incidence: A systematic review and meta-analysis. *Clinical Infectious Diseases*. 2019;68:S105-S116. doi:10.1093/cid/ciy1094

14. Global Burden of Disease Collaborative Network. Global Burden of Disease Study 2019 (GBD 2019) Results. Published 2020. Accessed February 12, 2023. https://vizhub.healthdata.org/gbd-results/.
15. Antillón M, Warren JL, Crawford FW, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. Published online 2017. doi:10.1371/journal.pntd.0005376
16. Mogasale V, Maskery B, Ochiai RL, et al. Burden of typhoid fever in low-income and middle-income countries: A systematic, literature-based update with risk-factor adjustment. *Lancet Glob Health*. 2014;2(10):e570-e580. doi:10.1016/S2214-109X(14)70301-8
17. Kim JH, Mogasale V, Im J, Ramani E, Marks F. Updated estimates of typhoid fever burden in sub-Saharan Africa. *Lancet Glob Health*. 2017;5(10):e969. doi:10.1016/S2214-109X(17)30328-5
18. World Health Organization. Typhoid vaccines: WHO position paper. *Weekly Epidemiological Record*. 2008;83(6):49-60. doi:10.1007/BF02752413
19. John A. Crump, Stephen P. Luby and EDM. The global burden of typhoid fever. Bull World Health Organ. *Bull World Health Organ*. 2004;82(5):346-353. doi:10.1590/S0042-96862004000500008
20. Marks F, von Kalckreuth V, Aaby P, et al. Incidence of invasive *Salmonella* disease in sub-Saharan Africa: a multicentre population-based surveillance study. *Lancet Glob Health*. 2017;5(3):e310-e323. doi:10.1016/S2214-109X(17)30022-0
21. World Health Organization. Typhoid vaccines: WHO position paper – March 2018. *Weekly epidemiological record*. 2018;13(93):153-172.
22. Garrett DO, Longley AT, Aiemjoy K, et al. Incidence of Typhoid and Paratyphoid Fever in Bangladesh, Nepal, and Pakistan: Results of the Surveillance for Enteric Fever in Asia Project. Vol 5. https://ssrn.com/abstract=3866551
23. Meiring JE, Shakya M, Khanam F, et al. Burden of enteric fever at three urban sites in Africa and Asia: a multicentre population-based study. *Lancet Glob Health*. 2021;9(12):e1688-e1696. doi:10.1016/S2214-109X(21)00370-3
24. Klemm EJ, Shakoor S, Page AJ, 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):1-10. doi:10.1128/mBio.00105-18
25. Qamar FN, Yousafzai MT, Khalid M, et al. Outbreak investigation of ceftriaxone-resistant *Salmonella enterica* serotype Typhi and its risk factors among the general population in Hyderabad, Pakistan: a matched case-control study. *Lancet Infect Dis*. Published online 2018. doi:10.1016/S1473-3099(18)30483-3
26. Eshaghi A, Zittermann S, Bharat A, Mulvey MR, Allen VG, Patel SN. Importation of Extensively Drug-Resistant *Salmonella enterica* Serovar Typhi Cases in Ontario, Canada. *Antimicrob Agents Chemother*. 2020;64(5). https://journals.asm.org/journal/aac
27. Watkins LKF, Winstead A, Appiah GD, et al. Update on Extensively Drug-Resistant *Salmonella* Serotype Typhi Infections Among Travelers to or from Pakistan and Report of Ceftriaxone-Resistant *Salmonella* Serotype Typhi Infections Among Travelers to Iraq-United States, 2018-2019. *Morbidity and Mortality Weekly Report* . 2020;69(20):618-622. https://www.ncbi.nlm.nih.gov.

28. Ingle DJ, Andersson P, Valcanis M, et al. Genomic epidemiology and antimicrobial resistance mechanisms of imported typhoid in australia. *Antimicrob Agents Chemother*. 2021;65(12). doi:10.1128/AAC.01200-21
29. Herdman MT, Karo B, Dave J, et al. Increasingly limited options for the treatment of enteric fever in travellers returning to England, 2014-2019: A cross-sectional analytical study. *J Med Microbiol*. 2021;70(8). doi:10.1099/JMM.0.001359
30. Bogomolnaya LM, Aldrich L, Ragoza Y, Talamantes M, Andrews KD. Identification of Novel Factors Involved in Modulating Motility of *Salmonella enterica* Serotype Typhimurium. *PLoS One*. 2014;9(11):111513. doi:10.1371/journal.pone.0111513
31. Hooda Y, Saha S, Sajib MSI, et al. Emergence and molecular basis of azithromycin resistance in typhoidal *Salmonella* in Dhaka, Bangladesh. *bioRxiv*. Published online 2019:594531. doi:10.1101/594531
32. Iqbal, Junaid, Dehraj, Irum F., Carey, Megan E., Dyson, Zoe A., Garrett, Denise, Seidman, Jessica C., Kabir, Furqan, Saha, Senjuti, Baker, Stephen, Qamar FN. A Race against Time : Reduced Azithromycin Susceptibility in *Salmonella enterica* Serovar Typhi in Pakistan. *mSphere*. 2020;5(4):e00215-20.
33. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal . *JAC Antimicrob Resist*. 2020;2(4). doi:10.1093/jacamr/dlaa109
34. Carey ME, Jain R, Yousuf M, et al. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of *Salmonella* Typhi in Northern India. *Clinical Infectious Diseases*. 2021;72(5):e120-127. doi:10.1093/cid/ciaa1773
35. Sikorski MJ, Hazen TH, Desai SN, et al. Persistence of rare *Salmonella* Typhi genotypes susceptible to first-line antibiotics in the remote islands of Samoa. *medRxiv*. Published online 2022. doi:10.1101/2022.03.22.22271657
36. Octavia S, Chew KL, Lin RTP, Teo JWP. Azithromycin-Resistant *Salmonella* enterica Serovar Typhi AcrB-R717Q/L, Singapore. *Emerging Infectious Diseases*. 2021;27(2):624-627. doi:10.3201/eid2702.203874
37. Marchello CS, Carr SD, Crump JA. A systematic review on antimicrobial resistance among *Salmonella* Typhi worldwide. *American Journal of Tropical Medicine and Hygiene*. 2020;103(6):2518-2527. doi:10.4269/ajtmh.20-0258
38. Laghari GS, Hussain Z, Hussain SZM, Kumar H, Uddin SMM, Haq A. Antimicrobial Susceptibility Patterns of *Salmonella* Species in Southern Pakistan. *Cureus*. Published online April 4, 2019. doi:10.7759/cureus.4379
39. Hardjo Lugito NP, Cucunawangsih. Antimicrobial resistance of *Salmonella enterica* serovars Typhi and Paratyphi isolates from a general Hospital in Karawaci, Tangerang, Indonesia: A five-year review. *Int J Microbiol*. 2017;2017. doi:10.1155/2017/6215136
40. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. 2022;399(10325):629-655. doi:10.1016/S0140-6736(21)02724-0
41. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. 2022;399(10325):629-655. doi:10.1016/S0140-6736(21)02724-0

42. Parkhill J, Dougan G, James KD, et al. Complete genome sequence of a multiple drug resistant *Salmonella enterica* serovar Typhi CT18. *Nature*. 2001;413(6858):848-852. doi:10.1038/35101607
43. Argimón S, Yeats CA, Goater RJ, et al. A global resource for genomic predictions of antimicrobial resistance and surveillance of *Salmonella* Typhi at pathogenwatch. *Nat Commun*. 2021;12(1). doi:10.1038/s41467-021-23091-2
44. Duchêne S, Holt KE, Weill FX, et al. Genome-scale rates of evolutionary change in bacteria. *Microb Genom*. 2016;2(11):e000094. doi:10.1099/mgen.0.000094
45. Wong VK, Baker S, Pickard DJ, 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):632-639. doi:10.1038/ng.3281
46. Typhi S, Esther da Silva K, Mohammad Tanmoy A, et al. The international and intercontinental spread and expansion of antimicrobial-resistant *Salmonella* Typhi. doi:10.1101/2021.09.03.21262852
47. Wong VK, Baker S, Connor TR, et al. An extended genotyping framework for *Salmonella enterica* serovar Typhi, the cause of human typhoid. *Nat Commun*. 2016;7:1-11. doi:10.1038/ncomms12827
48. Roumagnac P, Weill FX, Dolecke C, et al. Evolutionary History of *Salmonella* Typhi. *Science (1979)*. 2006;314(5803):1301-1304. doi:10.1126/science.1133649
49. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global *Salmonella* Typhi genotyping framework. doi:10.1093/infdis/jiab414/6358992
50. Anderson ES. The problem and implications of chloramphenicol resistance in the typhoid bacillus. *Journal of Hygiene*. 1975;74:289-299. doi:10.1017/S0022172400024360
51. Holt KE, Phan MD, Baker S, et al. Emergence of a globally dominant inchi1 plasmid type associated with multiple drug resistant typhoid. *PLoS Negl Trop Dis*. 2011;5(7). doi:10.1371/journal.pntd.0001245
52. da Silva KE, Tanmoy AM, Pragasam AK, et al. The international and intercontinental spread and expansion of antimicrobial-resistant *Salmonella* Typhi: a genomic epidemiology study. *Lancet Microbe*. Published online June 2022. doi:10.1016/s2666-5247(22)00093-3
53. Feasey NA, Gaskell K, Wong V, et al. Rapid Emergence of Multidrug Resistant, H58-Lineage *Salmonella* Typhi in Blantyre, Malawi. *PLoS Negl Trop Dis*. 2015;9(4):1-13. doi:10.1371/journal.pntd.0003748
54. Kariuki S, Revathi G, Kiiru J, et al. Typhoid in Kenya is associated with a dominant multidrug-resistant *Salmonella enterica* serovar Typhi haplotype that is also widespread in Southeast Asia. *J Clin Microbiol*. 2010;48(6):2171-2176. doi:10.1128/JCM.01983-09
55. Ingle DJ, Nair S, Hartman H, 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):1-20. doi:10.1371/journal.pntd.0007620
56. Tanmoy AM, Westeel E, de Bruyne K, et al. *Salmonella enterica* serovar Typhi in Bangladesh: Exploration of genomic diversity and antimicrobial resistance. *mBio*. 2018;9(6):1-17. doi:10.1128/mBio.02112-18

57. Rahman SIA, Dyson ZA, Klemm EJ, et al. Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in Urban Dhaka, Bangladesh from 2004 to 2016. *PLoS Negl Trop Dis*. 2020;14(2). doi:10.1371/journal.pntd.0008036
58. Chau TT, Campbell JI, Galindo CM, et al. Antimicrobial drug resistance of *Salmonella enterica* serovar Typhi in Asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. *Antimicrob Agents Chemother*. 2007;51(12):4315-4323. doi:10.1128/AAC.00294-07
59. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic resistance and typhoid. *Clinical Infectious Diseases*. 2019;68:S165-S170. doi:10.1093/cid/ciy1111
60. Day MR, Doumith M, do Nascimento V, et al. Comparison of phenotypic and WGS-derived antimicrobial resistance profiles of *Salmonella enterica* serovars Typhi and Paratyphi. Published online 2017. doi:10.1093/jac/dkx379
61. Pham Thanh D, Karkey A, Dongol S, et al. A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. *Elife*. 2016;5(e14003). doi:10.7554/eLife.14003.001
62. Wain J, Hoa NTT, Chinh NT, et al. Quinolone-Resistant *Salmonella* Typhi in Viet Nam: Molecular Basis of Resistance and Clinical Response to Treatment. *Clinical Infectious Diseases*. 1997;25:1404-1410. https://academic.oup.com/cid/article/25/6/1404/363746
63. Britto CD, Dyson ZA, Mathias S, et al. Persistent circulation of a fluoroquinolone-resistant *Salmonella enterica* Typhi clone in the Indian subcontinent. *Journal of Antimicrobial Therapy*. 2020;75:337-341. doi:10.1093/jac/dkz435
64. Balasegaram S, Potter AL, Grynszpan D, et al. Guidelines for the public health management of typhoid and paratyphoid in England. Practice guidelines from the National Typhoid and Paratyphoid Reference Group. *Journal of Infection*. 2012;65(3):197-213. doi:10.1016/j.jinf.2012.05.005
65. Rai S, Jain S, Prasad KN, Ghoshal U, Dhole TN. Rationale of azithromycin prescribing practices for enteric fever in India. *Indian J Med Microbiol*. 2012;30(1):30-33. doi:10.4103/0255-0857.93017
66. Basnyat B, Qamar FN, Rupali P, Ahmed T, Parry CM. Enteric fever. *BMJ*. 2021;372(n437). doi:10.1136/bmj.n437
67. Yousafzai MT, Qamar FN, Shakoor S, et al. Ceftriaxone-resistant *Salmonella* Typhi Outbreak in Hyderabad City of Sindh, Pakistan: High Time for the Introduction of Typhoid Conjugate Vaccine. *Clinical Infectious Diseases*. 2019;68(MIC):S16-S21. doi:10.1093/cid/ciy877
68. Nair S, Chattaway M, Langridge GC, et al. ESBL-producing strains isolated from imported cases of enteric fever in England and Wales reveal multiple chromosomal integrations of blaCTX-M-15 in XDR *Salmonella* Typhi. *Journal of Antimicrobial Chemotherapy*. 2021;76(6):1459-1466. doi:10.1093/jac/dkab049
69. Sah R, Donovan S, Seth-Smith HMB, et al. A Novel Lineage of Ceftriaxone-resistant *Salmonella* Typhi From India That Is Closely Related to XDR S. Typhi Found in Pakistan. *Clinical Infectious Diseases ®*. 2020;71(5):1327-1357. doi:10.1093/cid/ciz1204

70. Rodrigues C, Kapil A, Sharma A, et al. Whole genome shotgun sequencing of cephalosporin-resistant *Salmonella enterica* serovar Typhi. *Genome Announc*. 2017;5(10). doi:10.1128/genomeA.01639-16
71. Jacob JJ, Pragasam AK, Vasudevan K, et al. *Salmonella* Typhi acquires diverse plasmids from other *Enterobacteriaceae* to develop cephalosporin resistance. *Genomics*. 2021;113(4):2171-2176. doi:10.1016/j.ygeno.2021.05.003
72. Argimón S, Nagaraj G, Shamanna V, et al. Clinical Infectious Diseases Circulation of Third-Generation Cephalosporin Resistant *Salmonella* Typhi in Mumbai, India. *Clinical Infectious Diseases*. 2022;74(12):2234-2241. doi:10.1093/cid/ciab897
73. Levine MM, Simon R. The Gathering Storm: Is Untreatable Typhoid Fever on the Way? *mBio*. 2018;9(2):e00482-18. doi:10.1128/mBio
74. Jin C, Gibani MM, Pennington SH, et al. Treatment responses to Azithromycin and Ciprofloxacin in uncomplicated *Salmonella* Typhi infection: A comparison of Clinical and Microbiological Data from a Controlled Human Infection Model. *PLoS Negl Trop Dis*. 2019;13(12):e0007955. doi:10.1371/journal.pntd.0007955
75. Hooda Y, Sajib MSI, Rahman H, et al. Molecular mechanism of azithromycin resistance among typhoidal *Salmonella* stains in Bangladesh identified through passive pediatric surveillance. *PLoS Negl Trop Dis*. 2019;13(11):e0007868. doi:10.1371/journal.pntd.0007868
76. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant *Salmonella* Typhi in Nepal. *JAC Antimicrob Resist*. 2020;2(4):dlaa109-dlaa109. doi:10.1093/jacamr/dlaa109
77. Sajib MSI, Tanmoy AM, Hooda Y, et al. Tracking the emergence of azithromycin resistance in multiple genotypes of typhoidal *Salmonella*. *mBio*. 2021;12(1):1-12. doi:10.1128/mBio.03481-20
78. Chattaway MA, Gentle A, Nair S, et al. Phylogenomics and antimicrobial resistance of *Salmonella* Typhi and Paratyphi A, B and C in England, 2016–2019. *Microb Genom*. 2021;7(8). doi:10.1099/mgen.0.000633
79. Okeke IN, Feasey N, Parkhill J, et al. Leapfrogging laboratories: the promise and pitfalls of high-tech solutions for antimicrobial resistance surveillance in low-income settings Handling editor Seye Abimbola. *BMJ Glob Health*. 2020;5:3622. doi:10.1136/bmjgh-2020-003622
80. Steele AD, Hay Burgess DC, Diaz Z, Carey ME, Zaidi AKM. Challenges and Opportunities for Typhoid Fever Control: A Call for Coordinated Action. *Clinical Infectious Diseases*. 2016;62. doi:10.1093/cid/civ976
81. Hessel L, Debois H, Fletcher M, Dumas R. Experience with *Salmonella* Typhi Vi capsular polysaccharide vaccine. *European Journal of Clinical Microbiology and Infectious Diseases*. 1999;18(9):609-620. doi:10.1007/s100960050361
82. Germanier R, Fuerer E. Isolation and characterization of Gal E mutant Ty 21a of *Salmonella* Typhi: a candidate strain for a live, oral typhoid vaccine. *Journal of Infectious Diseases*. 1975;131(5):553-558. doi:10.1093/infdis/131.5.553
83. Bentsi-Enchill AD, Hombach J. Revised Global Typhoid Vaccination Policy. *Clinical Infectious Diseases*. 2019;68(Suppl 1):S31-S33. doi:10.1093/cid/ciy927
84. Carey ME, Diaz ZI, Zaidi AKM, Steele AD. A Global Agenda for Typhoid Control - A Perspective from the Bill & Melinda Gates Foundation. *Clinical Infectious Diseases*. 2019;68. doi:10.1093/cid/ciy928

85. Jin C, Gibani MM, Moore M, et al. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of *Salmonella* Typhi: a randomised controlled, phase 2b trial. *Lancet*. 2017;390(10111):2472-2480. doi:10.1016/S0140-6736(17)32149-9
86. Khan MI, Franco-Paredes C, Sahastrabuddhe S, Ochiai RL, Mogasale V, Gessner BD. Barriers to typhoid fever vaccine access in endemic countries. *Res Rep Trop Med*. 2017;Volume 8:37-44. doi:10.2147/rrtm.s97309
87. Lin FYC, Ho VA, Khiem HB, et al. The efficacy of a *Salmonella* Typhi Vi conjugate vaccine in two-to-five-year-old children. 2001;344(17):1263-1269.
88. Szu SC. Development of Vi conjugate - A new generation of typhoid vaccine. *Expert Rev Vaccines*. 2013;12(11):1273-1286. doi:10.1586/14760584.2013.845529
89. Qadri F, Khanam F, Liu X, et al. Protection by vaccination of children against typhoid fever with a Vi-tetanus toxoid conjugate vaccine in urban Bangladesh: a cluster-randomised trial. *Lancet*. 2021;398(10301):675-684. doi:10.1016/S0140-6736(21)01124-7
90. Patel PD, Patel P, Liang Y, et al. Safety and Efficacy of a Typhoid Conjugate Vaccine in Malawian Children. *New England Journal of Medicine*. 2021;385(12):1104-1115. doi:10.1056/nejmoa2035916
91. Shakya M, Voysey M, Theiss-Nyland K, et al. Efficacy of typhoid conjugate vaccine in Nepal: final results of a phase 3, randomised, controlled trial. *Lancet Glob Health*. 2021;9(11):e1561-e1568. doi:10.1016/S2214-109X(21)00346-6
92. Laurens MB, Sirima SB, Rotrosen ET, et al. A Phase II, Randomized, Double-blind, Controlled Safety and Immunogenicity Trial of Typhoid Conjugate Vaccine in Children Under 2 Years of Age in Ouagadougou, Burkina Faso: A Methods Paper. *Clinical Infectious Diseases*. 2019;68(Supplement_2):S59-S66. doi:10.1093/cid/ciy1104
93. Sirima SB, Ouedraogo A, Barry N, et al. Safety and immunogenicity of co-administration of meningococcal type A and measles–rubella vaccines with typhoid conjugate vaccine in children aged 15–23 months in Burkina Faso. *International Journal of Infectious Diseases*. 2021;102(September 2011):517-523. doi:10.1016/j.ijid.2020.10.103
94. Sirima SB, Ouedraogo A, Barry N, et al. Safety and immunogenicity of Vi-typhoid conjugate vaccine co-administration with routine 9-month vaccination in Burkina Faso: A randomized controlled phase 2 trial. *International Journal of Infectious Diseases*. 2021;108:465-472. doi:10.1016/j.ijid.2021.05.061
95. SAGE Working Group on Typhoid Vaccines & the WHO Secretariat. *Background Paper to SAGE on Typhoid Vaccine Policy Recommendations*. April 2022.
96. Yousafzai MT, Karim S, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed *Salmonella enterica* serotype Typhi in an extensively drug-resistant outbreak setting of Hyderabad, Pakistan: a cohort study. *Lancet Glob Health*. 2021;9(8):e1154-e1162. doi:10.1016/S2214-109X(21)00255-2
97. Thuluva S, Paradkar V, Matur R, Turaga K, Reddy S. A multicenter, single-blind, randomized, phase-2/3 study to evaluate immunogenicity and safety of a single intramuscular dose of biological E's Vi-capsular polysaccharide-CRM 197

conjugate typhoid vaccine (TyphiBEV TM) in healthy infants, children, and adults in comparison with a licensed comparator. *Hum Vaccin Immunother*. Published online 2022. doi:10.1080/21645515.2022.2043103
98. Date KA, Harvey P, Bhatnagar P, et al. LB-5213 - Field Effectiveness of a Typhoid Conjugate Vaccine — Navi Mumbai (India), 2018-2020. *American Society of Tropical Medicine and Hygiene 2020 National Meeting*; 2020. Accessed March 15, 2022. https://www.abstractsonline.com/pp8/#!/9181/presentation/4739
99. Batool R, Tahir Yousafzai M, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed typhoid in a peri-urban setting in Karachi: A case-control study. *Vaccine*. 2021;39(40):5858-5865. doi:10.1016/j.vaccine.2021.08.051
100. Yousafzai MT, Karim S, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed *Salmonella enterica* serotype Typhi in an extensively drug-resistant outbreak setting of Hyderabad, Pakistan: a cohort study. *Lancet Glob Health*. 2021;9(8):e1154-e1162. doi:10.1016/S2214-109X(21)00255-2
101. Mohan VK, Varanasi V, Singh A, et al. Safety and Immunogenicity of a Vi Polysaccharide–Tetanus Toxoid Conjugate Vaccine (Typbar-TCV) in Healthy Infants, Children, and Adults in Typhoid Endemic Areas: A Multicenter, 2-Cohort, Open-Label, Double-Blind, Randomized Controlled Phase 3 Study. *Clinical Infectious Diseases*. 2015;61(3):393-402. doi:10.1093/cid/civ295
102. Reddy R, Reddy B, Sarangi V, Reddy S, Ella R, Vadrevu KM. A multi-centre, post-marketing surveillance study of Vi polysaccharide–tetanus toxoid conjugate vaccine (Typbar TCV®) in India. *Hum Vaccin Immunother*. Published online 2021. doi:10.1080/21645515.2021.1947761
103. Neuzil KM, Basnyat B, Clemens JD, et al. Early insights from clinical trials of typhoid conjugate vaccine. *Clinical Infectious Diseases*. 2020;71:S155-S159. doi:10.1093/cid/ciaa370
104. Qamar FN, Yousafzai MT, Khaliq A, et al. Adverse events following immunization with typhoid conjugate vaccine in an outbreak setting in Hyderabad, Pakistan. *Vaccine*. 2020;38(19):3518-3523. doi:10.1016/j.vaccine.2020.03.028
105. Longley AT, Date K, Luby SP, et al. Evaluation of Vaccine Safety after the First Public Sector Introduction of Typhoid Conjugate Vaccine - Navi Mumbai, India, 2018. *Clinical Infectious Diseases*. 2021;73(4):E927-E933. doi:10.1093/cid/ciab059
106. Vadrevu KM, Raju D, Rani S, et al. Persisting antibody responses to Vi polysaccharide–tetanus toxoid conjugate (Typbar TCV®) vaccine up to 7 years following primary vaccination of children < 2 years of age with, or without, a booster vaccination. *Vaccine*. Published online 2021. doi:10.1016/j.vaccine.2021.07.073
107. Nampota-Nkomba N, Mbewe M, Nyirenda O, et al. *Safety and Long-Term Immunogenicity of a Novel Typhoid Conjugate Vaccine in Malawian Infants and Children (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses Meeting)*; 2021.

108. Qadri F, Khanam F, Babu G, et al. *Immune Responses Induced in Children after Vaccination with Vi-TT Vaccine: Immunogenicity Data from a Cluster-Randomised Controlled Trial in Bangladesh (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses)*; 2021.
109. Medise BE, Soedjatmiko S, Gunardi H, et al. A novel Vi-diphtheria toxoid typhoid conjugate vaccine is safe and can induce immunogenicity in healthy Indonesian children 2–11 years: a phase II preliminary report. *BMC Pediatr*. 2020;20(1). doi:10.1186/s12887-020-02375-4
110. Medise BE, Soedjatmiko S, Gunardi H, et al. A novel Vi-diphtheria toxoid typhoid conjugate vaccine is safe and can induce immunogenicity in healthy Indonesian children 2–11 years: a phase II preliminary report. *BMC Pediatr*. 2020;20(1). doi:10.1186/s12887-020-02375-4
111. Medise BE, Soedjatmiko S, Gunardi H, et al. One-month follow up of a randomized clinical trial-phase II study in 6 to <24 months old Indonesian subjects: Safety and immunogenicity of Vi-DT Typhoid Conjugate Vaccine. *International Journal of Infectious Diseases*. 2020;93:102-107. doi:10.1016/j.ijid.2020.01.045
112. Medise BE, Soedjatmiko S, Rengganis I, et al. Six-month follow up of a randomized clinical trial-phase I study in Indonesian adults and children: Safety and immunogenicity of Salmonella typhi polysaccharide-diphtheria toxoid (VI-DT) conjugate vaccine. *PLoS One*. 2019;14(2). doi:10.1371/journal.pone.0211784
113. Choi SK, Baik YO, Kim CW, et al. An open-label, comparative, single dose, clinical Phase Ⅰ study to assess the safety and immunogenicity of typhoid conjugate vaccine (Vi-CRM197) in healthy Filipino adults. *Vaccine*. 2021;39(19):2620-2627. doi:10.1016/j.vaccine.2021.03.089
114. Capeding MR, Teshome S, Saluja T, et al. Safety and immunogenicity of a Vi-DT typhoid conjugate vaccine: Phase I trial in Healthy Filipino adults and children. *Vaccine*. 2018;36(26):3794-3801. doi:10.1016/j.vaccine.2018.05.038
115. Capeding MR, Alberto E, Sil A, et al. Immunogenicity, safety and reactogenicity of a Phase II trial of Vi-DT typhoid conjugate vaccine in healthy Filipino infants and toddlers: A preliminary report. *Vaccine*. 2020;38(28):4476-4483. doi:10.1016/j.vaccine.2019.09.074
116. RITABRATA KUNDU, AMBROSE KUMAR KANDULNA, UMA NAYAK, et al. Immunogenicity and Safety of Typhoid Conjugate Vaccine in Healthy Indian Subjects: A Randomized, Active-controlled, Comparative Clinical Trial. *Indian Pediatr*. 2020;57:625-630.
117. Chan-hyuk K. SK Bioscience wins export nod for typhoid vaccine. Korea Biomedical Review.
118. Ali Syed K, Saluja T, Cho H, et al. Review on the Recent Advances on Typhoid Vaccine Development and Challenges Ahead. *Clinical Infectious Diseases* . 2020;71(S2):141-150. doi:10.1093/cid/ciaa504
119. Malhame M, Baker E, Gandhi G, et al. Shaping markets to benefit global health – A 15-year history and lessons learned from the pentavalent vaccine market. *Vaccine X*. 2019;2:100033. doi:10.1016/j.jvacx.2019.100033
120. WHO Expert Committee on Biological Standardization. *Annex 2 Recommendations to Assure the Quality, Safety and Efficacy of Typhoid*

Conjugate Vaccines Replacement of Annex 3 of WHO Technical Report Series, No. 987.
121. Capeding MR, Sil A, Tadesse BT, et al. Safety and immunogenicity of Vi-DT conjugate vaccine among 6-23-month-old children: Phase II, randomized, dose-scheduling, observer-blind Study. *EClinicalMedicine*. 2020;27. doi:10.1016/j.eclinm.2020.100540
122. Capeding MR, Tadesse BT, Sil A, et al. Immune persistence and response to booster dose of Vi-DT vaccine at 27.5 months post-first dose. *NPJ Vaccines*. 2022;7(1). doi:10.1038/s41541-022-00434-8
123. Saluja T, Giri BR, Chaudhary S, et al. Challenges and opportunities in setting up a phase III vaccine clinical trial in resource limited settings: Experience from Nepal. *Hum Vaccin Immunother*. 2021;17(7):2149-2157. doi:10.1080/21645515.2020.1855955
124. World Health Organization. Meeting of the Strategic Advisory Group of Experts on Immunization, April 2022: conclusions and recommendations. *Weekly Epidemiological Record*. 2022;97(24):261-276.
125. Date K, Shimpi R, Luby S, et al. Decision making and implementation of the first public sector introduction of typhoid conjugate vaccine-Navi Mumbai, India, 2018. *Clinical Infectious Diseases*. 2020;71:S172-S178. doi:10.1093/cid/ciaa597
126. World Health Organization Regional Office for the Eastern Mediterranean. Pakistan becomes first country to introduce new typhoid vaccine into routine immunisation program. 2019;(November):10-12. http://www.emro.who.int/pdf/pak/pakistan-news/pakistan-first-country-to-introduce-new-typhoid-vaccine-into-routine-immunization-programme.pdf?ua=1
127. World Health Organization Liberia. Liberia becomes the first country in sub-Saharan Africa to introduce Typhoid Conjugate Vaccine (TCV): Over 1 million children to be reached. WHO AFRO.
128. Oberman E. Typhoid conjugate vaccine arrives in Zimbabwe. Take on Typhoid (Blog).
129. Nepal introduces typhoid vaccine into routine immunisation across the country. UNICEF.
130. Sikorski M. Amidst a global pandemic, Samoa rolls out TCV and other new vaccines. Take on Typhoid (Blog).
131. Steele AD, Victor JC, Carey ME, et al. Experiences with rotavirus vaccines: can we improve rotavirus vaccine impact in developing countries? *Hum Vaccin Immunother*. 2019;15(6):1215-1227. doi:10.1080/21645515.2018.1553593
132. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. *CLSI*. Published online 2018.
133. Dyson ZA, Holt KE. Five Years of GenoTyphi: Updates to the Global Salmonella Typhi Genotyping Framework. *J Infect Dis*. 2021;224(S7):775-780. doi:10.1093/infdis/jiab414
134. Dyson ZA, Holt K. katholt/genotyphi: GenoTyphi v1.9.1. Zenodo.
135. Stamatakis A. RAxML-VI-HPC: Maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics*. 2006;22(21):2688-2690. doi:10.1093/bioinformatics/btl446

136. Garrett DO, Longley AT, Aiemjoy K, et al. Incidence of typhoid and paratyphoid fever in Bangladesh, Nepal, and Pakistan: results of the Surveillance for Enteric Fever in Asia Project. *Lancet Glob Health*. 2022;10:e978-e988. doi:10.1016/S2214-109X(22)00119-X
137. Britto CD, Dyson ZA, Duchene S, et al. Laboratory and molecular surveillance of paediatric typhoidal *Salmonella* in Nepal: Antimicrobial resistance and implications for vaccine policy. *PLoS Negl Trop Dis*. 2018;12(4):1-19. doi:10.1371/journal.pntd.0006408
138. Britto CD, Dyson ZA, Mathias S, et al. Persistent circulation of a fluoroquinolone-resistant *Salmonella enterica* Typhi clone in the Indian subcontinent. doi:10.1093/jac/dkz435
139. Pham Thanh D, Karkey A, Dongol S, et al. A novel ciprofloxacin-resistant subclade of H58 Salmonella Typhi is associated with fluoroquinolone treatment failure. Published online 2016. doi:10.7554/eLife.14003.001
140. Ingle DJ, Nair S, Hartman H, 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):1-20. doi:10.1371/journal.pntd.0007620
141. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9(4):357-359. doi:10.1038/nmeth.1923
142. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079. doi:10.1093/bioinformatics/btp352
143. Croucher NJ, Page AJ, Connor TR, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res*. 2015;43(3):e15. doi:10.1093/nar/gku1196
144. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics*. 2014;30(9):1312-1313. doi:10.1093/bioinformatics/btu033
145. Argimón S, Abudahab K, Goater RJE, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microb Genom*. 2016;2(11):e000093. doi:10.1099/mgen.0.000093
146. Yu G, Smith DK, Zhu H, Guan Y, Lam TTY. Ggtree: an R Package for Visualization and Annotation of Phylogenetic Trees With Their Covariates and Other Associated Data. *Methods Ecol Evol*. 2017;8(1):28-36. doi:10.1111/2041-210X.12628
147. Inouye M, Dashnow H, Raven LA, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. *Genome Med*. 2014;6(11):1-16. doi:10.1186/s13073-014-0090-6
148. Gupta SK, Padmanabhan BR, Diene SM, et al. ARG-annot, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother*. 2014;58(1):212-220. doi:10.1128/AAC.01310-13
149. Carattoli A, Zankari E, Garciá-Fernández A, et al. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother*. 2014;58(7):3895-3903. doi:10.1128/AAC.02412-14

150. Rahman SIA, Dyson ZA, Klemm EJ, et al. Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in Bangladesh from 2004 to 2016. *bioRxiv*. doi:http://dx.doi.org/10.1101/664136.
151. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*. 2006;23(1):127-128.
152. Pragasam AK, Pickard D, Wong V, et al. Phylogenetic Analysis Indicates a Longer Term Presence of the Globally Distributed H58 Haplotype of Salmonella Typhi in Southern India. *Clinical Infectious Diseases*. 2020;71(8):1856-1863. doi:10.1093/cid/ciz1112
153. G.J. Hannon. FASTX-Toolkit. Published online 2010.
154. Simon Andrews. FASTQC. A quality control tool for high throughput sequence data. Published online 2010.
155. Holt KE, Parkhill J, Mazzoni CJ, et al. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. *Nat Genet*. 2008;40(8):987-993. doi:10.1038/ng.195
156. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079. doi:10.1093/bioinformatics/btp352
157. Holt KE, Thomson NR, Wain J, et al. Pseudogene accumulation in the evolutionary histories of *Salmonella enterica* serovars Paratyphi A and Typhi. *BMC Genomics*. 2009;10(1):36. doi:10.1186/1471-2164-10-36
158. Yu G. Using ggtree to Visualize Data on Tree-Like Structures. *Curr Protoc Bioinformatics*. 2020;69(1). doi:10.1002/cpbi.96
159. Bradley P, den Bakker HC, Rocha EPC, McVean G, Iqbal Z. Ultrafast search of all deposited bacterial and viral genomic data. *Nat Biotechnol*. 2019;37(2):152-159. doi:10.1038/s41587-018-0010-1
160. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol*. 2018;4(1). doi:10.1093/ve/vey016
161. Rambaut A, Lam TT, Carvalho LM, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol*. 2016;2(1). doi:10.1093/ve/vew007
162. Firth C, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. *Mol Biol Evol*. 2010;27(9):2038-2051. doi:10.1093/molbev/msq088
163. Duchêne S, Duchêne D, Holmes EC, Ho SYW. The performance of the date-randomization test in phylogenetic analyses of time-structured virus data. *Mol Biol Evol*. 2015;32(7):1895-1906. doi:10.1093/molbev/msv056
164. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat Methods*. 2017;14(6):587-589. doi:10.1038/nmeth.4285
165. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32(1):268-274. doi:10.1093/molbev/msu300
166. Duchêne S, Holt KE, Weill FX, et al. Genome-scale rates of evolutionary change in bacteria. *Microb Genom*. 2016;2(11):e000094. doi:10.1099/mgen.0.000094

167. Xie W, Lewis PO, Fan Y, Kuo L, Chen MH. Improving marginal likelihood estimation for Bayesian phylogenetic model selection. *Syst Biol*. 2011;60(2):150-160. doi:10.1093/sysbio/syq085
168. Hill V, Baele G. Bayesian Estimation of Past Population Dynamics in BEAST 1.10 Using the Skygrid Coalescent Model. *Mol Biol Evol*. 2019;36(11):2620-2628. doi:10.1093/molbev/msz172
169. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol*. 2018;67(5):901-904. doi:10.1093/sysbio/syy032
170. Ahmad N, Hii SYF, Hashim R, Issa R. Draft genome sequence of *Salmonella enterica* serovar Typhi IMR_TP298/15, a strain with intermediate susceptibility to ciprofloxacin, isolated from a typhoid outbreak. *Genome Announc*. 2017;5(9). doi:10.1128/genomeA.01740-16
171. Katiyar A, Sharma P, Dahiya S, Singh H, Kapil A, Kaur P. Genomic profiling of antimicrobial resistance genes in clinical isolates of *Salmonella* Typhi from patients infected with Typhoid fever in India. *Sci Rep*. 2020;10(8299). doi:10.1038/s41598-020-64934-0
172. Underwood A. GHRU (Genomic Surveillance of Antimicrobial Resistance) Retrospective 1 Bioinformatics Methods V.4. *protocols.io*. Published online 2020. https://gitlab.com/cgps/ghru/pipelines/dsl2/pipelines/amr_prediction
173. Prjibelski A, Antipov D, Meleshko D, Lapidus A, Korobeynikov A. Using SPAdes De Novo Assembler. *Curr Protoc Bioinformatics*. 2020;70(1). doi:10.1002/cpbi.102
174. Li H. Aligning Sequence Reads, Clone Sequences and Assembly Contigs with BWA-MEM.; 2013. http://github.com/lh3/bwa.
175. Li H, Durbin R. Fast and accurate short read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2009;25(14):1754-1760. doi:10.1093/bioinformatics/btp324
176. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. *Bioinformatics*. 2010;26(5):589-595. doi:10.1093/bioinformatics/btp698
177. Matono T, Morita M, Yahara K, et al. Emergence of Resistance Mutations in *Salmonella enterica* Serovar Typhi Against Fluoroquinolones. *Open Forum Infect Dis*. 2017;4(4). doi:10.1093/ofid/ofx230
178. US Centers for Disease Control. Surveillance System Overview: National Typhoid and Paratyphoid Fever Surveillance (NTPFS).; 2011. http://www.cdc.gov/outbreaknet/nors/
179. Ingle DJ, Andersson P, Valcanis M, et al. Genomic epidemiology and antimicrobial resistance mechanisms of imported typhoid in australia. *Antimicrob Agents Chemother*. 2021;65(12). doi:10.1128/AAC.01200-21
180. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, et al. A study of typhoid fever in five Asian countries: Disease burden and implications for controls. *Bull World Health Organ*. 2008;86(4):260-268. doi:10.2471/BLT.06.039818
181. Azmatullah A, Qamar FN, Thaver D, Zaidi AKM, Bhutta ZA. Systematic review of the global epidemiology, clinical and laboratory profile of enteric fever. *J Glob Health*. 2015;5(2). doi:10.7189/jogh.05.020407

182. Qamar FN, Yousafzai MT, Sultana S, et al. A Retrospective Study of Laboratory-Based Enteric Fever Surveillance, Pakistan, 2012-2014. *Journal of Infectious Diseases*. 2018;218(Suppl 4):S201-S205. doi:10.1093/infdis/jiy205
183. Qamar FN, Azmatullah A, Kazi AM, Khan E, Zaidi AKM. A three-year review of antimicrobial resistance of *Salmonella enterica* serovars Typhi and Paratyphi A in Pakistan. *J Infect Dev Ctries*. 2014;8(8):981-986. doi:10.3855/jidc.3817
184. Hasan F, Lami A, Butt MH, et al. Rising XDR-Typhoid Fever Cases in Pakistan: Are We Heading Back to the Pre-antibiotic Era? *Frontiers in Public Health | www.frontiersin.org*. 2022;1:794868. doi:10.3389/fpubh.2021.794868
185. Qamar FN, Yousafzai MT, Dehraj IF, et al. Antimicrobial Resistance in Typhoidal Salmonella: Surveillance for Enteric Fever in Asia Project, 2016 - 2019. *Clinical Infectious Disease*. 2020;71(S3):S276-S284. doi:10.1093/cid/ciaa1323
186. Waddington C, Carey ME, Boinett CJ, Higginson E, Veeraraghavan B, Baker S. Exploiting genomics to mitigate the public health impact of antimicrobial resistance. *Genome Med*. 2022;14(1). doi:10.1186/s13073-022-01020-2
187. John J, Van Aart CJC, Grassly NC. The Burden of Typhoid and Paratyphoid in India: Systematic Review and Meta-analysis. *PLoS Negl Trop Dis*. 2016;10(4):1-14. doi:10.1371/journal.pntd.0004616
188. John J, Bavdekar A, Rongsen-Chandola T, Dutta S, Kang G. Estimating the incidence of enteric fever in children in India: A multi-site, active fever surveillance of pediatric cohorts. *BMC Public Health*. 2018;18(1):1-6. doi:10.1186/s12889-018-5498-2
189. Srinivasan M, Sindhu KN, John J, Kang G. Opportunities for Typhoid Vaccination in India. *Indian Pediatr*. 2019;56(6):453-458. doi:10.1007/s13312-019-1566-7
190. Cunrath O, Meinel DM, Maturana P, et al. Quantitative contribution of efflux to multi-drug resistance of clinical Escherichia coli and Pseudomonas aeruginosa strains. *EBioMedicine*. 2019;41:479-487. doi:10.1016/j.ebiom.2019.02.061
191. Duy PT, Dongol S, Giri A, et al. The Emergence of azithromycin-resistant Salmonella Typhi in Nepal. *medRxiv*. Published online 2020. doi:https://doi.org/10.1101/2020.08.07.20166389
192. Hooda Y, Tanmoy AM, Sajib MSI, Saha S. Mass azithromycin administration: Considerations in an increasingly resistant world. *BMJ Glob Health*. 2020;5(6):1-4. doi:10.1136/bmjgh-2020-002446
193. Klein EY, Van Boeckel TP, Martinez EM, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. *Proc Natl Acad Sci U S A*. 2018;115(15):E3463-E3470. doi:10.1073/pnas.1717295115
194. Britto, Carl D., John, Jacob, Verghese, Valsan P. , Pollard AJ. A systematic review of antimicrobial resistance of typhoidal *Salmonella* in India. *Indian Journal of Medical Research*. 2019;149(2):151-163.
195. Park SE, Pham DT, Boinett C, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. *Nat Commun*. 2018;9(1). doi:10.1038/s41467-018-07370-z
196. Bogoch II, Utzinger J, Lo NC, Andrews JR. Antibacterial mass drug administration for child mortality reduction: Opportunities, concerns, and possible next steps. *PLoS Negl Trop Dis*. 2019;13(5):1-6. doi:10.1371/journal.pntd.0007315

197. Parry CM, Thieu NTV, Dolecek C, et al. Clinically and microbiologically derived azithromycin susceptibility breakpoints for *Salmonella enterica* serovars Typhi and Paratyphi A. *Antimicrob Agents Chemother*. 2015;59(5):2756-2764. doi:10.1128/AAC.04729-14
198. Carey ME, Nguyen T, Nguyen T, et al. The origins of haplotype 58 (H58) *Salmonella enterica* serovar Typhi. *bioRxiv*. Published online October 3, 2022. doi:10.1101/2022.10.03.510628
199. Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively Drug-Resistant Typhoid - Are Conjugate Vaccines Arriving Just in Time? *New England Journal of Medicine*. 2018;379(16):1493-1495. doi:10.1056/NEJMp1803926
200. Olarte J, Galindo E. *Salmonella* Typhi resistant to chloramphenicol, ampicillin, and other antimicrobial agents: strains isolated during an extensive typhoid fever epidemic in Mexico. *Antimicrob Agents Chemother*. 1973;4(6):597-601. doi:10.1128/AAC.4.6.597
201. Mirza SH, Beeching NJ, Hart CA. Multi-drug resistant typhoid: A global problem. *J Med Microbiol*. 1996;44(5):317-319. doi:10.1099/00222615-44-5-317
202. Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in *Salmonella enterica* serovar Typhi, the etiological agent of typhoid. *PLoS Negl Trop Dis*. 2018;12(10):e0006779. doi:10.1371/journal.pntd.0006779
203. Mejia N, Qamar F, Yousafzai MT, et al. Typhoid and Paratyphoid Cost of Illness in Pakistan: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S319-S335. doi:10.1093/cid/ciaa1336
204. Mejia N, Pallas SW, Saha S, et al. Typhoid and Paratyphoid Cost of Illness in Bangladesh: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S293-S305. doi:10.1093/cid/ciaa1334
205. Mejia N, Abimbola T, Andrews JR, et al. Typhoid and Paratyphoid Cost of Illness in Nepal: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S306-S318. doi:10.1093/cid/ciaa1335
206. Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu Rev Microbiol*. 2008;62:53-70. doi:10.1146/annurev.micro.62.081307.162832
207. Holt KE, Baker S, Dongol S, et al. High-throughput bacterial SNP typing identifies distinct clusters of *Salmonella* Typhi causing typhoid in Nepalese children. *BMC Infect Dis*. 2010;10. doi:10.1186/1471-2334-10-144
208. Kariuki S, Dyson ZA, Mbae C, et al. Multiple introductions of multidrug-resistant typhoid associated with acute infection and asymptomatic carriage, Kenya. *Elife*. 2021;10. doi:10.7554/eLife.67852
209. Zahrt TC, Buchmeier N, Maloy S. Effect of mutS and recD Mutations on *Salmonella* Virulence. *Infect Immun*. 1999;67(11):6168-6172. https://journals.asm.org/journal/iai
210. Duy PT, Thieu NTV, Nguyen TNT, et al. Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi. *PLoS Pathog*. 2020;16(10):1-17. doi:10.1371/journal.ppat.1008998

211. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, et al. *Salmonella enterica* serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. *J Antimicrob Chemother*. 2021;76(5):1160-1167. doi:10.1093/jac/dkaa519
212. Park SE, Pham DT, Boinett C, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. *Nat Commun*. 2018;9(1):5094. doi:10.1038/s41467-018-07370-z
213. Maes M, Dyson ZA, Higginson EE, et al. Multiple introductions of *Salmonella enterica* serovar typhi H58 with reduced fluoroquinolone susceptibility into Chile. *Emerg Infect Dis*. 2020;26(11):2736-2740. doi:10.3201/eid2611.201676
214. Gunn JS, Marshall JM, Baker S, Dongol S, Charles RC, Ryan ET. Salmonella chronic carriage: Epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol*. 2014;22(11):648-655. doi:10.1016/j.tim.2014.06.007
215. Senthilkumar B, Senbagam D, Rajasekarapandian M. An epidemiological surveillance of asymptomatic typhoid carriers associated in respect to socioeconomic status in India. *Journal of Public Health (Germany)*. 2014;22(3):297-301. doi:10.1007/s10389-012-0545-4
216. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, et al. *Salmonella enterica* serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. *J Antimicrob Chemother*. 2021;76(5):1160-1167. doi:10.1093/jac/dkaa519
217. Deng W, Liou SR, Plunkett G, et al. Comparative genomics of Salmonella enterica serovar Typhi strains Ty2 and CT18. *J Bacteriol*. 2003;185(7):2330-2337. doi:10.1128/JB.185.7.2330-2337.2003
218. Boyd EF, Porwollik S, Blackmer F, McClelland M. Differences in gene content among Salmonella enterica serovar Typhi isolates. *J Clin Microbiol*. 2003;41(8):3823-3828. doi:10.1128/JCM.41.8.3823-3828.2003
219. Bastien S, Faucher P, Porwollik S, Dozois CM, Mcclelland M, Daigle F. Transcriptome of *Salmonella enterica* Serovar Typhi within Macrophages Revealed through the Selective Capture of Transcribed Sequences.; 2006. www.ncbi.nlm.nih.govgeo
220. Baker S, Holt K, van de Vosse E, et al. High-throughput genotyping of Salmonella enterica serovar Typhi allowing geographical assignment of haplotypes and pathotypes within an urban district of Jakarta, Indonesia. *J Clin Microbiol*. 2008;46(5):1741-1746. doi:10.1128/JCM.02249-07
221. Holt KE, Parkhill J, Mazzoni CJ, et al. High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi. *Nat Genet*. 2008;40(8):987-993. doi:10.1038/ng.195
222. Lagrada ML, Argimón S, Borlasa JB, et al. Genomic Surveillance of *Salmonella* spp. in the Philippines, 2013-2014. *bioRxiv*. Published online 2022. doi:10.1101/2022.02.14.480363
223. Argimón S, Masim MAL, Gayeta JM, et al. Integrating whole-genome sequencing within the National Antimicrobial Resistance Surveillance Program in the Philippines. *Nat Commun*. 2020;11(1). doi:10.1038/s41467-020-16322-5
224. Nadon C, van Walle I, Gerner-Smidt P, et al. Pulsenet international: Vision for the implementation of whole genome sequencing (WGS) for global foodborne

disease surveillance. *Eurosurveillance*. 2017;22(23). doi:10.2807/1560-7917.ES.2017.22.23.30544
225. Ikhimiukor OO, Oaikhena AO, Afolayan AO, et al. Genomic characterization of invasive typhoidal and non-typhoidal *Salmonella* in 1 southwestern Nigeria. *medRxiv*. Published online 2022. doi:10.1101/2022.01.28.22270011
226. Pham Thanh D, Thompson CN, Rabaa MA, Sona S, Sopheary S, Kumar V. The Molecular and Spatial Epidemiology of Typhoid Fever in Rural Cambodia. *PLoS Negl Trop Dis*. 2016;10(6):4785. doi:10.1371/journal.pntd.0004785
227. Maes M, Sikorski MJ, Carey ME, et al. Whole genome sequence analysis of *Salmonella* Typhi provides evidence of phylogenetic linkage between cases of typhoid fever in Santiago, Chile in the 1980s and 2010–2016. Bourret TJ, ed. *PLoS Negl Trop Dis*. 2022;16(6):e0010178. doi:10.1371/journal.pntd.0010178
228. Kuijpers LMF, Phe T, Veng CH, et al. The clinical and microbiological characteristics of enteric fever in Cambodia, 2008-2015. *PLoS Negl Trop Dis*. 2017;11(9). doi:10.1371/journal.pntd.0005964
229. Lagrada ML, Argimón S, Borlasa JB, et al. Genomic surveillance of *Salmonella* spp. in the Philippines during 2013–2014. *Trans R Soc Trop Med Hyg*. Published online August 23, 2022. doi:10.1093/trstmh/trac080
230. Wong VK, Holt KE, Okoro C, et al. Molecular Surveillance Identifies Multiple Transmissions of Typhoid in West Africa. *PLoS Negl Trop Dis*. 2016;10(9). doi:10.1371/journal.pntd.0004781
231. Guevara PD, Maes M, Thanh DP, et al. A genomic snapshot of *Salmonella enterica* serovar Typhi in Colombia. *PLoS Negl Trop Dis*. 2021;15(9). doi:10.1371/JOURNAL.PNTD.0009755
232. Dyson ZA, Malau E, Horwood PF, et al. Whole genome sequence analysis of *Salmonella* Typhi in Papua New Guinea reveals an established population of genotype 2.1.7 sensitive to antimicrobials. *PLoS Negl Trop Dis*. 2022;16(3). doi:10.1371/journal.pntd.0010306
233. Rasheed MK, Hasan SS, Babar ZUD, Ahmed SI. Extensively drug-resistant typhoid fever in Pakistan. *Lancet Infect Dis*. 2019;19(3):242-243. doi:10.1016/S1473-3099(19)30051-9
234. Doyle M, Fookes M, Ivens A, Mangan MW, Wain J, Dorman CJ. An H-NS–like Stealth Protein Aids Horizontal DNA Transmission in Bacteria. *Science (1979)*. 2007;315(251):236-238. doi:10.1126/science.1128794
235. Munir T, Lodhi M, Ansari JK, Andleeb S, Ahmed M. Extended Spectrum Beta Lactamase producing Cephalosporin resistant *Salmonella* Typhi, reported from Rawalpindi, Pakistan. *Journal of Pakistan Medical Association*. 2016;66(8).
236. Gul D, Potter RF, Riaz H, et al. Draft genome sequence of a *Salmonella enterica* serovar Typhi strain resistant to fourth-generation cephalosporin and fluoroquinolone antibiotics. *Genome Announc*. 2017;5(42). doi:10.1128/genomeA.00850-17
237. Phoba MF, Barbé B, Lunguya O, et al. *Salmonella enterica* serovar Typhi Producing CTX-M-15 Extended Spectrum β-Lactamase in the Democratic Republic of the Congo. *Clinical Infectious Diseases*. 2017;65(7):1229-1231. doi:10.1093/cid/cix342
238. Hendriksen RS, Leekitcharoenphon P, Mikoleit M, et al. Genomic dissection of travel-associated extended-spectrum-beta-lactamase-producing *Salmonella*

enterica serovar Typhi isolates originating from the Philippines: A one-off occurrence or a threat to effective treatment of typhoid fever? *J Clin Microbiol*. 2015;53(2):677-680. doi:10.1128/JCM.03104-14
239. Djeghout B, Saha S, Sajib MSI, et al. Ceftriaxone-resistant *Salmonella* Typhi carries an Inci1-ST31 plasmid encoding CTX-M-15. *J Med Microbiol*. 2018;67(5):620-627. doi:10.1099/jmm.0.000727
240. Godbole GS, Day MR, Murthy S, Chattaway MA, Nair S. First Report of CTX-M-15 *Salmonella* Typhi From England. *Clinical Infectious Diseases*. 2018;66(12):1975-1976. doi:10.1093/cid/ciy041
241. Ikhimiukor OO, Odih EE, Donado-Godoy P, Okeke IN. A bottom-up view of antimicrobial resistance transmission in developing countries. *Nat Microbiol*. 2022;7(6):757-765. doi:10.1038/s41564-022-01124-w
242. Griffiths EJ, Timme RE, Mendes CI, et al. Future-proofing and maximizing the utility of metadata: The PHA4GE SARS-CoV-2 contextual data specification package. *Gigascience*. 2022;11. doi:10.1093/gigascience/giac003
243. Browne AJ, Kashef Hamadani BH, Kumaran EAP, et al. Drug-resistant enteric fever worldwide, 1990 to 2018: A systematic review and meta-analysis. *BMC Med*. 2020;18(1). doi:10.1186/s12916-019-1443-1
244. World Health Organization. WHO Model List of Essential Medicines – 22nd List (2021). Published online 2021.
245. Aneley Getahun S, Parry CM, Crump JA, et al. A retrospective study of patients with blood culture-confirmed typhoid fever in Fiji during 2014-2015: Epidemiology, clinical features, treatment and outcome. *Trans R Soc Trop Med Hyg*. 2019;113(12):764-770. doi:10.1093/trstmh/trz075
246. Davies MR, Duchene S, Valcanis M. Genomic epidemiology of *Salmonella* Typhi in Central Division, Fiji, 2012 to 2016. *Lancet Reg Health West Pac*. 2022;24.
247. Baker S, Duy PT, Nga TVT, et al. Fitness benefits in fluoroquinolone-resistant Salmonella Typhi in the absence of antimicrobial pressure. *Elife*. 2013;2. doi:10.7554/elife.01229
248. Browne AJ, Chipeta MG, Haines-Woodhouse G, et al. Global antibiotic consumption and usage in humans, 2000–18: a spatial modelling study. *Lancet Planet Health*. 2021;5(12):e893-e904. doi:10.1016/S2542-5196(21)00280-1
249. Wang Y, Lu D, Jin Y, et al. Extensively Drug-Resistant (XDR) *Salmonella* Typhi Outbreak by Waterborne Infection-Beijing Municipality, China, January-February 2022. *China CDC Wkly*. 2022;4(12).
250. Hughes MJ, Birhane MG, Dorough L, et al. Extensively Drug-Resistant Typhoid Fever in the United States. *Open Forum Infect Dis*. 2021;8(12). doi:10.1093/ofid/ofab572
251. Nair S, Ashton P, Doumith M, et al. WGS for surveillance of antimicrobial resistance: A pilot study to detect the prevalence and mechanism of resistance to azithromycin in a UK population of non-typhoidal Salmonella. *Journal of Antimicrobial Chemotherapy*. 2016;71(12):3400-3408. doi:10.1093/jac/dkw318
252. Tack B, Phoba MF, Thong P, et al. Epidemiological cut-off value and antibiotic susceptibility test methods for azithromycin in a collection of multi-country invasive non-typhoidal *Salmonella*. *Clinical Microbiology and Infection*. 2022;28(12):1615-1623. doi:10.1016/j.cmi.2022.06.009

253. Baker KS, Dallman TJ, Field N, et al. Horizontal antimicrobial resistance transfer drives epidemics of multiple Shigella species. *Nat Commun*. 2018;9(1). doi:10.1038/s41467-018-03949-8
254. Gavi the Vaccine Alliance. New typhoid vaccine to receive Gavi support. Published 2018. https://www.gavi.org/library/news/statements/2018/new-typhoid-vaccine-to-receive-gavi-support/
255. Carey ME, McCann NS, Gibani MM. Typhoid fever control in the 21st century: where are we now? *Curr Opin Infect Dis*. 2022;35(5):424-430. doi:10.1097/QCO.0000000000000879
256. Ingle DJ, Hawkey J, Dyson ZA, Holt KE. *GenoTyphi Implementation in Mykrobe - Preliminary Technical Report.*; 2022.
257. Tacconelli E, Carrara E, Savoldi A, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. *Lancet Infect Dis*. 2018;18(3):318-327. doi:10.1016/S1473-3099(17)30753-3
258. Jorgensen JH, Ferraro MJ. Antimicrobial Susceptibility Testing: A Review of General Principles and Contemporary Practices. *Clinical Infectious Diseases*. 2009;49:1749-1755. doi:10.1086/647952
259. Syal K, Mo M, Yu H, et al. Current and emerging techniques for antibiotic susceptibility tests. *Theranostics*. 2017;7(7):1795-1805. doi:10.7150/thno.19217
260. Ashton PM, Nair S, Peters TM, et al. Identification of *Salmonella* for public health surveillance using whole genome sequencing. *PeerJ*. 2016;4:e1752. doi:10.7717/peerj.1752
261. Didelot X, Gardy J, Colijn C. Bayesian Inference of Infectious Disease Transmission from Whole-Genome Sequence Data. *Mol Biol Evol*. 2014;31(7):1869-1879. doi:10.1093/molbev/msu121
262. Price J, Gordon NC, Crook D, Llewelyn M, Paul J. The usefulness of whole genome sequencing in the management of *Staphylococcus aureus* infections. *Clinical Microbiology and Infection*. 2013;19(9):784-789. doi:10.1111/1469-0691.12109
263. Uzzell CB, Troman CM, Rigby J, et al. Environmental surveillance for *Salmonella* Typhi as a tool to estimate the incidence of typhoid fever in low-income populations. *medRxiv*. Published online 2021. doi:10.1101/2021.05.21.21257547
264. Field Epidemiology & Laboratory Training Program National Institute of Health Islamabad. Weekly Field Epidemiology Report Update on Extensive Drug Typhoid Fever. 2019;2(18):1-7.
265. Regional Office for the Eastern Mediterranean. Over 13 million children vaccinated with typhoid conjugate vaccine in Punjab and Islamabad.
266. Goay YX, Chin KL, Tan CLL, et al. Identification of Five Novel *Salmonella* Typhi-Specific Genes as Markers for Diagnosis of Typhoid Fever Using Single-Gene Target PCR Assays. *Biomed Res Int*. 2016;2016. doi:10.1155/2016/8905675
267. Saha S, Tanmoy AM, Andrews JR, et al. Evaluating PCR-based detection of *Salmonella* Typhi and Paratyphi A in the environment as an enteric fever surveillance tool. *American Journal of Tropical Medicine and Hygiene*. 2019;100(1):43-46. doi:10.4269/ajtmh.18-0428

268. Inzaule SC, Tessema SK, Kebede Y, Ogwell Ouma AE, Nkengasong JN. Genomic-informed pathogen surveillance in Africa: opportunities and challenges. *Lancet Infect Dis*. 2021;21(9):e281-e289. doi:10.1016/S1473-3099(20)30939-7
269. von Kalckreuth V, Konings F, Aaby P, et al. The Typhoid Fever Surveillance in Africa Program (TSAP): Clinical, Diagnostic, and Epidemiological Methodologies. *Clinical Infectious Diseases*. 2016;62(S1):S9-16. doi:10.1093/cid/civ693
270. Makungo UB, Ramutshila TE, Mabotja MC, et al. Epidemiological investigation of a typhoid fever outbreak in Sekhukhune District, Limpopo province, South Africa – 2017. *S Afr J Infect Dis*. 2020;35(1). doi:10.4102/sajid.v35i1.107
271. Smith AM, Tau NP, Ngomane HM, et al. Whole-genome sequencing to investigate two concurrent outbreaks of *Salmonella* enteritidis in South Africa, 2018. *J Med Microbiol*. 2020;69(11):1303-1307. doi:10.1099/jmm.0.001255
272. Ikhimiukor OO, Oaikhena AO, Afolayan AO, et al. Genomic characterization of invasive typhoidal and non-typhoidal *Salmonella* in southwestern Nigeria. *medRxiv*. Published online 2022. doi:10.1101/2022.01.28.22270011
273. Wilkinson E, Giovanetti M, Tegally H, et al. A year of genomic surveillance reveals how the SARS-CoV-2 pandemic unfolded in Africa. *Science (1979)*. 2021;374:423-431. https://www.science.org
274. Dyson ZA, Cerdeira L. zadyson/TyphiNET: v1.2.1. Zenodo.
275. Belman S, Saha S, Beale MA. SARS-CoV-2 genomics as a springboard for future disease mitigation in LMICs. *Nat Rev Microbiol*. 2022;20(1):3. doi:10.1038/s41579-021-00664-y
276. Mallapati S. Omicron border bans ignore the evidence, say scientists. *Nature*. 2021;600:199. doi:doi: https://doi.org/10.1038/d41586-021-03608-x
277. The COVID-19 Genomics UK (COG-UK) consortium. An integrated national scale SARS-CoV-2 genomic surveillance network. *Lancet Microbe*. 2020;1(3):e99-e100. doi:10.1016/S2666-5247(20)30054-9
278. Hadfield J, Megill C, Bell SM, et al. NextStrain: Real-time tracking of pathogen evolution. *Bioinformatics*. 2018;34(23):4121-4123. doi:10.1093/bioinformatics/bty407

Academic work published/pre-printed during this PhD

1. **Carey ME**, Macwright WR, Im J, et al. The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever St. *Clinical Infectious Diseases*. 2020;71(Suppl 2). doi:10.1093/cid/ciaa367
2. Steele AD, **Carey ME**, Kumar S, et al. Typhoid conjugate vaccines and enteric fever control: Where to next? *Clinical Infectious Diseases*. 2020;71(Suppl 2):S185-S190. doi:10.1093/cid/ciaa343
3. Iqbal J, Dehraj F, **Carey ME**, et al. A Race against Time: Reduced Azithromycin Susceptibility in *Salmonella enterica* Serovar Typhi in Pakistan. *mSphere*. 2020;5(4):e00215-20.
4. Duy PT, Thieu NTV, Nguyen TNT, et al. Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi. *PLoS Pathogens*. 2020;16(10):1-17. doi:10.1371/journal.ppat.1008998
5. **Carey ME,** Jain R, Yousuf M, et al. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of *Salmonella* Typhi in Northern India. *Clinical Infectious Diseases*. 2021;72(5):e120-127. doi:10.1093/cid/ciaa1773
6. Haselbeck AH, Tadesse BT, Park J, et al. Evaluation of Typhoid Conjugate Vaccine Effectiveness in Ghana (TyVEGHA) Using a Cluster-Randomized Controlled Phase IV Trial: Trial Design and Population Baseline Characteristics. Published online 2021. doi:10.3390/vaccines9030281
7. Guevara PD, Maes M, Thanh DP et al. A genomic snapshot of *Salmonella enterica* serovar Typhi in Colombia. *PLoS Negl Trop Dis*. 2021 Sep 16;15(9):e0009755. doi: 10.1371/journal.pntd.0009755. PMID: 34529660; PMCID: PMC8478212.
8. Park SE, Pham DT, Pak GD, et al. The genomic epidemiology of multi-drug resistant invasive non-typhoidal Salmonella in selected sub-Saharan African countries. *BMJ Global Health*. 2021;6(8). doi:10.1136/bmjgh-2021-005659
9. Maes M, Sikorski MJ, **Carey ME**, et al. Whole genome sequence analysis of *Salmonella* Typhi provides evidence of phylogenetic linkage between cases of typhoid fever in Santiago, Chile in the 1980s and 2010-2016. *PLoS Negl Trop Dis*. 2022 Jun 29;16(6):e0010178. doi: 10.1371/journal.pntd.0010178.
10. da Silva KE, Tanmoy AM, Pragasam AK, et al. The international and intercontinental spread and expansion of antimicrobial-resistant *Salmonella* Typhi: a genomic epidemiology study. *Lancet Microbe*. Published online June 2022. doi:10.1016/s2666-5247(22)00093-3
11. Waddington C, **Carey ME**, Boinett CJ, Higginson E, Veeraraghavan B, Baker S. Exploiting genomics to mitigate the public health impact of antimicrobial resistance. *Genome Medicine*. 2022;14(1). doi:10.1186/s13073-022-01020-2
12. **Carey ME**, McCann NS, Gibani MM. Typhoid fever control in the 21st century: where are we now? *Curr Opin in Infect Dis*. 2022. doi: 10.1097/QCO.0000000000000879
13. **Carey ME**, Dyson ZA, Argimòn S et al. Unlocking the potential of genomic data to inform typhoid fever control policy - supportive resources for genomic data generation, analysis, and visualisation. *Open Forum Infect Dis* (Accepted).

14. Nampota-Nkomba N, **Carey ME,** Jamka LP et al. Typhoid Conjugate Vaccines: Achieving their potential for disease prevention and health equity. *Open Forum Infect Dis* (Accepted).
15. **Carey ME**, Nguyen T, Nhu TDH et al. The origins of haplotype 58 (H58) *Salmonella enterica* serovar Typhi. *bioRxiv*. Published online October 3, 2022. doi:10.1101/2022.10.03.510628
16. **Carey ME**. Gateway to typhoid conjugate vaccine introduction in India and beyond – programmatic effectiveness of a public sector typhoid conjugate vaccine campaign in Navi Mumbai, *Clinical Infectious Diseases*, 2023; doi: https://doi.org/10.1093/cid/ciad134.
17. **Carey ME**, Dyson ZA, Ingle JI et al. Global diversity and antimicrobial resistance of typhoid fever pathogens: insights from 13,000 *Salmonella* Typhi genomes. *medRxiv* 2022; doi:https://doi.org/10.1101/2022.12.28.22283969

List of Appendices

Supplementary Tables

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data available from https://doi.org/10.5281/zenodo.7142499
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Table S1. List of isolates from Chapter 3 (Pakistan)

Table S2. List of isolates from Chapter 4 (Chandigarh)

Table S3. List of isolates from Chapter 5 (Origins of H58)

Table S4. List of published isolates from Chapter 6 (global update paper)

Table S5. Data on source of isolates included in global updated paper (specimen type and patient health status) in Chapter 6 (global update paper)

Appendix 1 – List of oral presentations during PhD

1. Typhoid Conjugate Vaccine Introduction (THECA) Annual Meeting – "Overview of Typhoid Conjugate Vaccine introduction in DRC (TyVECO) Study Design" – December 9, 2019.
2. Learning Session for Infectious Diseases & Microbiology Trainees, Imperial College. "Rotavirus Vaccines – An Overview." December 18, 2019.
3. Wolfson College Symposium, University of Cambridge. "Updates on Joint Global Health Programmes between International Vaccine Institute & University of Cambridge." January 23, 2020.
4. Cambridge Institute of Therapeutic Immunology and Infectious Disease (CITIID) PhD student and Post-doc Seminar Series – "Evidence to Policy – Addressing Typhoid Fever Burden in Sub-Saharan Africa." February 24, 2020.
5. Typhoid Conjugate Vaccine Introduction (THECA) Annual Meeting & General Assembly Meeting. "TyVECO Progress & Study Design Update."February 3, 2021.
6. University of Cambridge Centre for Science and Policy Horn Fellows Vaccines Event - "Evidence to Policy – Typhoid Conjugate Vaccine Introduction."– April 14, 2021
7. Abbott Enteric Fever Diagnostics Advisory Board & Expert Panel. "Use Cases for Enteric Fever Diagnostics." April 1 & 26, 2021
8. World Health Organization Stakeholder consultation to review and interpret emerging data on enteric fever disease burden Session 3 – "AMR overview and potential role of whole genome sequencing in informing typhoid control." May 19, 2021.
9. University of Sheffield Impact of a Typhoid Conjugate Vaccine Campaign on Antimicrobial Use in Harare, Zimbabwe Meeting. "Genomic Epidemiology of *S*. Typhi & Utility of WGS data informing policy." September 9, 2021.
10. World Health Organization TCV Research Agenda Scoping Meeting – "2nd Generation TCV Updates." September 20, 2021.
11. World Health Organization Stakeholder consultation to review and interpret emerging data on enteric fever disease burden Session 5 – "Overview of epidemiological research gaps and how to address." October 27, 2021.
12. 12th International Conference on Typhoid & Other Invasive Salmonelloses - "Origins of H58 *S*. Typhi." December 6, 2021.
13. 12th International Conference on Typhoid & Other Invasive Salmonelloses - "Supportive Resources for Genomic Data Generation, Analysis & Visualization." December 7, 2021.
14. 12th International Conference on Typhoid & Other Invasive Salmonelloses - "Twenty years of typhoid genomics – a global update paper." December 8, 2021.
15. Typhoid Conjugate Vaccine Introduction (THECA) Annual Meeting. "Translating Evidence to Policy – Global Typhoid Genomics Consortium". January 24, 2022.
16. World Health Organization Immunization and vaccines related implementation research advisory committee (IVIR-AC). "Development of guidance to measure the impact of vaccines on AMR." March 8, 2021.
17. World Health Organization Technical Advisory Group on Vaccines and AMR Meeting. "Development of guidance to measure the impact of vaccines on AMR." March 15, 2022.

18. World Health Organization Strategic Advisory Group of Experts Meeting - “Update on TCV pipeline and evidence to inform future SAGE sessions.” April 6, 2022.
19. 32nd European Congress of Clinical Microbiology and Infectious Diseases. “Twenty Years of Typhoid Genomics.” April 23, 2022.
20. Invited talk – 16th Asian Conference on Diarrheal Diseases and Nutrition (ASCODD). “Twenty Years of Typhoid Genomics.” November 12, 2022.
21. Invited talk – World Health Organization Technical Advisory Group on Vaccines and AMR Meeting. “Overview of landscape and needs analysis for developing a guidance on methodologies to measure the impact of vaccines on AMR.” December 7, 2022.

In addition, I presented on analytical progress to the Global Typhoid Genomics Consortium at Working Group and Advisory Group meetings on a quarterly basis and presented academic work at lab meetings every two months.

Appendix 2 – The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences (publication)

Clinical Infectious Diseases

SUPPLEMENT ARTICLE

The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences

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Building on previous multicountry surveillance studies of typhoid and others salmonelloses such as the Diseases of the Most Impoverished program and the Typhoid Surveillance in Africa Project, several ongoing blood culture surveillance studies are generating important data about incidence, severity, transmission, and clinical features of invasive *Salmonella* infections in sub-Saharan Africa and South Asia. These studies are also characterizing drug resistance patterns in their respective study sites. Each study answers a different set of research questions and employs slightly different methodologies, and the geographies under surveillance differ in size, population density, physician practices, access to healthcare facilities, and access to microbiologically safe water and improved sanitation. These differences in part reflect the heterogeneity of the epidemiology of invasive salmonellosis globally, and thus enable generation of data that are useful to policymakers in decision-making for the introduction of typhoid conjugate vaccines (TCVs). Moreover, each study is evaluating the large-scale deployment of TCVs, and may ultimately be used to assess post-introduction vaccine impact. The data generated by these studies will also be used to refine global disease burden estimates. It is important to ensure that lessons learned from these studies not only inform vaccination policy, but also are incorporated into sustainable, low-cost, integrated vaccine-preventable disease surveillance systems.

Keywords. blood culture; enteric fever surveillance; *Salmonella* Typhi; typhoid fever.

Enteric fever, the collective term for typhoid and paratyphoid fevers, describes a systemic infection caused by *Salmonella enterica* serovars Typhi or Paratyphi A, B, or C. Recent estimates suggest that these organisms cause 14.3 million infections (95% confidence interval [CI], 12 500 000–16 300 000) and 136 000 deaths (95% CI, 77 000–219 000) annually [1]. Invasive nontyphoidal *Salmonella* (iNTS) disease is caused by other *Salmonella* serovars, most frequently by *Salmonella* Typhimurium, *Salmonella* Enteritidis, or *Salmonella* Dublin. Invasive nontyphoidal *Salmonella* disease caused an estimated 535 000 infections (95% CI, 409 000–705 000) and 77 500 deaths (95% CI, 46 400–123 000) in 2017 [2], of which 18 400 were

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attributed to human immunodeficiency virus. While improved water treatment and sanitation infrastructure have eliminated enteric fever as a public health problem in high-income countries, invasive *Salmonella* infections, which include iNTS, remain a public health issue in many low- and lower-middle-income countries.

A major impediment to understanding the true burden of enteric fever and iNTS disease is the lack of appropriately sensitive diagnostics and inconsistent usage of existing tests. Bone marrow culture is considered the gold standard for diagnosis of typhoid and paratyphoid fever, but given the invasive and challenging nature of obtaining bone marrow aspirate, it is rarely performed [3]. Often, treating physicians rely on a serological test like the Widal test, which has limited utility in endemic settings [4]. Blood culture–based diagnostics are recommended for use in surveillance of typhoid fever and other invasive *Salmonella* infections by the World Health Organization (WHO) [5], but these tests are not available in most low-resource settings, which often lack adequate resources and trained personnel required to conduct routine blood culture tests [3]; when they are available, blood cultures are only 40%–60% sensitive, depending in part on the volume of blood collected and prior antibiotic usage, and results are not available for several days, so are not useful for decisions on empiric therapy [6].

Often, febrile patients will not present to healthcare facilities for diagnosis and treatment. Potential deterrents to healthcare seeking include distance to and accessibility of the closest healthcare facility, or costs associated with treatment and/or hospitalization, combined with ease of access and affordability of antimicrobials in the community. As a result, the true number of invasive *Salmonella* infections may be underestimated.

In 2009, the WHO highlighted the need for additional data on the burden of invasive *Salmonella* disease [7]. At that time, early estimates of disease burden relied on extrapolation of data obtained from surveillance studies conducted in limited geographical regions, which did not entirely reflect the diversity of epidemiological settings in which typhoid is encountered [8]. A historic lack of population-based surveillance studies has also contributed to uncertainty around disease burden, particularly in the African continent. A review of the global burden of enteric fever conducted in 2004 showed that only 2 countries in Africa had conducted systematic, population-based surveillance between 1954 and 2000 (South Africa and Egypt) [8].

To address the limitations of existing data sets, several surveillance studies have been established over the past decade, funded primarily by the Bill & Melinda Gates Foundation and the Wellcome Trust. One of the first studies funded was the Typhoid Fever Surveillance in Africa Program (TSAP), coordinated by the International Vaccine Institute (IVI). The TSAP study demonstrated higher overall incidence rates of typhoid fever in sub-Saharan Africa than previously suspected across both rural and urban sites, as well as high incidence rates of iNTS disease across multiple sites [9]. In the years that followed, additional surveillance studies were funded to provide more data on the burden of disease in diverse epidemiological settings and to answer additional questions about clinical features of enteric fever, such as the prevalence of severe manifestations of disease and chronic intestinal carriage. These included the Severe Typhoid Fever Surveillance in Africa program (SETA, IVI); the Surveillance for Enteric Fever in Asia Project (SEAP, Sabin Vaccine Institute); the Strategic Typhoid Alliance Across Africa and Asia (STRATAA, University of Oxford); and the Surveillance of Enteric Fever in India (SEFI, Christian Medical College, Vellore). Preliminary data from these studies have helped to inform the WHO Strategic Advisory Group of Experts' recommendation for typhoid conjugate vaccine (TCV) use in the control of typhoid fever in endemic settings [10], and additional data generated by these studies will help direct optimal use of TCVs going forward.

Each of these studies has been conducted across mutliple distinct epidemiological settings and aims to address subtly different questions relating to invasive *Salmonella* disease burden. In this article, we compare the methodological similarities and differences between these diverse and complementary studies. We also identify early lessons learned and outstanding data gaps, and issue recommendations for optimizing and sustaining surveillance systems going forward.

STUDY SETTINGS AND METHODS

Severe Typhoid Fever Surveillance in Africa (SETA)

The SETA program builds on the infrastructure established as part of the TSAP study to characterize the severity and long-term effects of typhoid fever and iNTS disease across Africa. TSAP collected blood culture data between 2010 and 2014 and generated typhoid incidence rates from sites in 10 sub-Saharan African countries: Burkina Faso, Ethiopia, Ghana, Guinea-Bissau, Kenya, Madagascar, Senegal, South Africa, Sudan, and Tanzania. The TSAP results showed a great deal of heterogeneity in typhoid incidence across sites, with crude rates ranging from 0 to 284 cases per 100 000 person-years, and that there is a high burden in both rural and urban sites [9]. SETA surveillance was continued at sites in Madagascar, Burkina Faso, and Ghana, and surveillance was extended to include additional sites in the Democratic Republic of Congo, Ethiopia, and Nigeria. These sites were selected based on existing evidence of typhoid transmission and clinical microbiology capacity, as well as to ensure geographical representativeness of key regions within the continent.

Within each of the SETA study areas, patients were recruited at healthcare facilities across multiple tiers. Each site included a referral hospital, which, to be included in SETA, had to be equipped with imaging and surgical capacity to identify and treat intestinal perforations, as well as primary or secondary healthcare centers, which enrolled less severe febrile subjects

using broader enrollment criteria [11]. Each recruitment center was assigned a geographic catchment area from which a defined population was identified. In all countries apart from Madagascar, the catchment area of the primary and/or secondary centers was "nested" within the catchment area of the tertiary center. A healthcare utilization survey (HCUS) was conducted in households randomly selected from the nested and broader study catchment areas to estimate incidence rates based on the proportion of the population seeking healthcare at the respective study facilities.

Screening for study eligibility was systematically conducted at all study facilities, including inpatient, outpatient, surgical, and emergency wards at referral facilities. To augment case detection, *Salmonella* bacteremia detected in SETA laboratories from patients who were not enrolled in the study were also included, as well as patients with intestinal (ileal) perforation suspected to be due to typhoid, from referral hospitals. Intestinal perforation cases were recruited into the study regardless of whether or not the patient resided in the study catchment area. Upon enrollment, blood, stool, oropharyngeal, and urine samples were collected. Blood was subjected to conventional microbiological culture for detection and identification of bacterial pathogens as well as other immunological investigations; stool was cultured to assess acute carriage status, and urine was examined for antibiotic residues to determine patterns in antimicrobial pretreatment.

Patients with blood culture–confirmed *Salmonella* Typhi, *Salmonella* Paratyphi, and non-Typhi *Salmonella* serotype infections were recruited into the long-term follow-up component of the study. Two healthy household members and 4 healthy neighborhood controls were enrolled for each case, and the entire cohort was followed for 1 year with contact points at predefined intervals to collect clinical information, blood and stool samples, and cost-of-illness and quality-of-life assessments [11].

Surveillance for Enteric Fever in Asia Project (SEAP)

SEAP is a multicountry, multisite, population-based surveillance study aimed at characterizing the burden of enteric fever in South Asia. The project had two phases: phase 1, a retrospective clinical record review of invasive *Salmonella* infections, and phase 2, a prospective surveillance study. Phase 1 showed that *Salmonella* Typhi and Paratyphoid A was isolated from 0.43% to 2% of blood cultures conducted at hospitals in Bangladesh, Nepal, Pakistan, and India [12].

The prospective component of the study—initiated in October 2016—was conducted at urban and periurban sites in Bangladesh, Nepal, and Pakistan. Sites were selected to represent diverse communities in South Asia, but choices were constrained by the availability of laboratories capable of performing high-quality blood cultures. In addition to patients meeting the inclusion criteria at the hospital sites, the SEAP study also recruited enteric fever cases from laboratory networks beyond the site hospitals. Blood specimens were collected for culture, and a subsample of participants provided urine samples to test for residual antibiotic metabolites to understand antibiotic usage patterns. Data were collected on clinical manifestations, markers of severity of illness, complications of illness, and antimicrobial resistance. In addition, in collaboration with the US Centers for Disease Control and Prevention, an economic study of enteric illness was implemented at all 3 sites [12].

SEAP used a hybrid surveillance approach, adapted from Luby et al [13, 14], combining facility-based surveillance with an HCUS, administered to a representative subset of households using a single-stage, cluster design; among patients with fever lasting 3 or more days, the proportion that sought care for the febrile episode at a study facility was used as an adjustment to the number of enteric fever cases at the facility, for the purpose of calculating incidence and disease severity rates. Geographic catchment areas were delineated at surveillance sites encompassing the majority of suspected enteric fever cases identified during phase 1. Catchment areas used administrative boundaries so that study staff could easily determine if patients resided in the catchment area [12]. SEAP used a hybrid surveillance approach, adapted from Luby et al [13, 14], combining facility-based surveillance with an HCUS, which was administered to a representative subset of households using a single-stage, cluster design [13]. The proportion within the surveyed group who reported a fever lasting 3 or more days and sought care for the febrile episode at a study facility was then used as an adjustment for care-seeking at the study facilities within the catchment area.

Surveillance of Enteric Fever in India (SEFI)

There has been a lack of nationally representative enteric fever incidence data in India, as highlighted by a 2016 meta-analysis [15]. To fill these data gaps, the SEFI program was established by the Christian Medical College, Vellore, and its design was developed in collaboration with the Indian Council of Medical Research, the Translational Health Sciences and Technology Institute, the Indian Academy of Pediatrics, and other public health stakeholders [16]. The SEFI team established a 3-tiered surveillance system to estimate the age-specific incidence of enteric fever in children, examine the heterogeneity of incidence in diverse settings across India, and generate information on antimicrobial resistance patterns and cost of illness. Study enrollment began in October 2017.

The tier 1 surveillance for estimating the incidence of enteric fever in children consists of active, community-based surveillance in 1 rural and 3 urban sites, which were selected to be broadly representative of different geographic settings and population densities. At each of the 4 sites, 6000 children between 6 months and 15 years are followed each week for 2 years to

estimate the age-specific incidence rates of enteric fever in children. At least 1 contact each month is in-person and the rest are either in-person or by telephone. Parents are also encouraged to reach out to the study team if a child has fever in between weekly follow-up points, and all participating families are given thermometers and diary cards. Any fever of 3 or more consecutive days is considered a suspected case, and the child is referred to a study facility on the fourth day of fever, where a blood culture is performed if the child has had fever in the past 12 hours. Risk factors and other demographic data are also collected from participating households to permit extrapolation of incidence estimates to other similar risk settings [17].

Tier 2 is passive, hospital-based surveillance, which has been harmonized across 6 secondary-care facilities in 6 settings (5 rural and 1 urban) with well-defined catchment populations between 100 000 and 400 000 each. All patients hospitalized at a study facility with a fever are considered eligible. Upon consent, a blood culture is collected irrespective of duration of fever or temperature. Those with blood culture–confirmed enteric fever are followed up for 28 days to capture costs and clinical complications. Biannual HCUSs are administered to 5000 randomly selected households in 100 clusters at each site. These are used to determine the probability of seeking healthcare at the study facility. The population denominators are estimated by projecting annual growth rates from the 2011 census for each village in the catchment area and validated by reviewing the population in the sampled clusters in the HCUS. The tier 2 surveillance system was designed to estimate the incidence of severe enteric fever (requiring hospitalization) across all age groups.

Tier 3 surveillance is being conducted at 8 laboratories linked to tertiary care hospitals. The aims of this component of the surveillance system are to generate estimates of the proportion of blood cultures that are positive for *Salmonella* Typhi or Paratyphi A and to characterize antimicrobial resistance patterns. Patients with nontraumatic ileal perforations are also enrolled in tier 2 and 3 surveillance and followed up for up to 90 days for clinical and health economic outcomes.

Strategic Typhoid Alliance Across Africa and Asia (STRATAA)

The STRATAA consortium, which includes key partners from the University of Oxford's Oxford Vaccine Group, the Malawi Liverpool Wellcome Trust Clinical Research Programme, the International Center for Diarrhoeal Disease Research, Bangladesh (icddr,b), and the Oxford University Clinical Research Unit (Vietnam and Nepal), has conducted a prospective multicomponent epidemiological study in 3 densely populated urban sites in Bangladesh, Nepal, and Malawi [18]. This study combines passive febrile illness surveillance with serological surveillance. A baseline population of approximately 100 000 was established at each site by demographic census. Field teams visited each individual house, recording GPS position and collecting epidemiological data at the household and individual level. A census update was conducted every 6 months at the Bangladeshi site and at 12 months in Nepal, and a final census was conducted at all 3 sites after 2 years. In addition, 2 HCUS and water, sanitation, and hygiene surveys were performed in each site during the surveillance period to generate additional data on healthcare-seeking behavior and to investigate potential disease risk factors and transmission routes.

Passive surveillance was conducted from June 2016 in referral hospitals and primary health centers. Patients living within the population catchment area who presented to a study facility reporting fever of 2 days or longer or having a documented temperature of $\geq$ 38°C were enrolled and consented and blood cultures were taken [18]. Age-stratified serosurveys were performed at each study site to assess seroincidence of typhoid infection. The resulting seroincidence rates will be used to validate and provide upper bounds on the incidence rates derived from blood culture surveillance, using host responses as a proxy for incidence of infection. Additionally, the serosurveys were designed to identify potential chronic carriers of *S.* Typhi. After identifying individuals with a high anti-Vi antibody response, follow-up with stool collection and culture was performed to identify stool shedding.

DISCUSSION

Study Similarities

There are important methodological similarities across these four studies. Each study includes passive, healthcare facility–based blood culture surveillance of febrile patients to generate crude age-stratified incidence rate estimates stratified by age. Each study team also conducts an HCUS and applies correction factors to these crude rates to account for the proportion of patients from within the catchment area or study population seeking care for febrile illness at a study facility. All 4 studies make an adjustment for eligible cases missed by the study (either patients who met eligibility criteria but were not approached for enrollment, or who were enrolled and chose not to consent, or both), as well as an adjustment for blood culture sensitivity. Antimicrobial resistance patterns from sites in all 4 studies are being analyzed, and whole-genome sequencing of selected *Salmonella* isolates will be available from all studies. Cost-of-illness studies are embedded within each of these studies, as is screening for chronic typhoid carriage.

The SEAP and SETA studies employ similar methods to calculate incidence, owing in part to efforts coordinated by the Scientific Advisory Process for Optimal Research on Typhoid Burden of Disease Project (SAPORT, Emory University) group to harmonize methods; therefore, the results are more easily compared. Both studies generate data on the incidence of severe disease and follow enteric fever patients for at least 6 weeks to characterize long-term sequelae. SEFI tier 2 surveillance

processes also closely resemble that of SEAP and SETA, although enrollment criteria differ across all 3 studies.

Each of the studies contributes data to advance broader enteric fever control objectives. Epidemiological data generated from all 4 have been shared with policymakers in relevant countries in support of decision-making around TCV introduction. The impact of TCV deployment is being evaluated or will be evaluated at site(s) from 3 of the 4 studies, although the designs of these studies and the delivery strategies under evaluation differ. A TCV impact assessment is being conducted through Kharadar General Hospital in Karachi, Pakistan (SEAP), in response to an extensively drug-resistant typhoid outbreak [19]. The University of Maryland and University of Oxford with Typhoid Vaccine Acceleration Consortium (TyVAC) partners are conducting 3 large-scale TCV trials at STRATAA surveillance sites: a cluster-randomized efficacy trial in Bangladesh [20] and 2 individually randomized efficacy studies in Nepal and Malawi [21, 22]. Interim analysis of the Nepal RCT showed that TCV had an 82% vaccine efficacy at 1 year after vaccination in children < 15 years of age [23]. Leveraging SETA surveillance, the University of Cambridge and partners are planning 2 TCV studies through the THECA (Typhoid Conjugate Vaccine Introductions in Africa) consortium: a cluster-randomized trial in Ghana using a similar methodology to the TyVAC trial in Bangladesh, as well as a mass-vaccination campaign with cohort effectiveness evaluation in the Democratic Republic of Congo [24]. In addition, alternatives to blood culture surveillance—namely, seroepidemiology and/or environmental sampling—are being validated at sites in all 4 studies.

Study Differences

There are several differences between the methodologies of these studies, which are important to consider when interpreting and comparing results. While there is some overlap, the objectives are not uniform across the 4 studies. Eligibility criteria differ, as do the type, number, and frequency of sample collections. The approach to estimating adjusted incidence rates differs between studies, and for SETA and SEFI, within study tiers as well. These and other general methodological differences are summarized in Table 1.

Each study generates both crude and adjusted incidence rates, but the approach to defining population denominators and the choice of adjustment factors used differ. Population denominators affect both crude and adjusted incidence rate calculations, so this distinction is important. The STRATAA approach to estimating the population denominator is different from the approach used by SEAP, SETA, and SEFI tier 2, as the demographic censuses provide precise population denominators. In SEAP, SETA, and SEFI tier 2, eligible cases come from predefined geographic catchment areas, which is arguably less precise but also less resource-intensive, and enables surveillance to cover larger catchment areas. Each study employs an adjustment for blood culture sensitivity, but STRATAA samples from probability distributions informed by a recent meta-analysis of blood culture sensitivity by volume of blood acquired per subject and reported prior antibiotic usage [25], whereas SEAP and SETA apply the same correction factor (assuming sensitivity of 59%) to each blood culture result [26]. For the healthcare-seeking adjustment, SETA assumes the same risk of typhoid infection for patients who seek care at a study facility and for patients who seek care elsewhere, whereas SEAP, SEFI tier 2, and STRATAA assume a differential typhoid risk for febrile patients who seek care at a study facility [13, 27]. SEFI tier 1 does not include a healthcare-seeking adjustment, since it employs active surveillance.

There are also key differences between these studies based on the geographies under surveillance, which are illustrated in Figure 1. The STRATAA sites are in densely populated, urban areas, whereas SEFI, SETA, and SEAP have a mixture of urban, periurban, and rural sites. There are observed differences in physician practices around administration of blood culture and clinical familiarity with typhoid fever among some South Asian sites, and differences in the availability of antimicrobials across all study sites. There are differences in preexisting capacities across sites to conduct routine blood culture surveillance, and the difference in blood volumes collected and antimicrobial usage across sites could lead to variability in the sensitivity of results. There is also a great deal of variability in accessibility of healthcare facilities, local water and sanitation behaviors infrastructure, and fecal sludge management.

There is value in the diversity of these study approaches. Some methodological differences are driven by rational, pragmatic choices made by study investigators that reflect differences between sites captured above. Each study addresses distinct data gaps, like potential transmission routes, prevalence of chronic carriers, and duration of immune response to natural infection, all of which will potentially inform optimal intervention strategies. Each study includes efforts to validate 1 or more low-cost alternatives to blood culture surveillance, which means that there is likely to be greater clarity in the near future about which approaches, if any, are viable, feasible, and cost-effective. The diversity of settings under surveillance broadens our understanding of the global incidence rates of typhoid fever and other invasive *Salmonella* infections, which will impact prioritization and targeting of combination vaccine development efforts. Having more broadly representative genomic data also facilitates monitoring the evolution and spread of different antimicrobial resistance genotypes, which also should inform more sophisticated targeting of interventions.

CONCLUSIONS

In a position paper from 2008, the WHO stated that the deployment of the then-available typhoid vaccines "should be based on detailed knowledge of the local epidemiological situation"

Table 1. Comparison of Surveillance Methods

	SETA	SEAP	SEFI	STRATAA
Design	Prospective passive, facility-based surveillance paired with population-based HCUS Prospective case-control cohort for long-term follow-up	Retrospective and prospective passive, facility-based surveillance paired with population-based HCUS	Tier 1: Prospective population-based cohort with active surveillance Tier 2: Prospective passive, hospital-based paired with population-based HCUS Tier 3: Laboratory-based surveillance	Prospective population-based cohort with passive surveillance paired with population-based HCUS and seroincidence surveys
Eligibility criteria	Primary/secondary health facilities • Objective fever of ≥ 38°C OR • Subjective fever ≥ 3 consecutive days in the last week • AND reside in the nested catchment area Referral hospitals • Subjective fever ≥ 3 consecutive days in the last week, OR • Clinically suspected typhoid fever • AND reside in the catchment area • OR pathognomonic gastrointestinal perforations even in the absence of laboratory confirmation and regardless of catchment area (special cases)	Outpatient • 3 days of consecutive fever in the last 7 days • AND reside in the study catchment area • AND physician must advise blood culture Inpatient • Clinical suspicion of enteric fever AND physician must advise blood culture OR • Confirmed diagnosis of enteric fever at any time during hospitalization OR • Nontraumatic ileal perforations, even in the absence of laboratory confirmation Laboratory: • Blood culture positive for *S.* Typhi or *S.* Paratyphi A only	Tier 1 • Subjective fever ≥ 3 consecutive days (families given thermometers and diary cards to record) • AND reside in census population area • AND fever in the last 12 hours before presentation, Tier 2 • All inpatients presenting with fever OR • Patient with nontraumatic ileal perforation • AND residing in geographic catchment area Tier 3 • Blood culture positive for *S.* Typhi or *S.* Paratyphi A only	• Objective fever of ≥38°C OR • Subjective fever of ≥ 2 days • AND reside in census population area
Sample collection and follow-up	• Blood samples taken from enrolled subjects at baseline • For blood culture–confirmed cases of *S.*Typhi and iNTS and associated controls, blood, urine, and stool samples and oropharyngeal swabs were taken at day 3-7, 14, 28, 90, 180, 270, and 360 • Ileal tissue or other surgical samples taken in cases of nontraumatic ileal perforation regardless of blood culture positivity • 1-year follow-up of blood culture–confirmed *S.*Typhi and iNTS cases and controls	• Blood samples taken from enrolled subjects at baseline • Urine samples taken from a sample of enrolled subjects at baseline • Ileal tissue samples taken in cases of nontraumatic ileal perforation regardless of blood culture positivity • 6-week phone call for blood culture–confirmed cases of *S.* Typhi or *S.* Paratyphi A—patients with complications followed up	• Blood samples taken from enrolled subjects at baseline • Ileal tissue samples taken in cases of nontraumatic ileal perforation regardless of blood culture positivity • Tier 1: Weekly follow-up, and in-person follow-up and blood collection at 28 days for enteric fever subcohort • Tier 2: Phone contact at 14 and 28 days postdischarge for cost-of-illness data	• Blood, plasma, and stool samples taken from enrolled subjects at baseline • Blood, plasma, and stool samples taken from cases and household members of culture-confirmed cases) • Day 8, 30, 180 follow-up
Incidence rate adjustment factors	• Probability of seeking care at a study facility, based on HCUS • Proportion of eligible patients enrolled in study • Proportion of eligible patients consenting to participate with a blood culture taken • Sensitivity of blood culture (assumed 60%)	• Probability of eligible patient seeking care at a study facility, based on HCUS • Proportion of eligible patients who consented and received a blood culture • Difference in healthcare-seeking according to socioeconomic status • Sensitivity of blood culture (assumed 59%)	• Probability of seeking care at a study facility, based on HCUS • Proportion of eligible patients who consented and received a blood culture • Sensitivity of blood culture (assumed 59%)	• Probability of seeking care at a study facility, based on HCUS; adjusted for the prevalence of previously identified typhoid risk factors • Proportion of eligible patients who consented and had blood drawn for culturing; adjusted for age, duration of fever, temperature at presentation, and clinical suspicion (Nepal and Bangladesh only) • Sensitivity of blood culture; adjusted for volume and reported prior antibiotic usage

Table 1. Continued

	SETA	SEAP	SEFI	STRATAA
Additional objectives	• Long-term sequelae, antimicrobial resistance, natural immune response, prevalence of chronic carriage, cost of illness, quality of life, long-term socioeconomic study	• Long-term sequelae, antimicrobial resistance, cost of illness	• Antimicrobial resistance • Cost of illness	• Prevalence of chronic carriage, seroincidence, antimicrobial resistance, household transmission

Abbreviations: HCUS, healthcare utilization survey; iNTS, invasive nontyphoidal Salmonella; SEAP, Surveillance for Enteric Fever in Asia Project; SEFI, Surveillance of Enteric Fever in India; SETA, Severe Typhoid Fever Surveillance in Africa; STRATAA, Strategic Typhoid Alliance Across Africa and Asia.

while simultaneously acknowledging the limitations of the existing data, particularly for the African continent [7]. In the intervening decade, the availability of safe, immunogenic, and efficacious TCVs [23, 28] has strengthened the need for accurate burden of disease data across diverse epidemiological settings. In this article, we have described the design and methodology of 4 landmark surveillance studies that collectively incorporate 44 surveillance facilities, 11 countries, and a total population under surveillance of > 20 million people. We argue that these studies have made large strides toward achieving the 2008 targets, and it is hoped that this article provides an important overview of the methodologies, strengths, and limitations of each individual study. Together, these data will provide key stakeholders and country-level decision makers with more accurate estimates of disease burden, allowing targeted, timely, and cost-effective deployment of new preventative strategies.

The TSAP study demonstrated that there was a significant incidence of typhoid fever and iNTS disease in Africa. SETA, SEAP, SEFI, and STRATAA are generating data on disease transmission, risk factors, cost of illness, and incidence of severe disease in Africa and some parts of Asia. Furthermore, these studies have provided baseline data in support of ongoing or planned phase 3/4 TCV trials. Data from these studies are now being used to enable national stakeholders to make informed decisions on optimal TCV delivery strategies, and to direct the development and prioritization of future *Salmonella* combination vaccine approaches. Methodological differences notwithstanding, each study has contributed important data to advance global typhoid control.

Each surveillance study will provide an estimate of disease burden for a particular setting at a particular point in time and will reflect transmission dynamics specific to a particular setting. We acknowledge that caution should be exercised when extrapolating these figures in an attempt to provide country-level estimates of disease burden, as the true distribution of disease is likely to display marked intra- and intercountry and temporal variation [29]. Furthermore, estimates of disease burden are unlikely to be static and will likely change in response to improvements or breakdown of sanitation infrastructure and the deployment of TCVs. Consideration should be given toward the establishment of ongoing surveillance programs to track changes in spatial and temporal trends in disease incidence. Such programs should be integrated into broader, vaccine-preventable disease surveillance efforts, and should potentially incorporate new, lower-cost alternatives to blood culture surveillance if and when these methods are validated, for maximal sustainability.

The updated 2018 WHO position paper on typhoid vaccination recommends that "endemic countries strengthen the surveillance of typhoid fever in all age groups, and monitor the presence of antimicrobial resistant strains of *S.* Typhi in endemic and epidemic disease, before and after introduction of typhoid vaccines"

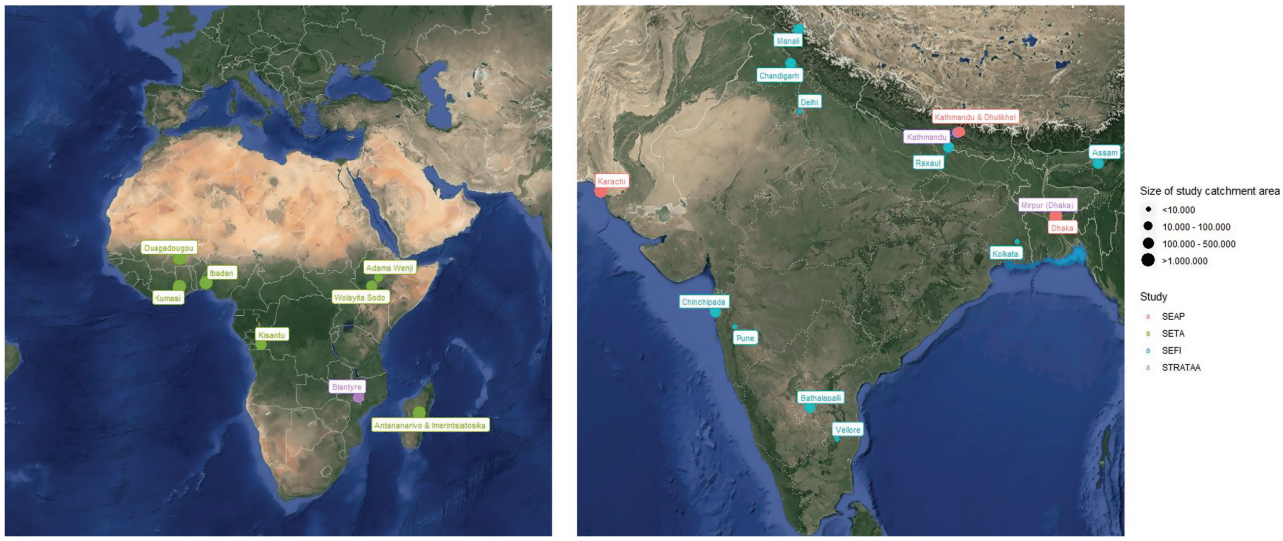

Figure 1. Locations and catchment area sizes for Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa program (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) surveillance sites.

[10]. Nevertheless, the large surveillance studies described herein are unlikely to be funded in perpetuity, and a shift in emphasis may need to be made toward strengthening routine national surveillance systems with a more targeted remit. It is therefore imperative that the existing studies achieve maximal utility by addressing outstanding questions relating to age-specific incidence, transmission, carriage, strain/serovar replacement, and incidence post-TCV introduction. In addition, the continued validation of alternative low-cost methods for typhoid surveillance at these sites could yield substantial benefit to the field.

Notes

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References

1. GBD 2017 Typhoid and Paratyphoid Collaborators. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis **2019**; 19:269–381.
2. GBD 2017 Non-Typhoidal *Salmonella* Invasive Disease Collaborators. The global burden of non-typhoidal *Salmonella* invasive disease: a systematic analysis for the Global Burden of Disease study 2017. Lancet Infect Dis **2019**; 19:1312–24.
3. Parry CM, Wijedoru L, Arjyal A, Baker S. The utility of diagnostics tests for enteric fever in endemic locations. Expert Rev Anti Infect Ther **2011**; 9:711–25.
4. Levine MM, Grads O, Gilman RH, Woodward WE, Solis-Plaza R, Waldman W. Diagnostics value of the Widal test in areas endemic to typhoid fever. Am J Trop Med Hyg **1978**; 27:795–800.
5. World Health Organization. Vaccine-preventable diseases surveillance standards: typhoid and other invasive salmonellosis. **2018**. Available at: https://www.who.int/immunization/monitoring_surveillance/burden/vpd/WHO_SurveillanceVaccinePreventable_21_Typhoid_R2.pdf?ua=1. Accessed 16 December 2019.
6. Antillon M, Saad NJ, Baker S, Pollard AJ, Pitzer VE. The relationship between blood sample volume and diagnostics sensitivity of blood culture typhoid and paratyphoid fever: a systematic review and meta-analysis. J Infect Dis **2018**; 218:S255–67.
7. World Health Organization. Typhoid vaccines: WHO position paper. Wkly Epidemiol Rec **2008**; 83:49–60.
8. Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. Bull World Health Organ **2004**; 82:346–53.
9. Marks F, von Kalckreuth V, Aaby P, et al. Incidence of invasive *Salmonella* disease in sub-Saharan Africa: a multicenter population-based surveillance study. Lancet Glob Health **2017**; 5:e310–23.
10. World Health Organization. Typhoid vaccines: WHO position paper—March 2018. Wkly Epidemiol Rec **2018**; 93:153–72.
11. Park SE, Toy T, Cruz Espinoza LM, et al. The Severe Typhoid Fever in Africa Program: study design and methodology to assess disease severity, host immunity, and carriage associated with invasive salmonellosis. Clin Infect Dis **2019**; 69:S422–34.
12. Barkume C, Date K, Saha SK, et al. Phase I of the Surveillance for Enteric Fever in Asia Project (SEAP): an overview and lessons learned. J Infect Dis **2018;** 218:S188–94.
13. Andrews JR, Barkume C, Yu AT, et al. Integrating facility-based surveillance with healthcare utilization surveys to estimate enteric fever incidence: methods and challenges. J Infect Dis **2018**; 218:S268–76.
14. Luby SP, Saha S, Andrews JR. Towards sustainable public health surveillance for enteric fever. Vaccine **2015**; 33:C3–7.

15. John J, Van Aart CJ, Grassly NC. The burden of typhoid and paratyphoid in India: systematic review and meta-analysis. PLoS Negl Trop Dis **2016**; 10:e0004616.
16. Carey ME, Diaz ZI, Broadstock M, Bailey R, Bentsi-Enchill AD, Larson HJ. Toward control? The prospects and challenges of typhoid conjugate vaccine introduction. Clin Infect Dis **2019**; 69:S408–11.
17. John J, Bavdekar A, Rongsen-Chandola T, Dutta S, Kang G; NSSEFI Collaborators. Estimating the incidence of enteric fever in children in India: a multi-site, active fever surveillance of pediatric cohorts. BMC Public Health **2018**; 18:594.
18. Darton TC, Meiring JE, Tonks S, et al. The STRATAA study protocol: a programme to assess the burden of enteric fever in Bangladesh, Malawi and Nepal using prospective census, passive surveillance, serological studies and healthcare utilization surveys. BMJ Open **2017**; 7:e016283.
19. World Health Organization Regional Office for the Eastern Mediterranean. Pakistan first country to introduce new typhoid vaccine into routine immunization programme. Available at: http://www.emro.who.int/pak/pakistan-news/pakistan-first-country-to-introduce-new-typhoid-vaccine-into-routine-immunization-programme.html. Accessed 23 January 2020.
20. Theiss-Nyland K, Qadri F, Colin-Jones R, et al. Assessing the impact of a Vi-polysaccharide conjugate vaccine in preventing typhoid infection among Bangladeshi children: a protocol for a phase IIIb trial. Clin Infect Dis **2019**; 68:S74–82.
21. Meiring JE, Laurens MB, Patel P, et al. Typhoid Vaccine Acceleration Consortium Malawi: a phase III, randomized, double-blind, controlled trial of the clinical efficacy of typhoid conjugate vaccine among children in Blantyre, Malawi. Clin Infect Dis **2019**; 68:S50–8.
22. Theiss-Nyland K, Shakya M, Colin-Jones R, et al. Assessing the impact of a Vi-polysaccharide conjugate vaccine in preventing typhoid infections among Nepalese children: a protocol for a phase III, randomized control trial. Clin Infect Dis **2019**; 68:S67–73.
23. Shakya M, Colin-Jones R, Theiss-Nyland K, et al; TyVAC Nepal Study Team. Phase 3 efficacy analysis of a typhoid conjugate vaccine trial in Nepal. N Engl J Med **2019**; 381:2209–18.
24. European and Developing Countries Clinical Trials Partnership. Strategic actions supporting large-scale clinical trials. **2017**. Available at: http://www.edctp.org/projects-2/edctp2%20-projects/strategic-actions-supporting-large-scale-clinical%20-trials-2017/. Accessed 18 December 2019.
25. Antillon M, Saad NJ, Baker S, Pollard AJ, Pitzer VE.. The relationship between blood sample volume and diagnostic sensitivity of blood culture for typhoid and paratyphoid fever: a systematic review and meta-analysis. J Infect Dis **2018**; 218:S255–67.
26. Mogasale V, Ramani E, Mogasale VV, Park J. What proportion of *Salmonella* Typhi cases are detected by blood culture? A systematic literature review. Ann Clin Microbiol Antimicrob **2016**; 15:32.
27. Voysey M, Pant D, Shakya M, et al. Under-detection of blood culture-positive enteric fever cases: the impact of missing data and methods for adjusting incidence estimates. PLoS Negl Trop Dis **2020**; 14:e0007805.
28. Jin C, Gibani MM, Moore M, et al. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of *Salmonella* Typhi: a randomised controlled, phase 2b trial. Lancet **2017**; 390:2472–80.
29. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, et al; Domi Typhoid Study Group. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ **2008**; 86:260–8.

Appendix 3 – A Race Against Time: Reduced Azithromycin Susceptibility in *Salmonella enterica* Serovar Typhi in Pakistan (publication)

OBSERVATION
Clinical Science and Epidemiology

A Race against Time: Reduced Azithromycin Susceptibility in *Salmonella enterica* Serovar Typhi in Pakistan

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ABSTRACT Antimicrobial resistance is an ongoing issue in the treatment of typhoid fever. Resistance to first-line antimicrobials and extensively drug resistant (XDR) *Salmonella* Typhi isolates in Pakistan have left azithromycin as the only remaining effective oral treatment. Here, we report the emergence of organisms with a single point mutation in *acrB* gene, implicated in azithromycin resistance, in a *S.* Typhi isolate from Pakistan. The isolation of this organism is worrisome and highlights the significance of the introduction of typhoid conjugate vaccine in South Asia.

IMPORTANCE The emergence of XDR *Salmonella* Typhi in Pakistan has left azithromycin as the only viable oral treatment option. Here, we report the detection of an azithromycin resistance-associated mutation in one *S.* Typhi isolate. This finding is important because any possible spread of azithromycin resistance in *S.* Typhi isolates would make it nearly impossible to treat in outpatient settings due to the need of injectable antibiotics. Our findings also signify the importance of introduction of typhoid conjugate vaccine in regions of endemicity such as Pakistan.

KEYWORDS *Salmonella* Typhi, typhoid fever, antimicrobial resistance, azithromycin higher MIC, Pakistan

Typhoid fever, the disease caused by the bacterium *Salmonella* Typhi, is responsible for an estimated 11.8 million infections and 128,200 deaths annually worldwide (1). *S.* Typhi is a human-restricted pathogen that is transmitted via the fecal-oral route. Typhoid mortality ranged from 10–30% of cases in the preantimicrobial era (2), but when treated with effective antimicrobials, typhoid has a case fatality rate of <1% (3). The rise of multidrug resistance (MDR) in the 1990s (4), followed by fluoroquinolone resistance (5), resulted in limited treatment options. The emergence and spread of an extensively drug-resistant (XDR) *S.* Typhi variant in Pakistan (6, 7), which is resistant to chloramphenicol, ampicillin, co-trimoxazole, streptomycin, fluoroquinolones, and third-generation cephalosporins, has left azithromycin as only realistic option for typhoid treatment in Pakistan (8). The recent report of azithromycin-resistant *S.* Typhi in Bangladesh highlights the issues associated with the reliance on this drug and signals the potential of untreatable typhoid (9).

Typhoid is notifiable in Pakistan, and the Aga Khan University has conducted standardized prospective facility and laboratory-based blood culture surveillance in outpatient and inpatient wards at Aga Khan University Hospital and Kharadar General Hospital between September 2016 and September 2019 through the Surveillance for Enteric fever in Asia Project (SEAP). These hospitals serve ~30 million people, including densely populated

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informal urban settlements. Subjects presenting to outpatient clinics living in predefined catchment areas with three consecutive days of fever for whom a study clinician recommended a blood culture were enrolled. Inpatients with clinical suspicion of typhoid or with nontraumatic ileal perforation were also enrolled. After blood culture, serologically confirmed *S.* Typhi isolates were subjected to antimicrobial susceptibility testing against azithromycin, ampicillin, co-trimoxazole, chloramphenicol, ciprofloxacin, levofloxacin, ceftriaxone, cefepime, cefixime, and ceftazidime by disk diffusion; resistant organisms (according to CLSI guidelines) were confirmed by Etest (bioMérieux, France) (10).

Between the specified dates, 10,080 patients were enrolled in SEAP in Karachi; 2,104 had a positive blood culture for *S.* Typhi, and 139 had a positive blood culture for *S.* Paratyphi A. Six *S.* Typhi isolates exhibited potential azithromycin resistance by disc diffusion (diameter $\leq$ 12 mm). Upon MIC testing, one failed to revive, four isolates had azithromycin MICs ranging between 1 and 2 μg/ml and one S. Typhi isolate had an MIC of 12 μg/ml (CLSI susceptibility breakpoint $\leq$ 16 μg/ml) (10). This places this isolate at the upper range of the wild-type azithromycin susceptibility distribution, with additional resistance to chloramphenicol, fluoroquinolones, and co-trimoxazole, but it was susceptible to third-generation cephalosporins.

We aimed to investigate the genetic basis of the higher azithromycin MIC and place this organism into phylogenetic context with contemporaneous *S.* Typhi through whole-genome sequencing (WGS). Genomic DNA was extracted and subjected to WGS on a Hiseq2500 (Illumina, San Diego, CA) to generate 125-bp paired-end reads. The resulting sequence data were mapped against the CT18 reference sequence (accession no. AL513382) using the RedDog mapping pipeline to identify single-nucleotide variants (SNVs) and to confirm the *S.* Typhi genomes were within H58 lineage I (4.3.1.1) (7, 9, 11–19). (https://github.com/katholt/genotyphi). After removing repetitive sequences and recombination (20), we generated a final alignment 7,661 chromosomal SNVs for 664 isolates (see Table S1 in the supplemental material). Maximum-likelihood phylogenetic trees were inferred from the chromosomal SNV alignments with RAxML (v8.2.9) (21) and visualized in Microreact (22) (https://microreact.org/project/8FjPCdisk) and the ggtree package in R (23). SRST2 (24) was used with ARGannot (25) and PlasmidFinder (26) to identify antimicrobial resistance genes and plasmid replicons, respectively. Mutations in *gyrA*, and *parC*, as well as the R717Q mutation in *acrB*, were detected using GenoTyphi (https://github.com/katholt/genotyphi).

This higher azithromycin MIC *S.* Typhi isolate (MIC of 12 μg/ml), was typed as genotype 4.3.1.1 (H58 lineage I), which is the same sublineage at the XDR clade circulating in Pakistan. The organism additionally had single mutation in *gyrA* (S83F), resulting in reduced fluoroquinolone susceptibility. The apparent mechanism of higher MIC against azithromycin was an R717Q mutation in the gene encoding AcrB, a mutation identical to the recently described azithromycin resistant (MIC of $\geq$32 μg/ml) *S.* Typhi 4.3.1.1 in Bangladesh (9). The identification of this mutation in *S.* Typhi in Pakistan raises the possibilities that this was either a *de novo* mutation in the Pakistan-specific 4.3.1.1 cluster or an organism that was part of larger, internationally disseminating, azithromycin-resistant clone. To determine which was more likely, we used a collection of 663 South Asian 4.3.1.1 (H58 lineage I) sequences to contextualize *S.* Typhi isolate FQ2181 (7, 9, 11–19). The resulting phylogenetic tree demonstrated that this was a spontaneous mutation which emerged in Pakistan, since it was distantly related (relative within H58 lineage I) to the organisms with *acrB* mutations in Bangladesh, and independent of the proximal XDR sublineage (Fig. 1).

Typically, the isolation of a single *S.* Typhi exhibiting resistance to the primary treatment would not be a major cause for concern. However, this isolate demonstrates an additional, independent acquisition of the same mutation that has been observed in Bangladesh (9). Given the reliance of azithromycin for the treatment of typhoid and other bacterial infections and the "fluoroquinolone experience," we predict that we are likely to see more of these homoplasies arising. It is too early to predict how these particular organisms may spread, and it is encouraging that these mutations have not yet been reported in XDR *S.* Typhi. However, given the nature of these mutations, one could arise in XDR *S.* Typhi, and/or the XDR plasmid may be mobilized into an azithromycin-resistant lineage.

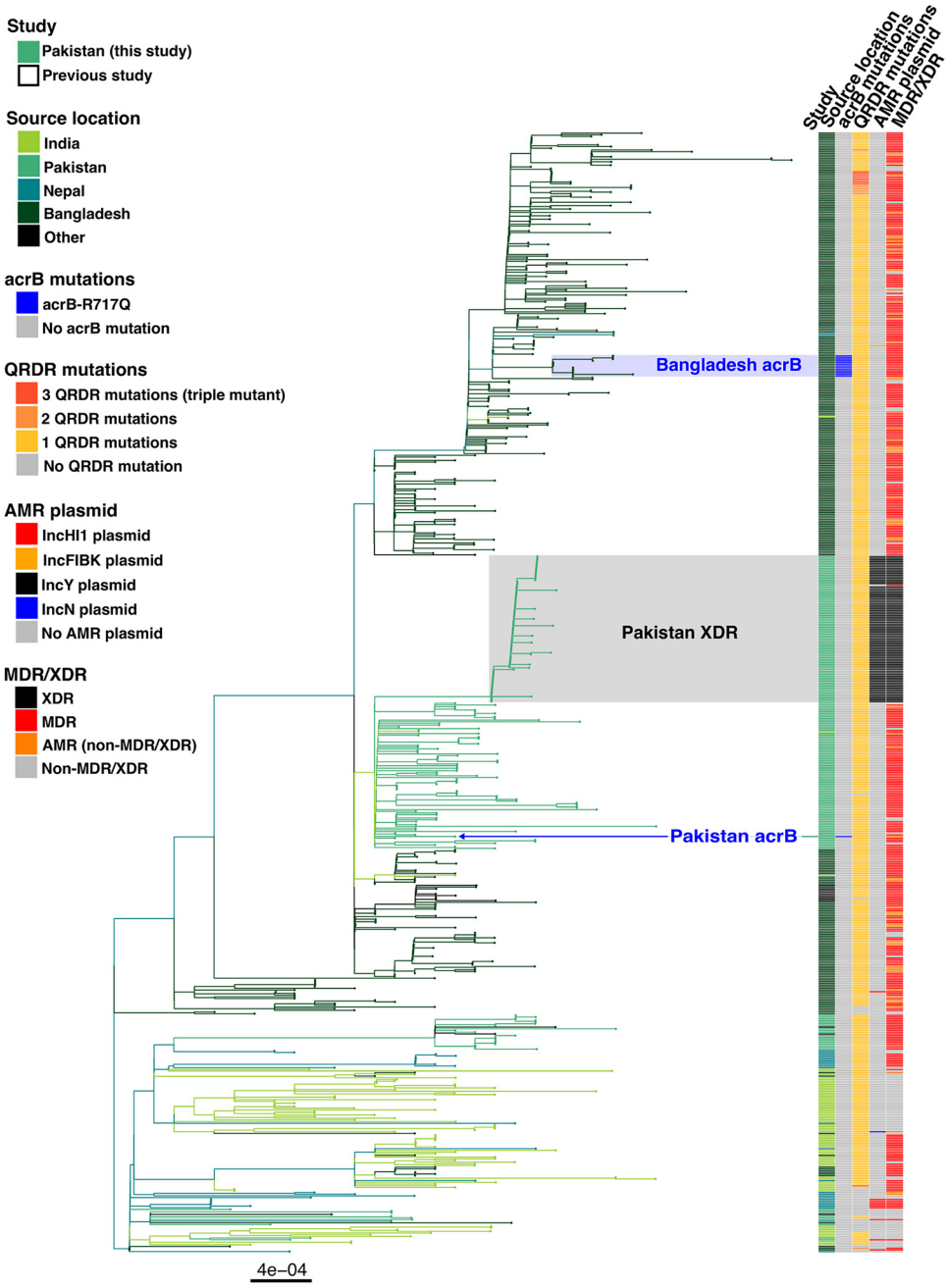

FIG 1 South Asian H58 lineage I (genotype 4.3.1.1) phylogenetic tree ($n = 664$ genomes). Branches are colored by source country according to the inset legend and first color bar. The second color bar indicates genomes containing the *acrB*-R717Q mutation. The third color bar indicates mutations in the quinolone resistance determining region (QRDR) of genes *gyrA*, and *parC*. The final color bar indicates MDR and XDR sequences.

Pakistan has initiated a nationwide typhoid conjugate vaccine (TCV) rollout program, which began with a mass vaccination in Sindh province in November 2019 (27). Now, there is in a race against time in the prevention of untreatable typhoid fever. With one World Health Organization prequalified manufacturer of TCV supplying vaccine for Gavi-eligible countries and several additional manufacturers in late-stage clinical development (28), there is reason to be optimistic about typhoid control. However, the vaccine is not yet available in all countries of endemicity, and effective treatment is still paramount for typhoid control. Consequently, we need to progress with additional intervention strategies and not overlook that antimicrobials have a substantial impact

on typhoid disease control. In addition, as part of this sustained effort, we need to continue to track phenotypic and genotypic antimicrobial resistance in *S.* Typhi to inform best practices for antimicrobial prescribing and the impact of TCV implementation.

SUPPLEMENTAL MATERIAL

Supplemental material is available online only.

TABLE S1, XLSX file, 0.1 MB.

REFERENCES

1. GBD Typhoid and Paratyphoid Collaborators. 2019. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis 19:369–381. https://doi.org/10.1016/S1473-3099(18)30685-6.
2. Butler T, Knight J, Nath SK, Speelman P, Roy SK, Azad MA. 1985. Typhoid fever complicated by intestinal perforation: a persisting fatal disease requiring surgical management. Rev Infect Dis 7:244–256. https://doi.org/10.1093/clinids/7.2.244.
3. Crump JA. 2019. Progress in typhoid fever epidemiology. Clin Infect Dis 68:S4–S9. https://doi.org/10.1093/cid/ciy846.
4. Mirza SH, Beeching NJ, Hart CA. 1996. Multidrug-resistant typhoid: a global problem. J Med Microbiol 44:317–319. https://doi.org/10.1099/00222615-44-5-317.
5. Britto CD, Wong VK, Dougan G, Pollard AJ. 2018. A systematic review of antimicrobial resistance in *Salmonella enterica* serovar Typhi, the etiological agent of typhoid. PLoS Negl Trop Dis 12:e0006779. https://doi.org/10.1371/journal.pntd.0006779.
6. Yousafzai MT, Qamar FN, Shakoor S, Saleem K, Lohana H, Karim S, Hotwani A, Qureshi S, Masood N, Rauf M, Khanzada JA, Kazi M, Hasan R. 2019. Ceftriaxone-resistant *Salmonella* Typhi outbreak in Hyderabad City of Sindh, Pakistan: high time for the introduction of typhoid conjugate vaccine. Clin Infect Dis 68:S16–S21. https://doi.org/10.1093/cid/ciy877.
7. Klemm EJ, Shakoor S, Page AJ, Qamar FN, Judge K, Saeed DK, Wong VK, Dallman TJ, Nair S, Baker S, Shaheen G, Qureshi S, Yousafzai MT, Saleem MK, Hasan Z, Dougan G, Hasan R. 2018. Emergence of an extensively drug-resistant *Salmonella enterica* serovar Typhi clone harboring a promiscuous plasmid encoding resistance to fluoroquinolones and third-generation cephalosporins. mBio 9:e00105-18. https://doi.org/10.1128/mBio.00105-18.
8. Levine MM, Simon R. 2018. The gathering storm: is untreatable typhoid fever on the way? mBio 9:e00482-18. https://doi.org/10.1128/mBio.00482-18.
9. Hooda Y, Sajib MSI, Rahman H, Luby SP, Bondy-Denomy J, Santosham M, Andrews JR, Saha SK, Saha S. 2019. Molecular mechanism of azithromycin resistance among typhoidal Salmonella strains in Bangladesh identified through passive pediatric surveillance. PLoS Negl Trop Dis 13:e0007868. https://doi.org/10.1371/journal.pntd.0007868.
10. CLSI. 2018. Performance standards for antimicrobial susceptibility testing, 28th ed. Clinical and Laboratory Standards Institute, Wayne, PA.
11. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, Murphy N, Holliman R, Sefton A, Millar M, Dyson ZA, Dougan G, Holt KE, International Typhoid Consortium. 2016. An extended genotyping framework for *Salmonella enterica* serovar Typhi, the cause of human typhoid. Nat Commun 7:12827. https://doi.org/10.1038/ncomms12827.
12. Britto CD, Dyson ZA, Duchene S, Carter MJ, Gurung M, Kelly DF, Murdoch DR, Ansari I, Thorson S, Shrestha S, Adhikari N, Dougan G, Holt KE, Pollard AJ. 2018. Laboratory and molecular surveillance of paediatric typhoidal *Salmonella* in Nepal: antimicrobial resistance and implications for vaccine policy. PLoS Negl Trop Dis 12:e0006408. https://doi.org/10.1371/journal.pntd.0006408.
13. Britto CD, Dyson ZA, Mathias S, Bosco A, Dougan G, Jose S, Nagaraj S, Holt KE, Pollard AJ. 2020. Persistent circulation of a fluoroquinolone-resistant *Salmonella enterica* Typhi clone in the Indian subcontinent. J Antimicrob Chemother 75:337–341. https://doi.org/10.1093/jac/dkz435.
14. Tanmoy AM, Westeel E, De Bruyne K, Goris J, Rajoharison A, Sajib MSI, van Belkum A, Saha SK, Komurian-Pradel F, Endtz HP. 2018. *Salmonella enterica* serovar Typhi in Bangladesh: exploration of genomic diversity and antimicrobial resistance. mBio 9:e02112-18. https://doi.org/10.1128/mBio.02112-18.
15. Pham Thanh D, Karkey A, Dongol S, Ho Thi N, Thompson CN, Rabaa MA, Arjyal A, Holt KE, Wong V, Tran Vu Thieu N, Voong Vinh P, Ha Thanh T, Pradhan A, Shrestha SK, Gajurel D, Pickard D, Parry CM, Dougan G, Wolbers M, Dolecek C, Thwaites GE, Basnyat B, Baker S. 2016. A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. Elife 5:e14003. https://doi.org/10.7554/eLife.14003.
16. Ingle DJ, Nair S, Hartman H, Ashton PM, Dyson ZA, Day M, Freedman J, Chattaway MA, Holt KE, Dallman TJ. 2019. Informal genomic surveillance of regional distribution of *Salmonella* Typhi genotypes and antimicrobial resistance via returning travellers. PLoS Negl Trop Dis 13:e0007620. https://doi.org/10.1371/journal.pntd.0007620.
17. Rahman SIA, Dyson ZA, Klemm EJ, Khanam F, Holt KE, Chowdhury EK, Dougan G, Qadri F. 2020. Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in urban Dhaka, Bangladesh, from 2004 to 2016. PLoS Negl Trop Dis 14:e0008036. https://doi.org/10.1371/journal.pntd.0008036.
18. Langmead B, Salzberg SL. 2012. Fast gapped-read alignment with Bowtie 2. Nat Methods 9:357–359. https://doi.org/10.1038/nmeth.1923.
19. Li H, Handsaker B, Wysoker A, Fennell T, Ruan J, Homer N, Marth G, Abecasis G, Durbin R, Genome Project Data Processing Study. 2009. The sequence alignment/map format and SAMtools. Bioinformatics 25:2078–2079. https://doi.org/10.1093/bioinformatics/btp352.
20. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, Parkhill J, Harris SR. 2015. Rapid phylogenetic analysis of large samples of recombinant bacterial whole-genome sequences using Gubbins. Nucleic Acids Res 43:e15. https://doi.org/10.1093/nar/gku1196.
21. Stamatakis A. 2006. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics 22:2688–2690. https://doi.org/10.1093/bioinformatics/btl446.
22. Argimon S, Abudahab K, Goater RJE, Fedosejev A, Bhai J, Glasner C, Feil EJ, Holden MTG, Yeats CA, Grundmann H, Spratt BG, Aanensen DM. 2016. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microb Genom 2:e000093. https://doi.org/10.1099/mgen.0.000093.
23. Yu G, Smith DK, Zhu H, Guan Y, Lam TT-Y. 2017. ggtree: an R package for visualization and annotation of phylogenetic trees with their covariates and other associated data. Methods Ecol Evol 8:28–36. https://doi.org/10.1111/2041-210X.12628.
24. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, Zobel J, Holt KE. 2014. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome Med 6:90. https://doi.org/10.1186/s13073-014-0090-6.
25. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, Rolain JM. 2014. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother 58:212–220. https://doi.org/10.1128/AAC.01310-13.
26. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, Møller Aarestrup F, Hasman H. 2014. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother 58:3895–3903. https://doi.org/10.1128/AAC.02412-14.
27. EMRO/WHO. 2019. Pakistan becomes first country to introduce new typhoid vaccine into routine immunization program, p 10–12. World Health Organization, Geneva, Switzerland.
28. Sahastrabuddhe S, Saluja T. 2019. Overview of the typhoid conjugate vaccine pipeline: current status and future plans. Clin Infect Dis 68:S22–S26. https://doi.org/10.1093/cid/ciy884.

Appendix 4. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of *Salmonella* Typhi in Northern India (publication)

Clinical Infectious Diseases

MAJOR ARTICLE

Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of *Salmonella* Typhi in Northern India

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Background. The emergence and spread of antimicrobial resistance (AMR) pose a major threat to the effective treatment and control of typhoid fever. The ongoing outbreak of extensively drug-resistant *Salmonella* Typhi (*S.* Typhi) in Pakistan has left azithromycin as the only remaining broadly efficacious oral antimicrobial for typhoid in South Asia. Ominously, azithromycin-resistant *S.* Typhi organisms have been subsequently reported in Bangladesh, Pakistan, and Nepal.

Methods. Here, we aimed to understand the molecular basis of AMR in 66 *S.* Typhi organisms isolated in a cross-sectional study performed in a suburb of Chandigarh in Northern India using whole-genome sequencing and phylogenetic analysis.

Results. We identified 7 *S.* Typhi organisms with the R717Q mutation in the *acrB* gene that was recently found to confer resistance to azithromycin in Bangladesh. Six out of the seven azithromycin-resistant *S.* Typhi isolates also exhibited triple mutations in *gyrA* (S83F and D87N) and *parC* (S80I) genes and were resistant to ciprofloxacin. These contemporary ciprofloxacin/azithromycin-resistant isolates were phylogenetically distinct from each other and from those reported from Bangladesh, Pakistan, and Nepal.

Conclusions. The independent emergence of azithromycin-resistant typhoid in Northern India reflects an emerging broader problem across South Asia and illustrates the urgent need for the introduction of typhoid conjugate vaccines in the region.

Keywords. *Salmonella* Typhi; typhoid fever; antimicrobial resistance; azithromycin resistance; India.

Salmonella enterica serovar Typhi (*S.* Typhi), the etiologic agent of typhoid fever, is associated with an estimated 10.9 million infections and 116 800 deaths globally [1]. The majority of this disease burden is concentrated in South Asia, which has a modeled incidence rate of 592 cases per 100 000 person-years [1]. A pooled estimate of typhoid fever incidence of 377 cases per 100 000 in India has also been calculated using limited population-based data; significant geographical heterogeneity was observed [2]. The ongoing Surveillance for Enteric Fever in India (SEFI) Study is generating geographically representative, age-specific incidence data, as well as additional information regarding cost of illness, range of clinical severity, and antimicrobial resistance (AMR) patterns associated with *S.* Typhi in India [3]. These data will undoubtedly provide a more comprehensive understanding of typhoid fever incidence rates across the Indian subcontinent and, ultimately, in supporting decision making concerning typhoid conjugate vaccine (TCV) introduction in India [4].

Growing rates of AMR have made typhoid control increasingly challenging, beginning with the rise of multidrug resistance (MDR; resistant to chloramphenicol, trimethoprim-sulfamethoxazole, ampicillin) in the 1990s [5] and the subsequent increase in fluoroquinolone resistance in the early 2000s, which was predominantly focused in South and Southeast Asia [6, 7]. Ultimately, these phenotypic changes led to the common use of third-generation cephalosporins for the treatment of typhoid fever. The emergence and spread of extensively drug-resistant (XDR; resistant to chloramphenicol, ampicillin, cotrimoxazole, streptomycin, fluoroquinolones, and third-generation cephalosporins) typhoid in Pakistan has left azithromycin as the only available oral antimicrobial for effective treatment of typhoid fever across South Asia [8]. Concerningly, azithromycin-resistant *S.* Typhi have subsequently been reported in Bangladesh, Pakistan, and Nepal, although this phenotype has not yet arisen in XDR organisms [9–11].

It is apparent that we have an escalating problem with drug-resistant *S.* Typhi in South Asia, due in part to empirical treatment of febrile patients and widespread community availability of antimicrobials. We are currently unsure of the regional

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distribution of azithromycin resistance or the potential for the emergence of a specific sublineage with this phenotype, as has been observed for MDR, XDR, and fluoroquinolone resistance. Here, we aimed to characterize the molecular basis of AMR in *S.* Typhi in a cross-sectional study performed in a suburb of Chandigarh in Northern India. Through whole-genome sequencing (WGS), we describe the distribution of a collection of azithromycin-resistant *S.* Typhi and show that these organisms have arisen independently of those in Pakistan and Bangladesh through the acquisition of an identical mutation in the *arcB* gene. Our data support the prioritization of TCV introduction to prevent the continued emergence and spread of drug-resistant *S.* Typhi in South Asia.

METHODS

Ethics

Ethical clearance was granted by the Institutional Ethics Committee of the Government Multi-specialty Hospital, Sector 16, Chandigarh (letter no. GMSH/2018/8763 dated 26 July 2018) and the Postgraduate Institute of Medical Education and Research (PGIMER) Institutional Ethics Committee (IEC-08/2018–285 dated 24 September 2018). Administrative approval to carry out this collaborative study on enteric fever was granted by the Chandigarh Health Department (CHMM-2017/2991 dated 28 August 2017). Approval was also granted by the Collaborative Research Committee of PGIMER (no. 79/227-Edu-18/4997 dated 12 December 2018). Informed consent was a prerequisite for inclusion in the study.

Study Design

The *S.* Typhi isolates were obtained from blood cultures taken from febrile patients presenting to Civil Hospital Manimajra in Chandigarh (CHMM) between September 2016 and December 2017, where passive blood culture surveillance has been conducted since November 2013. The CHMM is a 100-bed secondary healthcare facility located on the outskirts of Chandigarh, and serves a catchment area of approximately 200 000 people, including referrals from 4 primary care centers. Data from patients with blood culture–confirmed invasive *Salmonella* infection, which includes *Salmonella* serovars Typhi and Paratyphi A, B, and C, from both inpatient and outpatient wards were used in the study. Clinical history, laboratory test results, and risk factor data were recorded for each confirmed case of enteric fever for patients residing in Manimajra. This analysis focuses solely on confirmed cases of *S.* Typhi.

Identification and Antimicrobial Susceptibility Testing

Bacterial isolates were identified as *S.* Typhi using conventional biochemical tests; motility agar, Hugh–Leifson Oxidative-Fermentation test, the Triple Sugar Iron test, citrate test, urease test, phenyl pyruvic acid test, and indole test. All isolates were eventually confirmed using antisera from Central Research Institute, Kasauli. Antimicrobial susceptibility was determined for the following antimicrobials by disc diffusion: ampicillin (10 μg), chloramphenicol (30 μg), trimethoprim/sulfamethoxazole (1.25/23.75 μg), ceftriaxone (30 μg), azithromycin (15 μg), ciprofloxacin (5 μg), and pefloxacin (5 μg). Zone diameters were measured and interpreted as per Clinical Laboratory Standards Institute guidelines [12]. Minimum inhibitory concentration (MIC) testing was also conducted on all organisms showing resistance to any of the above antimicrobials by disc diffusion using E-tests (bioMerieux, France).

Whole-Genome Sequencing and Phylogenetic Analysis

All *S.* Typhi were stored and shipped to PGIMER Chandigarh. Isolates from patients residing outside of Manimajra were excluded, as were those for which there were inadequate clinical metadata, or the DNA yield was below the amount required for WGS. Total genomic DNA was extracted from the *S.* Typhi using the Wizard genomic DNA extraction Kit (Promega, Madison WI, USA) and subjected to WGS using the Illumina MiSeq platform (Illumina, San Diego, CA, USA) to generate 250-bp paired end reads. We then aimed to put these sequences into global and regional phylogenetic context. Subsequently, these reads were mapped against the CT18 reference sequence (accession no. AL513382) using the RedDog mapping pipeline (V1beta.10.4; available at: https://github.com/katholt/RedDog) to identify single nucleotide variants (SNVs) [13–15]. RedDog uses Bowtie (v2.2.9) [16] to map reads to the reference sequence; SAMtools (v1.9) [17] is used to identify SNVs with phred quality scores above 30; to filter out SNVs supported by less than 5 reads, or with more than 2.5 times the genome-wide average read depth (representing putative repeated sequences), or with ambiguous (heterozygous) consensus base calls. For each SNV position that passed these criteria in any single sequence, consensus base calls/alleles for that position were extracted from all genomes and used to construct an alignment of alleles across all SNV sites. Ambiguous base calls and those with a phred quality score of less than 20 were treated as unknown alleles and represented with a gap character in the SNV alignment. Read alignments were used to assign isolates to previously defined lineages according to the extended genotyping framework [13] by subjecting the alignments Binary Alignment Map (BAM format) to analysis using the GenoTyphi pipeline (available at: http://github.com/katholt/genotyphi).

Chromosomal SNVs with confident homozygous calls (phred score >20) in more than 95% of the genomes mapped (representing a "soft" core genome) were concatenated to form an alignment of alleles at 26 991 variant sites, and alleles from *S.* Paratyphi A str. AKU1_12601 (accession no. FM200053) [18] were also included using the same mapping approach for phylogenetic tree outgroup rooting. SNVs called in repetitive sequences and prophage regions were removed from the alignment using the parseSNPtable.py script from RedDog

(354 kbp; ~7.4% of bases in the CT18 reference chromosome, as defined previously) [13, 19, 20] and a pseudo-genome alignment inferred using the CT18 reference sequence with the snpTable2GenomeAlignment.py script (also from RedDog). Any further recombination was filtered from the resultant whole-genome pseudo-alignment using Gubbins (v2.3.2) [21], resulting in a final alignment length of 25 832 chromosomal SNVs for 3473 isolates.

RAxML (v8.9.2) [22] was used to infer maximum likelihood (ML) phylogenetic trees from the final chromosomal SNV alignment, with a generalized time-reversible model, a gamma distribution to model site-specific rate variation (the GTR+ Γ substitution model; GTRGAMMA in RAxML), and 100 bootstrap pseudo-replicates to assess branch support. Resultant ML trees were visualized with Microreact [23] (https://microreact.org/project/nniNzBL2uq3XZXYDKgG374) and the Interactive Tree of Life [24]. SRST2 (v0.2.0) [25] was used with ARGannot (available at: https://github.com/katholt/srst2/blob/master/data/ARGannot_r3.fasta) [26] and PlasmidFinder (available at: https://github.com/katholt/srst2/blob/master/data/PlasmidFinder.fasta) [27] databases to identify AMR genes and plasmid replicons, respectively. Mutations in *gyrA* and *parC*, as well as the R717Q mutation in *acrB*, were detected using GenoTyphi (https://github.com/katholt/genotyphi). Raw read data were deposited in the European Nucleotide Archive (ENA) under accession number ERP124488.

RESULTS

Epidemiological Observations

Typhoid fever is a major public health concern among children and young adults in this region of Northern India. It is thought that many cases in this area, a hub for the states of Punjab, Haryana, and Himachal Pradesh, are associated with the mixing of large populations of seasonal workers from the adjoining states. These workers generally live in informal dwellings with poor sanitation and limited access to safe water. Approximately 1500 patients with suspected enteric fever present to the CHMM facility annually and receive blood cultures, of which approximately 10% are positive for *S.* Typhi. There is a seasonal peak of typhoid fever in this facility during the monsoon months from May to September (Figure 1). As a component of a typhoid surveillance program at CHMM, study staff provided health education about safe drinking water and chlorine tablets to all households where there was a confirmed typhoid fever case from mid-2017 onwards. This likely accounts for the reduction in case numbers from 2017 to 2019.

The *S.* Typhi organisms interrogated here by WGS were isolated between September 2016 and December 2017 and all originated from blood cultures taken from febrile patients attending CHMM. All patients with a positive blood culture for *S.* Typhi resided within 12.9 km of the healthcare facility and were located in an area of approximately 28 km^2 (Figure 2). *S.* Typhi was isolated throughout the specified months, again with a higher number of cases observed between May and September. The median age of patients with typhoid included in this analysis was 7 years. The standard-of-care antimicrobials at this facility for patients with suspected enteric fever in outpatient settings are cotrimoxazole, cefixime, and/or azithromycin, and ceftriaxone for inpatients.

The Local Phylogenetic Structure of *S.* Typhi

Ultimately, after data quality control, we generated and analyzed 66 *S.* Typhi genome sequences from Chandigarh. We observed that the population structure of *S.* Typhi around Chandigarh exhibited a high level of genetic diversity with 8 co-circulating genotypes, indicative of population mixing and sustained introduction of organisms from a variety of locations across India (Figure 3). However, and in an analogous manner to other locations in Asia (and East Africa), most organisms (80%, 53/66) belonged to lineage 4.3.1 (H58), with the majority of those (66%, 35/53) belonging to 4.3.1.2. In total, 24% (16/66) of isolates were subclade 4.3.1 and 3% (2/66) were 4.3.1.1. Additional genotypes were composed of subclade 3.3 (7.5%, 5/66), clade 2.5 (7.5%, 5/66), clade 3.3.1 (1.5%, 1/66), clade 4.1 (1.5%, 1/66), and major lineage 2 (genotype 2; 1.5%, 1/66).

Fluoroquinolone Resistance

All (66/66) *S.* Typhi genome sequences, regardless of the genotype, possessed mutations in *gyrA*, conferring reduced susceptibility to fluoroquinolones. Notably, given that these mutations were observed in a range of genotypes, these had occurred independently, likely as a result of sustained antimicrobial pressure from widespread fluoroquinolone use. We further observed multiple *gyrA* mutation profiles in 4.3.1 organisms conferring intermediate resistance against fluoroquinolones (0.12 μg/mL < ciprofloxacin MIC < 1 μg/mL). These mutations included S83Y (29.1%, 16/55), S83F (16.4%, 9/55), and D87N (1.8%, 1/55). Additionally, we identified a subclade of organisms that represented 49.1% (27/55) of the 4.3.1 isolates, all of which belonged to 4.3.1.2, that contained the classical triple mutations associated with fluoroquinolone resistance (S83F and D87N in *gyrA* and S80I in *parC*) [14]. These organisms exhibited high-level fluoroquinolone resistance (ciprofloxacin MIC >24 μg/mL). Our observations with respect to ubiquitous fluoroquinolone resistance were concerning; however, none of the *S.* Typhi isolates were MDR, which may be associated with a reduced reliance on older classes of antimicrobials.

Azithromycin Resistance

We identified that 7 of 66 (10.6%) of the sequenced isolates contained a mutation in *acrB*, a gene encoding a component of the AcrAB efflux pump [28]. Mutations in *acrB* have been previously observed to be associated with resistance

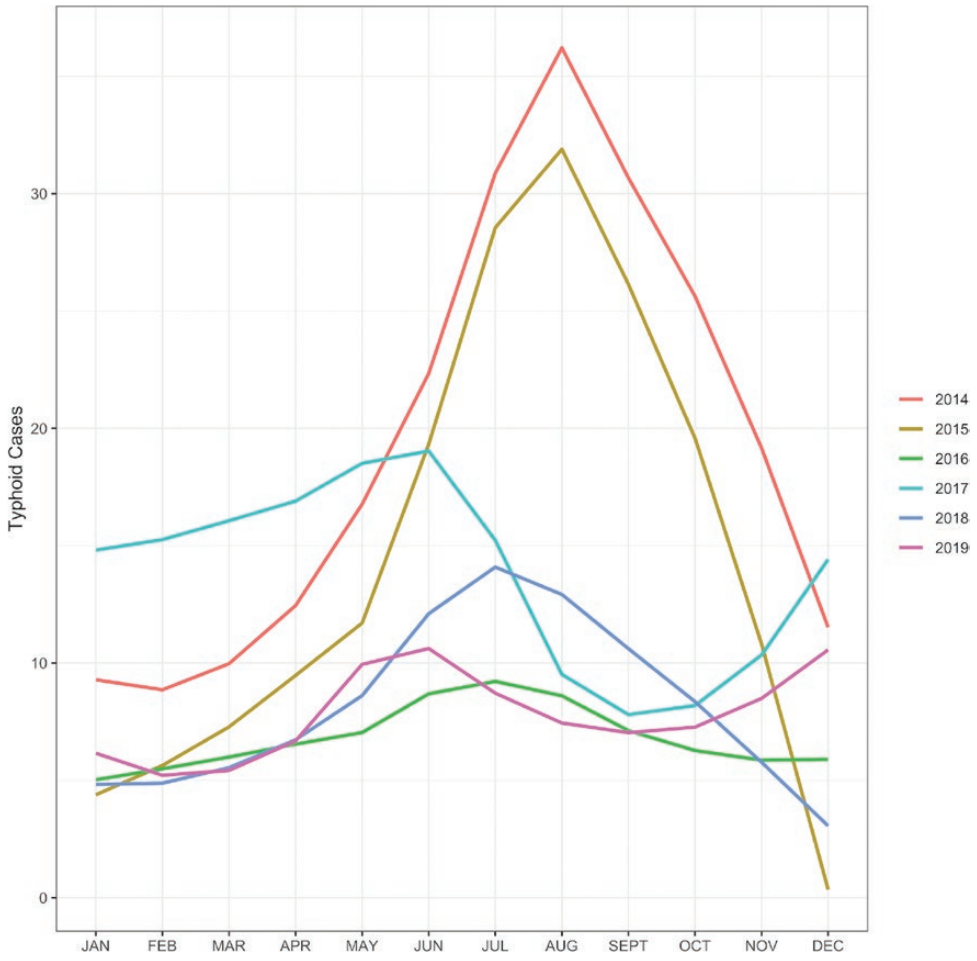

Figure 1. The annual seasonality of typhoid fever in Manimajra, Chandigarh. Plots showing the number of typhoid cases recorded the civil hospital in Manimajra from 2014 to 2019. The observed annual peak in typhoid cases corresponds with the monsoon season in Northern India (May to September).

to azithromycin [9]. There are currently no other described mechanisms of azithromycin resistance in *S*. Typhi. Here, the *acrB* mutation was nonsynonymous (R717Q) and identified in 6 genotype 4.3.1.2 organisms and in 1 genotype 3.3.1 organism. These data are indicative of convergent mutation in different lineages, highlighting a potential increasing reliance on azithromycin; this selective pressure is further accentuated by the small clonal expansion in genotype 4.3.1.2 (Figure 3).

The R717Q mutation in *acrB* has been linked to high azithromycin MICs in genotype 4.3.1.1 *S*. Typhi isolates from Bangladesh [8] and also in genotype 4.3.1.1 *S*. Typhi in Pakistan [9]. Here, 6 of 7 of the contemporary Indian *S*. Typhi isolates identified here with the same R717Q mutation in *acrB* showed nonsusceptibility to azithromycin with MICs greater than 16 μg/mL (Table 1). Our data suggested that these R7171Q mutations in the *acrB* gene have arisen spontaneously in India. To test this hypothesis, we constructed an expanded phylogenetic tree comprising a global *S*. Typhi collection, including organisms from across South Asia and the recently described azithromycin-resistant organisms from Bangladesh, Pakistan, and Nepal. We found that the azithromycin-resistant *S*. Typhi from India were phylogenetically distinct from those reported from Bangladesh, Pakistan, and Nepal (Figure 4). Additionally, we found that the azithromycin-resistant organisms associated with *acrB* mutations were dispersed around the tree and appear to have arisen on at least 6 different occasions, with a differing *acrB* mutation in organisms from Nepal (R717L) [11].

The azithromycin-resistant isolates from India described here were isolated in 2017, meaning that they are contemporaneous with those reported from Bangladesh and Pakistan, and arose independently in phylogenetically distinct lineages. Last, 6 of the 7 Indian isolates with the R717Q mutation in *arcB* (all 4.3.1.2) were also within the group of organisms with the triple mutation associated with high-level fluoroquinolone resistance, making these organisms highly resistant to these 2 key oral antimicrobials.

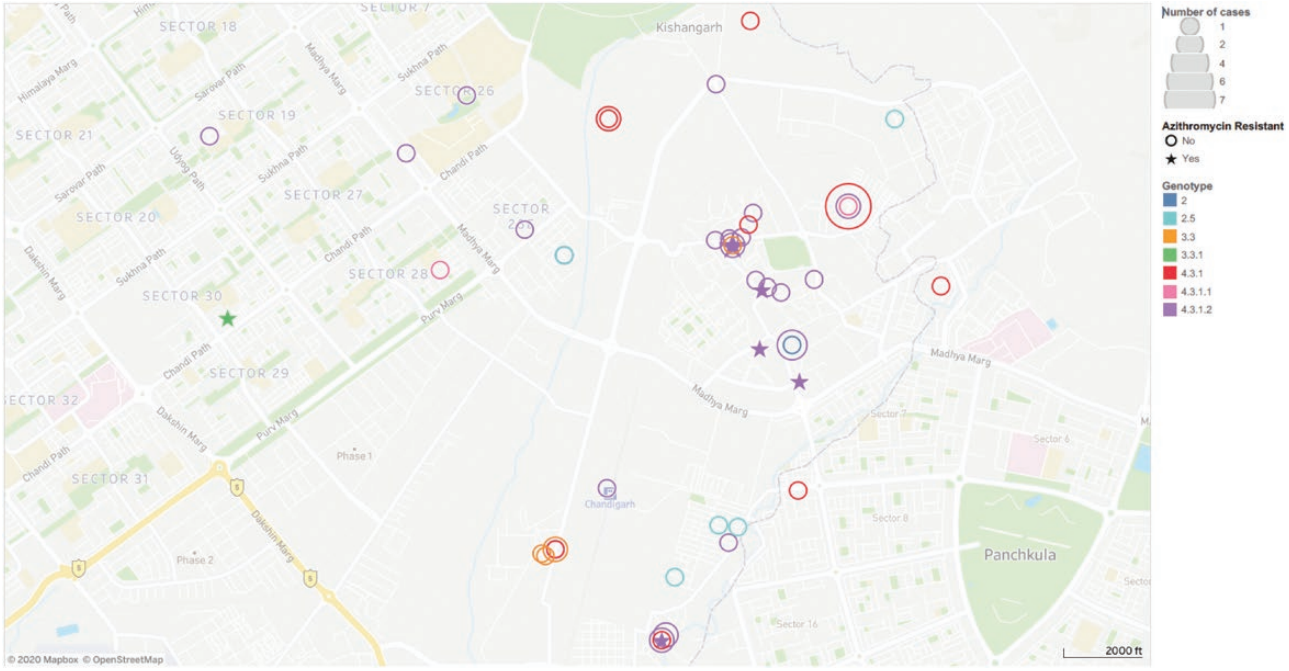

Figure 2. The spatial distribution of confirmed typhoid fever cases in Chandigarh. Map of Chandigarh (scale shown) of the residential locations of the *Salmonella* Typhi cases recorded at the civil hospital in Manimajra between September 2016 and December 2017. *S.* Typhi genotype is indicated by color (see key), azithromycin-resistant isolates are indicated with stars, and the number of cases in each coordinate is represented by the size of the circles. All cases were located within a 28-km^2 area. There is a cluster of cases of genotype 4.3.1.2 in a 0.25-km^2 area of central Manimajra, which includes 5 of the 6 closely related azithromycin-resistant isolates.

DISCUSSION

In this study, we aimed to describe the genomic aspects the *S.* Typhi causing disease in an endemic region in Northern India. We investigated antimicrobial susceptibility patterns using phenotypic testing and WGS data and then placed these data into a regional and global context using published genomic data. Notably, we identified 7 azithromycin-resistant *S.* Typhi isolates. These organisms belonged to 2 different lineages and were genetically distinct from azithromycin-resistant isolates recently reported from Bangladesh, Pakistan, and Nepal. Our observations suggest that azithromycin-resistance mutations at codon 717 in *arcB* are arising independently in locations where there is substantial selective pressure induced by azithromycin. At present, azithromycin resistance in *S.* Typhi is rare, as the 24 *acrB* mutants concatenated here represent all published *S.* Typhi sequences with this mutation. An increased reliance on azithromycin for treatment of typhoid fever (given in a 500-mg dose twice a day on day 1 and once a day for 2 weeks for adults and as a 10-mg/kg dose once a day for 2 weeks in children in this location) and other invasive bacterial infections in South Asia appears to be driving resistance. We also speculate that this may be facilitated by an interaction with fluoroquinolone-resistance mutations, which may stimulate the acquisition of further resistance phenotypes [29]. Additionally, ongoing clinical trials measuring the impact of prophylactic administration of azithromycin on growth and mortality of infants and young children in Pakistan, Bangladesh, and India [30] signal the inevitability of what Hooda and colleagues [30] have termed pan-oral drug-resistant Typhi. This scenario would necessitate inpatient intravenous drug administration for effective treatment of typhoid fever in the region at enormous cost to patients and to healthcare systems. Where intravenous drug administration is not an option, typhoid could once again become a disease with a high mortality rate, as was observed in the pre-antimicrobial era.

While the catchment area of this study is not representative of the entire Indian subcontinent, the phenomenon described herein is unlikely to be restricted to Chandigarh. India is currently the largest consumer of antimicrobials of all low- and middle-income countries, with a reported 6.5 billion defined daily doses (DDDs) in 2015, or 13.6 DDDs per 1000 inhabitants per day [31, 32]. With such widespread availability and use of antimicrobials nationally, selective pressure on circulating pathogens is likely immense. The SEFI study will soon yield additional concrete AMR data from multiple, geographically representative sites in India, which will further elucidate AMR patterns across the country. Ideally, these data will inform local antimicrobial stewardship practices and may be used as a basis for prioritization of future interventions.

How then to tackle this "red queen" dilemma? Drug-development efforts cannot keep pace with bacterial evolution. Therefore, there is an urgent need for preventative interventions, namely water, sanitation, and hygiene (WASH) interventions and TCV introduction, in India and across South

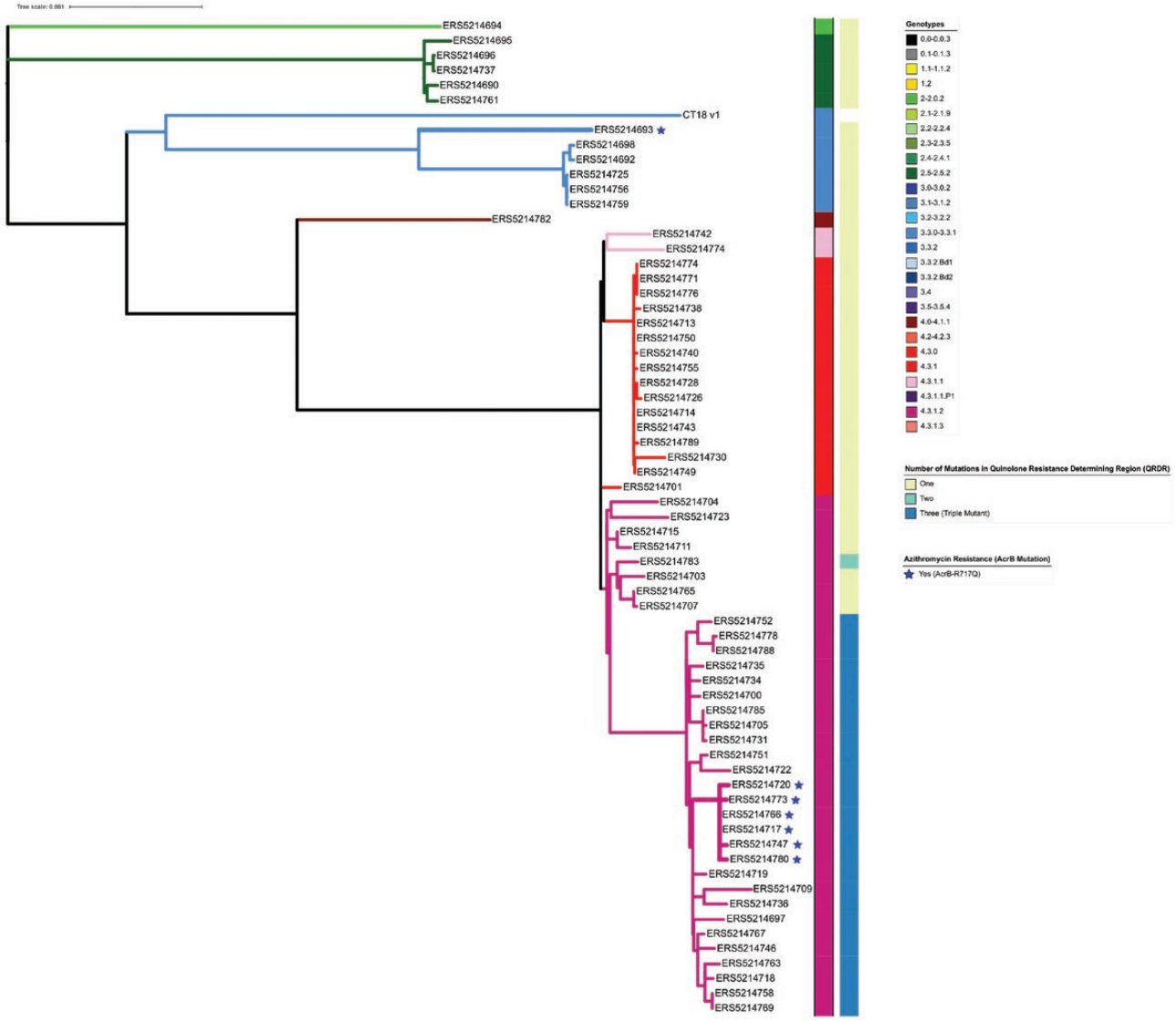

Figure 3. The phylogenetic distribution of *Salmonella* Typhi isolated at the civil hospital in Manimajra, Chandigarh. Phylogenetic tree made in RAxML of the 67 isolates genome sequenced. This collection shows considerable genetic diversity, with 8 genotypes represented (as color coded on branches and in the key). Mutations in the Quinolone Resistance Determining Region (QRDR) and presence of the acrB-R717Q mutation are shown for each organism. There are 2 distinct clusters of organisms with the *acrB* mutation that confers azithromycin resistance; each of these individual organisms are indicated with a star.

Table 1. *Salmonella* Typhi Considered Nonsusceptible to Azithromycin Identified in This Study

ENA Accession Number	Study ID[a]	MIC,[b] μg/mL
ERS5214693	B950_2017	>256
ERS5214717	B628_2017	24
ERS5214720	B550_2017	>256
ERS5214747	B396_2017	64
ERS5214766	B231_2017	16[c]
ERS5214773	B117_2017	>256
ERS5214778	B113_2017	128

Abbreviations: ENA, European Nucleotide Archive; MIC, minimum inhibitory concentration.

[a]All organisms contained *acrB* mutation.

[b]Clinical and Laboratory Standards Institute (CLSI) guidelines for azithromycin susceptibility (MIC): susceptible, ≤16 μg/mL; resistant, ≥32 μg/mL.

[c]Contained *acrB* mutation but borderline susceptible by MIC.

Asia. There is also a need for enhanced typhoid surveillance, in South Asia and globally, specifically to monitor the emergence and spread of this and other resistance phenotypes. Historic genomic data show us that drug-resistant *S.* Typhi lineages emerge in South Asia and then spread to East Africa and even Latin America [33]. With the widespread prophylactic deployment of azithromycin through clinical studies and public health programs in West Africa and South Asia [30, 34] it will be critical to monitor global AMR patterns to mitigate a public health catastrophe. Recently published data from the MORDOR (Macrolides Oraux pour Réduire les Décès avec un Oeil sur la Résistance) cluster-randomized trial in Niger showed that a random sample of children (ages 1–59 months) living in

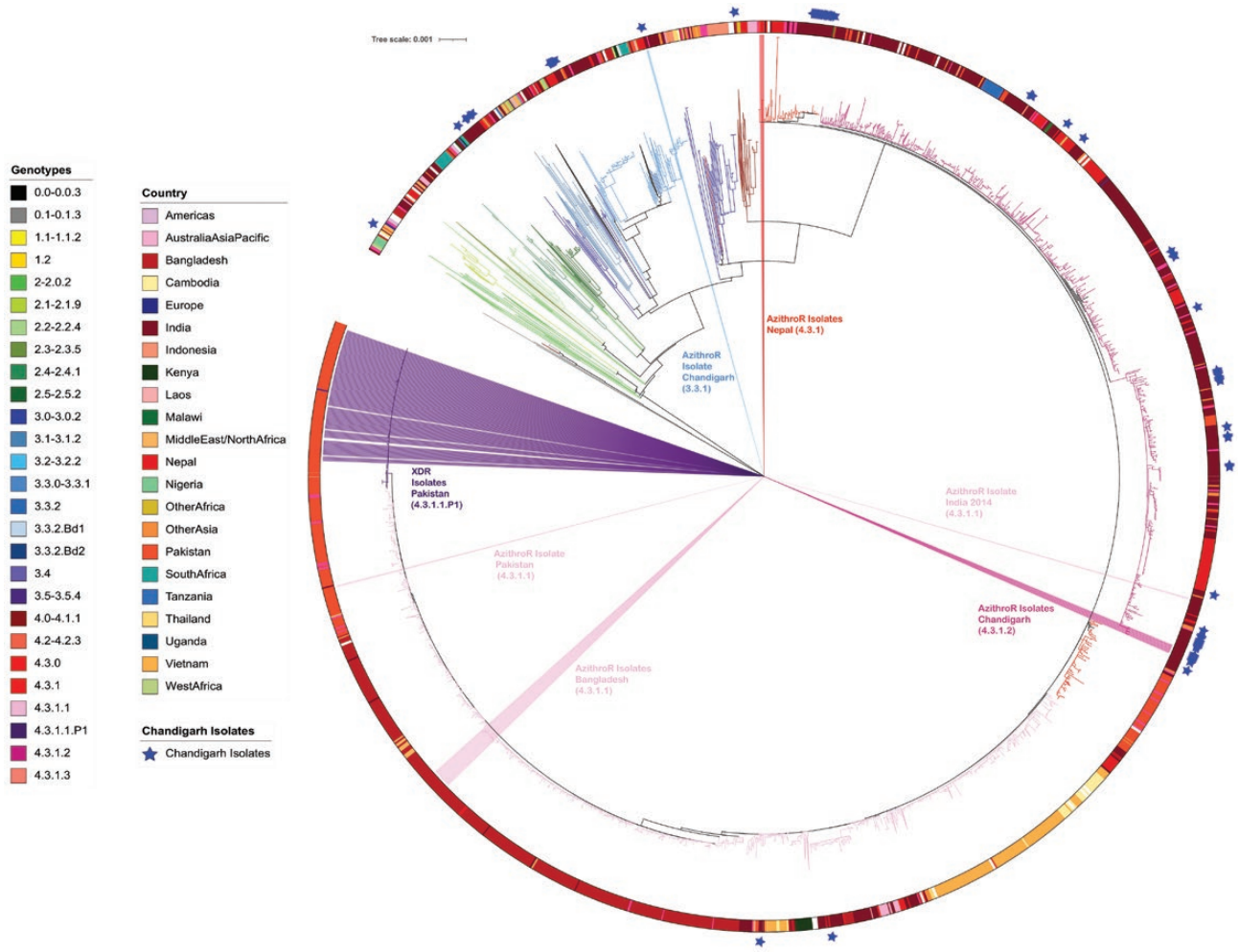

Figure 4. Azithromycin-resistant *Salmonella* Typhi in a global context. The diagram depicts a maximum likelihood rooted phylogenetic tree with a final alignment of 25 832 chromosomal SNVs for 3472 globally representative isolates, including all publicly available isolates from India. The color of the internal branches represents the genotype, the colored ring around the tree indicates the country or region of origin for each isolate, and the blue stars indicate which isolates originate from this study. Additionally, the tree contains each known *S.* Typhi isolate with an *acrB* mutation in public databases, these originate from India, Nepal, Bangladesh, and Pakistan. The location of the XDR isolates from Pakistan are added for context. Abbreviations: AzithroR, azithromycin resistant; SNV, single nucleotide variant; XDR, extensively drug-resistant.

villages randomized to receive a single dose of azithromycin twice annually through mass drug administration were 7.5 times more likely (95% confidence interval, 3.8–23.1) to have macrolide-resistance determinants in their gut microbiomes after 48 months than their peers residing in villages that were randomized to receive a placebo [35]. These data further underscore the need for continued and intensified AMR surveillance in these and other areas where community azithromycin use is high.

This emerging problem additionally represents an opportunity for the use of genomics to inform policy. Whole-genome sequencing data provide clear information regarding AMR in organisms where molecular mechanisms of resistance are understood. Genomic surveillance also enables the identification and characterization of new resistance phenotypes, as was the case for XDR typhoid [8] and the new azithromycin-resistant organisms identified in Bangladesh and described further here [9]. The outputs of antimicrobial susceptibility testing are not always straightforward, particularly in cases where susceptibility breakpoints have not been validated extensively using clinical data, as is the case for azithromycin [36]. Genomic AMR data can inform prioritization of TCV introduction, as well as implementation of WASH interventions. Genomic surveillance should also be an important component of long-term monitoring of the impact of widespread TCV deployment. Not only can genomic surveillance provide additional information on the impact of TCV on AMR it will also illustrate the impact of vaccine on bacterial population structures and enable the identification of any vaccine escape mutants. Such information is vital to understanding the long-term impact of vaccine and to facilitate any potential future efforts for global typhoid elimination.

Notes

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References

1. Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis **2019**; 19:369–81.
2. John J, Van Aart CJ, Grassly NC. The burden of typhoid and paratyphoid in India: systematic review and meta-analysis. PLoS Negl Trop Dis **2016**; 10:e0004616.
3. John J, Bavdekar A, Rongsen-Chandola T, Dutta S, Kang G; NSSEFI Collaborators. Estimating the incidence of enteric fever in children in India: a multi-site, active fever surveillance of pediatric cohorts. BMC Public Health **2018**; 18:594.
4. Srinivasan M, Sindhu KN, John J, Kang G. Opportunities for typhoid vaccination in India. Indian Pediatr **2019**; 56:453–8.
5. Mirza SH, Beeching NJ, Hart CA. Multi-drug resistant typhoid: a global problem. J Med Microbiol **1996**; 44:317–9.
6. Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in Salmonella enterica serovar Typhi, the etiological agent of typhoid. PLoS Negl Trop Dis **2018**; 12:e0006779.
7. Wong VK, Baker S, Pickard DJ, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter- and intracontinental transmission events. Nat Genet **2015**; 47:632–9.
8. Klemm EJ, Shakoor S, Page AJ, 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**; 9:1–10.
9. Hooda Y, Sajib MSI, Rahman H, et al. Molecular mechanism of azithromycin resistance among typhoidal Salmonella strains in Bangladesh identified through passive pediatric surveillance. PLoS Negl Trop Dis **2019**; 13:e0007868.
10. Iqbal J, Dehraj IF, Carey ME, et al. A race against time: reduced azithromycin susceptibility in Salmonella enterica Serovar Typhi in Pakistan. mSphere **2020**; 5:e00215–20.
11. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal. medRxiv **2020**. doi:10.1101/2020.08.07.20166389.
12. Clinical and Laboratory Standards Institute. Performance standards for antimicrobial susceptibility testing. Wayne, Pennsylvania, USA: Clinical and Laboratory Standards Institute, **2018**.
13. Wong VK, Baker S, Connor TR, et al.; International Typhoid Consortium. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nat Commun **2016**; 7:12827.
14. Britto CD, Dyson ZA, Duchene S, et al. Laboratory and molecular surveillance of paediatric typhoidal Salmonella in Nepal: antimicrobial resistance and implications for vaccine policy. PLoS Negl Trop Dis **2018**; 12:1–19.
15. Rahman SIA, Dyson ZA, Klemm EJ, et al. Population structure and antimicrobial resistance patterns of *Salmonella Typhi* isolates in urban Dhaka, Bangladesh from 2004 to 2016. PLoS Negl Trop Dis **2020**; 14:e0008036. doi: 10.1371/journal.pntd.0008036
16. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. Nat Methods **2012**; 9:357–9.
17. Li H, Handsaker B, Wysoker A, et al.; 1000 Genome Project Data Processing Subgroup. The sequence alignment/map format and SAMtools. Bioinformatics **2009**; 25:2078–9.
18. Holt KE, Thomson NR, Wain J, et al. Pseudogene accumulation in the evolutionary histories of Salmonella enterica serovars Paratyphi A and Typhi. BMC Genomics **2009**; 10:36.
19. Ingle DJ, Nair S, Hartman H, et al. Informal genomic surveillance of regional distribution of Salmonella Typhi genotypes and antimicrobial resistance via returning travellers. PLoS Negl Trop Dis **2019**; 13:e0007620.
20. Holt KE, Parkhill J, Mazzoni CJ, et al. High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nat Genet **2008**; 40:987–93.
21. Croucher NJ, Page AJ, Connor TR, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res **2015**; 43:e15.
22. Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. Bioinformatics **2014**; 30:1312–3.
23. Argimón S, Abudahab K, Goater RJE, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microb Genom **2016**; 2:e000093.
24. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics **2007**; 23:127–8.
25. Inouye M, Dashnow H, Raven LA, et al. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome Med **2014**; 6:90.
26. Gupta SK, Padmanabhan BR, Diene SM, et al. ARG-annot, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother **2014**; 58:212–20.
27. Carattoli A, Zankari E, García-Fernández A, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother **2014**; 58:3895–903.
28. Cunrath O, Meinel DM, Maturana P, et al. Quantitative contribution of efflux to multi-drug resistance of clinical Escherichia coli and Pseudomonas aeruginosa strains. EBioMedicine **2019**; 41:479–87.
29. Thanh Duy P, Thi Nguyen TN, Vu Thuy D, et al. Commensal Escherichia coli are a reservoir for the transfer of XDR plasmids into epidemic fluoroquinolone-resistant Shigella sonnei. Nat Microbiol **2020**; 5:256–64.
30. Hooda Y, Tanmoy AM, Sajib MSI, Saha S. Mass azithromycin administration: considerations in an increasingly resistant world. BMJ Glob Heal **2020**; 5:1–4.
31. Klein EY, Van Boeckel TP, Martinez EM, et al. Global increase and geographic convergence in antibiotic consumption between 2000 and 2015. Proc Natl Acad Sci USA **2018**; 115:E3463–70.
32. Britto CD, John J, Verghese VP, Pollard AJ. A systematic review of antimicrobial resistance of typhoidal Salmonella in India. Indian J Med Res **2019**; 149:151–63.
33. Park SE, Pham DT, Boinett C, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. Nat Commun **2018**; 9:5094.
34. Bogoch II, Utzinger J, Lo NC, Andrews JR. Antibacterial mass drug administration for child mortality reduction: opportunities, concerns, and possible next steps. PLoS Negl Trop Dis **2019**; 13:1–6.
35. Doan T, Worden L, Hinterwirth A, et al. Macrolide and nonmacrolide resistance with mass azithromycin distribution. N Engl J Med **2020**; 383:1941–50.
36. Parry CM, Thieu NT, Dolecek C, et al. Clinically and microbiologically derived azithromycin susceptibility breakpoints for Salmonella enterica serovars Typhi and Paratyphi A. Antimicrob Agents Chemother **2015**; 59:4364.

Appendix 5. The origins of haplotype 58 (H58) *Salmonella enterica* serovar Typhi (pre-print)

The origins of haplotype 58 (H58) *Salmonella enterica* serovar Typhi

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Running title

The Origins of H58 *Salmonella* Typhi

Key words

Salmonella Typhi (*S.* Typhi); antimicrobial resistance; H58; chronic carriage; genomics

Abstract

Antimicrobial resistance (AMR) poses a serious threat to the clinical management of typhoid fever. AMR in *Salmonella* Typhi (*S*. Typhi) is associated with the H58 lineage, which arose comparatively recently before becoming globally disseminated. To better understand when and how this lineage emerged and became dominant, we performed detailed phylogenetic and phylodynamic analyses on contemporary genome sequences from *S*. Typhi isolated in the period spanning the emergence. Our dataset, which contains the earliest described H58 *S*. Typhi, indicates that the prototype H58 organisms were multi-drug resistant (MDR). These organisms emerged spontaneously in India in 1987 and became radially distributed throughout South Asia and then globally in the ensuing years. These early organisms were associated with a single long branch, possessing mutations associated with increased bile tolerance, suggesting that the first H58 organism was generated during chronic carriage. The subsequent use of fluoroquinolones led to several independent mutations in *gyrA*. The ability of H58 to acquire and maintain AMR genes continues to pose a threat, as extensively drug-resistant (XDR; MDR plus resistance to ciprofloxacin and third generation cephalosporins) variants, have emerged recently in this lineage. Understanding where and how H58 *S*. Typhi originated and became successful is key to understand how AMR drives successful lineages of bacterial pathogens. Additionally, these data can inform optimal targeting of typhoid conjugate vaccines (TCVs) for reducing the potential for emergence and the impact of new drug-resistant variants. Emphasis should also be placed upon the prospective identification and treatment of chronic carriers to prevent the emergence of new drug resistant variants with the ability to spread efficiently.

Introduction

Salmonella enterica serovar Typhi (*S*. Typhi) is the etiologic agent of typhoid fever, a disease associated with an estimated 10.9 million new infections and 116,800 deaths annually.[1] The disease classically presents as a non-differentiated fever and can progress to more severe manifestations or even death.[2] Typhoid fever necessitates antimicrobial therapy, as the associated mortality rate in the pre-antimicrobial era ranged from 10–30%;[3] presently, typhoid has a case fatality rate (CFR) of <1% when treated with effective antimicrobials.[4] *S*. Typhi is spread via the faecal-oral route, typically through the ingestion of contaminated food or water.[2] Therefore, high prevalence rates of typhoid fever were historically associated with urban slums in South Asia with poor sanitation.[5] Recent multicentre surveillance studies have demonstrated that typhoid fever is also a major problem in both urban and rural areas in sub-Saharan Africa.[6–8]

Given the importance of antimicrobials for the management and control of typhoid, antimicrobial resistance (AMR) in *S*. Typhi has the potential to be a major public health issue. Indeed, the problem of AMR in *S*. Typhi first appeared in the 1950s with the emergence of resistance against the most widely used drug, chloramphenicol.[9] Multi-drug resistant typhoid (MDR; resistance to all first-line antimicrobials chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin) was first identified in the 1970s and became common in the early 1990s.[10,11] MDR in *S*. Typhi is frequently conferred by self-transmissible IncH1 plasmids carrying a suite of resistance genes, include resistance determinants for chloramphenicol (*catA1* or *cmlA*), ampicillin (*bla*TEM-1D, *bla*OXA-7), and co-trimoxazole (at least one *dfr*A gene and at least one *sul* gene).[12] Lower efficacy of first-line antimicrobials led to the increased use of fluoroquinolones, but decreased fluoroquinolone susceptibility became apparent in the mid-1990s, and was widespread in South and Southeast Asia in the early 2000s.[13,14] Inevitably, as treatment options have become limited, third-generation cephalosporins and azithromycin have been used more widely for effective treatment of typhoid fever.[15–17] However, newly circulating extensively-drug resistant variants of *S*. Typhi (XDR; MDR plus resistance to fluoroquinolones and third generation cephalosporins) has left azithromycin as the only feasible oral antimicrobial for the treatment of typhoid fever across South Asia.[18] We are arguably at a tipping point, as azithromycin-

resistant *S*. Typhi has since been reported in Bangladesh, Pakistan, Nepal, and India, thereby threatening efficacy of common oral antimicrobials for effective typhoid treatment.[19–22] If an XDR organism were to acquire azithromycin resistance (single bae pair mutation), this would lead to what Hooda and colleagues have referred to as pan-oral drug-resistant (PoDR) *S.* Typhi, which would require inpatient intravenous treatment.[23] This would come at substantial additional cost to patients and their families, and place additional strain on already overburdened health systems. [24–26]

In contrast to many other Gram-negative bacteria, *S*. Typhi is human restricted with limited genetic diversity that can be described by a comparatively straightforward phylogenetic structure.[27] Therefore, the phylogeny and evolution of *S.* Typhi provide a model for how AMR emerges, spreads, and becomes maintained in a human pathogen. AMR phenotypes in *S*. Typhi are typically dominated by a single lineage; H58 (genotype 4.3.1 and consequent sublineages), which was the 58th *S.* Typhi haplotype to be described in the original genome wide typing system.[28] This highly successful lineage is commonly associated with MDR phenotypes and decreased fluoroquinolone susceptibility.[14] Previous phylogeographic analysis suggested that H58 emerged initially in Asia between 1985 and 1992 and then disseminated rapidly to become the dominant clade in Asia and subsequently in East Africa.[14] H58 is currently subdivided into three distinct lineages – lineage I (4.3.1.1) and lineage II (4.3.1.2), which were first identified in a pediatric study conducted in Kathmandu,[29] and lineage III (4.3.1.3), which was identified in Dhaka, Bangladesh.[30] A recent study of acute typhoid fever patients and asymptomatic carriers in Kenya demonstrated the co-circulation of genotypes 4.3.1.1 and 4.3.1.2 in this setting, and closer analysis showed that these East African sequences had distinct AMR profiles and were the result of several introduction events.[31,32] These events led to the designation of three additional genotypes: H58 lineage I sublineage East Africa I (4.3.1.1.EA1), H58 lineage II sublineage East Africa II (4.3.1.2.EA2), and H58 lineage II sublineage East Africa III (4.3.1.2.EA3).[32] In addition, the XDR *S*. Typhi clone, which was caused by a monophyletic outbreak of genotype 4.3.1.1 organisms, was designated genotype 4.3.1.1.P1 to facilitate monitoring of its spread.

It is apparent from investigating the phylogeny of *S.* Typhi that H58 is atypical in comparison to other lineages. This lineage became dominant in under a decade, and first appeared on a long basal branch length, indicative of a larger number of single base pair mutations separating it from its nearest neighbour (Figure 1a). These observations suggest that there is something 'unique' about the evolution of this lineage, but we have limited understanding of how H58 emerged, what enabled its rapid spread, and when it initially appeared. Here, by collating new genome sequences of *S.* Typhi that were associated with travel to South Asia in the late 1980s and early 1990s and comparing them to a global population over the same period, we explore an expanded early phylogenetic dataset to resolve the origins and rapid success of this important and successful AMR clone.

Results and discussion

Sampling

The main questions that we aimed to address with this study were: i) when and where did H58 *S.* Typhi first emerge; ii) can we better resolve the evolutionary events that lead to the long branch length observed for H58 *S.* Typhi; and iii) how quickly did this lineage spread and why? Therefore, to investigate the origins of *S.* Typhi H58, data from United Kingdom Health Security Agency (UKHSA, formerly Public Health England) containing information on stored *S.* Typhi organisms isolated between 1980 and 1995 from travellers returning to the UK from overseas and receiving a blood culture were analysed.

The database was queried and organisms were selected from the following three categories: i) 126 *S.* Typhi with the E1 Phage type (which is considered to be associated with H58)[12] originating from South Asia (India, Nepal, Pakistan, and Bangladesh), ii) 159 *S.* Typhi organisms with a variety of non-E1 phage types originating from South Asia, and iii) 184 *S.* Typhi organisms with a variety of phage types (both E1 and non-E1) originating from locations outside of South Asia. A total of 470 *S.* Typhi organisms meeting these criteria were randomly selected, revived, subjected to DNA extraction and whole genome sequenced. Ultimately, our dataset was composed of 463 novel sequences generated as a component of this study and 305 existing sequences[30,33,34] known to belong to the H58

lineage and its nearest neighbours, yielding a total of 768 whole genome sequences on which to structure subsequent analysis.

Population structure, genotype distribution, and antimicrobial resistance profiles of historical S. Typhi

We inferred a maximum likelihood phylogenetic tree from the sequencing data to examine the population structure of this historic (1980 – 1995) collection of global *S.* Typhi sequences (Figure 1). Notably, unlike the extent global population of *S.* Typhi, which is largely dominated by a single lineage,[14,35] this historic population exhibited considerable genetic diversity, with 37 genotypes represented (Figure 1a). The majority of isolates belonged to primary clade 2 (194/463), of which clade 2.0 was most common (36%, 69/194) followed by subclades 2.3.3 (14%, 28/194) and 2.2.2 (13%, 25/194). An additional 23% of isolates belonged to primary clade 3 (108/463) and 3% of isolates were classified as major lineage 1 (12/463). Ultimately, 29% of isolates belonged to major lineage 4 (134/463), of which 63% (84/134) were H58. Among these, H58 lineage I (genotype 4.3.1.1) was most common (67%, 56/84), followed by genotype 4.3.1 (H58 not differentiated into any sublineage; 24%, 20/84). The earliest H58 isolates in our dataset are illustrated in higher resolution in Figure 1b.

Although we enriched our historical dataset for samples isolated from travellers returning from South Asia, our final dataset generated substantial geographic coverage, with 39 different countries represented. Therefore, these data are likely representative of the circulating *S.* Typhi in this pivotal period. Notably, the earliest H58 organism in our dataset that was classified as H58 according to the GenoTyphi scheme was isolated in 1983 from an individual entering the UK from India, followed by two additional Indian isolates (1988) that were also classified as H58. These organisms differed from the larger cluster of H58 organisms by 61-64 single nucleotide polymorphisms (SNPs) (Figure 1a). All H58 *S.* Typhi in this dataset were isolated from travellers returning from South Asia, with the majority (51/84; 61%) originating from Pakistan and the remainder from India and Bangladesh; 31/84 (37%) and 2/84 (2%), respectively.

Given that this dataset included isolates from the early MDR era and then following the emergence of reduced fluoroquinolone susceptibility, we analysed the data for genes associated with MDR and mutations in the DNA gyrase gene, *gyrA*. Overall, 7% (34/463) of the organisms in this historical dataset were genetically defined as being MDR; significantly, all were H58 (genotypes 4.3.1, 4.3.1.1, 4.3.1.2, and 4.3.1.3) and isolated between 1991 and 1995, and 97% (33/34) possessed an IncH1 plasmid (Figure 1b). Thirteen H58 organisms contained an IncH1 plasmid carrying AMR genes, but did not possess the genes conferring resistance to all three first-line antimicrobials, and thus were not genetically defined as MDR (Figure 1b, Table S1). The first mutation in the quinolone resistance determining region (QRDR) in our dataset was a *gyrA*-D87Y substitutions identified in a genotype 2.5 organism originating in South Africa in 1986 (Figure 1a). This was clearly a spontaneous mutation that evolved *de novo* and did not appear to become fixed in the population. Similarly, two single QRDR mutations (*gyrA*-S83F and *gyrA*-S83Y) occurred independently in H58 organisms (genotype 4.3.1) in India in 1995 (Figure 1b) and were not observed in descendant populations. No mutations in *gyrB* or *parC* were observed in this dataset.

In order to contextualise these isolates to understand the evolutionary events leading to this clone, we selected H58 and nearest neighbours (from genotypes 4.1 and 4.2) *S.* Typhi organisms (n=305) that were already available in the public domain from previous studies[30,33,34] (Table S2) and generated a phylogenetic tree combining these isolates with early H58 and nearest neighbour isolates from our unpublished dataset (n=117). In our H58 and nearest neighbour dataset (n= 422, Figure 2), which included both published data as well as our contemporary data, 17 countries were represented.[30,33,34] Of the non-H58 isolates (nearest neighbours), 42% were genotype 4.1 (32/76), 13% (10/76) were genotype 4.2, 28% (21/76) were 4.2.1, and 16% (12/76) were 4.2.3. These non-H58 nearest neighbour organisms were isolated between 1981 and 2000. None of them were MDR, and none carried an IncH1 plasmid. Of our H58 isolates (defined as having informative SNPs indicative of lineage 4.3.1), the earliest organism was isolated in 1983 in India, followed by two additional Indian isolates (1988) that were also classified as H58. However, we can observe that there were no more recent isolates

from this founder group (Figure 2), implying that this lineage became extinct; these isolates did not contain incH1 plasmids and were non-MDR. Within the H58 lineage, most of the organisms belonged to H58 sublineage I (4.3.1.1; 84%, 290/346), followed by sublineage II (4.3.1.2; 7%, 25/346), genotype 4.3.1 (6%, 21/346), and sublineage III (4.3.1.3; 3%, 10/346). Overall, 63% (219/346) of these H58 organisms were MDR, and 87% (191/219) of these MDR H58 organisms carried an IncH1 plasmid. All of the MDR H58 organisms lacking an IncH1 plasmid were genotype 4.3.1.1, the earliest of which was isolated in India in 1991.Within this group, the first single point mutation in the QRDR occurred comparatively early in an organism isolated in India in 1991.[33]

Evolutionary history of H58 S. Typhi

Using BEAST analysis, we determined that the median substitution rate of the H58 was 2.79×10^{-7} substitutions base^{-1} year^{-1} [95% highest posterior density (HPD): 2.40×10^{-7} - 3.24×10^{-7}), which is comparable to that observed in previous studies.[36] We found that the most recent common ancestor (MRCA) of the H58 was estimated to have emerged in late 1987 (95% HPD: 1986 – 1988). Two H58 sublineages (4.3.1.1 and 4.3.1.2) then emerged almost simultaneously in India in 1987 and 1988. The time-inferred phylogeny shows a clonal expansion of H58 that originated from South Asia, specifically in India, and then disseminated globally. As noted above, 63% (219/346) of isolates were MDR, and 87% (191/219) of those isolates contained an IncH1 plasmid known to carry AMR genes. Detailed genetic analysis of the IncH1 plasmids observed in most of these MDR isolates revealed high genetic similarity, with only 13 single nucleotide polymorphisms (SNPs) difference between them (Supplementary Figure 1). These data strongly suggest that the ancestral H58 organism that was the basis for the major clonal expansion was already MDR before undergoing clonal expansion and subsequent global dissemination; some ensuing H58 organisms then lost the MDR plasmid in certain settings, presumably because of decreased antimicrobial selection pressure with first-line antimicrobials, with a corresponding impact on fitness. The fact the three early precursor organisms that were not MDR appear to have become extinct supports our hypothesis that the presence of an MDR phenotype was a pivotal selective event.

It is also stark that QRDR mutations appeared in H58 organisms quickly and frequently. Within this H58 dataset, organisms with one or more QRDR mutations appeared within six independent lineages of H58, as illustrated in Figures 2 and 3. The earliest H58 lineage (lineage II) to develop QRDR mutations was observed in 1991 in India, with a single S83Y mutation in the *gyrA* gene, and the first isolate containing two QRDR mutations (*gyr*AS83Y; *par*C-S80R) appeared in a genotype 4.3.1.3 organism in Bangladesh in 1999 (Figure 3). The first "triple mutant" (mutations in *gyr*A-D87G, *gyr*A-S83F, and *parC*-E84K) was found in a 4.3.1.1 Bangladeshi isolate in 1999 (Figure 3).

Genetic variation associated with H58 S. Typhi

Our data support the hypothesis that H58 *S*. Typhi was successful specifically because of the acquisition and maintenance of an MDR plasmid. This selection meant that later *gyrA* mutations were more likely to occur in this lineage given its dominance (and therefore, higher rates of replication leading to additional opportunities for mutations to occur), as well as assumed frequent fluoroquinolone exposure, given its existing MDR phenotype. Based on the results of our mapping, we undertook further genetic analysis to identify non-synonymous SNPs unique to the early H58 isolates, as well as SNPs that were unique to early H58 isolates that were MDR. The motivation was to explain the origins of the long branch length illustrated on Figure 1a, to infer why this lineage was so globally successful, and identify genetic elements that may stabilize an MDR IncH1 plasmid.

We identified 16 unique non-synonymous SNPs that were exclusive to the early H58 isolates as compared to precursor 4.1 and 4.2 organisms, the majority of which were present in genes associated with central metabolism and outer membrane structures; one of which was associated with pathogenicity (Table 1). Within the early H58 isolates that were also MDR, we identified an additional 23 unique non-synonymous SNPs, most of which were found in genes encoding proteins predicted to regulate metabolism, degrade small molecules, membrane/surface structures, as well as regulators, pathogenicity adaptation, and information transfer (Table 2). We additionally identified mutations in a gene (t2518/STY0376) encoding a hypothetical protein with an EAL (diguanylate

phosphodiesterase) domain with a non-synonymous mutation, which has previously been identified as being associated with H58 organisms.[37] The homologous gene (STM0343) in *Salmonella* Typhimurium has been described as regulating motility and invasion, which suggests that SNPs in this gene might also contribute to virulence.[38,39] In addition, we identified SNPs in genes that have previously been associated with tolerance to bile in *S.* Typhi (*sirA*, *recB*, *wecF*, *dsdA*, and *yjjV*) among the early MDR H58 isolates.[40,41]

Clearly, there was a cascade of events that corresponded with the genesis of the first H58 *S.* Typhi. The cumulative mutations signified by the observed long branch length is uncommon in *S.* Typhi and has two feasible explanations. The first is that the progenitor organism was a hyper mutator, and that a key mutation in *mutS* was responsible for generating a large amount of genetic diversity in a short time frame.[42] However, no such informative SNPs were observed in the early H58 isolates, but any mutations may have reverted. The second, and more likely explanation, is that the organism was in an environment that created an atypical selective pressure to induce mutations that facilitated its ability become exposed to, and then accept, an MDR plasmid. Our previous data on *S.* Typhi carriage in the gallbladder determined that this environment creates an atypical selective pressure and stimulates mutations in metabolism and outer membrane structures.[31,43] This genetic variation was associated with organisms being located on signature long branches; our observations here are comparable. We suggest that H58 *S.* Typhi became successful due to its early ability to accept and stabilise a large MDR plasmid, which probably occurred whilst in the gallbladder; this one-off event and onward transmission then created this successful lineage. Therefore, we speculate that gallbladder carriage acts as a niche for generation of new variants with both modest (single SNPs) and large (plasmid acquisition) events capable of generating new lineages of *S.* Typhi with a selective advantage.

Implications

Our phylodynamic analysis of the origins of H58 *S.* Typhi has important consequences for how we understand the emergence and spread of new drug-resistant variants and can help inform optimal use of typhoid conjugate vaccines (TCVs). Our data suggest that whilst rare, these events can happen

given the specific selective pressures, allowing MDR organisms to arise and spread rapidly. This observation comes at a critical time in global typhoid control. Two TCVs have been prequalified by the World Health Organization, with additional candidates in late-stage clinical development, and promising clinical efficacy and effectiveness data.[44,45] The continued spread of drug-resistant H58 genotypes is clearly a major argument for the use of TCV, particularly as resistance to all oral antimicrobials has been reported in *S.* Typhi in South Asia.[19–22] We continue to observe a pattern in which drug-resistant variants emerge in South Asia and spread radially, with H58 being the only major genotype to do so to date. [14,35,46–49] H58 *S.* Typhi was first isolated in Kenya shortly after its estimated emergence in 1988, further illustrating the potential for rapid spread of this lineage.[48] This suggests that prioritization of widespread use of TCVs in South Asia can not only prevent significant morbidity in the region but should limit the continued emergence and spread of drug-resistant organisms elsewhere, which could expand the useful lifespan of existing therapeutic options in some parts of the world until TCVs become more widely available.

Our data strongly suggest that H58 *S.* Typhi, which is highly associated with being MDR and decreased fluoroquinolone susceptibility, is highly adept at acquiring and maintaining drug resistance determinants, which is likely facilitated by unique mutations that occurred in the earliest H58 organism. The rapid international dissemination of the H58 lineage, starting in South Asia and spreading throughout Southeast Asia,[14,33] into Africa[14,31,47,48,50–52] and more recently, Latin America,[53] suggest that expanded genomic surveillance is warranted to monitor its continued global spread. Such information can also help inform the development of transmission dynamics models that predict the spread of newer drug-resistant variants, like XDR, which can also inform TCV introduction decision-making.

Ultimately, we surmise that H58 *S.* Typhi likely emerged in India from a chronic carrier, which is supported by the indicative long branch length between early H58 organisms and its nearest non-H58 neighbours. This deduction is consistent with observations from previous studies conducted in Nepal and Kenya, in which higher mean branch lengths were observed in carriage isolates as compared to

isolates from symptomatic patients;[31,43] this phenomenon is to be expected, assuming that chronic carriers will have had a longer time from acquisition of infection to shedding and sampling. Notably, considering the structure of the phylogenetic tree and the loss of the early non-MDR H58 organisms from the population, it is likely that the MDR phenotype was the main catalysing factor for the success of this lineage. Additionally, we observed non-synonymous mutations in genes associated with outer membrane structures, metabolism and virulence, which we have been observed previously in organisms isolated directly from the gallbladder.[31,43] Our analysis also supports previous transcriptomic analysis showing that H58 *S.* Typhi has higher bile tolerance relative to other laboratory strains (Ty2 and CT18) and show increased virulence in the presence of bile, thereby increasing the potential of H58 organisms to colonize and persist in the gallbladder. These observations suggest that gallbladder is the ideal location for the generation of variants and highlights the potential for chronic carriage to lead to the emergence of novel *S.* Typhi (and other invasive *Salmonella*) that are genetically predisposed to express new phenotypes, which may include drug resistance. Therefore, emphasis should be placed upon the prospective identification and treatment of chronic carriers to prevent the emergence of new variants with the ability to spread. Contemporary data from returning travellers to the UK suggest that 1.4% of those infected with *S.* Typhi are chronic carriers, and 0.7% are carrying MDR *S.* Typhi.[54] A comparable frequency of carriage (1.1%) has been observed among children aged 16 years and younger in Mukuru, an informal settlement close to Nairobi, Kenya.[31] This prevalence rate is likely to be higher in older age groups,[55–57] and among people living in settings where typhoid is hyperendemic, and thus, may present a more substantial risk in terms of sustained transmission of drug-resistant *S.* Typhi and the potential emergence of additional drug-resistant variants. Scalable, low-cost assays to detect carriers will become vital if we aim to eliminate typhoid and prevent future resurgence.

We conclude that H58 *S.* Typhi likely emerged from a chronic carrier in India in 1987. The prototype organism of the successful clonal expansion was already MDR and became highly successful across South Asia in over a period of <10 years. Ultimately, sustained use of, and exposure to, fluoroquinolones led to selective mutations in *gyrA* on many independent occasions. The dominance

of this organism and its ability to maintain AMR genes has latterly meant it has become resistant to additional antimicrobials. Our work represents a blueprint of how such organisms can arise and become dominant, but also provides the justification and evidence for the introduction of new interventions for disease control; if we reduce disease burden by vaccination, we will additionally reduce the likelihood of comparable events occurring in other *S.* Typhi organisms and other pathogens. Widespread vaccine deployment, as well as screen and treat programs, can not only impact AMR directly through the prevention of drug-resistant infections, but also indirectly, as reduced transmission leads to decreased selection pressure on account of lower bacterial replication, and potentially decreased antimicrobial use following the prevention of clinical disease warranting treatment.

Methods

DNA extraction and Whole Genome Sequencing

Genomic DNA from *S.* Typhi isolates was extracted using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA), following standardized manufacturer's protocol. Two ng of genomic DNA from each organism was fragmented and tagged for multiplexing with Nextera DNA Sample Preparation Kits, followed by paired-end sequencing on an Illumina HiSeq2000 Platform to produce 101 bp paired-end reads (Illumina, Cambridge, UK). Raw reads were deposited in the European Nucleotide Archive (ENA) under study accession number PRJEB15284 (Table S1).

Read alignment and SNP analysis

FastQC and FASTX-Toolkit bioinformatics pipelines were used to check the quality of raw reads.[58,59] Six samples were excluded from the analysis, one was determined to not be *Salmonella*, one appeared to be comprised of multiple genotypes, and four samples were on a long branch length and were conclused to be contaminated. Paired end reads for the remaining 464 samples were mapped to the *S.* Typhi CT18 reference genome (accession number: AL513382)[60] using the RedDog mapping pipeline (v1beta.10b, available at http://githib.com/katholt/reddog). RedDog uses Bowtie2 v2.2.9[61] to map all raw reads to the CT18 reference genome and then uses SAMtools v1.3.1[62] to identify high quality

SNP calls. SNPs that did not meet predefined criteria (a minimal phred quality score of 30 and depth coverage of 5 were filtered out).[63] A failed mapping sequence was defined as when <50% of total reads mapped to the reference genome. 2 isolates were excluded from additional analysis after mapping failed, due to depth coverage of less than 10 (as per the RedDog pipeline default). A concatenation of core SNPs that were present in >95% of all genomes was generated and filtered to exclude all SNPs from phage regions or repetitive sequences in the genome reference CT18 as defined previously (Table S2).[60] Gubbins (v2.3.2)[64] was used to filter out SNPs in recombination regions. Finally, the alignment of 17,325 SNPs from mapping of the remaining 462 isolates was utilized for phylogenetic analysis. Resultant BAM files for all isolates from RedDog mapping were used to determine previously defined genotypes according to an extended genotyping framework using the GenoTyphi pipeline[65] (available: https://github.com/katholt/genotyphi).

Phylogenetic analysis

RAxML (v8.2.9)[26] was used to infer maximum likelihood (ML) phylogenetic trees from the final chromosomal SNP alignment, with a generalized time-reversible model, a gamma distribution to model site-specific rate variation (the GTR+ Γ substitution model; GTRGAMMA in RAxML), and 100 bootstrap pseudo-replicates to assess branch support. *Salmonella* Paratyphi A AKU1_12601 (accession no: FM200053)[66] was used as an outgroup. The resultant trees were visualized using Interactive Tree of Life (iTOL)[67] and the ggtree package in R.[68] An interactive visualisation of this phylogeny and associated metadata can be found in Microreact (https://microreact.org/project/hzELvWqY3UCvsyAw892fnd-origins-of-h58-s-typhi).[69]

Characterisation of AMR associated genes and mobile elements

SRST2 (v0.2.0) [70] was used to detect AMR genes and plasmid replicons using the ARGannot[71] and PlasmidFinder[72] databases, respectively. Mutations in the *gyrA* and *parC* genes, as well as the R717Q mutation in *acrB*, were detected using Mykrobe v0.10.0.[73]

Bayesian phylogenetic analysis of H58 and nearest neighbours

Our estimation of the temporal signal of our H58 and nearest neighbour data exhibited a strong correlation between the sampling dates and the root-to-tip distances, with a positive value for the slope and an R^2 value of 0.4743 (Supplementary figure 2). Additionally, the randomly reassigned sampling time of sequences 20 times to generate the mean rates indicated that there was no overlap between the 95% credible intervals of the mean rate of the real data set and that of the date randomization data (Supplementary figure 3). To infer where and when the first H58 (genotype 4.3.1) organism emerged, we conducted Bayesian phylogenetic analyses on a subset (n=345) of H58 (genotype 4.3.1) from our dataset and from published literature isolated between 1980 and 2000.[14,30,33] This analysis of 345 *S.* Typhi isolates was conducted in the BEAST v1.8.4.[74] The temporal signal of the data was checked initially. The maximum likelihood tree, constructed using the GTR+ Γ substitution model and GTRGAMMA, was subjected to TempEst v1.5 to test the best fit of linear regression between sampling dates and their root-to-tip genetic distances, using default TempEst parameters.[75] To further test temporal signal, the TipDatingBeast R package was used to randomly reassign the sampling dates of sequences 20 times to create date-randomized data sets. BEAST analyses were conducted for these randomized data sets and the mean rates were compared between runs. The data had sufficient temporal signal if the 95% credible interval of mean rates of the date-randomized datasets did not overlap with that of the original sampling dataset.[76,77]

An automatic model selection program (ModelFinder)[78] was implemented through IQ-TREE[79] and run on the non-recombinant SNP alignment (724 variable sites) to select the best-fit sequence evolution model for BEAST analysis. ModelFinder showed that GTR had the lowest Bayesian Information Criteria (BIC) score and thus it was chosen as the best-fit substitution model.

As part of the BEAST analysis, six different model combinations were run for six combinations, and the final analysis was conducted using the best fitting model The path sampling and stepping-stone sampling approaches were applied to compare the log marginal likelihoods of the different runs.[36,80] The GTR+Γ_4 with strict clock and Bayesian skyline was identified as the best-fit model for running BEAST. Finally, BEAST was run three independent times using the best-fit model, using a Bayesian Markov chain Monte Carlo (MCMC) parameter-fitting approach (generated 10^7 chains and sampled

every 1000 iterations). The log files after three runs were combined using LogCombiner v1.8.3[81] with a burn-in rate of 10%. The effective sample size (ESS) of all parameters was assessed by Tracer v1.8.3.[82] If the ESS of any parameters was less than 200, we increased the MCMC chain length by 50% and reduced the sampling frequency accordingly.[36] The trees were combined and summarized using LogCombiner v1.8.3 and TreeAnnotator v1.8.3.[74]

Table 1. Unique non-synonymous Single Nucleotide Polymorphisms detected in early H58 *S.* Typhi

Gene ID	SNP Position in CT18	Gene	Reference nucleotide	4.3.1 nucleotide	Ancestral codon	Derived codon	Ancestral amino acid	Derived amino acid	Functional Category	Product
STY0376	387595		C	T	ACC	ATC	T	I	Central/intermediary metabolism	putative rtn protein; diguanylate cyclase/ phosphodiesterase domain-containing protein
STY0452	461438	*yajI*	G	A	CGT	TGT	R	C	Membrane/surface structures	putative lipoprotein
STY0522	529155	*kefA/aefA*	A	G	AAA	GAA	K	E	Membrane/surface structures	integral membrane protein AefA
STY0698	693560	*rlpB*	C	T	ATG	ATA	M	I	Central/intermediary metabolism	rare lipoprotein B precursor
STY1458	1408039		C	T	CAG	TAG	Q	*		putative lipoprotein
STY1703	1629304	*ssaP*	G	A	GCG	GTG	A	V	Pathogenicity/adaptation/chaperones	putative type III secretion protein
STY2371	2202853		C	T	CGT	TGT	R	C	Membrane/surface structures	putative nucleoside permease
STY2513	2348633	*glpA*	G	A	GGC	AGC	G	S	Central/intermediary metabolism	anaerobic glycerol-3-phosphate dehydrogenase subunit A
STY2553	2388057	*nuoG*	G	A	ACT	ATT	T	I	Information transfer	NADH dehydrogenase I chain G
STY2564	2401233	*yfbT*	G	A	CGC	TGC	R	C	Central/intermediary metabolism	putative phosphatase
STY3518	3360344	*nanE2*	T	C	ATA	GTA	I	V	Pseudogenes	conserved hypothetical protein (pseudogene)
STY3795	3659647	*Ydey*	C	T	GGG	GAG	G	E	Membrane/surface structures	Putative ABC transporter protein, AI-2 transport system permease
STY4405	4273783	*metH*	C	A	CGT	AGT	R	S	Central/intermediary metabolism	B12-dependent homocysteine-N5-methyltetrahydrofolate transmethylase
STY4451	4321540		A	G	AAG	AGG	K	R		single-strand DNA-binding protein
STY4890	4754034		C	T	TGG	TAG	W	*		probable carbon starvation protein
STY4890	4754035		A	G	TGG	CGG	W	R		probable carbon starvation protein

Table 2. Unique non-synonymous SNPs unique to early MDR H58 *S.* Typhi

Gene ID	SNP Position in CT18	Gene	Reference nucleotide	4.3.1 nucleotide	Ancestral codon	Derived codon	Ancestral amino acid	Derived amino acid	Functional Category	Product
STY0042	40159	*betC*	G	A	GGG	GAG	G	E	Central/intermediary metabolism	putative secreted sulfatase
STY0085	89102	*etfB/ fixA*	A	G	AAA	AGA	K	R	Degradation of small molecules	FixA protein
STY0376	387082		G	A	CGA	CAA	R	Q	Central/intermediary metabolism	putative rtn protein; diguanylate cyclase/phosphodiesterase domain-containing protein
STY0886	880083	*ybiK*	A	G	ACC	GCC	T	A	Central/intermediary metabolism	putative L-asparaginase
STY1310	1270888		G	A	CGA	CAA	R	Q	Membrane/surface structures	Voltage-gated potassium channel
STY1328	1286044	*trpE*	T	C	GAC	GGC	D	G	Central/intermediary metabolism	Anthranilate synthase component
STY1410	1360939	*dbpA*	C	T	CAG	TAG	Q	*	Pseudogenes	ATP-dependent RNA helicase (pseudogene)
STY1917	1810914	*hyaE*	G	A	GCT	ACT	A	T	Information transfer	hydrogenase-1 operon protein HyaE
STY2155	2002943	*uvrY*	G	A	CTT	TTT	L	F	Regulators	invasion response-regulator
STY2875	2750755		G	A	GCG	ACG	A	T	Pathogenicity adaptation/chaperones	large repetitive protein
STY3001	2875160	*sptP*	G	A	CAA	TAA	Q	*	Pathogenicity adaptation/chaperones	Pathogenicity island 1 tyrosine phosphatase (associated with virulence)
STY3132	3004181	*recB*	T	C	GAA	GGA	E	G	Degradation of macromolecules	exonuclease V subunit
STY3297	3144053	*ordL*	A	C	GTG	GGG	V	G	Central/intermediary metabolism	Putative gamma-glutamylputrescine oxidoreductase
STY3555	3398551	*yhdA*	G	A	GCT	GTT	A	V	Membrane/surface structures	Putative lipoprotein
STY3628	3484294	*wecF*	C	T	GTA	ATA	V	I	Conserved hypothetical proteins	Putative 4-alpha-L-fucosyl transferase
STY3955	3824631	*torC*	T	G	TCA	GCA	S	A	Pseudogenes	Cytochrome c-type protein
STY3977	3843665	*dsdA*	C	T	GGC	GAC	G	D	Degradation of small molecules	D-serine dehydratase
STY4161	4020211	*yhjY*	C	T	ACG	ATG	T	M	Membrane/surface structures	putative membrane protein
STY4314	4192687	*gph*	C	T	GCG	GTG	A	V	Degradation of small molecules	phosphoglycolate phosphatase
STY4318	4196909	*bigA*	G	A	CCA	TCA	P	S	Pseudogenes	putative surface-exposed virulence protein (pseudogene)
STY4392	4253640	*dprA*	G	A	GCT	ACT	A	T	Conserved hypothetical proteins	Putative DNA protecting protein
STY4805	4665891		G	A	GCG	GTG	A	V	Regulators	arginine deiminase
STY4915	4775254	*yjjV*	C	A	CCG	CAG	P	Q	Conserved hypothetical proteins	TatD DNase family protein

Figure Legends

Figure 1. The phylogenetic structure of historical *S*. Typhi isolates.

a) A phylogenetic overview of historical (1980 – 1995) *S*. Typhi from the UKHSA. Maximum likelihood outgroup rooted phylogenetic tree depicting the genomic sequences of 463 *S*. Typhi isolated from returning travellers to the United Kingdom isolated between 1980 and 1995. Branch colour indicates genotype, and rings outside of the tree indicate country of origin, MDR, and presence of IncH1 plasmid, coloured as per the inset legend. Individual mutations in the quinolone resistance determining region (QRDR) indicated by stars outside the rings. b) A phylogenetic overview of historical H58 (4.3.1) *S*. Typhi isolates. Zoomed-in view of H58 *S*. Typhi isolates from historical collection. Genotype, country of origin, presence of MDR, AMR mutations, and presence of IncH1 plasmids are indicated by bars to the right of the tree and coloured as per the inset legend. Individual mutations in the quinolone resistance determining region (QRDR) indicated by stars to the right of the bars.

Figure 2. The phylogenetic structure of early H58 organisms and nearest neighbours.
Maximum likelihood rooted phylogenetic tree showing *S*. Typhi organisms (genotype 4.3.1) and nearest neighbours (genotypes 4.1 and 4.2) from our historical collection and from published literature (n=422 total). Genotype is indicated by branch colour, presence of QRDR mutation(s) are indicated by coloured circles at the end of the branches, and country of origin, presence of MDR, AMR mutations, and presence of incH1 plasmid are indicated by bars to the right of the tree and coloured as per the inset legend.

Figure 3. A dated phylogenetic structure of historical H58 *S*. Typhi isolates.
BEAST-generated dated phylogeny of H58 *S*. Typhi isolates from UKHSA collection and published literature (n=345). Tip colours indicate presence of specific mutation(s) in the QRDR as per inset legend. Branch colour and the first column to the right of the tree indicate genotype, the second column indicates country of origin, the third represents presence of MDR, and the final column indicates presence of an incH1 plasmid. Our analysis suggests that the Most Recent Common

Ancestor (MCRA) of H58 appeared in 1987, and that two sublineages (I and II) emerged almost simultaneously in India in 1987 and 1988. The first single point mutation in the QRDR was observed in 1990, and the first "triple mutant" was observed in Bangladesh in 1999.

Supplemental Figure Legends

Figure S1. A genetic comparison of IncH1 plasmid content of early H58 *S.* Typhi isolates.

Zoomed in view of maximum likelihood phylogenetic tree including historical and published H58 isolates (n=206). Country of origin is indicated by the coloured circles at the tips of the tree. Gene content of an almost identical IncH1 plasmid is indicated by the colour to the right of the tree.

Figure S2. An estimation of temporal signal in H58 *S.* Typhi data.

Path-O-Gen/TempEst results (analysis included n=345 isolates using an alignment of 724 non-recombinant SNPs), demonstrating strong correlation between sampling dates and root-to-tip distance ($R^2 = 0.4743$).

Figure S3. A TipDatingBeast estimation of temporal signal in H58 *S.* Typhi data.

TipDatingBeast results (analysis incorporated n=345 isolates and an alignment of 724 non-recombinant SNPs), demonstrating no overlap between the original mean rates of mutation and mean rates of date randomization.

Supplementary Tables

Table S1. Organism-level data and metadata for historical UKHSA *S.* Typhi isolates.

Table S2. Organism-level data and metadata for published H58 and nearest neighbours *S.* Typhi isolates.

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References

1. Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis*. 2019;19(4):369-381. doi:10.1016/S1473-3099(18)30685-6
2. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive Salmonella infections. *Clin Microbiol Rev*. 2015;28(4):901-937. doi:10.1128/CMR.00002-15
3. Andrews JR, Barkume C, Yu AT, et al. Integrating Facility-Based Surveillance with Healthcare Utilization Surveys to Estimate Enteric Fever Incidence: Methods and Challenges. *Journal of Infectious Diseases*. 2018;218(Suppl 4):S268-S276. doi:10.1093/infdis/jiy494
4. Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively Drug-Resistant Typhoid — Are Conjugate Vaccines Arriving Just in Time? *New England Journal of Medicine*. 2018;379(16):1491-1493. doi:10.1056/nejmp1805927
5. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, et al. A study of typhoid fever in five Asian countries: Disease burden and implications for controls. *Bull World Health Organ*. 2008;86(4):260-268. doi:10.2471/BLT.06.039818
6. Marks F, von Kalckreuth V, Aaby P, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. *Lancet Glob Health*. 2017;5(3):e310-e323. doi:10.1016/S2214-109X(17)30022-0
7. Baker S, Hombach J, Marks F. What Have We Learned from the Typhoid Fever Surveillance in Africa Program? *Clinical Infectious Diseases*. 2016;62(Suppl 1):s1-s3. doi:10.1093/cid/civ675

8. Carey ME, Steele AD. The Severe Typhoid Fever in Africa Program Highlights the Need for Broad Deployment of Typhoid Conjugate Vaccines. *Clinical Infectious Diseases*. 2019;69(6). doi:10.1093/cid/ciz637

9. Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively Drug-Resistant Typhoid - Are Conjugate Vaccines Arriving Just in Time? *New England Journal of Medicine*. 2018;379(16):1493-1495. doi:10.1056/NEJMp1803926

10. Olarte J, Galindo E. Salmonella typhi resistant to chloramphenicol, ampicillin, and other antimicrobial agents: strains isolated during an extensive typhoid fever epidemic in Mexico. *Antimicrob Agents Chemother*. 1973;4(6):597-601. doi:10.1128/AAC.4.6.597

11. Mirza SH, Beeching NJ, Hart CA. Multi-drug resistant typhoid: A global problem. *J Med Microbiol*. 1996;44(5):317-319. doi:10.1099/00222615-44-5-317

12. Holt KE, Phan MD, Baker S, et al. Emergence of a globally dominant inchi1 plasmid type associated with multiple drug resistant typhoid. *PLoS Negl Trop Dis*. 2011;5(7). doi:10.1371/journal.pntd.0001245

13. Britto CD, Wong VK, Dougan G, Pollard AJ. A systematic review of antimicrobial resistance in Salmonella enterica serovar Typhi, the etiological agent of typhoid. *PLoS Negl Trop Dis*. 2018;12(10):e0006779. doi:10.1371/journal.pntd.0006779

14. Wong VK, Baker S, Pickard DJ, 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):632-639. doi:10.1038/ng.3281

15. Balasegaram S, Potter AL, Grynszpan D, et al. Guidelines for the public health management of typhoid and paratyphoid in England. Practice guidelines from the National Typhoid and Paratyphoid Reference Group. *Journal of Infection.* 2012;65(3):197-213. doi:10.1016/j.jinf.2012.05.005

16. Rai S, Jain S, Prasad KN, Ghoshal U, Dhole TN. Rationale of azithromycin prescribing practices for enteric fever in India. *Indian J Med Microbiol*. 2012;30(1):30-33. doi:10.4103/0255-0857.93017

17. Basnyat B, Qamar FN, Rupali P, Ahmed T, Parry CM. Enteric fever. *BMJ*. 2021;372(n437). doi:10.1136/bmj.n437

18. Klemm EJ, Shakoor S, Page AJ, 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):1-10. doi:10.1128/mBio.00105-18

19. Iqbal, Junaid, Dehraj, Irum F., Carey, Megan E., Dyson, Zoe A., Garrett, Denise, Seidman, Jessica C., Kabir, Furqan, Saha, Senjuti, Baker, Stephen, Qamar FN. A Race against Time : Reduced Azithromycin Susceptibility in Salmonella enterica Serovar Typhi in Pakistan. *mSphere*. 2020;5(4):e00215-20.

20. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal . *JAC Antimicrob Resist*. 2020;2(4). doi:10.1093/jacamr/dlaa109

21. Carey ME, Jain R, Yousuf M, et al. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of Salmonella Typhi in Northern India . *Clinical Infectious Diseases*. 2021;72(Xx Xxxx):1-8. doi:10.1093/cid/ciaa1773

22. Hooda Y, Sajib MSI, Rahman H, et al. Molecular mechanism of azithromycin resistance among typhoidal Salmonella stains in Bangladesh identified through passive pediatric surveillance. *PLoS Negl Trop Dis*. 2019;13(11):e0007868. doi:10.1371/journal.pntd.0007868

23. Hooda Y, Tanmoy AM, Sajib MSI, Saha S. Mass azithromycin administration: Considerations in an increasingly resistant world. *BMJ Glob Health*. 2020;5(6):1-4. doi:10.1136/bmjgh-2020-002446

24. Mejia N, Qamar F, Yousafzai MT, et al. Typhoid and Paratyphoid Cost of Illness in Pakistan: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S319-S335. doi:10.1093/cid/ciaa1336

25. Mejia N, Pallas SW, Saha S, et al. Typhoid and Paratyphoid Cost of Illness in Bangladesh: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S293-S305. doi:10.1093/cid/ciaa1334

26. Mejia N, Abimbola T, Andrews JR, et al. Typhoid and Paratyphoid Cost of Illness in Nepal: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S306-S318. doi:10.1093/cid/ciaa1335

27. Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu Rev Microbiol*. 2008;62:53-70. doi:10.1146/annurev.micro.62.081307.162832

28. Roumagnac P, Weill FX, Dolecke C, et al. Evolutionary History of Salmonella Typhi. *Science (1979)*. 2006;314(5803):1301-1304. doi:10.1126/science.1133649

29. Holt KE, Baker S, Dongol S, et al. High-throughput bacterial SNP typing identifies distinct clusters of Salmonella Typhi causing typhoid in Nepalese children. *BMC Infect Dis*. 2010;10. doi:10.1186/1471-2334-10-144

30. Tanmoy AM, Westeel E, de Bruyne K, et al. Salmonella enterica serovar typhi in Bangladesh: Exploration of genomic diversity and antimicrobial resistance. *mBio*. 2018;9(6):1-17. doi:10.1128/mBio.02112-18

31. Kariuki S, Dyson ZA, Mbae C, et al. Multiple introductions of multidrug-resistant typhoid associated with acute infection and asymptomatic carriage, Kenya. *Elife*. 2021;10. doi:10.7554/eLife.67852

32. Dyson ZA, Holt KE. Five Years of GenoTyphi: Updates to the Global Salmonella Typhi Genotyping Framework. *J Infect Dis*. 2021;224(S7):775-780. doi:10.1093/infdis/jiab414

33. Pragasam AK, Pickard D, Wong V, et al. Phylogenetic Analysis Indicates a Longer Term Presence of the Globally Distributed H58 Haplotype of Salmonella Typhi in Southern India. *Clinical Infectious Diseases*. 2020;71(8):1856-1863. doi:10.1093/cid/ciz1112

34. Wong VK, Baker S, Connor TR, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. *Nat Commun*. 2016;7:1-11. doi:10.1038/ncomms12827

35. da Silva KE, Tanmoy AM, Pragasam AK, et al. The international and intercontinental spread and expansion of antimicrobial-resistant Salmonella Typhi: a genomic epidemiology study. *Lancet Microbe*. Published online June 2022. doi:10.1016/s2666-5247(22)00093-3

36. Duchêne S, Holt KE, Weill FX, et al. Genome-scale rates of evolutionary change in bacteria. *Microb Genom*. 2016;2(11):e000094. doi:10.1099/mgen.0.000094

37. Johnson R. *Investigating the Globally Dominant Salmonella Typhi Haplotype H58-an Insight into the Pathogenesis of Typhoid Fever*. 2018. Accessed May 30, 2022. https://spiral.imperial.ac.uk/bitstream/10044/1/72893/1/Johnson-R-2018-PhD-Thesis.pdf

38. Ahmad I, Lamprokostopoulou A, le Guyon S, et al. Complex c-di-GMP signaling networks mediate transition between virulence properties and biofilm formation in

salmonella enterica serovar typhimurium. *PLoS One*. 2011;6(12). doi:10.1371/journal.pone.0028351

39. Bogomolnaya LM, Aldrich L, Ragoza Y, Talamantes M, Andrews KD. Identification of Novel Factors Involved in Modulating Motility of Salmonella enterica Serotype Typhimurium. *PLoS One*. 2014;9(11):111513. doi:10.1371/journal.pone.0111513
40. Langridge GC, Phan MD, Turner DJ, et al. Simultaneous assay of every Salmonella Typhi gene using one million transposon mutants. *Genome Res*. 2009;19(12):2308-2316. doi:10.1101/gr.097097.109
41. Prieto AI, Ramos-Morales F, Casadesú J. Repair of DNA Damage Induced by Bile Salts in Salmonella enterica. *Genetics*. 2006;174:575-584. doi:10.1534/genetics.106.060889
42. Zahrt TC, Buchmeier N, Maloy S. Effect of mutS and recD Mutations on Salmonella Virulence. *Infect Immun*. 1999;67(11):6168-6172. https://journals.asm.org/journal/iai
43. Duy PT, Thieu NTV, Nguyen TNT, et al. Gallbladder carriage generates genetic variation and genome degradation in Salmonella Typhi. *PLoS Pathog*. 2020;16(10):1-17. doi:10.1371/journal.ppat.1008998
44. SAGE Working Group on Typhoid Vaccines & the WHO Secretariat. *Background Paper to SAGE on Typhoid Vaccine Policy Recommendations*.
45. World Health Organization. Meeting of the Strategic Advisory Group of Experts on Immunization, April 2022: conclusions and recommendations. *Weekly epidemiological record*. 2022;97(24):261-276.
46. Britto CD, Dyson ZA, Mathias S, et al. Persistent circulation of a fluoroquinolone-resistant Salmonella enterica Typhi clone in the Indian subcontinent. *Journal of Antimicrobial Therapy*. 2020;75:337-341. doi:10.1093/jac/dkz435

47. Feasey NA, Gaskell K, Wong V, et al. Rapid Emergence of Multidrug Resistant, H58-Lineage Salmonella Typhi in Blantyre, Malawi. *PLoS Negl Trop Dis*. 2015;9(4):1-13. doi:10.1371/journal.pntd.0003748

48. Kariuki S, Revathi G, Kiiru J, et al. Typhoid in Kenya is associated with a dominant multidrug-resistant Salmonella enterica serovar Typhi haplotype that is also widespread in Southeast Asia. *J Clin Microbiol*. 2010;48(6):2171-2176. doi:10.1128/JCM.01983-09

49. Chau TT, Campbell JI, Galindo CM, et al. Antimicrobial drug resistance of Salmonella enterica serovar Typhi in Asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. *Antimicrob Agents Chemother*. 2007;51(12):4315-4323. doi:10.1128/AAC.00294-07

50. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, et al. Salmonella enterica serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. *J Antimicrob Chemother*. 2021;76(5):1160-1167. doi:10.1093/jac/dkaa519

51. Park SE, Pham DT, Boinett C, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. *Nat Commun*. 2018;9(1):5094. doi:10.1038/s41467-018-07370-z

52. Ingle DJ, Nair S, Hartman H, 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):1-20. doi:10.1371/journal.pntd.0007620

53. Maes M, Dyson ZA, Higginson EE, et al. Multiple introductions of salmonella enterica serovar typhi H58 with reduced fluoroquinolone susceptibility into Chile. *Emerg Infect Dis*. 2020;26(11):2736-2740. doi:10.3201/eid2611.201676

54. Chattaway MA, Gentle A, Nair S, et al. Phylogenomics and antimicrobial resistance of Salmonella Typhi and Paratyphi A, B and C in England, 2016–2019. *Microb Genom*. 2021;7(8). doi:10.1099/mgen.0.000633

55. Gunn JS, Marshall JM, Baker S, Dongol S, Charles RC, Ryan ET. Salmonella chronic carriage: Epidemiology, diagnosis, and gallbladder persistence. *Trends Microbiol*. 2014;22(11):648-655. doi:10.1016/j.tim.2014.06.007

56. Senthilkumar B, Senbagam D, Rajasekarapandian M. An epidemiological surveillance of asymptomatic typhoid carriers associated in respect to socioeconomic status in India. *Journal of Public Health (Germany)*. 2014;22(3):297-301. doi:10.1007/s10389-012-0545-4

57. Levine MM, Black RE, Lanata C, Committee CT. Precise Estimation of the Numbers of Chronic Carriers of Salmonella typhi in Santiago, Chile, an Endemic Area. *Journal of Infectious Diseases*. 1982;146(6). http://jid.oxfordjournals.org/

58. G.J. Hannon. FASTX-Toolkit. Published online 2010.

59. Simon Andrews. FASTQC. A quality control tool for high throughput sequence data. Published online 2010.

60. Holt KE, Parkhill J, Mazzoni CJ, et al. High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. *Nat Genet*. 2008;40(8):987-993. doi:10.1038/ng.195

61. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods*. 2012;9(4):357-359. doi:10.1038/nmeth.1923

62. Li H, Handsaker B, Wysoker A, et al. The Sequence Alignment/Map format and SAMtools. *Bioinformatics*. 2009;25(16):2078-2079. doi:10.1093/bioinformatics/btp352

63. Rahman SIA, Dyson ZA, Klemm EJ, et al. Population structure and antimicrobial resistance patterns of salmonella typhi isolates in Urban Dhaka, Bangladesh from 2004 to 2016. *PLoS Negl Trop Dis*. 2020;14(2). doi:10.1371/journal.pntd.0008036

64. Croucher NJ, Page AJ, Connor TR, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res.* 2015;43(3):e15. doi:10.1093/nar/gku1196

65. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global Salmonella Typhi genotyping framework. doi:10.1093/infdis/jiab414/6358992

66. Holt KE, Thomson NR, Wain J, et al. Pseudogene accumulation in the evolutionary histories of Salmonella enterica serovars Paratyphi A and Typhi. *BMC Genomics*. 2009;10(1):36. doi:10.1186/1471-2164-10-36

67. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics*. 2006;23(1):127-128.

68. Yu G. Using ggtree to Visualize Data on Tree-Like Structures. *Curr Protoc Bioinformatics*. 2020;69(1). doi:10.1002/cpbi.96

69. Argimón S, Abudahab K, Goater RJE, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microb Genom.* 2016;2(11):e000093. doi:10.1099/mgen.0.000093

70. Inouye M, Dashnow H, Raven LA, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. *Genome Med*. 2014;6(11):1-16. doi:10.1186/s13073-014-0090-6

71. Gupta SK, Padmanabhan BR, Diene SM, et al. ARG-annot, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother*. 2014;58(1):212-220. doi:10.1128/AAC.01310-13

72. Carattoli A, Zankari E, Garciá-Fernández A, et al. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother*. 2014;58(7):3895-3903. doi:10.1128/AAC.02412-14

73. Bradley P, den Bakker HC, Rocha EPC, McVean G, Iqbal Z. Ultrafast search of all deposited bacterial and viral genomic data. *Nat Biotechnol*. 2019;37(2):152-159. doi:10.1038/s41587-018-0010-1

74. Suchard MA, Lemey P, Baele G, Ayres DL, Drummond AJ, Rambaut A. Bayesian phylogenetic and phylodynamic data integration using BEAST 1.10. *Virus Evol*. 2018;4(1). doi:10.1093/ve/vey016

75. Rambaut A, Lam TT, Carvalho LM, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol*. 2016;2(1). doi:10.1093/ve/vew007

76. Firth C, Kitchen A, Shapiro B, Suchard MA, Holmes EC, Rambaut A. Using time-structured data to estimate evolutionary rates of double-stranded DNA viruses. *Mol Biol Evol*. 2010;27(9):2038-2051. doi:10.1093/molbev/msq088

77. Duchêne S, Duchêne D, Holmes EC, Ho SYW. The performance of the date-randomization test in phylogenetic analyses of time-structured virus data. *Mol Biol Evol*. 2015;32(7):1895-1906. doi:10.1093/molbev/msv056

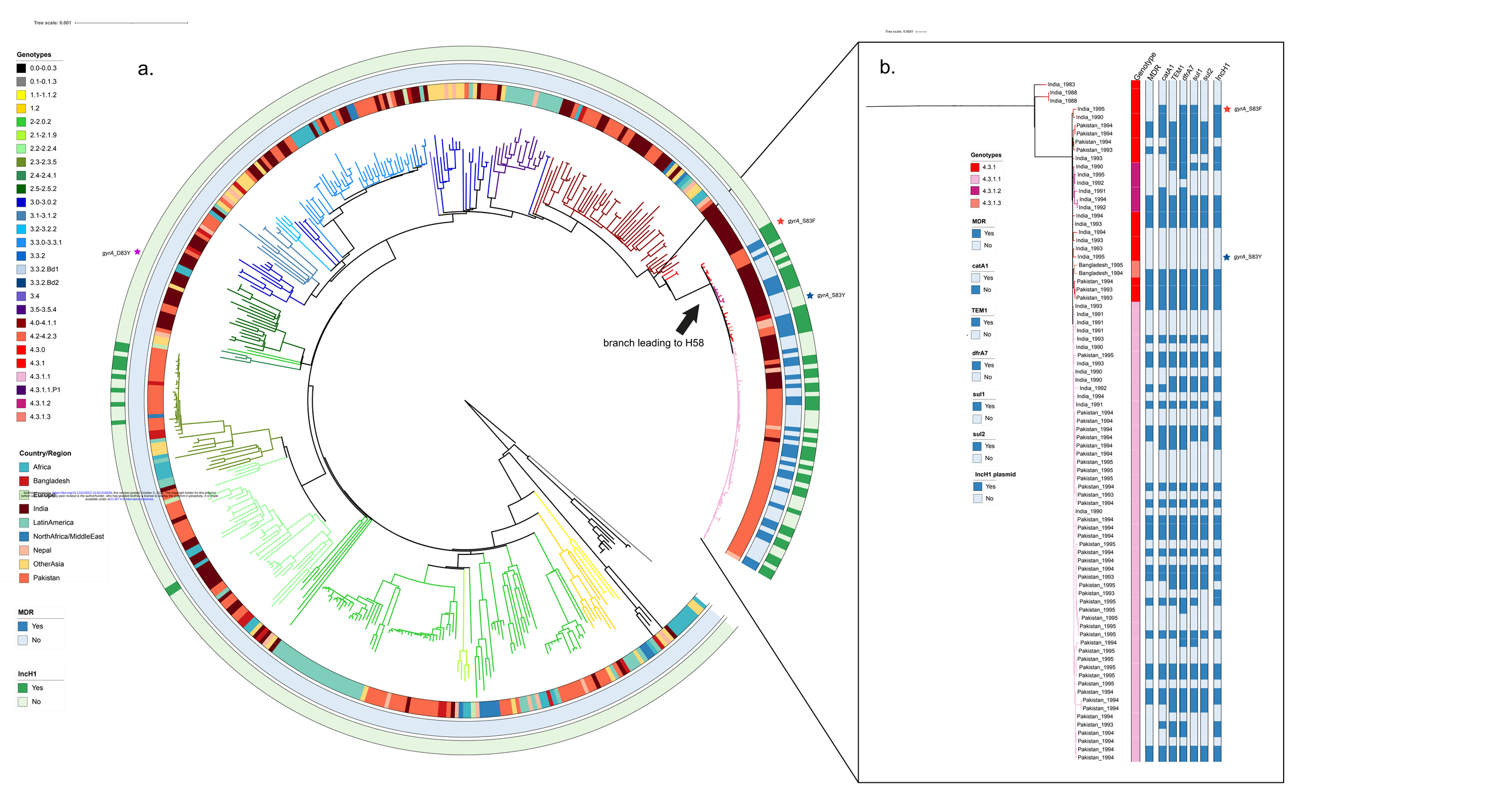
78. Kalyaanamoorthy S, Minh BQ, Wong TKF, von Haeseler A, Jermiin LS. ModelFinder: Fast model selection for accurate phylogenetic estimates. *Nat Methods*. 2017;14(6):587-589. doi:10.1038/nmeth.4285

79. Nguyen LT, Schmidt HA, von Haeseler A, Minh BQ. IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Mol Biol Evol*. 2015;32(1):268-274. doi:10.1093/molbev/msu300

80. Xie W, Lewis PO, Fan Y, Kuo L, Chen MH. Improving marginal likelihood estimation for bayesian phylogenetic model selection. *Syst Biol*. 2011;60(2):150-160. doi:10.1093/sysbio/syq085

81. Hill V, Baele G. Bayesian Estimation of Past Population Dynamics in BEAST 1.10 Using the Skygrid Coalescent Model. *Mol Biol Evol*. 2019;36(11):2620-2628. doi:10.1093/molbev/msz172

82. Rambaut A, Drummond AJ, Xie D, Baele G, Suchard MA. Posterior summarization in Bayesian phylogenetics using Tracer 1.7. *Syst Biol*. 2018;67(5):901-904. doi:10.1093/sysbio/syy032



Tree scale: 0.001

Country
MDR
catA1
TEM1
dfrA7
sul1
sul2
IncH1

Lorem ipsum

Country of origin
- LatinAmerica
- Africa
- Bangladesh
- India
- Pakistan
- Vietnam
- Oceania
- OtherAsia
- MiddleEast

MDR
- Yes
- No

catA1
- Yes
- No

QRDR
- gyrA-S83F
- gyrA-D87N
- gyrA-S83Y
- gyrA-S83Y+parC-S80R
- gyrA-D87G+gyrA-S83F+parC-E84K

TEM1
- Yes
- No

dfrA7
- Yes
- No

Genotypes
- 4.3.1
- 4.3.1.1
- 4.3.1.2
- 4.3.1.3

sul1
- Yes
- No

sul2
- Yes
- No

IncH1
- Yes
- No

Tree scale: 10

1968.33
(1962.38 - 1973.57)

1953.53
(1944.83 - 1961.94)

1st double QRDR mutation
1995

1987
Emergence of H58 lineage II
(4.3.1.2)

1st QRDR mutation
1990

1st “triple mutant”
1995

1987.63
(1986.32 - 1988.76)

1988
Emergence of H58 lineage I
(4.3.1.1)

Genotype
Country
MDR
IncH1
Source

Genotypes
- 4.3.1
- 4.3.1.1
- 4.3.1.2
- 4.3.1.3

Countries
- Bangladesh
- Fiji
- India
- Laos
- Nepal
- Pakistan
- Vietnam

MDR
- Yes
- No

IncH1
- Yes
- No

Source
- UKHSA
- Published

QRDR
- gyrA-S83F
- gyrA-D87N
- gyrA-S83Y
- gyrA-S83Y+parC-S80R
- gyrA-D87G+gyrA-S83F+parC-E84K

Appendix 6. Exploiting genomics to mitigate the public health impact of antimicrobial resistance (publication)

Genome Medicine

REVIEW

Open Access

Exploiting genomics to mitigate the public health impact of antimicrobial resistance

Claire Waddington[1,2], Megan E. Carey[1,2], Christine J. Boinett[3], Ellen Higginson[1,2], Balaji Veeraraghavan[4] and Stephen Baker[1,2*]

Abstract

Antimicrobial resistance (AMR) is a major global public health threat, which has been largely driven by the excessive use of antimicrobials. Control measures are urgently needed to slow the trajectory of AMR but are hampered by an incomplete understanding of the interplay between pathogens, AMR encoding genes, and mobile genetic elements at a microbial level. These factors, combined with the human, animal, and environmental interactions that underlie AMR dissemination at a population level, make for a highly complex landscape. Whole-genome sequencing (WGS) and, more recently, metagenomic analyses have greatly enhanced our understanding of these processes, and these approaches are informing mitigation strategies for how we better understand and control AMR. This review explores how WGS techniques have advanced global, national, and local AMR surveillance, and how this improved understanding is being applied to inform solutions, such as novel diagnostic methods that allow antimicrobial use to be optimised and vaccination strategies for better controlling AMR. We highlight some future opportunities for AMR control informed by genomic sequencing, along with the remaining challenges that must be overcome to fully realise the potential of WGS approaches for international AMR control.

Keywords: Antimicrobial resistance, Public health, Genomics, Surveillance, Vaccines, Diagnostics

Background

Antimicrobial resistance (AMR) is one of the greatest current threats in international public health [1]. The rapid increase and worldwide spread of AMR threaten the advances in modern medicine, compromising the treatment of common infections such as pneumonia, urinary tract infections, and tuberculosis as well as the care of patients needing organ transplantation, complex surgery, cancer chemotherapy, and intensive care [2]. There are also significant economic and societal costs associated with AMR infections [3, 4]. These costs are attributed with longer hospital stays, higher medical bills, and increased mortality. Although AMR is a global problem, the burden of AMR falls disproportionately on low- and middle-income countries (LMICs), where it threatens sustainable development [4–6].

The first wave of antimicrobials was derived from naturally occurring compounds; therefore, the emergence of AMR is largely a natural process, and AMR genes have been detected in samples originating millions of years before the widespread use of antimicrobials [7–9]. However, an exponential growth in antimicrobial use in recent decades has exerted enormous selective pressures on bacterial populations, which has dramatically accelerated the evolution of AMR [10]. Indeed, as soon as mankind develops a new class of small molecules to kill bacteria, organisms evolve to resist their action, leading to an increasing prevalence of multi-drug-resistant (MDR), extended-drug-resistant (XDR), and pan-drug-resistant (PDR) organisms.

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The detection of AMR has been traditionally reliant on culture-based antimicrobial susceptibility testing (AST), which remains the mainstay of clinical microbiology and patient management. Whilst phenotyping provides direct visual evidence of how a bacterium will interact with an antimicrobial, it generally provides little or no data regarding the resistance mechanisms, with disparate genetic clones often demonstrating identical resistance profiles [11]. Genetic typing methods, such as multi-locus sequence typing (MLST), provide a higher level of pathogen resolution compared to AST but are highly restrictive as they only describe a small fraction of a genome. Whole-genome sequencing (WGS), in contrast, provides genome-wide information at the single nucleotide level that can be used to identify the presence and mechanisms of AMR, as well as pathogen identity, virulence, and ancestry [12–14]. The advent of next-generation sequencing (NGS), via high-throughput, parallel sequencing of DNA fragments, has allowed pathogen genomes to be determined rapidly and at comparatively low cost [13, 15, 16]. The universality of the genetic code allows a unified approach to be applied to all organisms, and a range of technologies and platforms can provide the same data output [11]. Comparative phylogenetic analysis can be exploited to determine the degree of relatedness between different isolates based on the extent of the similarity between genomes and, when overlaid with epidemiological and clinical data, can inform our understanding of the specific temporospatial dynamics of AMR and transmission [11]. Additionally, the recent optimisation of metagenomic sequencing approaches circumvents the necessity for culture entirely. Therefore, by incorporating all available genomic material in a sample, metagenomic analysis facilitates a shift in focus from an individual pathogen to the community, microbiome landscape, generating a highly detailed model of how pathogens interact, and how they mobilise and access AMR genes [17].

The power of WGS is being increasingly employed to address the public health challenge of AMR, supporting surveillance, outbreak investigation, and contributing to improved diagnostics and therapeutics as highlighted in Table 1 [12, 27]. This review highlights some of the successes and advances supported by WGS in these areas and outlines future directions and remaining challenges associated with using WGS technologies to support public health efforts for AMR.

AMR surveillance

Surveillance is the cornerstone of public health efforts in controlling AMR. AMR surveillance has traditionally relied on phenotypic AST, but different testing methods, variation in interpretation, the extent to which thresholds are clinically validated, and changes in interpretive guidelines limit standardisation. WGS data overcomes many of these limitations, providing detailed insights that can greatly augment the value of AMR surveillance. Such data can inform an understanding of AMR evolution and spread, inform control strategies, facilitate the detection of new and emerging threats, and support new diagnostic and therapeutic approaches [4, 10, 12, 28–30].

Underlying the expansion of AMR is a dynamic and complex interaction between microbes, AMR encoding genes, and mobile genetic elements that act as vehicles for AMR via horizontal gene transfer (HGT) [7, 10, 11, 31]. Once horizontally transferred AMR genes have become chromosomally integrated, clonal expansion can lead to the rapid dissemination of these genes. This phenomenon has been observed with methicillin-resistant *Staphylococcus aureus* (MRSA), penicillin-resistant *Streptococcus pneumoniae*, vancomycin-resistant enterococci, and fluroquinolone-resistant *Clostridium difficile* [11, 32–37]. The granular resolution afforded by WGS allows inferences on the nature of AMR evolution and dissemination [38], providing insights that can help contain AMR and protect public health.

International surveillance

Increased accessibility to WGS has significantly enhanced our understanding of the global evolution and spread of AMR. High-throughput WGS methods have facilitated the sequencing of large, geographically representative collections of isolates, overcoming many of the biases associated with historic, small-scale studies that were often skewed by the domination of local clonal dissemination. This paradigm is illustrated by a modified understanding of AMR in *E. coli* ST131, which has rapidly spread to become a frequent cause of healthcare and community-acquired infection since it was first described in 2008 [33]. ST131 *E. coli* frequently exhibit cephalosporin (most commonly due to a CTX-M-15 encoding gene) and fluroquinolone resistance [33, 39]. The development of AMR in ST131 *E. coli* was initially speculated to have arisen from frequent and independent acquisitions of mobile genetic elements [40]. However, a study from the USA concluded that the success of AMR in ST131 *E. coli* was associated with a sustained clonal expansion. However, it was not until a more comprehensive global study that the true diversity of AMR in ST131 was revealed, which identified the chromosomal integrations of various resistance genes, the persistence and evolution of mobile elements within sub-lineages, and the sporadic acquisition of several different resistance elements [41]. As well as enhancing the mechanistic understanding of AMR, this study highlighted the need for multifaceted control strategies that could limit the spread

Table 1 Use cases for whole-genome sequencing (WGS) in mitigating the public health impact of antimicrobial resistance (AMR)

Trigger	Uses of WGS/workflow	Main findings	Advantages of using WGS
Use case 1: International surveillance—determining the population structure and epidemiology of carbapenem-resistant *K. pneumoniae* (CR-Kp) across Europe [18]			
Primary reservoirs and transmission dynamics of CR-Kp across Europe are incompletely understood.	Hospital laboratories across Europe submitted consecutive clinical isolates of CR-Kp, along with a comparator susceptible isolate for sequencing.	CR-Kp largely resulted from carbapenemase acquisition; nosocomial acquisition was the main cause of CR-Kp spread.	Provided a benchmark for ongoing surveillance of CR-Kp. Highlighted the role of nosocomial spread.
Use case 2: Enhancing the national surveillance of antimicrobial resistance in the Philippines [11]			
National laboratory-based surveillance had shown increasing AMR prevalence over the10 years previously, but understanding of the epidemiology and drivers of AMR were lacking.	WGS capability was introduced to the existing surveillance programme. Retrospective sequencing of MDR GNB obtained prior to the introduction was undertaken and analysed with phenotypic and epidemiological data to provide baseline data and inform control measures.	Drivers of carbapenem resistance at different levels of the healthcare system were identified, including a localised outbreak of plasmid-driven CR-Kp affecting a specific hospital, through the detection of the introduction and country-wide spread of a high-risk epidemic clone, *E. coli* ST410.	Detailed understanding of the epidemiology and drivers of AMR enabled the introduction of effective infection control measures. Data were contributed to international AMR surveillance efforts, improving the global coverage.
Use case 3: Investigating an MRSA outbreak in a neonatal unit [19]			
Phenotypically similar MRSA isolates were identified from patients on a neonatal unit over a 6-month period but could not be linked temporally or geographically, suggesting that the full extent of the outbreak had not been identified.	All MRSA isolates obtained from patients on the neonatal unit over a 6-month period underwent WGS regardless of phenotypic characteristics. MRSA isolates with antibiograms similar to the outbreak strain, identified from the community, and screening samples taken elsewhere in the hospital were also sequenced.	Two previously excluded isolates were identified as being part of the outbreak by phylogenetic analysis, allowing temporal links between cases to be established. A wide transmission network beyond the neonatal unit was identified.	WGS allowed a large number of isolates to be tested and related strains to be accurately identified, thereby enabling full outbreak reconstruction. Combining WGS data with clinical and epidemiological data enabled the identification of outbreak source and successful instigation of infection control measures.
Use case 4: Investigating the direction of transmission in an *A. baumannii* outbreak in a UK hospital [20]			
Molecular typing of a cluster of *A. baumannii* isolates obtained in a UK hospital suggested a clonal outbreak, but the chain of transmission between cases could not be established from the existing laboratory, clinical, and epidemiological data.	A cluster of isolates obtained from patients with identical molecular typing profiles and antibiograms underwent WGS analysis to inform the understanding of the direct transmission between patients.	Phylogenetic analysis enabled the identification of the index case and the subsequent chain of transmission to be determined. One patient/isolate was shown to be unrelated and was excluded from the outbreak investigation.	WGS-enabled directionality of transmission can be determined, allowing accurate reconstruction of the outbreak.
Use case 5: Contact tracing and detection of secondary cases of TB [21]			
Screening and detection of secondary cases of TB are essential for TB control. Accurate identification of case clusters and transmission networks is hampered by the limited resolution provided by molecular typing.	Clinical TB isolates in the Netherlands in 2016 were analysed by both molecular typing and WGS. The degree of discrimination and accuracy in identifying potentially related cases was compared between the two methods.	WGS was better able to decimate the relatedness of isolates, clustering a smaller proportion of isolates as related compared with molecular typing (25% vs. 14%) and increasing proportion confirmed as epidemiologically linked (57% vs. 31%).	WGS facilitated the identification of transmission events, facilitating contact tracing as well as informing the wider understanding of TB control.
Use case 6: Identifying the drivers of AMR in atypical enteropathogenic *E. coli* (aEPEC) strains isolated from children < 5 years in four sub-Saharan African countries and three South Asian countries [22]			
The frequency, mechanisms, and drivers of AMR in intestinal isolates of *E. coli* in children in the community in many countries worldwide were unknown.	Phenotypic susceptibility and WGS of isolates were analysed and correlated with antimicrobial use, disease status (symptomatic/asymptomatic), phylogenetic lineage, and geographic location.	High rates of AMR were shown, with 65% of isolates resistant to at least 3 antimicrobial drug classes. A diverse range of genetic mechanisms of AMR was shown, with geographic location and the associated antimicrobial use pattern being the strongest predictors of AMR.	WGS was used to provide a detailed analysis of AMR across a large geographical area, providing insights into the AMR epidemiology, spread, and drivers.

Table 1 (continued)

Trigger	Uses of WGS/workflow	Main findings	Advantages of using WGS
Use case 7: Investigating colistin resistance detected in commensal *E. coli* in food stock animals in China [23]			
Routine surveillance had detected a sharp increase in the rates of colistin resistance in colonising bacteria from pigs in China, but the mechanism of this resistance was not known.	Conjugation experiments were undertaken to confirm the presence of plasmid-associated, transmissible colistin resistance. WGS of the plasmids was used to identify the gene responsible.	The sequence of the plasmid-associated colistin resistance gene was identified and designated *mcr-1*.	The genetic basis of a new, AMR mechanism was identified and described, allowing ongoing surveillance, as well as informing investigation and detection of this emerging threat in other settings.
Use case 8: Detecting of transmitted drug resistance (TDR) in newly diagnosed, treatment naïve HIV-1-positive patients [24]			
Multiple genetic mutations contribute to HIV drug resistance. Mutations often only affect a fraction of the viral population in any given patient (low-level variants), but, if present in combination with other mutations or at key sites, likely contribute to treatment failure. Existing methods are insufficiently sensitive to detect low-level variants that can compromise treatment.	NGS was used to detect TDR, including low-level variants affecting $\geq$ 2% or more of the viral population, in treatment-naïve, clinical trial participants enrolled across 35 countries worldwide with a HIV viral load > 1000 copies/ml.	NGS revealed many low-level variants that were undetected by existing methods. Significant geographic diversity was seen in the prevalence of different TDR mutations.	NGS provided a more comprehensive assessment of TDR prevalence in individual patients, and in different regions of the world, helping guide empiric treatment choice and understanding of clinical outcomes in different patients and settings.
Use case 9: Understanding the epidemiology of MDR and XDR pathogens amenable to control by vaccination [25, 26]			
AMR is increasingly threatening the success of treatment for typhoid fever. Resistance to the last effective oral agent, azithromycin, was detected in Bangladesh and subsequently in Pakistan, but the genetic mechanism and the likely hood of dissemination were unknown.	Clinical isolates of azithromycin-resistant *S.* Typhi were analysed by WGS. The phylogenetic analysis enabled the contextualisation of the strains within contemporaneous *S.* Typhi isolates in both settings.	Phylogenetic analysis showed that resistant isolates in Bangladesh and Pakistan resulted from the independent acquisition of mutations in the same gene highlighting the extent of selection pressure on azithromycin and the imperative need for disease control by vaccination.	WGS was used to identify and investigate two separate outbreaks of azithromycin-resistant *S.* Typhi. These data helped provide the impetus to roll out novel typhoid conjugate vaccines to control infection.

of ST131, as well as the spread of mobile genetic elements to other pathogens [42]. These studies were foundational for additional work specifically investigating risk factors for ST131 infection and spread. ST131 is now known to be a common gut commensal, with opportunistic infections occurring mainly in functionally compromised hosts such as the elderly, particularly those having prior antimicrobial use and living in long-term care facilities [43–47]. Infection control measures for ST131 *E. coli* in long-term care facilities focusing on contact precautions have been largely ineffective [45, 48], leading to the strategy of limiting multi-bedded rooms and communal dining facilities [49]. The targeted screening of hospitalised patients with specific risk factors for ST131 facilitated patient isolation to prevent nosocomial transmission, as well as better management of empirical broad-spectrum antimicrobial therapy in specific high-risk patient groups [46, 47].

Global AMR surveillance has been bolstered by programmes such as the Global Antimicrobial Resistance and Use Surveillance System (GLASS) from the World Health Organization (WHO) [4], and the European Antimicrobial Resistance Surveillance Network (EARS-Net) [50]. These networks encourage data sharing and provide standardised templates for reporting AST data to facilitate comparative analysis. Recently, such networks have documented a rapid increase in MDR Gram-negative bacteria (GNB) that have become recognised as leading AMR threats, including third-generation cephalosporin-resistant and carbapenem-resistant *Enterobacterales* (CRE), carbapenem-resistant *Acinetobacter baumannii* and *Pseudomonas aeruginosa*, [1, 22, 51–55]. These networks are expanding their evidence base for other key threats, such as *Klebsiella pneumoniae*, which can cause a wide range of infections, including pneumonia, bacteraemia, and urinary tract infections [56], and have been identified as a crucial entry point for MDR into *Enterobacterales* [57–59]. Carbapenem-resistant *K. pneumoniae* (CR-Kp) is considered to be the fastest-growing AMR threat in Europe [60] and has a high attributable mortality rate (estimated as 30–70%) [61, 62]. Systematic surveillance of CR-Kp across Europe demonstrated that nosocomial spread was driving this epidemic, with carbapenemase acquisition occurring across diverse phylogenetic backgrounds [18]. As well as describing the epidemiology of CR-Kp, this study found that isolates were concentrated into four clonal lineages: sequence types (ST) 11, 15, 101, and 258/512. These data contrast with studies in other settings that found high genomic plasticity in CRE, with frequent poly-clonal and poly-species horizontal gene transfer accounting for much of the acquired resistance [54, 63, 64]. This inconsistency highlights the complex interplay between bacteria and MDR genes, with the role of clonal expansion [18] and HGT [54, 63, 64] potentially contributing variably across different timeframes and geographies.

Embedding WGS into prospective, cross-national surveillance will further strengthen AMR surveillance, as highlighted by the recent outbreak genomic investigation of $bla_{\text{NDM-1}}$ and $bla_{\text{OXA-48}}$ CR-Kp in Germany [65]. In the absence of routine collection and reporting of European-wide WGS data, investigators pooled data from 13 national surveillance systems to investigate the spread of the CR-Kp outbreak, identifying cross-border transmission of several unrelated clusters of $bla_{\text{NDM-1}}$- and $bla_{\text{OXA-48}}$-positive CR-Kp. It is anticipated that the planned incorporation of WGS into the European Antimicrobial Resistance Genes Surveillance Network (EURGen-Net) will facilitate the routine detection of such transmission events and inform targeted control measures [65].

National surveillance

The inclusion of WGS can similarly strengthen national AMR surveillance. The introduction of WGS into AMR surveillance in the Philippines, for example, showed that HGT was key for driving carbapenem resistance in *Klebsiella*, with mobile genetic element (MGE) acquisition often leading to MDR [11]. WGS data also identified a localised plasmid-driven outbreak of CR-Kp, where various infection control measures, including patient isolation, were initiated [11]. By contributing WGS data to the WHO international surveillance network, national surveillance data also enhanced the global understanding of some high-risk clones of interest, notably ST147 CR-Kp, of which a previously uncharacterised clade was described [11].

National surveillance can directly guide the need for AMR control measures that can be driven, resourced, and monitored centrally, as was demonstrated in Israel following the introduction of CR-Kp ST258 in 2005. The initial measures were unsuccessful at controlling the spread of CR-Kp ST258, and infections rapidly rose to a peak incidence of 41.9 per 100,000 patient-days [61]. Nationally mandated control measures were subsequently introduced in 2007, successfully reducing the incidence of infections by 79%, as well as the rate of asymptomatic carriage [52, 66–69].

The routine inclusion of WGS into national surveillance can also be used to evaluate the risk from emerging AMR threats, such as transferable colistin resistance [70]. Phenotypic colistin resistance testing is not routinely performed and therefore is not readily detectable without WGS [70]. Routine WGS of GNB in the UK generated a substantial data resource over time, which was mined to determine the extent of the spread of plasmid-encoded colistin resistance after it emerged as an AMR threat [70].

Although colistin resistance can be detected in organisms from a range of environments, this retrospective study found that it remained uncommon in the UK, leading to the monitoring of colistin prescribing in a bid to minimise any potential selective pressure within the UK.

Local surveillance

Healthcare facilities house patients with serious and complex infections in proximity to patients with compromised immunity, in settings that are characterised by high antimicrobial use [71, 72]. In this context, healthcare-associated infections (HCAIs), frequently with pathogens that are highly drug-resistant, are a significant challenge requiring rigorous infection prevention and control practices to reduce their incidence [72]. The ability to perform WGS in a clinically relevant turnaround time has made genomics an actionable front-line tool to investigate HCAIs [73].

A key strength of WGS is that it permits the degree of relatedness between selected isolates to be determined and, in the context of HCAIs, allows the reconstruction of nosocomial outbreaks [14, 39]. One of the earliest applications of this process was in a retrospective investigation of a protracted outbreak of MRSA in a neonatal unit [73]. By reconstructing the full outbreak, the source was eventually identified, ultimately, supporting infection control measures that eventually led to its resolution [19]. Other studies of *S. aureus* HCAI have similarly shown the improved granularity of WGS compared to other approaches, with more conventional *S. aureus*-specific staphylococcal protein A (*spa*) typing failing to detect transmission events, as well as the false attribution of unrelated isolates as HCAIs [74].

By exploiting a 'molecular clock' (the mutation rate in the genome of a specific organism), WGS can also infer directionality in outbreaks [13, 14, 19, 27]. This approach was key to understanding an outbreak of MDR *Acinetobacter baumannii* in a UK hospital treating civilian patients and injured military personnel returning from the Middle East [20]. Pre-admission colonisation of the wounds of military personnel with distinct lineages of *A. baumannii* was described, with the onward transmission of one specific clone leading to the infection of a civilian. Importantly, an additional civilian infection was not associated with the military-related infection, highlighting that other distantly related *A. baumannii* were also circulating. Similarly, the utility of WGS in determining the chains of transmission has also been demonstrated for MRSA and CR-Kp [75].

By generating detailed insights into HCAI, WGS surveillance can help identify the main sources of AMR, providing an opportunity for targeted infection control measures that can reduce the risk of further transmission events [73]. A large study of HCAI CR-Kp showed that the majority of inpatient infections resulted from the transfer of a relatively small number of patients from specific, high-risk facilities and that targeting interventions at such facilities could significantly reduce HCAIs across the healthcare system [75]. WGS-based investigations can similarly limit the use of unnecessary or ineffective measures that can be costly to implement. For example, studies of MRSA transmission have shown that patient-to-patient transmission is relatively uncommon and that persistently colonised staff are more frequent sources of infection than patients [74]. Targeting infection control at the staff rather than patients may therefore be a more efficient and more cost-effective control mechanism [76].

Prospectively applying WGS for local HCAI control has also been suggested for early outbreak detection, and thereby early intervention and source control, with a substantial cost saving [4]. This approach may be especially true for problematic pathogens in high-risk settings, such as MDR *K. pneumoniae* in ICUs [77]. WGS of routinely collected MRSA samples, obtained from both hospital and community settings over a year in the US identified extensive and previously unrecognised transmission events, highlighting the utility of routine WGS screening in high-risk patients and/or settings [76].

Community surveillance and outbreak investigation

WGS is also contributing to community public health efforts, such as contact tracing and the detection of secondary cases of TB. Contact tracing is difficult as there is often a long interval between the initial infection and the diagnosis. Several countries, such as the UK, have established a nationwide WGS database of TB to facilitate the identification of transmission events. This system enhances contact tracing as well as informs a wider understanding of TB control. Using WGS in preference to variable number tandem repeat (MIRU-VNTR) typing has been shown to reduce the number of false case clusters and has improved the detection of cases where potential clusters are identified [21, 78–81].

Understanding the drivers of AMR

The paradigm that the rapid expansion in AMR is a response to selective pressure from antimicrobial use drives AMR is well accepted [82]. WGS is being used to interrogate this selective evolution at the molecular level, across wide geographical areas, and between genetically diverse organisms. WGS was exploited to investigate AMR and the clinical epidemiology of atypical enteropathogenic *E. coli* isolated from symptomatic and asymptomatic children in South Asia and sub-Saharan Africa [22]. Despite broad geographical, symptomatic, and phylogenetic diversity, 65% of the bacterial isolates

were resistant to three or more classes of antimicrobials. In this study, the best predictor of resistance profile was not the presence or absence of clinical symptoms or genetic lineage, but the geographical patterns of antimicrobial usage [22].

On an individual patient level, the likelihood of developing infection with extended-spectrum beta-lactamase-producing bacteria is associated with an increased length of hospital stay prior to infection, exposure to antimicrobials, and recent overseas travel [83]. Predictors of MDR GNB infection more broadly include male sex, older age, and co-morbidities [84]. Changes in the human microbiota occur in response to illness, particularly when associated with frequent and/or prolonged antimicrobial exposure. The *Enterobacterales* are habitual colonisers of the gastrointestinal tract, where they can act as a major reservoir for mobile AMR genes [22]. Metagenomic studies have shown that commensal bacteria in healthy individuals help maintain pathogenic bacteria at a low density, meaning that carriage is rarely problematic [71]. However, when patients undergo an invasive procedure, there is a loss in microbial diversity followed by colonisation with pathogenic bacteria [85]. This effect is can be exacerbated by antimicrobial use, which frequently results in the selection of drug-resistant pathogens [71] and facilitates HGT of AMR genes between bacterial lineages and species [7, 86]. WGS studies are now being used to determine the colonisation factors that facilitate the rapid growth and persistence of certain pathogens in such circumstances, with the hypothesis that therapeutics targeting persistent organisms may be developed to control pathogen colonisation.

AMR and 'One Health'

One Health focuses on understanding the interconnectivity between ecosystems [87], recognising that human health is connected with, and dependent on, the health of animals, plants, and the wider environment [4, 17, 88]. AMR transmission occurs both within and across different ecosystems, facilitated by close animal and human contact, food, and water systems, all of which are influenced by culture and economics [87]. Understanding this complex interplay is crucial for the control of AMR. WGS is being increasingly used to support One Health aspects of AMR, such as by interrogating the spread of AMR via food and farming [88, 89], building on an established food-borne disease surveillance [27], and outbreak investigation [90, 91]. A WGS-based investigation of commensal *E. coli* isolates in livestock in China after a rapid increase of colistin resistance, for example, showed that this increase was due to the emergence of a plasmid associated colistin resistance gene in *E. coli*, designated MCR-1, and led to international efforts to control the dissemination of MCR-1 via food supply networks [23].

Optimising antimicrobial use through WGS-based rapid diagnostic tests

Rapid, accurate, low-cost diagnostic tests can aid in optimising and limiting antimicrobial use, thereby minimising the potential selective pressures. Culture-based microbiological diagnostics and AST are widely utilised for bacterial infections but are not rapid and not applicable to viral infections or fastidious organisms. WGS-based diagnostic approaches are being used to overcome these limitations, notably for HIV and tuberculosis, and offer the prospect of improved outcomes.

The prognosis of HIV infection has been transformed by combination anti-retroviral therapy (cART), but these treatments are complicated by the emergence of viral resistance. Genomic replication of RNA viruses utilises a reverse transcriptase lacking proofreading capacity, leading to the accumulation of mutations, with in host infection existing as a population of closely related genomes (quasispecies) [92]. Further in host genetic variability can result from the recombination of viruses infecting the same cell and the accumulation of variants over time [93]. WGS can detect viral variants at a prevalence of ~ 1%, and WGS-based resistance testing is used prior to initiating cART to detect mutations conferring drug resistance in the infecting quasispecies [94–97]. The full clinical implications of minority variants are unclear [98], but they predict failure of first-line regimens [99] and have been shown to be increasing in some locations [24], highlighting the need for the global monitoring of drug resistance in HIV.

Treatment success in *M. tuberculosis* declines from 83% for susceptible isolates to 54% and 30% for MDR- and XDR-TB isolates, respectively [100]. *M. tuberculosis* is slow growing, with culture-based diagnosis and AST often taking weeks and requiring a high-level biosafety laboratory. Poor reproducibility, uncertainty regarding appropriate drug concentrations for susceptibility testing [101], and a propensity for laboratory contamination can lead to false positives [102–104]. The *M. tuberculosis* genome comprised a single chromosome with drug resistance mediated through mutations in core genes or promoters [105], making the organism perfectly suited to WGS diagnostic approaches [105]. WGS diagnostics significantly decrease the time to a confirmatory TB diagnosis [106, 107], plus it reliably predicts the drug resistance profile [108], whilst facilitating the detection, monitoring, and diagnosis of de novo drug resistance [109]. These approaches have revolutionised TB control and are being increasingly used routinely in TB management.

Informing vaccination strategies to control AMR

Vaccination has an established role in AMR reduction. Conjugate vaccines for *Haemophilus influenzae* and *Streptococcus pneumoniae* have been shown to not only decrease the incidence of disease, but also reduce AMR [110, 111]. Increasing access to these vaccines would likely have a further impact on decreasing both disease and AMR [112, 113]. Vaccines against viral infections including influenza, rotavirus, and varicella zoster have also been shown to reduce antimicrobial use by decreasing the incidence of secondary bacterial infections and syndromic presentations for illnesses that would lead to antimicrobial use [112–114].

More recently, vaccination has been used as a direct mechanism for controlling AMR in typhoid fever, the result of many years of effort at understanding typhoid fever, in which WGS has played a significant role. Typhoid fever is caused by the bacterium *Salmonella enterica* serovar Typhi (*S.* Typhi) and causes an estimated 10.9 million cases and 116,800 deaths annually [115]. Whilst most cases of typhoid fever can be treated with antimicrobials, the emergence and spread of AMR have posed an increasing threat to typhoid control, particularly in South Asia, where resistance to each class of oral antimicrobials used to treat typhoid fever has emerged [25]. *S.* Typhi is highly clonal [116], and MDR *S.* Typhi is heavily associated with a single haplotype (H58) [117]. The global spread of H58 has largely replaced other non-H58 haplotypes and is causing sustained ongoing typhoid transmission in east and southern Africa [117]. MDR within H58 is linked with a single mobile element that was introduced via a specific plasmid (IncHI1-PST6) but has since transferred to the chromosome. As new resistance phenotypes have emerged, WGS has facilitated the further investigation of the underlying molecular mechanisms, including the recent emergence of azithromycin resistant typhoid in Bangladesh, Pakistan, Nepal, and India [26, 118, 119]. WGS-based investigation of an outbreak of ceftriaxone-resistant typhoid in Hyderabad, Pakistan, revealed the emergence of an extensively drug-resistant (XDR) variant that was not only resistant to the first-line antimicrobials chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole, but also fluoroquinolones and third-generation cephalosporins [120]. This variant, which was within the H58 clade, had acquired a plasmid encoding additional resistance elements, including a $bla_{\text{CTX-M-15}}$ and a *qnrS* fluoroquinolone resistance gene [121].

Following the initial reports of XDR typhoid, intensified surveillance was undertaken to monitor its spread of typhoid into Karachi and beyond [122]. Reactive vaccination campaigns were initiated in Hyderabad and Karachi, and vaccine safety and effectiveness data were generated [123, 124]. All of these efforts played a major role in the decision of the Federal Expanded Program on Immunization to introduce typhoid conjugate vaccine (TCV) into their national immunisation programme, making Pakistan the first country to do so [26, 125]. The phased introduction began with a vaccination campaign targeting ~ 10 million children in the urban areas of Sindh province in November 2019, which were the areas hardest hit by XDR typhoid, and a subsequent campaign was conducted in Islamabad and Punjab province, where XDR had spread, that covered over 13 million children [126]. Consequently, WGS and phenotypic AMR data had a direct impact on the decision to introduce TCV and on the vaccine introduction strategy itself.

In addition to elucidating the molecular mechanisms of AMR and providing well-defined, standardised AMR data where mechanisms of resistance are known, WGS data can also provide important information regarding typhoid transmission pathways. The 2018 WHO Position Paper on typhoid vaccines indicated that TCV introduction should be prioritised "in countries with the highest burden of typhoid disease or a high burden of antimicrobial-resistant *S.* Typhi" [127]. Given the ubiquitous nature of drug-resistant typhoid, the anticipated country demand for TCVs, and the presence of only two WHO prequalified manufacturers of TCV, additional selection criteria may be required [127]. Globally representative WGS data show that drug-resistant typhoid typically emerges and spreads from South Asia [117]; therefore, prioritising TCV introduction in this region may reduce the burden of AMR in the region and prevent the spread of new resistance phenotypes internationally. Such phylodynamic data can inform optimal vaccine introduction strategies for other key highly drug-resistant pathogens, as and when new vaccines become available.

Limitations, challenges, and future directions

Technical limitations

The concept of relatedness between bacterial isolates is based on a process of continuous evolution, but prospectively identifying genetic signals of AMR evolution from the background noise of genetic variation and sequencing error is difficult. Using sequence data to infer relatedness and transmission is highly variable, and mutation rates can vary because of different lifestyles/conditions such as biofilm formation, antimicrobial exposure, disease states, and environmental pressures such as starvation [128–130]. The threshold number of SNPs above which relatedness is unlikely is therefore highly context-dependent [21, 80, 131]; if not recognised, then selection biases within isolates in collections can lead to false epidemiological inferences. Despite these challenges, defined cut-off points for the relatedness of specific pathogens, such

as MRSA and *M. tuberculosis*, have been suggested in order to establish a threshold beyond which recent transmission is unlikely [132].

Inferring or excluding transmission events for pathogens that accumulate mutations slowly can be difficult [133], which presents a further challenge of differentiating genuine variants from sequencing error [134]. Interpreting the significance of WGS variation for pathogens that can establish asymptomatic carriage, have a period of latency, or cause a chronic, indolent illness can also prove challenging, as a degree of in-host genetic variation occurs with time, and different lineages may be intermittently transmitted at different points from a single host [13]. Bayesian mathematical approaches that integrate and model pathogen evolution with contact and temporal data on symptom onset, and that permit sequencing error, are being used to improve the inference of transmission chains, but understanding the sequence variance in real-world scenarios remains a major challenge [13, 133, 134].

Highly transmissible MGEs (such as plasmids) facilitate non-mutational AMR gene acquisition by HGT and are highly implicated in AMR dissemination [135]. In comparison, AMR that is driven by mutations on the bacterial chromosome is stable, and transmission is generally confined largely to progeny [136]. Determining the genetic location of AMR genes is therefore a key aspect of understanding AMR evolution and transmission, but is not readily determined by short-read sequencing [137–139]. Due to an inability to sequence long stretches of DNA and a failure to generate sufficient overlap in DNA fragments to allow accurate assembly of repetitive DNA sequences that are longer than the read length, these approaches are less well suited to describe the genetic environments of AMR genes [138, 139]. Additionally, AMR genes are commonly associated with long, repetitive insertion sequences, and short-read sequencing techniques cannot resolve if these are carried on a plasmid or chromosomally located [38, 54, 140, 141]. Most outbreak investigations have largely assumed the relative conservation of plasmid structures over the limited time period of an outbreak and the extent to which HGT events contribute to outbreaks of AMR is not fully understood [54]. However, more affordable long-read sequencing technology is becoming increasingly available and can generate reads that can span repetitive areas, allowing for the complete reconstruction of genome structure. This approach, however, is generally less accurate and has lower throughput compared to short-read sequencing [139, 140]. Hybrid approaches whereby isolates are sequenced en masse with short-read sequencing to determine identity, gene content, and relatedness, with selective addition of long-read sequencing to resolve the structure of MGEs, have been used to harness the benefits of both approaches [135, 139, 140, 142, 143].

Challenges in implementing WGS for routine use

The routine, prospective inclusion of WGS into microbiological surveillance has the potential to greatly enhance and strengthen public health efforts to combat AMR but comes with significant logistical and financial implications [144]. Unfortunately, prospective data demonstrating and quantifying the beneficial impact of routine WGS implementation are limited [145, 146]; most data are derived from retrospective studies, specifically from studies reconstructing chains of transmission during outbreak investigations [145]. Cost-effectiveness is even harder to determine, reflecting marked uncertainties and challenges in estimating the costs for the implementation of WGS. Generating accurate, contemporary cost estimates is difficult in the context of the marked decrease in costs that has accompanied the rapid technological advances in recent years, a problem compounded by the challenge of estimating costs for complex workflows that include several steps, including downstream analysis [147]. Further, routine diagnostics frequently requires a rapid turnaround time to support the management of individual cases, necessitating the processing of individual samples. This approach contrasts with many research and surveillance studies that lend themselves to batch processing of samples [147]. Despite these challenges, studies showing the cost-effectiveness of WGS for infection control and outbreak detection and management are starting to emerge and will help support implementation decisions [148–151].

Ensuring the reproducibility and validity of results through standardisation and quality assurance (QA) processes is an essential part of routine medical laboratory processing [152]. However, implementing QA processes for WGS is challenging. Variations in DNA extraction methods and reagents, sequencing technologies, analysis pipelines, and bioinformatic approaches can all impact WGS analysis [152], making the standardisation and quality assurance methods challenging [147, 153]. Nevertheless, efforts at quality assurance and result standardisation across laboratories are currently being attempted for WGS workflows [154]. Ring trials showing high performance and consistency across laboratories of WGS methods applied to *S. aureus* are highly encouraging [152, 153] and suggest that developing isolate collections that can act as biological standards, against which reproducibility and robustness of methods can be measured, might be feasible.

The ability to share, integrate, and compare the vast wealth of data derived from WGS across laboratories, settings, and time is key to harnessing the power

of WGS for global AMR surveillance [4, 154]. Barriers to data sharing include the reluctance of academics to share data before publication (a process that is itself often slow and time-consuming), political sensitivities when competing interests are at play (for example tourism), legal and requirements to protect personal data [154], and the need for standardised methodological and analytical approaches to generate comparable data [155]. Databases such as GenBank facilitate sharing of existing genomes, but their utility is again compromised by a lack of data standardisation, as well as deficiencies in accompanying metadata [154]. The Global Microbial Identifier (GMI) initiative is in the early stages of trying to address these issues, aiming to develop standardised identification and characterisation approaches in order to form a global interactive network of genomic databases [154]. The absence of validated globally standardised systems for genomic typing, defining clusters and determining AMR genes, further intensifies the challenges of interpreting genomic data [4, 11, 147]. Clinical and epidemiological metadata are essential for public health solutions to AMR, but are associated with standardisation, computational, and technical challenges [154]. The collaborative approach to sharing of the SARS-CoV-2 genomic data demonstrates that such an approach is possible [156, 157], but to date, scalable solutions to genomic data sharing have remained elusive.

Introducing WGS in LMICs

Despite AMR being a major problem in LMICs, there is currently limited understanding of AMR dynamics in these settings [22, 28] and a notable lack of sustained access to WGS surveillance [11]. The capital investment required to establish and maintain WGS platforms is substantial. Supply, procurement, and maintenance of WGS reagents and equipment can be highly challenging [11, 158], particularly, in locations with inconsistent supply chains, where short shelf-life consumables and long intervals between procurement and delivery may create wastage or significant project delays. Support for data analysis is also indispensable, along with investment in training and retention of people to develop and sustain the necessary proficiency. Even simple analyses of bacterial WGS data require access to high-performance compute (HPC) clusters, stable internet access, and expertise [11].

Conclusions

The rapid emergence and spread of AMR in recent decades are a major threat to global public health, and current antimicrobial development efforts cannot keep pace with pathogen evolutionary dynamics. Addressing this threat will require diverse, cross-sectoral interventions at all levels of public health and political systems. Improvements in genomic technologies in recent years have made these systems more widely accessible and affordable, and they are now being implemented as frontline tools in the battle against AMR. By enhancing AMR surveillance, WGS has greatly improved understanding of how, when, where, and why AMR emerges and spreads and has helped quantify temporal and geographical variations in AMR epidemiology. Genomic insights have also been applied to facilitate outbreak detection and control, improve diagnostic tests, optimise antimicrobial use, and inform vaccination strategies. Although logistical and technological barriers to the universal implementation of genomics in public health remain (particularly in LMICs), the use of these approaches is likely to further expand in the coming years and will hopefully help restrict the adverse public health impacts of AMR.

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Authors' contributions

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References

1. World Health Organization. Antimicrobial resistance: global report on surveillance. World Health Organization. 2014. https://apps.who.int/iris/handle/10665/112642.
2. Baker S. Infectious disease. A return to the pre-antimicrobial era? Science (New York, NY). 2015;347(6226):1064–6.

3. Jonas O, Team W: Drug-resistant infections: a threat to our economic future; 2017.
4. Global antimicrobial resistance and use surveillance system (GLASS) report 2021. Geneva: World Health Organization; 2021. Licence: CC BY-NC-SA 3.0 IGO.
5. Jasovsky D, Littmann J, Zorzet A, Cars O. Antimicrobial resistance-a threat to the world's sustainable development. Ups J Med Sci. 2016;121(3):159–64.
6. Bloom G, Merrett GB, Wilkinson A, Lin V, Paulin S. Antimicrobial resistance and universal health coverage. BMJ Glob Health. 2017;2(4):e000518.
7. van Schaik W. The human gut resistome. Philos Trans R Soc Lond Ser B Biol Sci. 2015;370(1670):20140087.
8. Forsberg KJ, Patel S, Gibson MK, Lauber CL, Knight R, Fierer N, et al. Bacterial phylogeny structures soil resistomes across habitats. Nature. 2014;509(7502):612–6.
9. Davies J, Davies D. Origins and evolution of antibiotic resistance. Microbiol Mol Biol Rev. 2010;74(3):417–33.
10. Baker S, Thomson N, Weill F-X, Holt KE. Genomic insights into the emergence and spread of antimicrobial-resistant bacterial pathogens. Science (New York, NY). 2018;360(6390):733–8.
11. Argimón S, Masim MAL, Gayeta JM, Lagrada ML, Macaranas PKV, Cohen V, et al. Integrating whole-genome sequencing within the National Antimicrobial Resistance Surveillance Program in the Philippines. Nat Commun. 2020;11(1):2719.
12. NIHR Global Health Research Unit on Genomic Surveillance of AMR. Whole-genome sequencing as part of national and international surveillance programmes for antimicrobial resistance: a roadmap. BMJ Glob Health. 2020;5(11):e002244.
13. Didelot X, Gardy J, Colijn C. Bayesian inference of infectious disease transmission from whole-genome sequence data. Mol Biol Evol. 2014;31(7):1869–79.
14. Price J, Gordon NC, Crook D, Llewelyn M, Paul J. The usefulness of whole genome sequencing in the management of Staphylococcus aureus infections. Clin Microbiol Infect. 2013;19(9):784–9.
15. Ashton PM, Nair S, Peters TM, Bale JA, Powell DG, Painset A, et al. Identification of Salmonella for public health surveillance using whole genome sequencing. PeerJ. 2016;4:e1752.
16. Wainwright C, Altamirano L, Cheney M, Cheney J, Barber S, Price D, et al. A multicenter, randomized, double-blind, controlled trial of nebulized epinephrine in infants with acute bronchiolitis. N Engl J Med. 2003;349(1):27–35.
17. Blake KS, Choi J, Dantas G. Approaches for characterizing and tracking hospital-associated multidrug-resistant bacteria. Cell Mol Life Sci. 2021;78(6):2585–606.
18. David S, Reuter S, Harris SR, Glasner C, Feltwell T, Argimon S, et al. Epidemic of carbapenem-resistant Klebsiella pneumoniae in Europe is driven by nosocomial spread. Nat Microbiol. 2019;4(11):1919–29.
19. Harris SR, Cartwright EJ, Török ME, Holden MT, Brown NM, Ogilvy-Stuart AL, et al. Whole-genome sequencing for analysis of an outbreak of meticillin-resistant Staphylococcus aureus: a descriptive study. Lancet Infect Dis. 2013;13(2):130–6.
20. Lewis T, Loman NJ, Bingle L, Jumaa P, Weinstock GM, Mortiboy D, et al. High-throughput whole-genome sequencing to dissect the epidemiology of Acinetobacter baumannii isolates from a hospital outbreak. J Hosp Infect. 2010;75(1):37–41.
21. Jajou R, Neeling A, Hunen R, Vries G, Schimmel H, Mulder A, et al. Epidemiological links between tuberculosis cases identified twice as efficiently by whole genome sequencing than conventional molecular typing: a population-based study. PLoS One. 2018;13(4):e0195413.
22. Ingle DJ, Levine MM, Kotloff KL, Holt KE, Robins-Browne RM. Dynamics of antimicrobial resistance in intestinal Escherichia coli from children in community settings in South Asia and sub-Saharan Africa. Nat Microbiol. 2018;3(9):1063–73.
23. Liu YY, Wang Y, Walsh TR, Yi LX, Zhang R, Spencer J, et al. Emergence of plasmid-mediated colistin resistance mechanism MCR-1 in animals and human beings in China: a microbiological and molecular biological study. Lancet Infect Dis. 2016;16(2):161–8.
24. Baxter JD, Dunn D, Tostevin A, Marvig RL, Bennedbaek M, Cozzi-Lepri A, et al. Transmitted HIV-1 drug resistance in a large international cohort using next-generation sequencing: results from the Strategic Timing of Antiretroviral Treatment (START) study. HIV Med. 2021;22(5):360–71.
25. Hooda Y, Saha S, Sajib MSI, Rahman H, Luby SP, Bondy-Denomy J, 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.
26. Iqbal J, Dehraj IF, Carey ME, Dyson ZA, Garrett D, Seidman JC, et al. A race against time: reduced azithromycin susceptibility in *Salmonella enterica* serovar Typhi in Pakistan. mSphere. 2020;5(4):e00215–20 / msphere/00215/00214/mSphere00215-00220.atom.
27. Besser J, Carleton HA, Gerner-Smidt P, Lindsey RL, Trees E. Next-generation sequencing technologies and their application to the study and control of bacterial infections. Clin Microbiol Infect. 2018;24(4):335–41.
28. Perez F, Villegas MV. The role of surveillance systems in confronting the global crisis of antibiotic-resistant bacteria. Curr Opin Infect Dis. 2015;28(4):375–83.
29. O'Brien TF, Stelling J. Integrated multilevel surveillance of the world's infecting microbes and their resistance to antimicrobial agents. Clin Microbiol Rev. 2011;24(2):281–95.
30. Mendelson M, Matsoso MP. The World Health Organization global action plan for antimicrobial resistance. South Afr Med J. 2015;105(5):325.
31. Partridge SR, Kwong SM, Firth N, Jensen SO. Mobile Genetic Elements Associated with Antimicrobial Resistance. Clin Microbiol Rev. 2018;31(4):e00088–17.
32. Loo VG, Poirier L, Miller MA, Oughton M, Libman MD, Michaud S, et al. A predominantly clonal multi-institutional outbreak of Clostridium difficile-associated diarrhea with high morbidity and mortality. N Engl J Med. 2005;353(23):2442–9.
33. Banerjee R, Johnson JR. A new clone sweeps clean: the enigmatic emergence of Escherichia coli sequence type 131. Antimicrob Agents Chemother. 2014;58(9):4997–5004.
34. McDonald LC, Killgore GE, Thompson A, Owens RC Jr, Kazakova SV, Sambol SP, et al. An epidemic, toxin gene-variant strain of Clostridium difficile. N Engl J Med. 2005;353(23):2433–41.
35. Bozdogan B, Bogdanovich T, Kosowska K, Jacobs MR, Appelbaum PC. Macrolide resistance in Streptococcus pneumoniae: clonality and mechanisms of resistance in 24 countries. Curr Drug Targets-Infect Disord. 2004;4(3):169–76.
36. Stefani S, Varaldo P. Epidemiology of methicillin-resistant staphylococci in Europe. Clin Microbiol Infect. 2003;9(12):1179–86.
37. Willems RJ, Top J. Marga van Santen D, Coque TM, Baquero F, Grundmann H, Bonten MJ: Global spread of vancomycin-resistant Enterococcus faecium from distinct nosocomial genetic complex. Emerg Infect Dis. 2005;11(6):821.
38. Orlek A, Stoesser N, Anjum MF, Doumith M, Ellington MJ, Peto T, et al. Plasmid classification in an era of whole-genome sequencing: application in studies of antibiotic resistance epidemiology. Front Microbiol. 2017;8:182.
39. Woodford N, Turton JF, Livermore DM. Multiresistant Gram-negative bacteria: the role of high-risk clones in the dissemination of antibiotic resistance. FEMS Microbiol Rev. 2011;35(5):736–55.
40. Naseer U, Sundsfjord A. The CTX-M conundrum: dissemination of plasmids and Escherichia coli clones. Microb Drug Resist. 2011;17(1):83–97.
41. Stoesser N, Sheppard AE, Pankhurst L, De Maio N, Moore CE, Sebra R, et al. Evolutionary history of the global emergence of the Escherichia coli epidemic clone ST131. mBio. 2016;7(2):e02162.
42. Lemmen S, Lewalter K. Antibiotic stewardship and horizontal infection control are more effective than screening, isolation and eradication. Infection. 2018;46(5):581–90.
43. Banerjee R, Johnston B, Lohse C, Chattopadhyay S, Tchesnokova V, Sokurenko EV, et al. The clonal distribution and diversity of extraintestinal Escherichia coli isolates vary according to patient characteristics. Antimicrob Agents Chemother. 2013;57(12):5912–7.
44. Johnson JR, Thuras P, Johnston BD, Weissman SJ, Limaye AP, Riddell K, et al. The pandemic H 30 subclone of Escherichia coli sequence type 131 is associated with persistent infections and adverse outcomes independent from its multidrug resistance and associations with compromised hosts. Clin Infect Dis. 2016;62(12):1529–36.
45. Martischang R, François P, Cherkaoui A, Gaïa N, Renzi G, Agostinho A, et al. Epidemiology of ESBL-producing Escherichia coli from repeated

prevalence studies over 11 years in a long-term-care facility. Antimicrob Resist Infect Control. 2021;10(1):148.
46. Rodríguez-Villodres Á, Martín-Gandul C, Peñalva G, Guisado-Gil AB, Crespo-Rivas JC, Pachón-Ibáñez ME, et al. Cisneros JM: Prevalence and Risk Factors for Multidrug-Resistant Organisms Colonization in Long-Term Care Facilities Around the World: A Review. Antibiotics (Basel). 2021;10(6):680.
47. Kanamori H, Parobek CM, Juliano JJ, Johnson JR, Johnston BD, Johnson TJ, et al. Genomic analysis of multidrug-resistant Escherichia coli from North Carolina community hospitals: ongoing circulation of CTX-M-producing ST131-H30Rx and ST131-H30R1 strains. Antimicrob Agents Chemother. 2017;61(8):e00912–17.
48. Tschudin-Sutter S, Lucet J-C, Mutters NT, Tacconelli E, Zahar JR, Harbarth S. Contact precautions for preventing nosocomial transmission of extended-spectrum β lactamase–producing Escherichia coli: a point/counterpoint review. Clin Infect Dis. 2017;65(2):342–7.
49. Terveer E, Fallon M, Kraakman M, Ormond A, Fitzpatrick M, Caljouw M, et al. Spread of ESBL-producing Escherichia coli in nursing home residents in Ireland and the Netherlands may reflect infrastructural differences. J Hosp Infect. 2019;103(2):160–4.
50. European Antimicrobial Resistance Surveillance Network [https://ecdc.europa.eu/en/about-us/partnerships-and-networks/disease-and-laboratory-networks/ears-net].
51. Tacconelli E, Carrara E, Savoldi A, Harbarth S, Mendelson M, Monnet DL, et al. Discovery, research, and development of new antibiotics: the WHO priority list of antibiotic-resistant bacteria and tuberculosis. Lancet Infect Dis. 2018;18(3):318–27.
52. Munoz-Price LS, Poirel L, Bonomo RA, Schwaber MJ, Daikos GL, Cormican M, et al. Clinical epidemiology of the global expansion of Klebsiella pneumoniae carbapenemases. Lancet Infect Dis. 2013;13(9):785–96.
53. Cornaglia G. Fighting infections due to multidrug-resistant Gram-positive pathogens: Elsevier; 2009.
54. Sheppard AE, Stoesser N, Wilson DJ, Sebra R, Kasarskis A, Anson LW, et al. Nested Russian doll-like genetic mobility drives rapid dissemination of the carbapenem resistance gene blaKPC. Antimicrob Agents Chemother. 2016;60(6):3767.
55. Boucher HW, Talbot GH, Bradley JS, Edwards JE, Gilbert D, Rice LB, et al. Bad bugs, no drugs: no ESKAPE! An update from the Infectious Diseases Society of America. Clin Infect Dis. 2009;48(1):1–12.
56. Broberg CA, Palacios M, Miller VL. Klebsiella: a long way to go towards understanding this enigmatic jet-setter. F1000Prime Rep. 2014;6:64.
57. Yigit H, Queenan AM, Anderson GJ, Domenech-Sanchez A, Biddle JW, Steward CD, et al. Novel carbapenem-hydrolyzing β-lactamase, KPC-1, from a carbapenem-resistant strain of Klebsiella pneumoniae. Antimicrob Agents Chemother. 2001;45(4):1151.
58. Poirel L, Héritier C, Tolün V, Nordmann P. Emergence of oxacillinase-mediated resistance to imipenem in Klebsiella pneumoniae. Antimicrob Agents Chemother. 2004;48(1):15–22.
59. Yong D, Toleman MA, Giske CG, Cho HS, Sundman K, Lee K, et al. Characterization of a new metallo-β-lactamase gene, bla NDM-1, and a novel erythromycin esterase gene carried on a unique genetic structure in Klebsiella pneumoniae sequence type 14 from India. Antimicrob Agents Chemother. 2009;53(12):5046–54.
60. Cassini A, Högberg LD, Plachouras D, Quattrocchi A, Hoxha A, Simonsen GS, et al. Attributable deaths and disability-adjusted life-years caused by infections with antibiotic-resistant bacteria in the EU and the European Economic Area in 2015: a population-level modelling analysis. Lancet Infect Dis. 2019;19(1):56–66.
61. Borer A, Saidel-Odes L, Riesenberg K, Eskira S, Peled N, Nativ R, et al. Attributable mortality rate for carbapenem-resistant Klebsiella pneumoniae bacteremia. Infect Control Hosp Epidemiol. 2009;30(10):972–6.
62. DeLeo FR, Chen L, Porcella SF, Martens CA, Kobayashi SD, Porter AR, et al. Molecular dissection of the evolution of carbapenem-resistant multilocus sequence type 258 Klebsiella pneumoniae. Proc Natl Acad Sci. 2014;111(13):4988–93.
63. Marimuthu K, Venkatachalam I, Khong WX, Koh TH, Cherng BPZ, Van La M, et al. Clinical and molecular epidemiology of carbapenem-resistant Enterobacteriaceae among adult inpatients in Singapore. Clin Infect Dis. 2017;64(suppl_2):S68–s75.
64. Stoesser N, Phan HTT, Seale AC, Aiken Z, Thomas S, Smith M, et al. Genomic Epidemiology of Complex, Multispecies, Plasmid-Borne bla KPC Carbapenemase in Enterobacterales in the United Kingdom from 2009 to 2014. Antimicrob Agents Chemother. 2020;64(5):e02244–19.
65. Ludden C, Lötsch F, Alm E, Kumar N, Johansson K, Albiger B, et al. Cross-border spread of blaNDM-1-and blaOXA-48-positive Klebsiella pneumoniae: a European collaborative analysis of whole genome sequencing and epidemiological data, 2014 to 2019. Eurosurveillance. 2020;25(20):2000627.
66. Ben-David D, Masarwa S, Adler A, Mishali H, Carmeli Y, Schwaber MJ. A national intervention to prevent the spread of carbapenem-resistant Enterobacteriaceae in Israeli post-acute care hospitals. Infect Control Hosp Epidemiol. 2014;35(7):802–9.
67. Schwaber MJ, Lev B, Israeli A, Solter E, Smollan G, Rubinovitch B, et al. Containment of a country-wide outbreak of carbapenem-resistant Klebsiella pneumoniae in Israeli hospitals via a nationally implemented intervention. Clin Infect Dis. 2011;52(7):848–55.
68. Schwaber MJ, Carmeli Y. An ongoing national intervention to contain the spread of carbapenem-resistant enterobacteriaceae. Clin Infect Dis. 2014;58(5):697–703.
69. Marchaim D, Navon-Venezia S, Schwaber MJ, Carmeli Y. Isolation of imipenem-resistant Enterobacter species: emergence of KPC-2 carbapenemase, molecular characterization, epidemiology, and outcomes. Antimicrob Agents Chemother. 2008;52(4):1413–8.
70. Doumith M, Godbole G, Ashton P, Larkin L, Dallman T, Day M, et al. Detection of the plasmid-mediated mcr-1 gene conferring colistin resistance in human and food isolates of Salmonella enterica and Escherichia coli in England and Wales. J Antimicrob Chemother. 2016;71(8):2300–5.
71. Jung H-J, Littmann ER, Seok R, Leiner IM, Taur Y, Peled J, et al. Genome-wide screening for enteric colonization factors in carbapenem-resistant ST258 Klebsiella pneumoniae. mBio. 2019;10(2):e02663–18.
72. Morris S, Cerceo E. Trends, Epidemiology, and Management of Multi-Drug Resistant Gram-Negative Bacterial Infections in the Hospitalized Setting. Antibiotics (Basel). 2020;9(4):196.
73. Köser CU, Holden MT, Ellington MJ, Cartwright EJ, Brown NM, Ogilvy-Stuart AL, et al. Rapid whole-genome sequencing for investigation of a neonatal MRSA outbreak. N Engl J Med. 2012;366(24):2267–75.
74. Price JR, Golubchik T, Cole K, Wilson DJ, Crook DW, Thwaites GE, et al. Whole-genome sequencing shows that patient-to-patient transmission rarely accounts for acquisition of Staphylococcus aureus in an intensive care unit. Clin Infect Dis. 2014;58(5):609–18.
75. Snitkin ES, Won S, Pirani A, Lapp Z, Weinstein RA, Lolans K, et al. Integrated genomic and interfacility patient-transfer data reveal the transmission pathways of multidrug-resistant Klebsiella pneumoniae in a regional outbreak. Sci Transl Med. 2017;9(417):eaan0093.
76. Coll F, Harrison EM, Toleman MS, Reuter S, Raven KE, Blane B, et al. Longitudinal genomic surveillance of MRSA in the UK reveals transmission patterns in hospitals and the community. *Sci Transl Med*. 2017;9(413).
77. Ferrari C, Corbella M, Gaiarsa S, Comandatore F, Scaltriti E, Bandi C, et al. Multiple Klebsiella pneumoniae KPC clones contribute to an extended hospital outbreak. Front Microbiol. 2019;10:2767.
78. Robinson ER, Walker TM, Pallen MJ. Genomics and outbreak investigation: from sequence to consequence. Genome Med. 2013;5(4):1–9.
79. Walker TM, Ip CL, Harrell RH, Evans JT, Kapatai G, Dedicoat MJ, et al. Whole-genome sequencing to delineate Mycobacterium tuberculosis outbreaks: a retrospective observational study. Lancet Infect Dis. 2013;13(2):137–46.
80. Walker TM, Lalor MK, Broda A, Ortega LS, Morgan M, Parker L, et al. Assessment of Mycobacterium tuberculosis transmission in Oxfordshire, UK, 2007–12, with whole pathogen genome sequences: an observational study. Lancet Respir Med. 2014;2(4):285–92.
81. Gardy JL, Johnston JC, Sui SJH, Cook VJ, Shah L, Brodkin E, et al. Whole-genome sequencing and social-network analysis of a tuberculosis outbreak. N Engl J Med. 2011;364(8):730–9.
82. Turnidge J, Christiansen K. Antibiotic use and resistance--proving the obvious. Lancet (London, England). 2005;365(9459):548–9.
83. Osthoff M, McGuinness SL, Wagen AZ, Eisen DP. Urinary tract infections due to extended-spectrum beta-lactamase-producing Gram-negative bacteria: identification of risk factors and outcome predictors in an Australian tertiary referral hospital. Int J Infect Dis. 2015;34:79–83.
84. Leal HF, Azevedo J, Silva GEO, Amorim AML, de Roma LRC, Arraes ACP, et al. Bloodstream infections caused by multidrug-resistant

gram-negative bacteria: epidemiological, clinical and microbiological features. BMC Infect Dis. 2019;19(1):1–11.
85. Taur Y, Xavier JB, Lipuma L, Ubeda C, Goldberg J, Gobourne A, et al. Intestinal domination and the risk of bacteremia in patients undergoing allogeneic hematopoietic stem cell transplantation. Clin Infect Dis. 2012;55(7):905–14.
86. Bell BG, Schellevis F, Stobberingh E, Goossens H, Pringle M. A systematic review and meta-analysis of the effects of antibiotic consumption on antibiotic resistance. BMC Infect Dis. 2014;14(1):1–25.
87. Hernando-Amado S, Coque TM, Baquero F, Martínez JL. Defining and combating antibiotic resistance from One Health and Global Health perspectives. Nat Microbiol. 2019;4(9):1432–42.
88. Collineau L, Boerlin P, Carson CA, Chapman B, Fazil A, Hetman B, et al. Integrating whole-genome sequencing data into quantitative risk assessment of foodborne antimicrobial resistance: a review of opportunities and challenges. Front Microbiol. 2019;10:1107.
89. Madec J-Y, Haenni M. Antimicrobial resistance plasmid reservoir in food and food-producing animals. Plasmid. 2018;99:72–81.
90. Moura A, Tourdjman M, Leclercq A, Hamelin E, Laurent E, Fredriksen N, et al. Real-time whole-genome sequencing for surveillance of Listeria monocytogenes, France. Emerg Infect Dis. 2017;23(9):1462.
91. Jackson BR, Tarr C, Strain E, Jackson KA, Conrad A, Carleton H, et al. Implementation of nationwide real-time whole-genome sequencing to enhance listeriosis outbreak detection and investigation. Rev Infect Dis. 2016;63(3):380–6.
92. Andino R, Domingo E. Viral quasispecies. Virology. 2015;479-480:46–51.
93. Angus B, Brook G, Awosusi F, Barker G, Boffito M, Das S, Dorrell L, Dixon-Williams E, Hall C, Howe B. (2019 interim update). https://www.bhiva.org/file/DqZbRxfzlYtLg/Monitoring-Guidelines pdf.2019.
94. Baxter JD, Mayers DL, Wentworth DN, Neaton JD, Hoover ML, Winters MA, et al. A randomized study of antiretroviral management based on plasma genotypic antiretroviral resistance testing in patients failing therapy. CPCRA 046 Study Team for the Terry Beirn Community Programs for Clinical Research on AIDS. Aids. 2000;14(9):F83–93.
95. Geretti AM, Paredes R, Kozal MJ. Transmission of HIV drug resistance: lessons from sensitive screening assays. Curr Opin Infect Dis. 2015;28(1):23–30.
96. Van Laethem K, Theys K, Vandamme AM. HIV-1 genotypic drug resistance testing: digging deep, reaching wide? Curr Opin Virol. 2015;14:16–23.
97. Cingolani A, Antinori A, Rizzo MG, Murri R, Ammassari A, Baldini F, et al. Usefulness of monitoring HIV drug resistance and adherence in individuals failing highly active antiretroviral therapy: a randomized study (ARGENTA). Aids. 2002;16(3):369–79.
98. Ávila-Ríos S, Parkin N, Swanstrom R, Paredes R, Shafer R, Ji H, et al. Next-generation sequencing for HIV drug resistance testing: laboratory, clinical, and implementation considerations. Viruses. 2020;12(6):617.
99. Li JZ, Paredes R, Ribaudo HJ, Svarovskaia ES, Metzner KJ, Kozal MJ, et al. Low-frequency HIV-1 drug resistance mutations and risk of NNRTI-based antiretroviral treatment failure: a systematic review and pooled analysis. Jama. 2011;305(13):1327–35.
100. Chakaya J, Khan M, Ntoumi F, Aklillu E, Fatima R, Mwaba P, et al. Global Tuberculosis Report 2020 - Reflections onthe Global TB burden, treatment and prevention efforts. Int J Infect Dis. 2021;113 Suppl 1(Suppl 1):S7–S12.
101. Heyckendorf J, Andres S, Köser CU, Olaru ID, Schön T, Sturegård E, et al. What is resistance? Impact of phenotypic versus molecular drug resistance testing on therapy for multi-and extensively drug-resistant tuberculosis. Antimicrob Agents Chemother. 2018;62(2):e01550–17.
102. Lange C, Aarnoutse R, Chesov D, Van Crevel R, Gillespie SH, Grobbel H-P, et al. Perspective for precision medicine for tuberculosis. Front Immunol. 2020;11:2442.
103. Cabibbe AM, Trovato A, De Filippo MR, Ghodousi A, Rindi L, Garzelli C, et al. Countrywide implementation of whole genome sequencing: an opportunity to improve tuberculosis management, surveillance and contact tracing in low incidence countries. Eur Respir J. 2018;51(6):1800387.
104. Cabibbe AM, Sotgiu G, Izco S, Migliori GB. Genotypic and phenotypic M. tuberculosis resistance: guiding clinicians to prescribe the correct regimens. Eur Respir J. 2017;50(6):1702292.
105. Meehan CJ, Goig GA, Kohl TA, Verboven L, Dippenaar A, Ezewudo M, et al. Whole genome sequencing of Mycobacterium tuberculosis: current standards and open issues. Nat Rev Microbiol. 2019;17(9):533–45.
106. Pankhurst LJ, Del Ojo EC, Votintseva AA, Walker TM, Cole K, Davies J, et al. Rapid, comprehensive, and affordable mycobacterial diagnosis with whole-genome sequencing: a prospective study. Lancet Respir Med. 2016;4(1):49–58.
107. Doyle RM, Burgess C, Williams R, Gorton R, Booth H, Brown J, et al. Direct whole-genome sequencing of sputum accurately identifies drug-resistant mycobacterium tuberculosis faster than MGIT culture sequencing. J Clin Microbiol. 2018;56(8):e00666–18.
108. Allix-Beguec C, Arandjelovic I, Bi L, Beckert P, Bonnet M, Bradley P, et al. Prediction of susceptibility to first-line tuberculosis drugs by DNA sequencing. N Engl J Med. 2018;379(15):1403–15.
109. Trauner A, Liu Q, Via LE, Liu X, Ruan X, Liang L, et al. The within-host population dynamics of Mycobacterium tuberculosis vary with treatment efficacy. Genome Biol. 2017;18(1):1–17.
110. Tomczyk S, Lynfield R, Schaffner W, Reingold A, Miller L, Petit S, et al. Prevention of antibiotic-nonsusceptible invasive pneumococcal disease with the 13-valent pneumococcal conjugate vaccine. Clin Infect Dis. 2016;62(9):1119–25.
111. Hampton LM, Farley MM, Schaffner W, Thomas A, Reingold A, Harrison LH, et al. Prevention of antibiotic-nonsusceptible Streptococcus pneumoniae with conjugate vaccines. J Infect Dis. 2012;205(3):401–11.
112. Jansen KU, Knirsch C, Anderson AS. The role of vaccines in preventing bacterial antimicrobial resistance. Nat Med. 2018;24(1):10–9.
113. Doherty TM, Hausdorff WP, Kristinsson KG. Effect of vaccination on the use of antimicrobial agents: a systematic literature review. Ann Med. 2020;52(6):283–99.
114. Lewnard JA, Lo NC, Arinaminpathy N, Frost I, Laxminarayan R. Childhood vaccines and antibiotic use in low- and middle-income countries. Nature. 2020;581(7806):94–9.
115. Stanaway JD, Reiner RC, Blacker BF, Goldberg EM, Khalil IA, Troeger CE, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis. 2019;19(4):369–81.
116. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill FX, Goodhead I, et al. High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nat Genet. 2008;40(8):987–93.
117. 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.
118. 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.
119. Duy PT, Dongol S, Giri A, Nguyen To NT, Dan Thanh HN, Nhu Quynh NP, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal. JAC-Antimicrobial Resistance. 2020;2(4):dlaa109.
120. Yousafzai MT, Qamar FN, Shakoor S, Saleem K, Lohana H, Karim S, et al. Ceftriaxone-resistant *Salmonella* Typhi outbreak in Hyderabad City of Sindh, Pakistan: high time for the introduction of typhoid conjugate vaccine. Clin Infect Dis. 2019;68(Supplement_1):S16–21.
121. 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.
122. Field Epidemiology & Laboratory Training Program National Institute of Health Islamabad: Weekly field epidemiology report update on extensive drug typhoid fever. 2019;2(18):1–7.
123. Qamar FN, Yousafzai MT, Khaliq A, Karim S, Memon H, Junejo A, et al. Adverse events following immunization with typhoid conjugate vaccine in an outbreak setting in Hyderabad, Pakistan. Vaccine. 2020;38(19):3518–23.
124. Batool R. Story of Lyari. Am J Trop Med Hyg. 2020;102(4):705–6.
125. World Health Organization. Regional Office for the Eastern Mediterranean. The work of WHO in the Eastern Mediterranean Region: annual report of the Regional Director 2019. World Health Organization.

Regional Office for the Eastern Mediterranean. 2020. https://apps.who.int/iris/handle/10665/335963. License: CC BY-NCSA 3.0 IGO.
126. Over 13 million children vaccinated with typhoid conjugate vaccine in Punjab and Islamabad [http://www.emro.who.int/pak/pakistan-news/over-13-million-children-vaccinated-with-typhoid-conjugate-vaccine-in-punjab-and-islamabad.html].
127. Typhoid vaccines. WHO position paper, March 2018–Recommendations. Vaccine. 2019;37(2):214–6.
128. Fitzgerald DM. Bacterial evolution: the road to resistance. Elife. 2019;8:e52092.
129. Croucher NJ, Didelot X. The application of genomics to tracing bacterial pathogen transmission. Curr Opin Microbiol. 2015;23:62–7.
130. Croucher NJ, Harris SR, Fraser C, Quail MA, Burton J, Van Der Linden M, et al. Rapid pneumococcal evolution in response to clinical interventions. Science (New York, NY). 2011;331(6016):430–4.
131. Schürch A, Arredondo-Alonso S, Willems R, Goering R. Whole genome sequencing options for bacterial strain typing and epidemiologic analysis based on single nucleotide polymorphism versus gene-by-gene–based approaches. Clin Microbiol Infect. 2018;24(4):350–4.
132. Coll F, Raven KE, Knight GM, Blane B, Harrison EM, Leek D, et al. Definition of a genetic relatedness cutoff to exclude recent transmission of meticillin-resistant Staphylococcus aureus: a genomic epidemiology analysis. Lancet Microbe. 2020;1(8):e328–35.
133. Campbell F, Cori A, Ferguson N, Jombart T. Bayesian inference of transmission chains using timing of symptoms, pathogen genomes and contact data. PLoS Comput Biol. 2019;15(3):e1006930.
134. De Maio N, Worby CJ, Wilson DJ, Stoesser N. Bayesian reconstruction of transmission within outbreaks using genomic variants. PLoS Comput Biol. 2018;14(4):e1006117.
135. Juraschek K, Borowiak M, Tausch SH, Malorny B, Käsbohrer A, Otani S, et al. Outcome of different sequencing and assembly approaches on the detection of plasmids and localization of antimicrobial resistance genes in commensal Escherichia coli. Microorganisms. 2021;9(3):598.
136. Thomas CM, Nielsen KM. Mechanisms of, and barriers to, horizontal gene transfer between bacteria. Nat Rev Microbiol. 2005;3(9):711–21.
137. Inouye M, Dashnow H, Raven L-A, Schultz MB, Pope BJ, Tomita T, et al. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. 2014;6(11):90.
138. Mardis ER. DNA sequencing technologies: 2006–2016. Nat Protoc. 2017;12(2):213–8.
139. De Maio N, Shaw LP, Hubbard A, George S, Sanderson ND, Swann J, et al. Comparison of long-read sequencing technologies in the hybrid assembly of complex bacterial genomes. Microb Genom. 2019;5(9):e000294.
140. Ashton PM, Nair S, Dallman T, Rubino S, Rabsch W, Mwaigwisya S, et al. MinION nanopore sequencing identifies the position and structure of a bacterial antibiotic resistance island. Nat Biotechnol. 2015;33(3):296–300.
141. Arredondo-Alonso S, van Schaik W, Willems R, Schürch A. On the (im)possibility of reconstructing plasmids from whole-genome short-read sequencing data. Microb Genom. 2017;3(10):e000128.
142. Wick RR, Judd LM, Gorrie CL, Holt KE. Completing bacterial genome assemblies with multiplex MinION sequencing. Microb Genom. 2017;3(10):e000132.
143. George S, Pankhurst L, Hubbard A, Votintseva A, Stoesser N, Sheppard AE, et al. Resolving plasmid structures in Enterobacteriaceae using the MinION nanopore sequencer: assessment of MinION and MinION/Illumina hybrid data assembly approaches. Microb Genom. 2017;3(8):e000118.
144. Peacock SJ, Parkhill J, Brown NM. Changing the paradigm for hospital outbreak detection by leading with genomic surveillance of nosocomial pathogens. Microbiology. 2018;164(10):1213–9.
145. Köser CU, Ellington MJ, Peacock SJ. Whole-genome sequencing to control antimicrobial resistance. Trends Genet. 2014;30(9):401–7.
146. Köser CU, Ellington MJ, Cartwright EJ, Gillespie SH, Brown NM, Farrington M, et al. Routine use of microbial whole genome sequencing in diagnostic and public health microbiology. PLoS Pathog. 2012;8(8):e1002824.
147. Rossen JWA, Friedrich AW, Moran-Gilad J. Practical issues in implementing whole-genome-sequencing in routine diagnostic microbiology. Clin Microbiol Infect. 2018;24(4):355–60.
148. Dymond A, Davies H, Mealing S, Pollit V, Coll F, Brown NM, et al. Genomic Surveillance of Methicillin-resistant Staphylococcus aureus: a mathematical early modeling study of cost-effectiveness. Clin Infect Dis. 2020;70(8):1613–9.
149. Gordon LG, Elliott TM, Forde B, Mitchell B, Russo PL, Paterson DL, et al. Budget impact analysis of routinely using whole-genomic sequencing of six multidrug-resistant bacterial pathogens in Queensland, Australia. BMJ Open. 2021;11(2):e041968.
150. Kumar P, Sundermann AJ, Martin EM, Snyder GM, Marsh JW, Harrison LH, et al. Method for economic evaluation of bacterial whole genome sequencing surveillance compared to standard of care in detecting hospital outbreaks. Clin Infect Dis. 2021;73(1):e9–e18.
151. Elliott TM, Lee XJ, Foeglein A, Harris PN, Gordon LG. A hybrid simulation model approach to examine bacterial genome sequencing during a hospital outbreak. BMC Infect Dis. 2020;20(1):72.
152. Mellmann A, Andersen PS, Bletz S, Friedrich AW, Kohl TA, Lilje B, et al. High interlaboratory reproducibility and accuracy of next-generation-sequencing-based bacterial genotyping in a ring trial. J Clin Microbiol. 2017;55(3):908–13.
153. Dylus D, Pillonel T, Opota O, Wüthrich D, Seth-Smith H, Egli A, et al. NGS-based S. aureus typing and outbreak analysis in clinical microbiology laboratories: lessons learned from a Swiss-wide proficiency test. Front Microbiol. 2020;11:2822.
154. Wielinga PR, Hendriksen RS, Aarestrup FM, Lund O, Smits SL, Koopmans MP, et al. Global microbial identifier. In: Applied genomics of foodborne pathogens: Springer; 2017. p. 13–31.
155. Connor TR, Barker CR, Baker KS, Weill F-X, Talukder KA, Smith AM, et al. Species-wide whole genome sequencing reveals historical global spread and recent local persistence in Shigella flexneri. eLife. 2015;4:e07335.
156. Snell LB, Fisher CL, Taj U, Stirrup O, Merrick B, Alcolea-Medina A, et al. Combined epidemiological and genomic analysis of nosocomial SARS-CoV-2 infection early in the pandemic and the role of unidentified cases in transmission. Clin Microbiol Infect. 2022;28(1):93–100.
157. du Plessis L, McCrone JT, Zarebski AE, Hill V, Ruis C, Gutierrez B, et al. Establishment and lineage dynamics of the SARS-CoV-2 epidemic in the UK. Science (New York, NY). 2021;371(6530):708–12.
158. Okeke IN, Feasey N, Parkhill J, Turner P, Limmathurotsakul D, Georgiou P, et al. Leapfrogging laboratories: the promise and pitfalls of high-tech solutions for antimicrobial resistance surveillance in low-income settings. BMJ Glob Health. 2020;5(12):e003622.

Publisher's Note

Appendix 7. Executive Summary – Approaches to measuring impact of typhoid conjugate vaccine on antimicrobial resistance (prepared for WHO IVIR-AC meeting)

Measuring the Impact of Typhoid Conjugate Vaccines on Antimicrobial Resistance – a Conceptual Framework

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Introduction

The introduction of vaccines can reduce the overall burden of antimicrobial resistance (AMR) through the prevention of infections with drug-resistant pathogens as well as the reduction of antimicrobial use following from lower disease incidence[1]. Developing guidance to help countries introducing vaccines to measure their impact on AMR can help policymakers to understand the full public health value of a given vaccination program. Encouraging vaccine manufacturers to consider inclusion of AMR-related outcomes in clinical trial design can also help make the case for prioritization of vaccine development programs that can have major future public health impact. These types of results can also help make the case for vaccine introduction in the future for policymakers in endemic countries.

Typhoid Conjugate Vaccines (TCVs) provide a timely and relevant use case for developing guidance to demonstrate the potential impact of a vaccination program on AMR. *Salmonella* enterica serovar Typhi (*S*. Typhi), the etiological agent that causes typhoid fever, was responsible for an estimated 10.9 million (95%CI 9.3, 12.6) infections and 116,800 (95%CI 65,4, 187.7) deaths in 2017[2], most of which occurred in low- and middle-income countries (LMICs) with limited access to microbiologically safe drinking water and functional sanitation systems. Antimicrobial resistance poses an increasingly large threat to effective typhoid fever control, particularly in South Asia, where resistance to all oral microbials used to treat typhoid has been reported[3].

Two typhoid conjugate vaccines (TCVs) have been prequalified by the WHO in recent years:

- **Typbar-TCV®** (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Bharat Biotech India Limited, Hyderabad, India, Prequalified December 2017)

- **TYPHIBEV®** (Vi polysaccharide conjugated to CRM_{197} carrier protein, Biological E. Limited, Pune, India, Prequalified December 2020).

In addition, the World Health Organization (WHO) Strategic Advisory Group of Experts (SAGE) has recommended introduction of TCVs in endemic countries as part of their updated guidance on use of typhoid vaccines[4], and Gavi, the Vaccine Alliance has opened a funding window to support TCV introduction in Gavi-eligible countries[5]. Four countries have introduced TCVs into their national immunization programs with support from Gavi, the Vaccine Alliance (Pakistan, Liberia, Zimbabwe, Nepal) and one country has introduced TCV without funding support (Samoa), with several additional countries planning introductions in the coming years. In addition, there is a robust pipeline of typhoid conjugate vaccine candidates in late-stage clinical development.

Given the ongoing introduction planning and vaccine development activities, this is opportune timing to develop guidance targeted to both vaccine manufacturers and public health policymakers about how to measure the impact of vaccines on AMR, beginning with TCVs. In addition, the high burden of typhoid fever in selected settings and the high proportion of drug-resistance, will facilitate the potential measurement of the impact of TCV on AMR.

Potential Approach

One can understand the potential of TCVs to reduce the burden of AMR along two pathways, as alluded to above: 1) through the prevention of drug-resistant *S*. Typhi infections, and 2) through the decreased use of antimicrobials following reduced incidence of febrile episodes requiring antimicrobial intervention, as illustrated in Figure 1 below. The first of these is reliant on the measurement of pathogens, primarily in people, and the second relies on measuring antimicrobial use and/or consumption (at individual patient or aggregate levels). For each pathway, we can consider potential methods to measure impact of AMR in both clinical trial settings, which may be more controlled and better resourced, as well as in public health contexts, where more pragmatic approaches may be necessary, but

in which the sample sizes (of patients, febrile episodes, and courses of antimicrobial therapy) will likely be much higher.

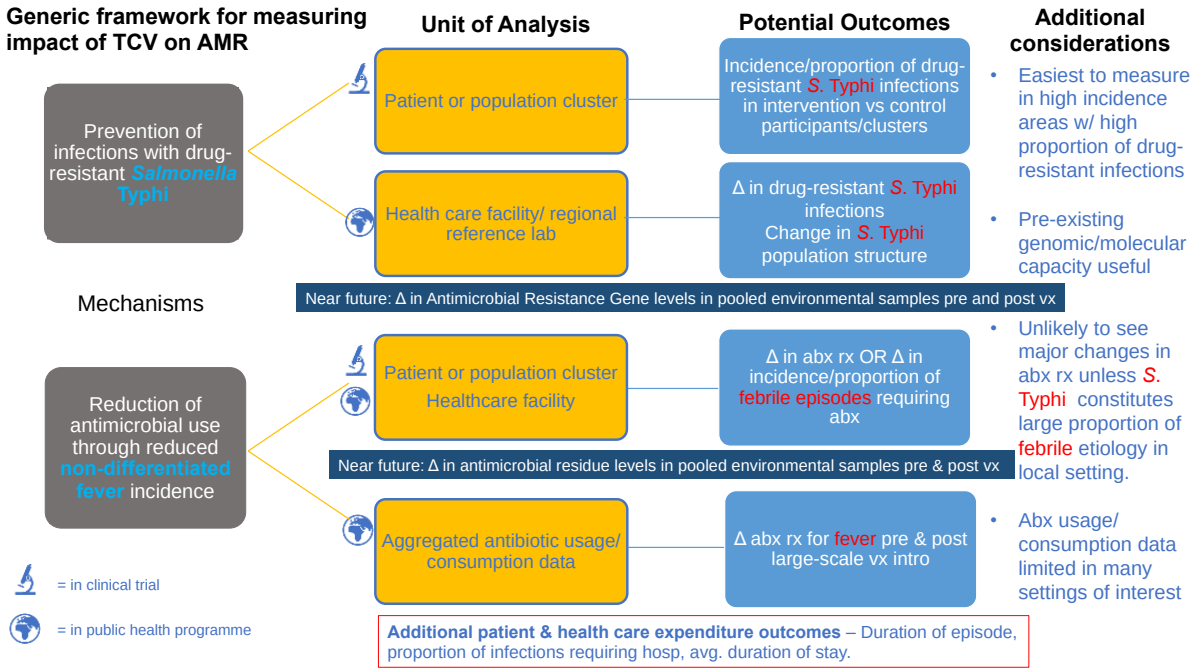

Figure 1. Generic framework for measuring impact of TCV on AMR

In a randomized-controlled clinical trial, it may be possible to compare the incidence of drug-resistant *S*. Typhi infections between intervention (TCV) and control arms at the level of randomization (individual or cluster). In a public health setting, one could measure the change in number of drug-resistant *S*. Typhi infections at the level of a regional reference laboratory or health care facility (comparing numbers of drug-resistant infections before and after vaccination, using a case-control or case-cohort design, stepped wedge, or other method). One could also measure the impact of TCV introduction in a public health context by looking at the impact of vaccination on the *S*. Typhi population structure (before and after vaccination) through genomic analysis to investigate whether there is differential impact of vaccine on different strains or lineages of S. Typhi (particularly drug-resistant strains) and to monitor for the potential emergence of drug-resistant "escape mutants." In the near future, one could attempt to measure changes in antimicrobial resistance genes (ARGs) in pooled environmental samples before and after vaccination. It worth considering that these analyses will be most feasible in settings where *S*. Typhi incidence is high and the proportion of bacteria that are drug-resistant is moderate, so it is possible to distinguish the

impact of vaccine on drug-resistant strains as compared to sensitive ones. These analyses will also be more straightforward to conduct in areas where blood culture capacity and antimicrobial susceptibility testing are already routinely conducted. If attempting any molecular or genomic analysis, pre-existing molecular and genomic capacity would also be very useful.

To measure a change in antimicrobial use in a clinical trial scenario, one could compare the incidence of febrile episodes requiring antibiotic treatment in intervention vs. control clusters or individuals. This analysis could also be undertaken in a public health context at the level of a tertiary care facility. Public health officials may also be able to analyse temporal trends in aggregated antimicrobial use or consumption data from large-scale community surveys like Multiple Indicator Cluster Surveys (MICs) or Demographic and Health Surveys (DHS), as was done in Lewnard et to estimate the potential impact of Pneumococcal conjugate vaccines (PCVs) against 10 and 13 serotypes of *Streptococcus pneumoniae* and live attenuated rotavirus in reduction of antimicrobial use in several LMICs[6]. Measuring changes in antimicrobial residue levels in pooled environmental samples could also be a useful approach in the context of public health programs.

The impact of TCV on patient outcomes and costs-associated with drug-resistant infections should also be considered, and measures like average duration of illness, proportion of infections requiring hospitalization, proportion of infections requiring antimicrobials, and cost-of-illness can be compared between infections caused by drug-resistant and drug-sensitive *S*. Typhi strains to estimate the potential economic impact of reducing AMR through TCV introduction.

Next Steps

A series of expert stakeholder interviews and a literature review will help inform the development of high-level methodological recommendations for several ways that countries introducing TCV could consider measuring its impact on AMR, which will be resource and context dependent. Future directions could include the development of a more detailed, standardized protocol clearly outlining specific methods for measuring impact of TCV on AMR.

References

1. Vekemans J, Hasso-Agopsowicz M, Kang G, et al. Leveraging Vaccines to Reduce Antibiotic Use and Prevent Antimicrobial Resistance: A World Health Organization Action Framework. *Clinical Infectious Diseases*. 2021;73(4):E1011-E1017. doi:10.1093/cid/ciab062
2. Stanaway JD, Parisi A, Sarkar K, et al. The global burden of non-typhoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*. 2019;19(December). doi:10.1016/s1473-3099(19)30418-9
3. Carey ME, Jain R, Yousuf M, et al. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of Salmonella Typhi in Northern India. *Clinical Infectious Diseases*. 2021;72(5):e120-127. doi:10.1093/cid/ciaa1773
4. WHO. Meeting of the Strategic Advisory Group of Experts on immunization, October 2017–conclusions and recommendations. *Weekly Epidemiological Record*. 2017;48:729-748. http://www.who.int/wer2017,92,729-748No48
5. Soble A, Patel Z, Sosler S, Hampton L, Johnson H. Gavi Support for Typhoid Conjugate Vaccines: Moving From Global Investments to Country Introduction. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America*. 2020;71(2):S160-S164. doi:10.1093/cid/ciaa342
6. Lewnard JA, Lo NC, Arinaminpathy N, Frost I, Laxminarayan R. Childhood vaccines and antibiotic use in low- and middle-income countries. *Nature*. 2020;581(7806):94-99. doi:10.1038/s41586-020-2238-4

Appendix 8. WHO TCV Research Agenda Meeting Pre-Reads and Summary (September 2021) (report)

World Health Organization

DEPARTMENT OF IMMUNIZATION, VACCINES AND BIOLOGICALS

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SCOPING MEETING FOR TYPHOID VACCINE RESEARCH AGENDA – Pre-Reading Materials

1. BACKGROUND:

In October 2017, WHO SAGE issued updated policy recommendations for the programmatic use of typhoid vaccines for controlling endemic and epidemic typhoid fever following extensive review of new epidemiological and vaccine performance data. WHO recommended routine programmatic administration of a single dose of typhoid conjugate vaccines (TCVs) for infants and children at 9 months of age, or in the 2nd year of life, and administration via catch-up campaigns targeting children up to 15 years of age where feasible and epidemiologically appropriate. WHO also recommended that TCV introduction be prioritized in settings with the highest burden of typhoid disease or a high burden of antimicrobial resistant *S*. Typhi. Following this recommendation, Gavi, the Vaccine Alliance approved funding support to eligible countries for TCV introduction and WHO prequalified the first TCV (Typbar-TCV®, Bharat Biotech India Ltd, India), in December 2017. A second conjugate vaccine (TYPHIBEV®, BiologicalE, India), was prequalified in 2020. As of 2021, a few endemic countries (Pakistan, Liberia, Zimbabwe) have introduced TCVs into their routine immunization program with Gavi support, with several additional countries planning Gavi-supported introductions. TCV introduction without Gavi support has also been implemented in the Navi Mumbai Municipal Authority of Maharashtra State in India and most recently in Samoa.

Several clinical studies have been conducted by the Typhoid Vaccine Acceleration Consortium (TyVAC), or in conjunction with the above routine introductions, to generate additional evidence on TCV performance (immunogenicity, efficacy/effectiveness, safety, duration of immune response), including co-administration with other routine vaccines and other parameters of TCV use. Effectiveness of TCV use in the context of outbreak response has also been demonstrated in Pakistan and Zimbabwe. All of these studies have been conducted using Typbar-TCV®, and data from these studies will be presented during the consultation. In addition, substantial progress with the clinical development of additional TCV candidate vaccines portends licensure and use of several 2nd generation TCVs in the near term. Several additional studies (planned and ongoing) will generate further relevant evidence on the epidemiology of typhoid fever, including prevalence of drug-resistant *S*. Typhi, and potential impact of vaccine use on AMR.

This brief consultation is being held to review the currently available evidence on TCV performance, including operational aspects of early TCV introductions, and to identify additional research gaps to guide further evidence generation to support policy and implementation of TCV use. It is anticipated that the outcomes of this meeting with a broad range of stakeholders will help inform the timing and scope for development of a broad research agenda and roadmap to accelerate typhoid fever control through vaccination.

2. KEY QUESTIONS TO GUIDE TCV USE:

Text in blue italics indicates where work is planned or ongoing to address the listed question(s).

Note: the question below is out of scope for this meeting (*20 September) but covered under the ongoing WHO Stakeholder Consultation on Enteric Fever Burden of Disease; key highlights from the consultation, including burden-specific research questions will be presented at this meeting.*

What is the burden of disease in the specific setting where TCV is to be introduced?

- Which data are available, and how credible are they considered to be as evidence of burden and risk of typhoid fever? For example:
    - Incidence of disease (modeled or derived from population-based studies), age and geographic distribution of disease, severity/mortality, antimicrobial resistance (AMR) patterns, cost of illness, proportion of blood cultures positive for S. Typhi, presence of non-traumatic ileal perforations without other obvious etiology, risk factor data.
- What novel, low-cost alternatives to blood culture surveillance can be established in in different settings (e.g. low vrs high endemic, different levels of resources), and how can these data be interpreted?
    - *Need for validation of approaches like environmental surveillance & sero-epidemiology (ideally together) in settings of varying endemicity where blood culture surveillance is also being conducted.*

What is the likely impact of introduction of TCV (via routine immunization & /outbreak response)?

- On clinical disease?
    - Efficacy & effectiveness
        - *Efficacy & effectiveness data from variety of settings available/forthcoming for Typbar-TCV® and under discussion for TyphiBEV® but not yet for other TCVs.*
            - What additional data will support country acceptability of/demand for 2nd gen TCVs? What data should inform WHO and regional Technical Advisory Group policy recommendations for use of 2nd gen TCVs?
        - How can impact of TCV (including 2nd generation vaccines) be measured in different settings?
            - Are there less resource-intensive methods for estimating TCV impact (i.e. serosurveys, passive lab-based surveillance, case control, test-negative design), particularly in places without background data and functional surveillance systems?
            - What is impact on non-specific febrile illness (partially accounts for low sensitivity of blood culture)?
            - Is there a need for impact studies during early introductions (mostly Vi-TT) to showcase early benefit, as well as impact studies using 2nd generation TCVs to assure countries of their clinical protection?
    - What is duration of protection (age-specific)?
    - Suitability for use in special populations
        - Safety & immunogenicity in immune compromised individuals, malnourished individuals, pregnant women
    - Will replacement be observed (e.g. Paratyphi A)?
    - What is impact of vaccination on transmission by chronic carriers?

- On AMR?
    - Impact on prevalence of resistance observed in clinical/lab settings? (AST data)
    - Impact on prescribing practices/antibiotic consumption?
    - Impact on prevalence of AMR genes in environmental samples?
    - Impact on bacterial population structures/circulating genotypes? (genomics)
        - *Planned in both Pakistan and Zimbabwe*
- Health economic impact?
    - *Free online tool available to estimate cost-effectiveness of different introduction strategies in different countries*
    - *Cost of illness and cost-effectiveness of TCV campaigns being evaluated in SEFI, SEAP, TyVAC, THECA studies*
- Potential for elimination of disease?
    - How far does widespread introduction of TCV go in terms of reduction of burden of disease at different coverage levels, age groups vaccinated, and different epidemiological and demographic contexts?
        - With or without other interventions (e.g., WaSH, health education)?
        - Will the vaccine work equally well against all AMR variants and genotypes?
            - *Recent data from Pakistan report high effectiveness of Typbar-TCV® against XDR typhoid*
            - *How can elimination be measured/certified (environmental surveillance, sero-epidemiology and/or clinical surveillance)?*
    - What is the burden of chronic carriers in different settings? What screening tools are available to identify carriers?

Which introduction strategy should be used by each country considering TCV introduction?

- Considerations of heterogeneity of disease distribution, budget, feasibility
- What lessons have been learned from previous introductions of TCV that can inform introduction in each country setting? (operational research questions)
    - Can these lessons be extrapolated to different country settings?
- What additional data will inform introduction strategy that informs optimal use of mass vaccination campaigns at time of introduction into routine schedule (also mass campaign vs. introduction into routine immunization only)?

What is the optimal TCV dosing schedule?

- Is a booster dose needed?
    - *Data forthcoming from TyVAC studies on duration of protection & immune response three years after initial dose of Typbar-TCV®*
- If so, what is the optimal timing of a booster?
    - When does efficacy/effectiveness/anti-Vi IgG titer wane?
    - Does protective effect (efficacy and duration) vary by age at initial vaccination?
- Can different TCV products be used interchangeably?
    - Are safety & immunogenicity data sufficient to demonstrate interchangeability?
- Might a heterologous boost confer some additional immunological benefit?

Other important considerations for TCV introduction (not part of research agenda discussion):
Which product should I use?

- Are there differences in vaccine performance (safety, immunogenicity, efficacy/effectiveness) between products?
- What is available supply? Procurement mechanism/financing (if not Gavi-eligible)?
- Are there key differences in delivery profiles (packaging, thermostability, etc.) between products? Are there differences in price? In total systems effectiveness?

3. INFORMATION ABOUT CLINICAL STUDIES UNDER DISCUSSION

Additional information about planned and clinical trials using TCV will be presented during the meeting and will be incorporated into the meeting report.

4. INFORMATION ABOUT KEY PRIORITIES FROM FUNDING ORGANIZATIONS

Additional information about strategic priorities from key funding organizations will be presented during the meeting and will be incorporated into the meeting report.

SCOPING MEETING FOR TYPHOID VACCINE RESEARCH AGENDA – Key Takeaways

- **TCV Performance & Optimal Dosing Schedule**
  - A single dose of Typbar-TCV has been shown to be highly and consistently efficacious and effective in diverse settings (Malawi, Nepal, Bangladesh, Pakistan), including in children younger than 2 years of age
  - TCVs are safe & immunogenic in children as young as 9 months of age across a variety of settings. Immunogenicity results are comparable across settings where Typbar-TCV efficacy has been demonstrated, and non-interference with multiple EPI vaccines across multiple settings has also been demonstrated.
  - Data are expected that will help us understand duration of **immune response** for multiple TCV products/candidates, but this is not the same as duration of **protection** (will have up to three years of duration of protection from the TyVAC trials). It would be great to identify a correlate of protection and this work is ongoing, but it may not be feasible in the short-term.
  - **Additional data on duration of protection and how it varies by age of initial administration are critical to assess need for and timing of booster doses of TCV**.
    - Longer-term immunogenicity data for single dose vs. booster dose are being generated for several TCVs but understanding duration of protection and need for booster dose will require longer-term efficacy/effectiveness evaluations, potentially comparing different dosing schedules.
- **TCV Introduction Decision-Making & Country Demand**
  - While burden of typhoid fever was considered out of scope for this meeting, gaps in our understanding of burden and additional work required to address those gaps were highlighted from the Enteric Fever Burden of Disease consultation and will inform the research agenda. These include validation of new alternative surveillance tools to blood culture (e.g. environmental surveillance, serosurveillance) and the development of frameworks for interpretation of other sources of data (including proportion of blood cultures that are *S*. Typhi positive, AMR data, intestinal perforation data) in support of decision-making around TCV introduction.
    - The point was raised that people living in resource-constrained settings with limited surveillance should not be penalized for this, as this creates greater health inequity, so we should be removing barriers to access to life-saving preventative vaccines. Put another way, capacity for blood culture surveillance is not equitable; therefore, if we base vaccine introductions on blood culture surveillance, then equity will not be achieved.
  - Consistency of immunogenicity results across settings and products notwithstanding, effectiveness data from newer TCVs may be more useful in generating country demand. These data could be generated by conducting post-introduction impact assessments for prequalified TCVs in one of many settings where there is well-established typhoid endemicity.

        - Ideally, such impact assessments would look at impact on AMR as well as clinical disease to help illustrate the full value of a TCV program.
- **<u>Operational Questions</u>**
    - Evaluations of combining TCV campaigns with other vaccination campaigns and/or targeting different age ranges could be useful.
        - Previous recommendations for catch-up campaigns targeting children 9 months to 15 years of age were informed in part by cost-effectiveness analyses. It could be more cost-effective to immunize a broader age group in some settings – this could be driven by local epidemiology (as was the decision to vaccinate 9-month-olds – 45-year-olds in TCV outbreak response campaign in Harare).
    - Given high observed rates of migration and dynamic nature of risk, "risk-based" campaigns may have limited value. This could be partially mitigated by vaccinating more broadly (geographically and/or target age range).

Appendix 9. April 2022 WHO SAGE Background Document – Typhoid (published report)

BACKGROUND PAPER TO SAGE: UPDATE ON TYPHOID VACCINES

Prepared by the WHO/IVB Secretariat

Updated 16 March 2022

Table of Contents

Abbreviations

AMR	Antimicrobial resistance
AST	Antimicrobial susceptibility testing
CRT	Cluster-randomized trial
ELISA	Enzyme-linked immunosorbent assay
EPI	Expanded Programme on Immunization
GMT	Geometric mean titer
LMIC(s)	Low- and middle-income countries
MDR	Multi-drug resistant (in the context of *S*. Typhi, resistant to traditional first-line antibiotics ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole)
RCT	Randomized controlled trial
SEAP	Surveillance of Enteric Fever in Asia Project
STRATAA	Strategic Typhoid Alliance across Africa and Asia
TCV	Typhoid conjugate vaccine
Ty21a	Orally administered typhoid vaccine based on live-attenuated Ty2 *Salmonella* Typhi strain
TyVAC	Typhoid Vaccine Acceleration Consortium
Vi-CRM_{197}	Vi polysaccharide conjugated to CRM_{197} (genetically detoxified form of diphtheria toxin) carrier protein
Vi-DT	Vi polysaccharide conjugated to diphtheria toxoid carrier protein
Vi-PS	Unconjugated Vi polysaccharide vaccine
Vi-rEPA	Vi polysaccharide antigen conjugated to the recombinant exoprotein A of *Pseudomonas aeruginosa* (carrier protein)
Vi-TT	Vi polysaccharide conjugated to tetanus toxoid carrier protein
WASH	Water Sanitation and Hygiene
WGS	Whole genome sequencing
XDR	Extensively-drug resistant (in the context of *S*. Typhi, MDR as defined above and fully resistant to fluoroquinolones and third generation cephalosporins)

1. Executive Summary

Purpose of session

SAGE will be updated on new evidence from prequalified typhoid conjugate vaccines (TCVs) and late-stage vaccine candidates on vaccine efficacy and effectiveness, immunogenicity and safety; data from special populations (e.g., HIV-infected individuals); as well as data on tolerability and non-interference in immune responses with routine EPI vaccines. The data available support the current TCV policy recommendation, and a revision is not being considered at this time. However, SAGE is being requested to make a statement in its report on the new evidence from prequalified TCVs (particularly on vaccine efficacy), to inform country decision making for ongoing TCV introductions.

Background

In October 2017, SAGE issued updated policy recommendations for use of typhoid vaccines in typhoid-endemic countries and the WHO Position Paper for typhoid vaccines was subsequently updated in March 2018.[1] Specifically, this included the first recommendation for the use of TCV in children less than 2 years of age, followed by an open funding window from Gavi, the Vaccine Alliance.[2] There are now two WHO prequalified TCVs, both licensed for use in infants and children from 6 months of age and in adults up to 45 years:

- **Typbar-TCV®** (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Bharat Biotech India Limited, Hyderabad, India, WHO PQ date December 2017), and
- **TYPHIBEV®** (Vi polysaccharide conjugated to CRM_{197} carrier protein, Biological E. Limited, Pune, India, WHO PQ date December 2020).

The 2017 SAGE evidence review was based primarily on immunogenicity data, supported by results of a human challenge trial in immunologically naive adult volunteers (18–60 years of age). There are now new data on Typbar-TCV® from large-scale efficacy studies in Malawi,[3] Nepal,[4] Bangladesh,[5] which show that a single dose of this vaccine is safe, immunogenic, and highly efficacious (81-85%) in preventing symptomatic typhoid fever in children 9 months to 15 years of age over a 18-24 month follow-up period. In addition, Typbar-TCV® was shown to be 95% effective against blood culture confirmed *Salmonella* Typhi (*S*. Typhi) and 97% effective against extensively drug-resistant (XDR) *S*. Typhi in 6 month – 10 year olds in Hyderabad, Pakistan.[6] Additional studies assessing longer-term (≥3 years) effectiveness and potential herd effects of a single dose of Typbar-TCV® are in planning or early execution stages in Ghana, Democratic Republic of Congo, and Fiji, and effectiveness studies

using TYPHIBEV® are being planned in India and Madagascar. Three countries (Pakistan, Liberia, Zimbabwe) have so far introduced Typbar-TCV® into their national immunization programmes.

There is also a rich vaccine development pipeline, with four additional programmes having a candidate in Phase III clinical trials ongoing or completed – **BioTCV** (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, PT Biofarma, Indonesia, WHO PQ submission expected 2025), **EuTYPH-C** (VI polysaccharide conjugated to CRM_{197} carrier protein, EuBiologics, South Korea, PQ submission expected 2023), **SKYTyphoid** (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, SK Bioscience, South Korea, PQ submission expected 2022), and **ZYVAC TCV** (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Zydus Cadila, India, PQ submission expected 2022). There are additional manufacturers with TCVs in clinical development. The diversity of potential TCV manufacturers bodes well for future supply security. However, licensure and prequalification of these vaccines will be based on safety and immunogenicity data (non-inferiority compared to Typbar-TCV®), and manufacturers do not have any current plans to conduct additional studies demonstrating efficacy or effectiveness of their candidate vaccines against clinical disease, which may have an impact on country product preferences.

In addition to the evidence on vaccine performance, there is also new information on operational challenges and lessons learned about the optimal programmatic delivery of TCVs based on early introduction experiences. There are outstanding questions about duration of protection, the potential need for a booster dose, and how this varies by age of initial administration. While data are being generated to address these questions, they will likely need to be deferred to a future SAGE session when additional data will be available to be considered for policy updates. Key outstanding research questions for TCVs are expected to inform the timing of future SAGE policy discussions.

2. Background

The summary below of background epidemiological and vaccine data reflects the evidence that was available to the World Health Organization (WHO) Strategic Advisory Group of Experts (SAGE) for the 2017 typhoid vaccine policy recommendations.

2.1 Epidemiological data

Epidemiological data on the global incidence, age distribution, and geographic distribution of infections caused by *Salmonella* Typhi (*S*. Typhi) were presented to SAGE.[3,7] Modelled estimates suggested that *S.* Typhi was responsible for between 11 and 21 million cases and 148,000-161,000 deaths in 2015.[9,10]

Age-distribution of typhoid fever cases (stratified by month of age) was estimated from primary surveillance data (published and unpublished) based on >10,000 blood culture-confirmed episodes of typhoid fever from 1998 to 2017 in Africa, Asia, and the Americas. Among these cases, 27% occurred in infants and children ages 0-4 years. Within this youngest age group, 29.7% of cases occurred in infants and children <2 years of age. These data suggested that vaccination against typhoid fever in children less than two years of age would have significant public health impact.[7]

While consistently high burden of typhoid fever in South and South-East Asia had previously been described,11–13 new data suggested higher than previously thought incidence rates in parts of sub-Saharan Africa.14 In addition, substantial typhoid fever incidence was demonstrated in rural sites in African and Asian settings, challenging the idea that typhoid fever was primarily a disease of urban slums. Increasingly frequent outbreaks of *S*. Typhi, particularly drug-resistant *S*. Typhi, in Africa and South-East Asia were described.15 Increasing prevalence of antimicrobial resistance (AMR), particularly the international proliferation and spread of the H58 haplotype,16 which is highly associated with multidrug resistance (MDR) and increasingly resistant to fluoroquinolones, and the emergence of extensively drug-resistant (XDR; MDR, fluoroquinolone-resistant, and third-generation cephalosporin-resistant) *S*. Typhi in Hyderabad, Pakistan[16] were also discussed.

2.2 Vaccines

As at the previous SAGE evidence review for policy in October 2017, the most robust evidence for a licensed typhoid conjugate vaccine (TCV) was from immunogenicity data on Typbar-TCV® (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Bharat Biotech India Limited, Hyderabad, India) and efficacy estimates from a Controlled Human Infection Model (CHIM) study in immunologically naïve, healthy adult volunteers in Oxford, England. Vaccine efficacy of Typbar-TCV®

in the CHIM was estimated as 87.1% (95% CI 47.2, 96.9%) against a definition of typhoid fever (fever ≥38˚C lasting for >12 hours followed by blood culture *S*. Typhi infection), and 54.6% (95% CI 26.8, 71.8) efficacious against blood culture-confirmed *S*. Typhi infection, which was the primary endpoint of the study.[17] Several additional TCVs were in early-stage clinical trials.[18]

While no field efficacy data were available for Typbar-TCV® or other TCVs with active clinical development programmes, a large-scale Phase III efficacy study evaluating a previous TCV construct (Vi polysaccharide linked to the recombinant exoprotein A of *Pseudomonas aeruginosa*, or Vi-rEPA) developed by the US National Institutes of Health (NIH) was conducted in Vietnam in the early 2000s. In this study, 1,091 Vietnamese children received two doses of Vi-rEPA vaccine, which provided 91.5% (95% CI 77.1, 96.6) protective efficacy over 27 months of follow-up (per protocol analysis). In addition, among 771 children who only received one dose of Vi-rEPA (intention to treat analysis), 87.7% efficacy was observed.[19] 75 children who received a single dose of Vi-rEPA still had anti-Vi IgG antibody levels above an assumed protective threshold eight years later,[20] strengthening the assertion that conjugate vaccines could confer longer duration of protection than previously licensed polysaccharide vaccines (Vi-PS) or Ty21a vaccines. This vaccine was never commercialized.

2.3 SAGE recommendation

Following SAGE's evidence review (including the geographic and age distribution of typhoid fever, the increasing threat posed by antimicrobial resistance, performance of Vi-PS and Ty21a vaccines as well as TCV, and cost-effectiveness of routine vaccination) and recommendations, WHO issued a revised global policy on typhoid vaccines in March 2018.[1] WHO recommends the introduction of a single dose of TCV for infants and children 6 months of age and over in typhoid-endemic countries, noting that it is likely to be most feasible at existing vaccine visits at 9 months of age or in the second year of life. TCV is recommended as the preferred vaccine against typhoid fever at all ages in view of its improved immunological properties, suitability for use in younger children and expected longer duration of protection. The recommendation further notes that TCV should be prioritized for countries with the highest burden of disease or a high burden of antimicrobial resistant *S.* Typhi and indicates that, for maximal public health impact, catch-up vaccination in children up to 15 years of age should be conducted at the time of introduction routine immunization.

2.4 Scope of April 2022 session

SAGE will be updated on new data on the global burden of typhoid fever (including geographic and age-specific incidence and AMR). SAGE will also be updated on new evidence from prequalified TCVs and late-stage vaccine candidates on vaccine efficacy and effectiveness (including duration of protection), immunogenicity, and safety; data from special populations (e.g., HIV-infected

individuals); as well as data on tolerability and non-interference in immune responses with routine EPI vaccines. The data available support the current TCV policy recommendation, and a revision is not being considered at this time. However, a SAGE statement on the new evidence from prequalified TCVs (particularly on vaccine efficacy), will be valuable to inform country decision-making for ongoing TCV introductions.

3. New Data on Typhoid Disease Burden and Vaccines

Understanding the true global incidence of typhoid fever has long been complicated by lack of sensitive diagnostics and poor global diagnostic availability. Blood culture is only 55% sensitive, depending on volume of blood sampled, and requires substantial resources, time, training, and reagents to execute, so blood culture surveillance has not been established in many parts of the world.[21–23] As such, typhoid fever burden has likely been underestimated, particularly in infants and young children, from whom it is not feasible to draw adequate volumes of blood. Several additional studies (modelled and population-based surveillance studies) have augmented understanding of the age and geographic distribution of typhoid fever, but substantial regional and local data gaps remain. New approaches to surveillance, including environmental surveillance (with detection of *S*. Typhi in sewage and drinking water) and serological surveillance, once validated may provide less resource-intensive, more sustainable approaches for demonstrating community prevalence,.[24–26]

Additional post-licensure TCV data have been generated, primarily through large-scale studies and public health programmes evaluating Typbar-TCV®, which received WHO prequalification in December 2017. These studies have shown that a single dose of this vaccine is safe, immunogenic, and efficacious (81-85%) in preventing symptomatic blood culture-confirmed typhoid fever in infants and children 9 months to 15 years of age over an 18–24-month follow-up period. Questions remain about longer-term duration of protection and the potential need for a booster dose. Additional safety and immunogenicity data have also been generated for TYPHIBEV®, which received WHO prequalification in December 2020. Further data have been generated for additional TCV candidates in clinical development. The pathway to regulatory approval for additional TCVs is through the demonstration of immunogenicity non-inferiority as compared to Typbar-TCV®.[27]

3.1 Epidemiology

Substantial additional epidemiological data have been generated and published since the last SAGE discussion in October 2017 from systematic reviews, modelling studies, and population-based blood culture surveillance studies.

3.1.1 Systematic reviews and modelled estimates

A 2019 review conducted by Marchello et al provides an overview of 33 surveillance studies reporting estimated typhoid fever incidence rates between 1954 and 2018, stratified by method (population-based or those using so-called hybrid surveillance that uses one of several multipliers to account for under-ascertainment of cases at sentinel surveillance sites).[28] High incidence rates were reported in Africa and Asia, but with considerable variation in rates over time (including in the same location over time) and geography, and key regional gaps were noted. Figures 1 and 2 show forest plots of incidence rates from population-based studies conducted in Asia and Africa, while Figure 3 shows incidence rate estimates from multiplier studies from Africa (including from the pivotal Typhoid Surveillance in Africa Project (TSAP).[13]

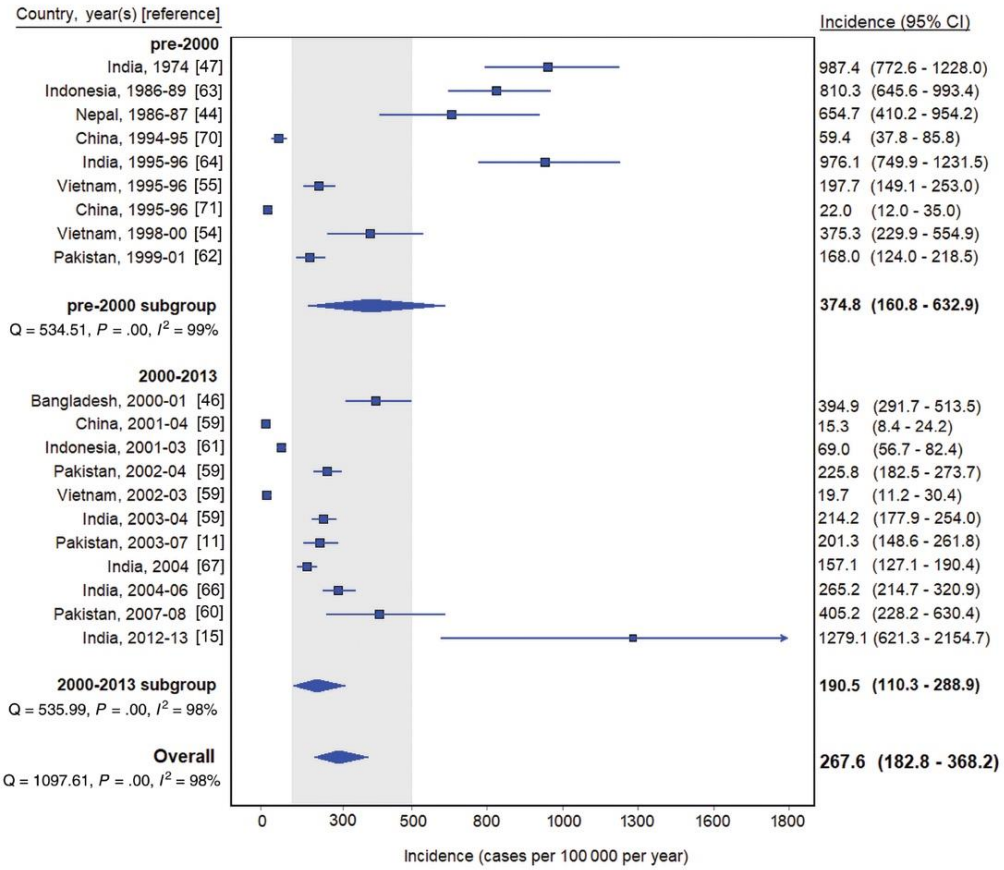


Figure 1. Typhoid incidence estimates among population-based studies in Asia, 1954 -2018. Grey shading indicates 100-500 per 100,000 per year. CI = confidence interval.[28]

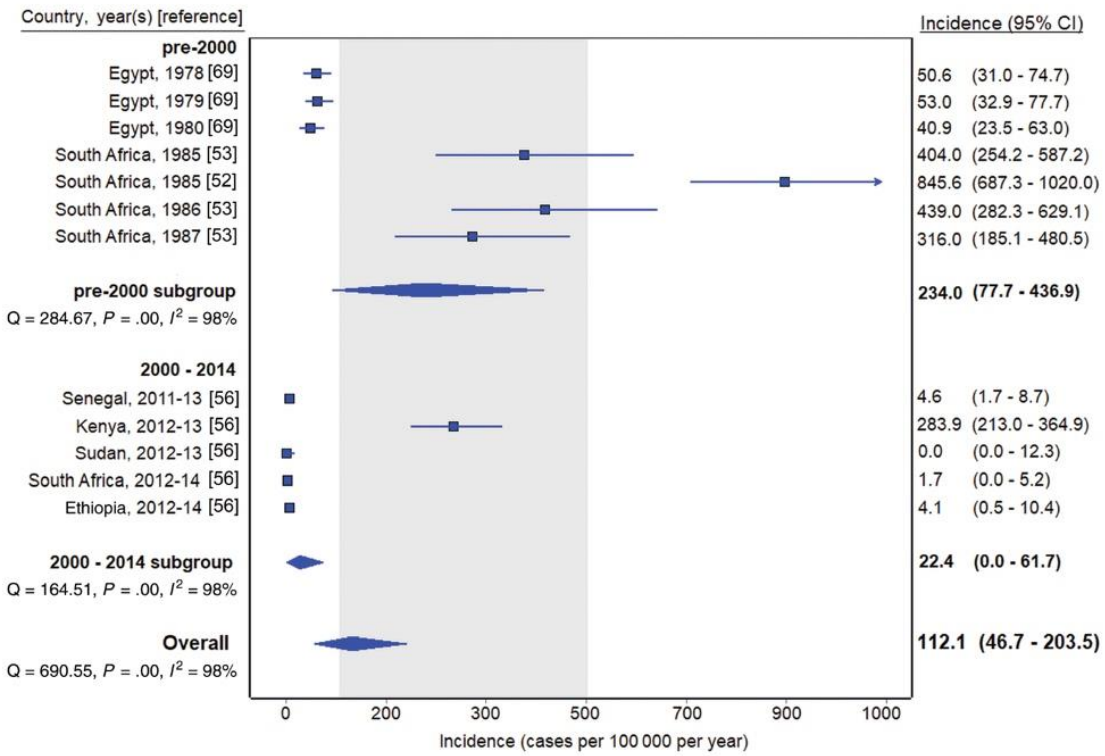


Figure 2. Typhoid incidence estimates among population-based studies in Africa, 1954 -2018. Grey shading indicates 100-500 per 100,000 per year. CI = confidence interval.[28]

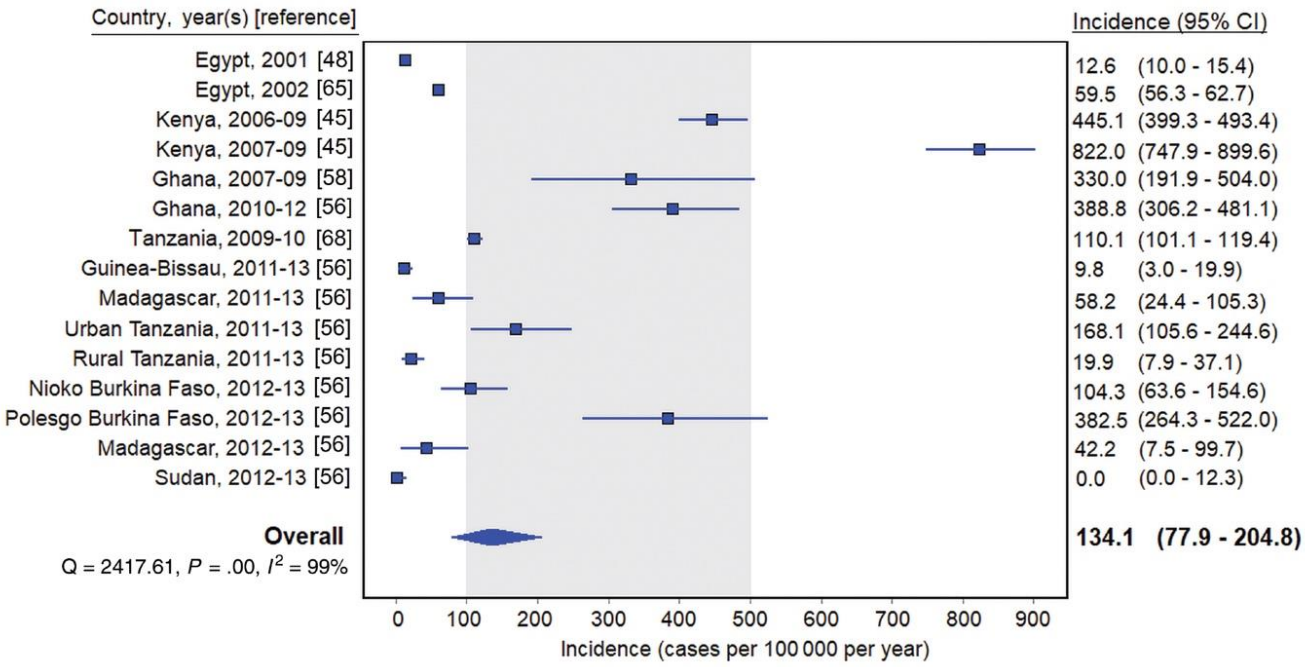


Figure 3. Typhoid incidence estimates among multiplier studies in Africa, 1954 – 2018 (from Marchello et al 2019). Grey shading indicates 100-500 per 100,000 per year. CI = confidence interval.[28]

The Institute for Health Metrics and Evaluation at the University of Washington and collaborators have generated updated modelled estimates of typhoid fever burden as part of the Global Burden of Disease Study 2019. Authors estimated that *S*. Typhi was responsible for 9.24 million (95% CI 5.94, 14.13) cases, 8.05 million (95% CI 3.86, 13.93) Disability-Adjusted Life Years (DALYs), and 110,029 (95% CI 52,810, 191,205) deaths in 2019.[29]

In the previously published GBD 2017 study, authors estimated that South Asia had the highest estimated incidence rate, followed by South-East Asia, western sub-Saharan Africa, eastern sub-Saharan Africa, and Oceania.[30] Authors noted that while overall estimated numbers of cases and deaths were similar to previously published estimates,[31–33] there were significant differences in regional estimates, particularly in Oceania and central sub-Saharan Africa, from which areas data are relatively scarce. The highest incidence of disease was estimated to be in children 5-9 years of age, followed by 10–14-year-olds and 1–4-year-olds (see Figure 4).

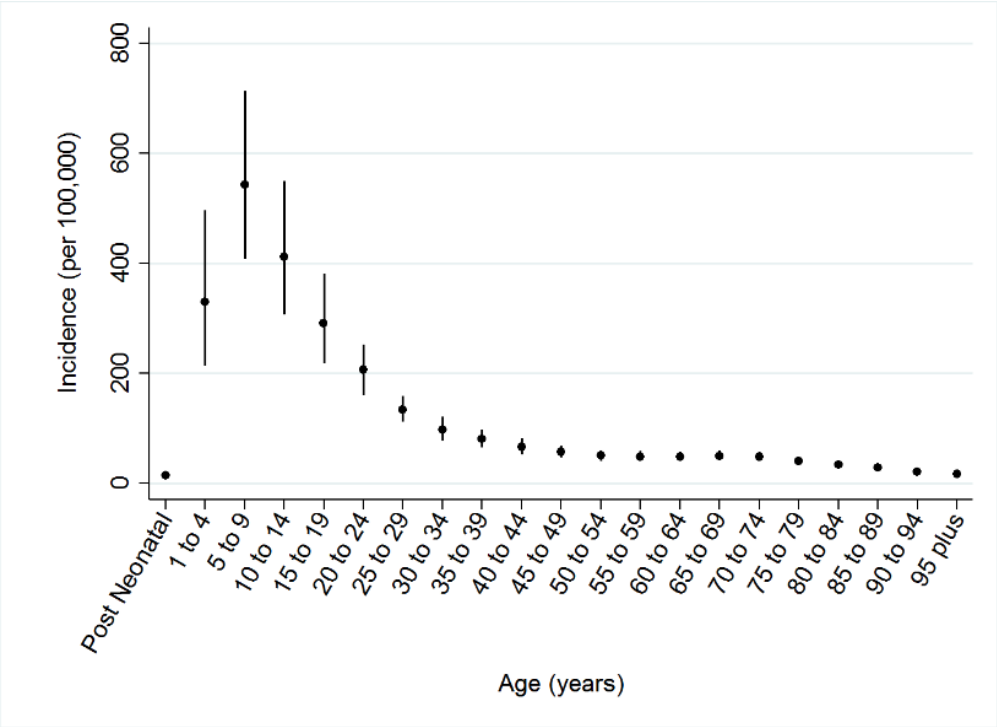


Figure 4. Typhoid fever incidence rates (per 100,000) by age, GBD 2017.[30]

3.1.2 Population-based blood culture surveillance studies

Multiple population-based blood culture studies have been completed during the period following the last SAGE discussion. Published data from two large multi-country studies, with several South Asian sites and one African site reported high overall incidence of typhoid fever, with significant

burden in children less than 4 years of age. This confirms what was described in previously published population-based surveillance studies in Asia and Africa.[10,13]

Surveillance for Enteric Fever in Asia Project (SEAP) , 2016 – 2019

Standardized prospective blood culture surveillance was conducted at five hospitals in pre-defined catchment areas in Dhaka, Bangladesh; Kathmandu and Kavrepalanchok, Nepal; and Karachi, Pakistan. [34] A hybrid surveillance model that combined facility-based blood culture surveillance with healthcare utilization surveys was used to estimate overall and age-specific adjusted incidence rates for *S.* Typhi and *S.* Paratyphi A. Age distribution of disease varied by site (see Appendix 1)[34] but high crude and adjusted incidence rates were observed among young children at all sites, particularly in Bangladesh and Pakistan. Overall adjusted incidence rates of *S*. Typhi by site were:

- Dhaka, Bangladesh: 1,110 cases per 100,000 per-years (p-y) (95% CI: 949, 1,305)
- Kathmandu, Nepal: 330 cases per 100,000 p-y (95% CI: 232, 476)
- Kavrepalanchok, Nepal: 271 cases per 100,000 p-y (95% CI:205, 365)
- Karachi, Pakistan: 195 cases per 100,000 p-y at Aga Khan University Hospital (95% CI:163, 236) and 126 cases per 100,000 p-y at Kharadar General Hospital (95% CI:106, 151).

Strategic Typhoid Alliance Across Africa and Asia (STRATAA), 2016-2018

A prospective multi-component passive febrile illness surveillance study was conducted in three densely populated urban sites in Bangladesh, Nepal, and Malawi (~100,000 each) that were pre-defined by a demographic census. This also included serological surveillance and stool screening for chronic carriers. Overall and age-adjusted incidence rates were estimated for each site (see Table 1).[35]

Ongoing STRATAA surveillance was leveraged to support TCV efficacy studies led by the Typhoid Vaccine Acceleration Consortium (TyVAC). Blood culture-confirmed typhoid incidence rates from the control arms of these studies have been included in published efficacy analyses and are generally higher than the crude incidence reported in the passive surveillance studies at each respective location. (These are described later in this document).

Table 1. Incidence of blood culture-confirmed typhoid fever by site and age (from Meiring et al 2021)

	Blantyre, Malawi			Kathmandu, Nepal			Dhaka, Bangladesh		
	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)	Crude incidence (95% CI)	Adjusted incidence* (95% CrI)	Incidence ratio (adjusted/ observed)
0–4 years	83 (53–124)	632 (398–965)	7·6	72 (33–136)	764 (307–1921)	10·7	417 (337–511)	2625 (1764–4244)	6·3
5–9 years	146 (103–201)	861 (599–1203)	5·9	341 (250–455)	6713 (3085–18 730)	19·7	554 (456–666)	3228 (2276–4757)	5·8
10–14 years	88 (56–132)	602 (377–915)	6·9	191 (128–275)	3750 (1653–10 559)	19·6	268 (203–348)	1564 (1050–2384)	5·8
15–29 years	32 (20–48)	361 (219–567)	11·4	92 (71–119)	1457 (684–3918)	15·8	98 (76–124)	956 (603–1635)	9·8
≥30 years	21 (10–37)	248 (124–447)	12·0	6 (2–13)	92 (29–301)	15·0	29 (19–42)	279 (157–514)	9·7
All ages	58 (48–70)	444 (347–717)	7·7	74 (62–87)	1062 (683–1839)	14·4	161 (145–179)	1135 (898–1480)	7·0

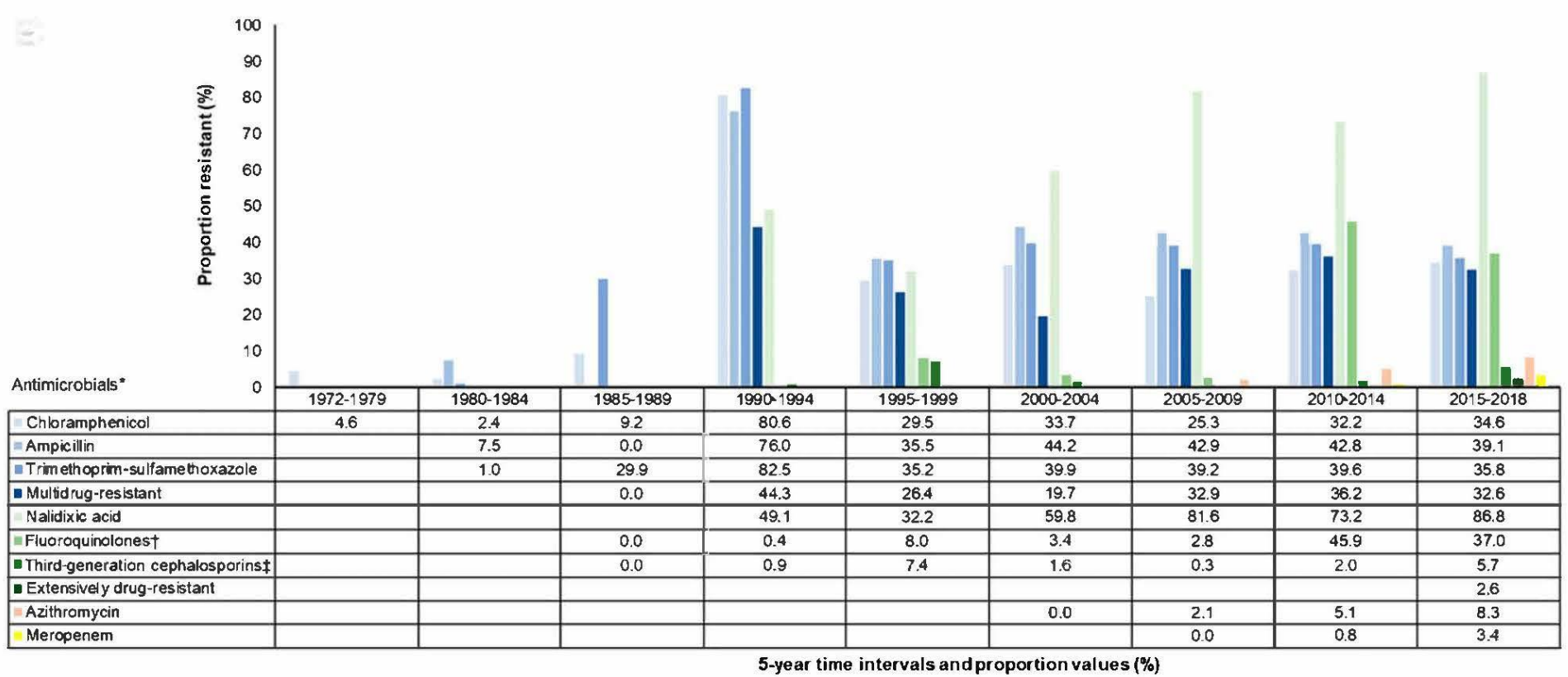
Rates are per 100 000 person-years of observation. CrI=credible interval. *Adjusted for blood-culture sensitivity, probability of receiving a blood culture diagnostic test, and probability of health-care seeking.

3.2 AMR

The increasing prevalence of AMR has long posed a major threat to effective typhoid fever control, beginning with widespread chloramphenicol resistance in the early 1970s.[36] By the late 1980s, multidrug resistance (MDR; resistance to first-line antimicrobials chloramphenicol, trimethoprim–sulfamethoxazole, and ampicillin) had become common, which led to widespread use of fluoroquinolones as first-line therapy in typhoid fever treatment. Decreased fluoroquinolone susceptibility soon emerged and became common, particularly in South and South-East Asia. The rise of fluoroquinolone non-susceptibility led to broader use of azithromycin or third-generation cephalosporins in typhoid fever treatment. Reports of ceftriaxone treatment failure in 2016 in Hyderabad, Pakistan led to the discovery of an extensively drug-resistant (XDR; defined as MDR plus resistance to fluoroquinolones and third-generation cephalosporins) strain of *S.* Typhi (genotype 4.3.1.1.P1), which subsequently spread throughout Pakistan.[37,38] Azithromycin-resistant *S.* Typhi has now been reported in Bangladesh, Pakistan, Nepal, and India.[39–42] There is an urgent need to track the emergence and spread of drug-resistant *S.* Typhi in order to inform empirical treatment guidelines and to inform decisions around use of preventative interventions like TCVs.

3.2.1 Systematic reviews

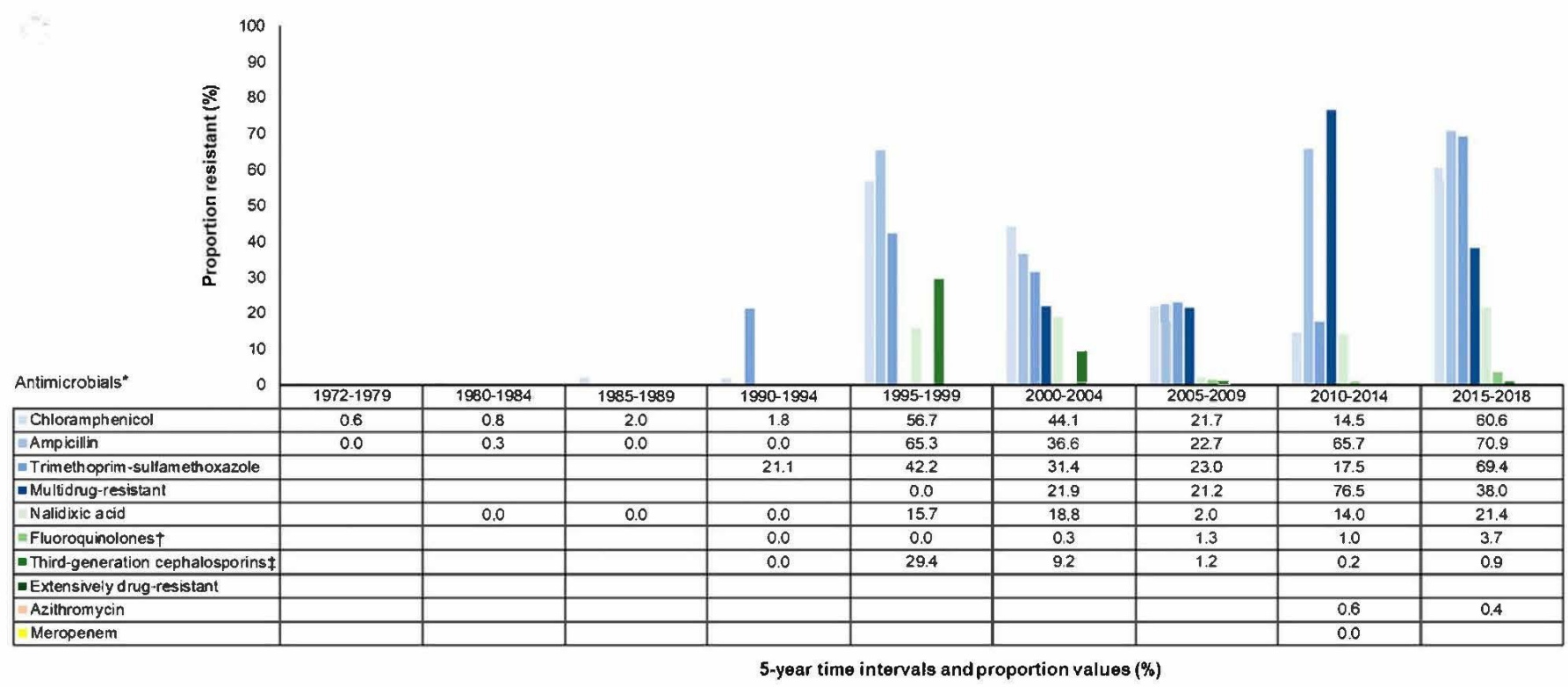
A 2019 systematic review provides an overview of antimicrobial susceptibility testing (AST) data from 1974 – 2018 from published literature.[43] These data show increasing prevalence rates of AMR over time, beginning with resistance to traditional first line antimicrobials, followed by widespread fluoroquinolone non-susceptibility, particularly in South and Southeast Asia, and at lower prevalence levels in Africa (see Figure 5a and 5b). More recently, resistance to third-generation cephalosporins and azithromycin has also emerged in Asia (see Figure 5b below).[43] This includes the XDR *S.* Typhi strain that emerged in Pakistan in late 2016. Phenotypic resistance to carbapenems has also been



Antimicrobials*	1972-1979	1980-1984	1985-1989	1990-1994	1995-1999	2000-2004	2005-2009	2010-2014	2015-2018
Chloramphenicol	4.6	2.4	9.2	80.6	29.5	33.7	25.3	32.2	34.6
Ampicillin		7.5	0.0	76.0	35.5	44.2	42.9	42.8	39.1
Trimethoprim-sulfamethoxazole		1.0	29.9	82.5	35.2	39.9	39.2	39.6	35.8
Multidrug-resistant			0.0	44.3	26.4	19.7	32.9	36.2	32.6
Nalidixic acid				49.1	32.2	59.8	81.6	73.2	86.8
Fluoroquinolones†			0.0	0.4	8.0	3.4	2.8	45.9	37.0
Third-generation cephalosporins‡			0.0	0.9	7.4	1.6	0.3	2.0	5.7
Extensively drug-resistant									2.6
Azithromycin						0.0	2.1	5.1	8.3
Meropenem							0.0	0.8	3.4

5-year time intervals and proportion values (%)

Figure 5a. Antimicrobial resistant *Salmonella* Typhi isolates in Asia, 1972–2018[43]



Antimicrobials*	1972-1979	1980-1984	1985-1989	1990-1994	1995-1999	2000-2004	2005-2009	2010-2014	2015-2018
Chloramphenicol	0.6	0.8	2.0	1.8	56.7	44.1	21.7	14.5	60.6
Ampicillin	0.0	0.3	0.0	0.0	65.3	36.6	22.7	65.7	70.9
Trimethoprim-sulfamethoxazole				21.1	42.2	31.4	23.0	17.5	69.4
Multidrug-resistant					0.0	21.9	21.2	76.5	38.0
Nalidixic acid		0.0	0.0	0.0	15.7	18.8	2.0	14.0	21.4
Fluoroquinolones†				0.0	0.0	0.3	1.3	1.0	3.7
Third-generation cephalosporins‡				0.0	29.4	9.2	1.2	0.2	0.9
Extensively drug-resistant									
Azithromycin								0.6	0.4
Meropenem								0.0	

Figure 5b. Antimicrobial resistant *Salmonella* Typhi isolates in Africa, 1972–2018[43]

reported from one study in Pakistan and one study in Indonesia,[44,45] but these results have not been confirmed with additional testing.

Murray and colleagues from the Global Research on Antimicrobial Resistance (GRAM) Project developed a model to estimate the effect of AMR on disease incidence, mortality, duration of stay, and healthcare costs for 88 pathogen-antimicrobial combinations in 204 countries and territories. The authors estimated that there were 4.95 million deaths (95% CI: 3.62, 6.57) associated with bacterial AMR, including 1.27 million deaths (95% CI: 911,000, 1,710,000) attributable to bacterial AMR in 2019.[46] Of the 23 pathogens evaluated, *S*. Typhi was estimated to be the 11th highest in terms of global deaths attributable to and associated with bacterial AMR.

3.2.2 Whole genome sequencing data

Broader generation and analysis of whole genome sequencing (WGS) data has facilitated investigation of molecular mechanisms of resistance, the development of a phylogenetic classification scheme (GenoTyphi)[47,48], and information about key international transmission events as well as *de novo* AMR emergence events. This includes identification of the emergence of H58 (Haplotype 58) *S*. Typhi in South Asia and its rapid intercontinental spread throughout Asia, to East and Southern Africa, Oceania, and Latin America,[15,49–54] as well as describing the emergence and molecular mechanism of XDR *S*. Typhi in Pakistan.[37] Phylogenetic analysis also facilitated the identification of a molecular mechanism for azithromycin resistance[55], which was first observed among *S*. Typhi isolates in Bangladesh, and subsequently detected in Pakistan, Nepal, and India.[40,42,56]

The Global Typhoid Genomics Consortium (https://www.typhoidgenomics.org) was established in 2021 with a mandate to collaborate with the broader international typhoid research community to aggregate as much *S*. Typhi WGS data and standardized metadata as possible, and to facilitate the analysis and visualization of those data to inform public health policy. Analysis to be published soon will provide a contemporary view of global typhoid genome diversity and AMR frequency and distribution based on 12,951 isolates from 83 countries. These data demonstrate increasing overall prevalence of AMR, particularly fluoroquinolone non-susceptibility, with high prevalence of multiple AMR phenotypes in several South Asian countries.

4. New Vaccine Data

4.1 Typbar-TCV®

Typbar-TCV® has been evaluated in three large-scale post-licensure efficacy/effectiveness studies in Bangladesh, Malawi, and Nepal by the Typhoid Vaccine Acceleration Consortium (TyVAC), which is led by the Center for Vaccine Development and Global Health at the University of Maryland School of Medicine, the Oxford Vaccine Group at the University of Oxford, and PATH.[3–5] TyVAC investigators also conducted an immunogenicity and co-administration study in infants and toddlers in Burkina Faso.[57] The results of these studies are described in additional detail below.

In addition to the TyVAC studies, Typbar-TCV® effectiveness has been evaluated in the context of public health programmes in Navi Mumbai, India;[58] in Hyderabad[59] and Karachi,[60]Pakistan; and in Harare, Zimbabwe (results not yet available). The results of these studies are summarized in Table 2 and described in additional detail in the subsequent sections.

Table 2. Protective efficacy/effectiveness of a single TCV dose (Typbar-TCV®) against primary endpoint of blood culture confirmed typhoid fever

Study, Design	**Age (# vaccinated)**	**Control vaccine**	**Follow up**	**Vaccine efficacy or effectveness (95% CI)**
TyVAC TRIALS				
Nepal, Individually randomized[4]	9 mths – 16 yrs (20,019)	Group A meningitis	24 months	79.1% (62.0, 88.5)
Malawi, Individually randomized[3]	9 mths – 12 yrs (28,130)	Group A meningitis	18-24 months	83.7% (68.1, 91.6)
Bangladesh, Cluster randomized[5]	9 mths – 16 yrs (67,395)	SA-14-14-2 JE	24 months	85.0% (76, 91)
ADDITIONAL STUDIES				
Navi Mumbai, India (routine immunization), Case-control[61]	9 mths – 14 yrs (160,000)	None	15 months	80.2% (53.2, 91.6)
Karachi, Pakistan (outbreak response campaign) Case control[60]	6 mths – 15 yrs (87,993)	None	4 months	72% (34, 88)

Hyderabad, Pakistan (outbreak response campaign) Cohort study[59]	6 mths – 10 yrs (207,000)	None	18 months	95% (93, 96)

4.1.1 Efficacy & effectiveness data – TyVAC studies

One of the major bodies of work undertaken by TyVAC was to demonstrate the impact of TCVs in typhoid-endemic areas through two individually randomized, controlled efficacy studies conducted in Nepal and Malawi, and one controlled cluster-randomized effectiveness trial conducted in Bangladesh. High-level results are described below.

Nepal

A phase 3, double-blinded, individually randomized efficacy study was conducted in Lalitpur, Nepal, in which 20,019 children were randomized to receive either a single dose of TCV or MenA and were followed for two years. During that time, 62 cases of typhoid fever were recorded in the MenA group (incidence rate of 342 cases [95% CI 262, 438] per 100,000 person years), and 13 cases in the TCV group (incidence rate of 72 [95% CI 38, 132] cases per 100,000 person-years).[4] The protective efficacy of TCV against blood culture-confirmed typhoid fever after two years was 79% (95% CI 61.9, 88.5). A subgroup analysis showing age, sex, and time-dependent specific efficacy estimates is included below (Table 3).

Table 3. Protective efficacy of TCV against blood culture-confirmed typhoid fever in Nepal[4]

	Typhoid conjugate vaccine group (n=10 005)	Incidence per 100 000 person-years* (95% CI)	Group A meningococcal vaccine group (n=10 014)	Incidence per 100 000 person-years (95% CI)	Vaccine efficacy (95% CI)	p value (interaction)
Age, years	..	..	..	..	..	0·13†
<2	2	167 (20–604)	0	..	..	..
2 to <5	5	127 (41–296)	14	362 (198–607)	64·9% (2·5–87·3)	..
≥5	6	46 (17–100)	47	369 (272–489)	87·5% (70·8–94·5%)	..
Sex	..	..	..	..	..	0·12
Male	5	54 (18–126)	39	417 (297–570)	87·0% (67·1–94·9)	..
Female	8	90 (39–180)	23	262 (166–392)	65·6% (23·1–84·6)	..
Time since vaccination, months	..	..	..	..	..	0·43
≤12	6	61 (23–134)	36	370 (259–512)	83·4% (60·5–93·0)	..
>12	7	83 (34–172)	26	310 (202–454)	73·0% (37·9–88·3%)	..

Data are n (%) unless stated otherwise. *Blood culture confirmed typhoid fever after 14 days. †p value for interaction inclusive of 2 to <5 years and 5 years and older.

Malawi

A phase 3, double-blinded, individually randomized trial was conducted in Blantyre, Malawi, to assess the efficacy of TCV against blood culture confirmed typhoid fever. Children ages 9 months to 12 years of age were randomized to receive either a single dose of Typbar-TCV® or meningococcal capsule group A conjugate (MenA) and followed for 24 months. The intention to treat analysis included 28,130 children, (14,069 received TCV, 14,061 received MenA)[3]. 12 cases of blood culture-confirmed typhoid fever occurred among TCV recipients (46.9 cases/100,000 person-years) and 62 cases occurred among MenA recipients (243.2 cases/100,000 person-years). The overall efficacy 83.7% (95% CI 68.1, 91.6) in the per-protocol analysis. Age-specific efficacy (<5 years or >5 years) was also estimated in both the intention-to-treat and per-protocol analysis groups (see Table 4). This study remains blinded through 4 years of follow-up, and additional efficacy data are anticipated in the 4th quarter 2022.

Table 4. Blood culture-confirmed typhoid fever and vaccine efficacy in Malawi[3]

Variable	Vi-TCV			MenA Vaccine			Protective Efficacy of Vi-TCV (95% CI)	Absolute Risk Reduction (95% CI)*	Number Needed to Vaccinate (95% CI)†
	Children (total follow-up time)	Cases of Typhoid	Incidence of Typhoid	Children (total follow-up time)	Cases of Typhoid	Incidence of Typhoid			
	no. (person-yr)	*no.*	*cases/100,000 person-yr (95% CI)*	*no. (person-yr)*	*no.*	*cases/100,000 person-yr (95% CI)*	*percent*	*cases/1000 children*	
Intention-to-treat population‡	14,069 (25,577)	12	46.9 (24.2–82.0)	14,061 (25,493)	62	243.2 (186.5–311.8)	80.7 (64.2–89.6)§	3.6 (2.4–4.8)§	277.8 (208.3–416.7)
Age <5 yr	5,058 (9086)	5	55.0 (17.9–128.4)	5,179 (9305)	20	215.0 (131.3–332.0)	74.4 (31.7–90.4)	2.9 (1.0–4.8)	344.8 (208.3–1000.0)
Age ≥5 yr	9,011 (16,491)	7	42.5 (17.1–87.5)	8,882 (16,188)	42	259.5 (187.0–350.7)	83.7 (63.6–92.7)	4.0 (2.4–5.5)	250.0 (181.8–416.7)
Per-protocol population¶	13,945 (25,323)	10	39.5 (18.9–72.6)	13,937 (25,239)	61	241.7 (184.9–310.5)	83.7 (68.1–91.6)§	3.7 (2.5–4.8)§	270.3 (208.3–400.0)
Age <5 yr	5,044 (9057)	5	55.2 (17.9–128.8)	5,158 (9261)	20	216.0 (131.9–333.5)	74.4 (31.8–90.4)	2.9 (1.0–4.8)	344.8 (208.3–1000.0)
Age ≥5 yr	8,901 (16,267)	5	30.7 (10.0–71.7)	8,779 (15,978)	41	256.6 (184.1–348.1)	88.0 (69.7–95.3)	4.1 (2.6–5.6)	243.9 (178.6–384.6)

* The absolute risk reduction (the risk in the MenA group minus the risk in the Vi-TCV group) is the total reduction in the risk of blood culture–confirmed typhoid fever that resulted from vaccination with Vi-TCV.
† The number needed to vaccinate is the number of children that would be needed to be vaccinated to prevent one case of blood culture–confirmed typhoid fever.
‡ Shown are data in the intention-to-treat population from the time of randomization.
§ P<0.001.
¶ Shown are data in the per-protocol population beginning 14 days after randomization.

Bangladesh

In a double-blinded cluster randomized trial conducted in an urban, typhoid-endemic setting in Dhaka, Bangladesh, 150 geographically distinct population clusters were defined (each with ±1350 residents) and randomly assigned to receive either TCV (Typbar-TCV®, Vi-TT) or SA 14-14-2 Japanese encephalitis (JE) vaccine.[5] Children 9 months to <16 years of age received a single dose of parenteral vaccine (vaccine determined by cluster of residence) and were followed for an average of 17.1

months. 41,344 children were vaccinated April-May 2018, and an additional 20,412 children were vaccinated during catch-up campaigns September-December 2018 and April-May 2019. The incidence of typhoid fever was 635 cases per 100,000 person-years in JE recipients and 96 in TCV recipients. Thus, total protection (comparison of incidence of typhoid fever in TCV recipients to incidence of typhoid fever among JE vaccine recipients) was 85% (95% CI 67, 91); vaccine effectiveness varied by age of participant (see Table 5). Indirect vaccine protection was evaluated by comparing the incidence of typhoid fever among non-recipients of TCV in the TCV clusters to that of non-recipients of JE vaccine in the JE clusters. No significant indirect protection conferred by TCV was observed; however, the authors noted that the trial was not adequately powered to evaluate the assumed level (20%) of herd protection and noted that the boundaries of the predefined clusters were unlikely to represent boundaries of short-cycle transmission of typhoid given the densely populated nature of the study area, which may have diluted estimated herd protection. Overall protection was also assessed through the comparison of typhoid fever incidence among all residents of the TCV clusters to that of all residents of the JE clusters. Age-specific indirect, total, and overall protection estimates are listed in Table 5.

Table 5. Incidence of blood culture-confirmed typhoid fever and protective effectiveness of TCV by age group in Bangladesh[5]

	Events/person-years†		Incidence, per 100 000 person-years		Protective effectiveness	p value	p value for interaction
	SA 14-14-2 group	Vi-TT group	SA 14-14-2 group	Vi-TT group			
Total vaccine protection							
9 months to <2 years	23/2804	4/2800	820 (520 to 1231)	143 (39 to 366)	81% (39 to 94)	0·0052	0·49
2 to 4 years	62/6413	12/6173	967 (741 to 1239)	194 (100 to 340)	80% (62 to 89)	<0·0001	..
5 to <16 years†	107/21 037	13/21 375	509 (417 to 615)	61 (32 to 104)	88% (78 to 93)	<0·0001	..
Overall vaccine protection							
<2 years	35/7779	13/7861	450 (313 to 626)	165 (88 to 283)	63% (20 to 83)	0·011	0·056
2 to 4 years	86/9295	34/9041	925 (740 to 1143)	376 (260 to 526)	59% (40 to 73)	<0·0001	..
5 to <16 years	141/32 316	50/32 462	436 (367 to 515)	154 (114 to 203)	65% (50 to 75)	<0·0001	..
≥16 years†	69/106 069	47/105 085	65 (51 to 82)	45 (33 to 59)	33% (−2 to 55)	0·061	..
Indirect vaccine protection							
<2 years	12/4846	8/4913	248 (128 to 433)	163 (70 to 321)	32% (−127 to 80)	0·53	0·38
2 to 4 years	24/2884	23/2880	832 (533 to 1238)	799 (506 to 1198)	6% (−78 to 51)	0·84	..
5 to <16 years	34/11 415	37/11 227	298 (206 to 416)	330 (232 to 454)	−13% (80 to 29)	0·60	..
≥16 years†	69/106 061	47/105 081	65 (51 to 82)	45 (33 to 59)	33% (−2 to 55)	0·060	..

Data are incidence rate/person-years, n (95% CI), or % (95% CI). SA 14-14-2=Japanese encephalitis vaccine. Vi-TT=Vi-tetanus toxoid conjugate vaccine. *Age at vaccination for the subgroup analyses of total vaccine protection, and age at date of residence for that of overall and indirect vaccine protection analyses; protective effectiveness, p values and CIs were adjusted for the stratifying variables for randomisation, including geographical ward, distance to study clinics, number of eligible children at baseline, and other baseline covariates prespecified in the statistical analysis plan, including age, sex, toilet type in the house, drinking water source, treatment of drinking water, handwashing before meals, and handwashing after defecation. †11 vaccinees were ≥16 years at vaccination and were included in the total vaccination analysis of the 5 to <16 years age group, resulting in a 12 person-years difference between overall and indirect vaccine protection analysis of the ≥16 years age group.

4.1.2 Immunogenicity data

Long-term immunogenicity of Typbar-TCV (7 years post-primary vaccination in boosted vs non-boosted participants)

Mohan et al reported duration of immune responses 7 years after primary vaccination in children who received either a single dose of Typbar-TCV® or a single dose plus booster at day 720. Serum IgG anti-Vi titres were measured at 3, 5, and 7 years after primary vaccination using three different enzyme-linked immunosorbent assays (ELISAs).[1]

Four subgroups were compared:

1. among boosted participants, an "All Specimens Cohort" (ASC), which included 86 children who provided sera at each follow-up time point (days 0, 42, 720 [booster], 762 [42 days post-booster], 1095, 1825, and 2555);
2. among non-boosted participants, an All Specimens Cohort, which included 25 children who provided sera at all follow-up points (days 0, 42, 720, 1095, 1825, 2555);
3. a boosted "Any Available Specimen" (AAS) subgroup, which included children who received a booster and provided sera on days 0, 42, and 720, and one or more (but not all) of the other time points (days 762, 1095, 1825, or 2555); and
4. an Any Available Specimen subgroup of 47 children who had not received a booster and who provided sera on days 0 and 42, 41 of whom also contributed sera at one or more additional timepoints (days 1095, 1825, 2555).

GMTs as measured using the commercial Vacczyme ELISA kit increased significantly among boosted ASC children (n=86) at day 762, and were 32-fold, 14-fold, and 10-folder higher than baseline levels at 3, 5, and 7 years after primary vaccination.[62] In unboosted ASC children (n=25), GMTs remained 21-fold, 14-fold, and 10-fold over baseline at 3, 5, and 7 years following primary vaccination. Post primary vaccination, 72% and 44% of unboosted ASC participants showed persistent seroconversion as measured by Vacczyme™ at 5 and 7 years, as compared to 84% and 71% of boosted ASC participants. Results were also compared between ASC participants who received initial vaccination at 6-11 months of age vs 12-23 months of age (see Appendix 2 for details of GMT analysis). The authors suggested that primary immunization could be followed by a booster vaccination after five

[1] The three ELISA assays used were the Vacczyme™ ELISA kit, US NIH ELISA ("Szu" method), and the National Institute of Biological Standards (NIBSC) ELISA. Persistent seroconversion (defined as serum anti-Vi IgG levels remaining ≥ 4-fold above baseline), geometric mean titer (GMT), geometric mean-fold rise post-vaccination, and percent exhibiting putative protective anti-Vi level (≥2 μg_{Szu}/ml) using the Szu method and National Institutes of Health IgG reference standard were evaluated.

years to maintain protection against clinical infection. Authors also noted that there was evidence of "natural" boosting caused by exposure to *S*. Typhi, as both boosted and non-boosted children showed increased levels of anti-Vi IgG between days 720 and 1095. The authors point to reports and published literature that suggest that typhoid fever was highly prevalent during the time of the study.[63,64]

Immunogenicity studies – TyVAC studies

An immunogenicity and non-interference study was conducted in infants and young children in Burkina Faso. Age-stratified immunogenicity sub-studies were embedded in the TyVAC trials in Nepal (n=1500), Malawi (n=600), and Bangladesh (n=1500). These studies are summarized in Table 6. (Additional site-specific details are provided in Appendix 3).

Table 6. Immunogenicity and co-administration studies for Typbar-TCV conducted by TyVAC

Immunogenicity and co-administration studies for Typbar-TCV conducted by TyVAC														
Country	**Design**	**Control vaccine**	**Study period**	**Ages**	**TCV (Number)**	**Control (Number)**	**Co-admin-istered vaccines**	**Postvaccination Vi GMT (EU/ml), 28 days (95% CI)[a]**				**Rate of Seroconversion (%) (95% CI)[b]**	
								TCV or other test vaccine		**Control**		**TCV**	**Control**
								Pre	**Post**	**Pre**	**Post**		
Burkina Faso[65]	Individually randomized	Inactivated poliovirus	Nov 2018-Aug 2019	9-11 mos	49	51	MR, YF	8.9 (5.9-13.6)	1204 (747-1940)	8.5 (5.6-12.7)	8.9 (6.1-13.1)	87.8 (75.2-95.4)	8.0 (2.2-19.2)
Burkina Faso[66]	Individually randomized	Inactivated poliovirus	Nov 2018-Aug 2019	15-23 mos	99	51	MenAfriVac, MR	4.9 (3.9-6.1)	3231 (2085-5078)	4.8 (3.8-6.2)	5.3 (4.1-6.9)	94.8 (84.4-99.1)	3.9 (0.5-13.5)
Nepal[4,c]	Individually randomized	Meningococcal serogroup A conjugate	Nov 2017-Mar 2020	< 5 years	55	46	-	4.6 (3.8-5.6)	1469 (1104-1955)	4.6 (3.8-5.5)	4.3 (3.8-4.9)	98.2	0.0
				5-<10 yrs	225	116		4.9 (4.5-5.4)	2113 (1890-2363)	4.4 (4.0-4.9)	5.4 (4.3-6.8)	100.0	4.3
				≥ 10 yrs	403	218		10.6 (9.4-12.1)	2133 (1947-2336)	8.7 (7.4-10.2)	8.9 (7.5-10.5)	98.8	1.4
Bangladesh [68c]	Cluster randomized	Live-attenuated JE	Feb 2018-Oct 2019	9 mos - <16 yrs	1010	505	-	3.7 (3.7-3.7)	3222 (1757-5472)	3.7 (3.7-3.7)	3.7 (3.7-3.7)	99.6	1.7
Malawi[67]	Individually randomized	Meningococcal serogroup A conjugate	Feb 2018-Apr 2021	9-11 mos	106	94	MR	3.9 (3.7-4.1)	2685 (2165-3330)	4.0 (3.7-4.4)	4.1 (3.7-4.4)	98.9 (94.0-100.0)	0.0 (0.0-4.7)
				1-5 yrs	99	102	-	4.2 (3.8-4.7)	2089 (1622-2691)	4.4 (3.9-4.9)	4.6 (3.9-5.5)	97.7 (91.9-99.7)	1.1 (0.0-6.0)
				6-12 yrs	100	101	-	4.5 (4.0-5.0)	2447 (1914-3129)	4.4 (3.9-4.9) -	4.4 (4.0-4.9) -	98.9 (94.2-100.0)	0.0 (0.0-4.0)

[a]VaxcZyme [b]≥ 4-fold rise from day 0 to 28 days after vaccination [c] Day 28 Median (Interquartile range, IQR)
Abbreviations: CI= confidence interval EU/ml= ELISA units per milliliter GMT=geometric mean titre
Source: Typhoid Vaccine Acceleration Consortium

4.1.3 Effectiveness data – other studies

Navi Mumbai, India

In 2018, the Navi Mumbai Municipal Corporation (NMMC), the local government body for Navi Mumbai, India, implemented the first phase of a public-sector pediatric typhoid conjugate vaccine (TCV) campaign with Typbar-TCV®. The original plan was to immunize all children 9 months – 14 years of age within NMMC (population--±320,000) through two vaccination phases.68 Each campaign phase was planned to cover 11 of 22 target areas (based on the city's 22 Urban Health Posts). Phase I was conducted July – August 2018.

Vaccine effectiveness was estimated using a case control design November 2018-March 2020. Cases were defined as blood-culture confirmed typhoid fever among NMMC residents who were age-eligible for vaccination and who sought treatment at one of the surveillance sites. 7 (16%) cases and 92 (53%) controls reported having received TCV, yielding an adjusted odds ratio (relative odds of being vaccinated among confirmed typhoid fever cases vs being vaccinated among controls) of 0.198 (95% CI: 0.084, 0.468; p=0.0002), which was equivalent to an effectiveness estimate of 80.2% (95% CI: 53.2%, 91.6%).

Pakistan

A matched case-control study was conducted to assess the effectiveness of a mass vaccination campaign using Typbar-TCV® in Lyari Town (**Karachi**), Pakistan.[60] Surveillance was conducted at three hospitals from August to December 2019, and children aged 6 months to 15 years presenting to a study facility with blood culture-confirmed *S*. Typhi were counted as cases. For each case, at least one age-matched afebrile facility control and two age-matched afebrile community controls were enrolled. Of the 82 confirmed typhoid patients who were enrolled, 8 (9.8%) had received TCV. Of the 163 community controls and 82 facility controls, 23.2% and 32.9% had received TCV, respectively. The age and sex-adjusted vaccine effectiveness was 72% (95% CI: 34 – 88%), and the consumption of meals prepared outside of the household more than once a month was found to be associated with increased risk of culture-confirmed typhoid fever (adjusted odds ratio: 3.72, 95% CI: 1.55 – 9.94, p=0.003).

Following an outbreak of extensively drug-resistant (XDR) *S*. Typhi in **Hyderabad**, Pakistan, a reactive vaccination campaign using Typbar-TCV® was conducted from February – December 2018, covering 207,000 children 6 months to 10 years of age.[59] A household census was conducted at baseline in the Qasimabad and Latifabad subdistricts, covering 174,005 households. Active surveillance was established in hospitals, clinics, and laboratories. 24,407 children from the census registry and

surveillance system were included in the vaccine effectiveness analysis, 13,436 of whom were vaccinated. Outcomes of interest were suspected *S*. Typhi, blood-culture confirmed *S*. Typhi, and XDR *S*. Typhi. Within this cohort, vaccine effectiveness was 55% (95% CI: 52, 57) against suspected *S*. Typhi, 95% (93-96%) against blood culture confirmed *S.* Typhi, and 97% (95-98%) against XDR *S*. Typhi during the study period (February 2018 – December 2019).

4.1.4 Safety data (prequalified TCVs)

Extensive safety data has been generated as part of manufacturer-led pre-licensure clinical trials, TyVAC clinical trials, and as part of in India and Pakistan.69,70 These safety data include a wide age range, as well as special populations (HIV-exposed, HIV-infected, malnourished in TyVAC Malawi). Safety data from >100,000 participants from the TyVAC trials were presented to the Global Advisory Committee on Vaccine Safety (GACVS) in December 2018, along with safety data from early public-sector use of TCV in India (Navi Mumbai Municipal Corporation) and Pakistan.[73] GACVS indicated that the "safety profile of the Typbar-TCV® vaccine is reassuring, and no signals of serious adverse events [AEs] were presented." In the TyVAC trials, solicited and systemic adverse reactions were reported with a similar frequency between TCV and control vaccine arms, and most events were of mild or moderate severity. Fever and pain were reported in 3-8% and 1-7% of vaccinees in each group, respectively, while other non-specific local and systemic reactions occurred in 0-3% of vaccinees in each group. In the mass immunization campaigns in Navi Mumbai and Hyderabad, Pakistan, low rates of mild-to-moderate local and systemic events were reported, with fever, pain, and swelling at site of injection being most commonly reported. The adverse event profile was determined to be similar to that of other routine injectable vaccines.

4.*2* TYPHIBEV®

TYPHIBEV® (Vi polysaccharide conjugated to CRM_{197} carrier protein [Vi-CRM_{197}], Biological E. Limited, Pune, India) was licensed in India for use in infants, children, adolescents and adults aged ≥ 6 months to ≤ 45 years, and received WHO prequalification in December 2020. Three prelicensure studies were conducted:

1. a Phase I open-label, single arm study to assess the safety, reactogenicity and immunogenicity of a single dose of Vi-CRM197 conjugate vaccine in healthy 18-45 year olds (n=30) in India;
2. a Phase II/III multicentre single-blind, randomized controlled study to assess the safety and immunogenicity of a single dose of Vi-CRM197 in healthy infants, children, and adults ages ≥ 6 months to < 64 years (n=622) in comparison to Typbar-TCV®;
3. a multicentre single-arm Phase III study to evaluate the safety and tolerability of Vi-CRM197 in healthy infants, children, and adults ages ≥ 6 months to < 45 years of age (n=1770).

The vaccine was safe and well tolerated, with no related severe and/or serious adverse events reported in these three studies. A Phase IV safety study in healthy infants, children, and adults ages ≥ 6 months to < 45 years with an embedded immunogenicity coadministration study with MR vaccine in infants 9-12 months of age (n=1252) is planned.

In the Phase II/III study, immune responses at day 0 (pre-vaccination) and day 42 were assessed and compared. The seroconversion rates in the age groups ≥ 6 months to < 2 years, ≥ 2 years to < 18 years and ≥ 18 years to < 64 years were 99.22%, 100.0% and 97.62% using the short-term threshold value of ≥ 2.0 µg/mL and 96.90%, 95.12% and 94.05% using long-term threshold value of ≥ 4.3 µg/mL respectively. Overall, the proportion of subjects achieving ≥ 4-fold increase in anti-Vi IgG antibody concentrations between Day 0 and Day 42 was 96.95%.[2]

An overview of the two WHO prequalified TCVs is presented in Table 7.

[2] Values in EU/ml have been requested from the manufacturer.

Table 7. Overview of WHO Prequalified TCVs

	Typbar-TCV® (Vi-TT, Bharat Biotech India Ltd., India)	TYPHIBEV® (Vi-CRM$_{197}$, Biological E Ltd., India)
Composition	*Salmonella* Typhi Vi polysaccharide conjugated to tetanus toxoid	Vi polysaccharide prepared from *Citrobacter freundii sensu latoa* conjugated to CRM
Phase of Development	WHO PQ 2017, post-licensure studies ongoing	WHO PQ 2020, post-licensure studies being planned
Indication/Target Ages	≥6 months- ≤45 years. ≥6 months-≤65 years DCGI approved, WHO filing pending	≥ 6 months to ≤ 45 years
Safety Data	>500,000 subjects (pre-licensure studies & post-marketing surveillance in India, TyVAC studies, Pakistan impact studies, Navi Mumbai)[71,72,88–90]	3000+ subjects (licensure studies in India)
Immunogenicity data (geographic representation, special populations, age range, duration of response)	Ph III (India) • 6 mo – 45 yrs: 3-, 5- & 7-years post immunization[62] TyVAC (2-3 yrs post-vaccination follow-up underway) • Burkina n=250, 9-11 months & 15-23 months[65,66] • Malawi n=600, 9 months – 12 years, HIV-infected & malnourished[69] • Nepal n=1500, 9 months – 16 years[4] • Bangladesh n=1300, 9 months – 16 years[68]	Ph II/III (India, n=622) • Immunogenicity non-inferiority compared to Typbar-TCV, participants ages 6 months - <64 years (not published) • Immunogenicity data 3 years post primary immunization expected July 2022
Efficacy and/or Effectiveness Data?	Efficacy data (TyVAC) • Nepal - 79% (95%CI 61.9, 88.5) efficacy, 24 months follow-up[4] • Malawi - 83.7% (68.1, 91.6) efficacy, 24 months follow-up[3] • Bangladesh - 85% (67, 91) total protection, 18-24 months follow-up[5] Effectiveness • Hyderabad, Pakistan - 95% (93-96%) effective against *S.* Typhi, and 97% (95-98%) against XDR *S.* Typhi, 18 months follow-up[6] • Lyari, Pakistan - 72% (34, 88) effective, 4 months follow-up[60] • Navi Mumbai– 80.2% (53.2, 91.6) effective, 15 months follow-up[61]	Planned evaluations in effectiveness studies in Madagascar (TyCOMA) & India
Coadministration/non-interference data?	• MCV & MMR in India @ 9 & 15 mo (DCGI approved, to be submitted to WHO, publication under review at IJID) • Yellow fever & measles-rubella (9 mo) & MCV-A (15 mo) in Burkina Faso	• MR coadministration study planned to start March 2022 in India
2 dose schedule tested?	No, 1 dose only assessed in primary series	No, 1 dose only assessed in primary series
Booster dose schedules tested?	Comparison of single dose vs booster after 2 yr in 6-23 mo cohort of PhIII 3-, 5- & 7-years post primary immunization in India [62]	Evaluation of booster dose 3 years post primary immunization planned for extended Ph II/III study in India

MCV – measles-containing vaccine. MMR – measles-mumps-rubella vaccine. MCV-A – meningococcal conjugate serogroup A vaccine.

4.3 Pipeline vaccines

An overview of the status of the clinical development of additional TCV candidates is presented in Table 8. Four candidates are in Phase III clinical trials ongoing or completed:

1. BioTCV (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, PT Biofarma, Indonesia, WHO PQ submission expected 2025)
2. EuTYPH-C (VI polysaccharide conjugated to CRM197 carrier protein, EuBiologics, South Korea, PQ submission expected 2023)
3. SKYTyphoid (Vi polysaccharide conjugated to diphtheria toxoid carrier protein, SK Bioscience, South Korea, PQ submission expected 2022)
4. ZYVAC TCV (Vi polysaccharide conjugated to tetanus toxoid carrier protein, Zydus Cadila, India, PQ submission expected 2022).

Further details of these four late stage candidates are described in Appendix 4. There are additional manufacturers with TCVs in early clinical development. The diversity of potential TCV manufacturers bodes well for future supply security. However, licensure and prequalification of these vaccines will be based on safety and immunogenicity data (non-inferiority compared to Typbar-TCV®), and manufacturers do not have any current plans to conduct additional studies demonstrating efficacy or effectiveness of their candidate vaccines against clinical disease, which may have an impact on country product preferences.

4.4 Additional TCV studies (planned and ongoing)

Several additional studies are planned or ongoing that will help address some key outstanding knowledge gaps. These include several studies being conducted as part of the Typhoid Conjugate Vaccine Introduction in Africa (THECA) Consortium (https://www.thecaproject.net), which is coordinated by the University of Cambridge (United Kingdom) and includes partners from Kwame Nkrumah University of Science and Technology (Ghana), University of Antananarivo (Madagascar), University of Ouagadougou (Burkina Faso), Institut National de Recherche Biomédicale (Democratic Republic of Congo), International Vaccine Institute (Republic of Korea), Foundation Mérieux (France), Institute of Tropical Medicine Antwerp (Belgium), International Center for Diarrhoeal Disease Research, Bangladesh, and the Center for Vaccine Development and Global Health at the University of Maryland School of Medicine (USA). These and additional studies are described at a high level in Table 9.

Table 8. Summary table of clinical data for pipeline typhoid conjugate vaccines.
All information shared by manufacturers with WHO unless otherwise indicated.

	Bio-TCV (Vi-DT, PT Bio Farma, Indonesia)	SKYTyphoid (Vi-DT, SK Bioscience, Korea)	ZYVAC TCV (Vi-TT, Zydus Cadila, India)	EuTYPH-C (Vi-CRM_{197}, EuBiologics, Korea)
Phase of Development	Phase III study completed (Indonesia)	Two parallel phase III studies completed (Philippines & Nepal)	Phase III, Phase IV, & active Post-Marketing Surveillance ongoing (India)	Phase II/III study completed (Philippines), Phase III starting (Kenya & Senegal)
Manufacturer's target WHO PQ date	2025	2023	2023 (submission planned Sept 2022)	2023
Indication/ Target Ages	≥ 6 months to ≤ 60 years	≥ 6 months	≥ 6 months to ≤ 45 years. Extended age indication (to 65 years) to be sought.	≥6 months to ≤45 years
Safety Data	3000+ subjects	3000+ subjects	<3000 subjects, but PMS study (n=3000) fully enrolled	<3000 subjects
Immunogenicity data (geographic representation, special populations, age range, duration of response)	Phase I (Indonesia) • n=30, 18-45 years, 6 month follow-up72 Ph II (Indonesia) • n=600, 6 months – 40 years73,74 • Long-term follow-up (5 years) of subjects aged 6-23 months at initial vaccination ongoing Phase III (Indonesia) • Non-inferiority to Typbar-TCV, n=3071, 6 months – 60 years (completed)	Phase I (Philippines) • n=144, 2-45 years75 Phase II (Philippines) • n=285, 6-23 months76,77 • 27.5 month follow-up published,78 longer-term (5 years) follow-up ongoing Ph III (Philippines) • n=1800, 6 months- 45 years Phase III (Nepal) • Non-inferiority vs Typbar-TCV, n=1800, 6 months–45 years79	Phase I (India) • n= 24, 18-45 years Phase II/III (India) • Non-inferiority to Typbar-TCV, n=240 6 mo -45 years80 • 3 years post vx data submitted & manuscript under review Phase III (India) • Immunogenicity non-inferiority to Typbar-TCV, n = 238, 45-65 years (completed)	Phase I (Philippines) • Non-inferiority to Typbar-TCV & Vi-PS, n=75, 18 -45 years81 Phase II/III (Philippines) • Non-inferiority to Typbar-TCV, n=444, 6 months – 45 years (completed) Phase III (Kenya & Senegal) Immunogenicity non-inferiority to Typbar-TCV, 6 months – 45 years, n= 3255
Coadmin non-interference data w/ routine vaccines?	• Measles-rubella included in Ph III study	• Coadministration with measles-rubella & measles-mumps-rubella included in Ph III in Nepal (complete)	• Coadministration study with measles-rubella vaccine (n=900) in infants (9-10 months) started March 2022	• Coadministration with measles-rubella & yellow fever will be assessed in Ph III (Kenya & Senegal)
2 dose schedule tested?	2 dose schedule (4 weeks apart) assessed in Ph I	2 dose schedule (4 weeks apart) was assessed in Ph I & II	No, 1 dose only in primary series	No, 1 dose only in primary series
Booster dose schedules tested?	Booster 3 years ±6 months post primary immunization tested in extended Ph II study.	Boosters 6 months and 2 years post primary immunization compared in extended Ph II study (out to 5 years post primary immunization)	Booster 3 years post primary immunization tested in extended Ph II/III subset (manuscript under review)	No

Table 9. Planned/Ongoing TCV Studies

Study Acronym	Site(s)	Vaccine Used	Study Design	Key research questions to be addressed	Data Expected
TyVEGHA (THECA)	Agogo, Ghana	Typbar-TCV®	Cluster-randomized trial	3 years of effectiveness of Typbar-TCV in West African setting, potential indirect protection	Q3 2024
TyVECO (THECA)	Kisantu, DRC	Typbar-TCV®	Mass vaccination followed by effectiveness (case control)	3 years of effectiveness (expected Feb 2025) of Typbar-TCV in Central African setting	Q1 2025
MITIMA (TyVAC)	Blantyre, Malawi	Typbar-TCV®		Post-introduction impact in vaccinated and unvaccinated populations	Study not yet started
TyCOMA (THECA)	Madagascar	TYPHIBEV®	Mass vaccination followed by effectiveness (case control)	2 years effectiveness data TYPHIBEV, feasibility of joint campaign with COVID-19 vaccination	Q2 2024 (study start expected Q2 2022)
TyFIVE – Fiji Intervention and Elimination Program International Vaccine Institute, University of Melbourne, Fiji Ministry of Health	Fiji	Typbar-TCV®	Under discussion	3 years of effectiveness data for Typbar-TCV in all ages > 9 months, link to environmental surveillance	Q3 2026 (1 year baseline surveillance starts Q3 2022, vaccination starts Q3 2023)
Vellore Typhoid Vaccine Impact Study (Christian Medical College, Vellore, India)	Vellore, India	TYPHIBEV®	Cluster-randomized trial, test-negative design	2 years effectiveness data for TYPHIBEV, potential indirect protection, extended age group, coordination with environmental surveillance	Q4 2024

5. Early TCV Introduction Experiences

TCV introduction to routine childhood immunization programmes has been implemented in a small number of countries to date. The first public sector TCV introduction globally took place at a subnational level in India in the Navi Mumbai Municipal Corporation and included an effectiveness and safety evaluation (as described in section 6.1). Gavi-funded programmatic introductions have taken place in Pakistan, Liberia, and Zimbabwe. The first and only non-Gavi country to have introduced TCV is Samoa. Key details of these introduction activities are described in Table 10 while best practices and lessons learned from early TCV introductions are summarized in Table 11.

Table 10: Key information on TCV introductions into routine immunization programmes (implemented or planned)

Country	Introduction strategy	Campaign and routine introduction* date(s)	Campaign target population (children 9 months to <15 years)	Post-campaign coverage achieved**	Age of vaccination in EPI schedule
India, Navi Mumbai Municipal Corporation	Phased	**Phase 1**: July-Aug 2018 in 11 out of 22 Urban Public Health Center (UPHC) areas.	160,000	**Phase 1**: 70.9% (range from 46% in high-income to 92% in low-income areas)	*Routine Immunization has not begun*
		Phase 2: Delayed due to COVID pandemic			
Pakistan	Phased	**Phase 1**: Sindh Nov 2019	**Phase 1**: 10,013,569	**Phase 1**: 82%	9 months
		Phase 2: A) Punjab & Islamabad, Feb 2021 B) Broader Punjab, June 2021	**Phase 2a**: 12,383,108 **Phase 2b**: 6,609,204 **Total Phase 1 & 2**: 29,005,881	**Phase 2a**: 88% **Phase 2b**: 95%	
		Phase 3: Planned Sept-Oct 2022			
Liberia	Nationwide	April 2021	1,900,000	63%	9 months
Zimbabwe	Nationwide (with Integrated TCV/IPV/HPV and vit A supplementation campaign)	May 2021	5,861,235	77.5% based on administrative data *Representative coverage survey not yet conducted.*	9 months

Samoa	Phased	Phase 1: Aug-Sept 2021 Upolu, Apia Urban Area, school-aged children (5-19 years)	26,358	84%	12 months *Routine immunizatio n is ongoing in Apia Urban Area, Upolo*
		Phase 2: Delayed due to COVID pandemic, Nationwide campaign (age 1-45 years)			
Nepal (planned 2022)	Phased	Gavi-approved Phase 1: Province 1, 2, and 5	4,607,503	NA	15 months
		Phase 2: Province 3, 4, 6, and 7	3,872,137 Total, Phase 1 & 2: 8,479,640	NA	
Malawi (planned 2022)	Nationwide	Gavi-approved	9,066,990	NA	9 months

* Unless otherwise specified, routine immunization in the Expanded Programme of Immunization immediately followed (or is planned to follow) the campaign
** Based on a post-campaign coverage survey

5.1 Navi Mumbai, India

The Navi Mumbai Municipal Corporation (NMMC) area represents a total population of 1.12 million of which the TCV introduction targeted 14.2%. The planning and implementation of the TCV campaign were led by NMMC with support from multiple partners. The Phase 1 campaign took place successfully in 2018 over a six-week period on weekends and public holidays to avoid disruption of routine immunization. Catch-up was offered on weekdays at public health centers. Coverage was highest in slums and low income areas.[58] The major challenge with this introduction was the delay in subsequent phases beyond the Phase 1 campaign. Though initially planned, routine immunization did not follow the Phase 1 campaign due to delays and administrative hurdles making vaccine procurement difficult within the NMMC. Phase 2, planned to take place in 2020, has been delayed due to the COVID-19 pandemic.

5.2 Pakistan

Pakistan has implemented the first and second phases of TCV introduction with campaigns targeting more than 29 million children in Sindh (Phase 1) and in Islamabad and Punjab (Phase 2) combined.

Through routine immunization in the EPI, roughly 7 million children are eligible for TCV each year. Phase 1 and 2 have rolled out as planned but with delays in the latter due to the COVID-19 pandemic. Assessment of the impact of the Pakistan TCV introduction is ongoing. In terms of antimicrobial resistance, review of routine typhoid surveillance data in Sindh shows a 71% decrease of the XDR attack rate in 2021 compared to that of 2019.82 The third phase of vaccination has been delayed but implementation is planned for Q4 of 2022.

5.3 Liberia

After multiple delays due to the COVID-19 pandemic, TCV was introduced in Liberia during a national campaign in April 2021. Since the campaign, TCV has been given as part of routine immunization, co-administered at 9 months of age with dose 1 of measles-containing vaccine, and yellow fever vaccine. The post-campaign coverage survey showed low coverage at 63% without significant variation in coverage by age. The most common reasons for non-vaccination were lack of awareness of the campaign (43.6%) and not being present when vaccinators passed through the village (37.4%). Concerns about rumors of TCV being harmful were noted by 5.4% of respondents.

5.4 Zimbabwe

Zimbabwe conducted a nationwide TCV campaign in May 2021, which was unique in the early introduction because the campaign was integrated to include TCV, IPV, HPV as well as vitamin A supplementation. During the campaign, a qualitative evaluation including key-informant interviews was conducted to document priority learnings from the integrated introduction process.

5.5 Samoa

Samoa is a small island nation in the Polynesian region of the South Pacific Ocean. The typhoid disease burden and epidemiology have been very well-defined in terms of subnational and age stratification. The highest incidence age group for typhoid fever in Samoa is school-aged children (5-19 years old), followed by adults 20-45 years of age. The two census regions with the highest incidence and burden are Northwest Upolu and the Apia Urban Area (the capital town and environs, on the North central coast of the island of Upolu). The Samoa Typhoid Fever Control Program and the Samoa EPI designed a phased vaccination strategy targeting the populations at greatest risk with mass campaigns. School-aged children in the Apia Urban Area were vaccinated first in August-September 2021. The second phase of vaccination will target a very broad age range including children and adults 1 to 45 years of age.

Routine vaccination of 12-month-old children in conjunction with measles-containing vaccine has been ongoing. Based on routine surveillance, as of March 2022, no case of typhoid fever in a

vaccinated school-aged child has been reported in the Apia Urban Area since the campaign took place. The continuing rollout of TCV to adults 1 to 45 years nationwide has been delayed three times because of arrival of COVID-19 vaccine shipments and diversion of immunization resources (personnel, vehicles, tablets for data collection) toward COVID-19 vaccination activities.

Table 11: Key best practices and lessons learned from early TCV introductions*

BEST PRACTICES & SUCCESSES	
Coordination	• **Strong government political commitment** • **Strong coordination among stakeholders with a high level of political and financial commitment at all levels.** • Strong national and global partner support for evidence generation to gain learnings from the roll-out
Capacity-building among immunization staff	• Training on strategies for conducting a wide-age injectable campaign • Additional AEFI trainings and AEFI surveillance strengthening
Private sector engagement	• Private pediatricians and general practitioners as key influencers • Private practitioners involved in AEFI committees
Integration	• **Integrated with planned vaccination sessions at schools** • **Leveraged other EPI and public health interventions: co-administration initiatives, mop-up vaccination sessions, national immunization weeks**
School-age vaccination as part of wide age-range campaign	• **Early/strong coordination with the Department of Education** • **Early engagement/orientation/advocacy with schools, school health coordinators and parents** • School-based vaccination prioritized in the first week of vaccination • Vaccination of students according to their class level • Leveraged EPI experience with delivery of other vaccines in the school-age group (e.g., human papillomavirus vaccine)
Microplanning, reporting, monitoring	• Capitalized on prior measles-rubella vaccination campaign experience for microplanning and readiness assessment (e.g., WHO tool adapted from measles campaigns) • Intensified real-time administrative data reporting and monitoring through use of mobile technologies
Advocacy, communication, and social mobilization	• Strengthened awareness in communities in advance of TCV campaigns; strong media involvement instrumental • Used effective social mobilization: strategies may be informed by key input from focus group discussions with pediatricians, parents and health workers • Strong health worker interpersonal skills
CHALLENGES & LESSONS LEARNED	
Competing priorities related to the COVID-	• **Delays due to COVID-19 priorities** • **Introduction of COVID-19 vaccines heightened vaccine misinformation and confusion; community members raised concerns that their children would be administered a COVID-19 vaccine disguised as TCV**

19 pandemic and COVID-19 vaccination	• Changing campaign dates may have reduced awareness and caused confusion
Resource limitations	• Insufficient human resources for large campaigns, especially skilled vaccinators • Lack of supplies including vaccination cards specific to TCV • Nonpayment of vaccinators for previous campaigns
School-based vaccination	• **School closures disrupted microplans** • School reluctance to vaccinate students without written parental consent • Crowd control and maintenance of physical distance at schools and other busy vaccination sites • Out-of-school children were difficult to target in microplanning • Need for increased engagement of both public and private schools, including training and communication materials to ensure consistent messaging
Lack of acceptance and hesitancy	• **Vaccination refusals and misinformation circulating on social media; need for more robust AEFI and vaccine safety messaging** • **Myths and misconceptions about the vaccines being administered (campaign vaccines were assumed to be COVID-19 vaccines)** • Insufficient distribution of education, and communication materials and inadequate training of county-level advocacy, communication, and social mobilization focal points. • Vaccine hesitancy among school-aged children manifested as absence from school during the dates of the campaign

*Findings as reported by one or more countries after TCV introduction. Bolded items were reported by multiple countries.

5.6 Global drivers and challenges for TCV introduction

Drivers for country TCV introduction include the availability of Gavi funding since 2017 and increasing evidence on typhoid disease and economic burden, impact and cost effectiveness of vaccination as a control strategy, AMR and potential impact of TCVs. In general, countries with reliable evidence of lab-confirmed typhoid appear to be earlier adopters of TCV (e.g., Pakistan, Zimbabwe, Nepal, Malawi). As awareness of the public health problem increases, so does political commitment to typhoid fever (and enteric fever) control in endemic countries.

A sufficient supply-demand balance for TCV is expected based on the WHO Market Information for Access to Vaccines (MI4A) global study on typhoid vaccines, assuming all anticipated manufacturers enter the market.83 There is a potential risk of oversupply in the long-term once all countries have conducted their multi-age campaigns. However, a significant level of uncertainty exists in how demand will materialize and at what levels.

Despite the existing drivers for introduction as adequate supply, the evolution of TCV use has been slower than expected for Gavi countries, and even slower for non-Gavi countries. COVID-19

competing priorities and disruptions to immunization programmes (TCV and other) have led to postponements in all of the early introducing countries as described above. COVID-19 vaccination competing priorities have generally led to postponement of new vaccine introduction decision-making in many countries and regions.

Another issue that may hinder countries considering a decision to introduce TCV is related to product selection; countries may be more hesitant to introduce with the newest product(s) with fewer efficacy/effectiveness, safety and impact data. This concern is common to all new vaccines as newer products become available. Programmatic challenges for TCV routine introduction include considerations for integration and potential co-administration with other new vaccines - MR, IPV, MenA, COVID-19 and malaria vaccines. The EPI vaccination schedule is increasingly crowded, and there is a need for additional data on TCV co-administration with other routine vaccines

Lastly, countries may struggle with limited ability to articulate the value proposition of TCV introduction to NITAGs due to challenges with burden assessment. The Burden and Risk Assessment of Typhoid (BRAT) framework and tool, developed by WHO and US CDC and currently in pilot phase, has the potential to assist typhoid endemic countries to conduct a standardized retrospective assessment of existing data to inform decision-making on TCV use.

6. Outstanding Questions and Data Needs

6.1 TCV performance and optimal dosing schedule

A single dose of Typbar-TCV® has been shown to be highly and consistently efficacious and effective in diverse settings (Malawi, Nepal, Bangladesh, Pakistan), including in children younger than 2 years of age, over an 18-24 month period. TCVs are safe & immunogenic in children as young as 9 months of age across a variety of settings. Immunogenicity results are comparable across settings where Typbar-TCV® efficacy has been demonstrated, and non-interference with multiple EPI vaccines across multiple settings has also been demonstrated.

Data are expected on duration of **immune responses** for multiple TCV products/candidates, but this is not the same as duration of **protection**, as a protective immunological threshold has not yet been established. Four years of duration of protection data are expected from the TyVAC trial in Malawi and eventually the THECA trials. Work is ongoing to identify a correlate of protection.[84]

Additional data on duration of protection and how it varies by age of initial administration are critical to assess need for and optimal timing of booster doses of TCV. Longer-term immunogenicity data for single dose and 2-dose primary vaccination as compared to primary + booster doses (on

various intervals) are being generated for several TCVs but understanding duration of protection and need for booster dose will require longer-term efficacy/effectiveness evaluations, potentially comparing different booster dosing schedules. In addition, such data may be crucial to generating country demand, particularly for newer TCVs.

6.2 Operational research questions

Evaluation of different campaign strategies such as combined campaigns with TCV and other vaccines and/or targeting different target age ranges could be useful. The current recommendation for catch-up campaigns targeting children 9 months to 15 years of age was informed in part by cost-effectiveness analyses. It could be more cost-effective to immunize a broader age group in some settings, depending on local epidemiology.

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8. References

1. World Health Organization. Typhoid vaccines: WHO position paper – March 2018. *Weekly epidemiological record*. 2018;13(93):153-172.
2. Gavi the Vaccine Alliance. New typhoid vaccine to receive Gavi support. Published 2018. https://www.gavi.org/library/news/statements/2018/new-typhoid-vaccine-to-receive-gavi-support/
3. Patel PD, Patel P, Liang Y, et al. Safety and Efficacy of a Typhoid Conjugate Vaccine in Malawian Children. *New England Journal of Medicine*. 2021;385(12):1104-1115. doi:10.1056/nejmoa2035916
4. Shakya M, Voysey M, Theiss-Nyland K, et al. Efficacy of typhoid conjugate vaccine in Nepal: final results of a phase 3, randomised, controlled trial. *The Lancet Global Health*. 2021;9(11):e1561-e1568. doi:10.1016/S2214-109X(21)00346-6
5. Qadri F, Khanam F, Liu X, et al. Protection by vaccination of children against typhoid fever with a Vi-tetanus toxoid conjugate vaccine in urban Bangladesh: a cluster-randomised trial. *Lancet (London, England)*. 2021;398(10301):675-684. doi:10.1016/S0140-6736(21)01124-7
6. Yousafzai MT, Karim S, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed Salmonella enterica serotype Typhi in an extensively drug-resistant

outbreak setting of Hyderabad, Pakistan: a cohort study. *The Lancet Global health*. 2021;9(8):e1154-e1162. doi:10.1016/S2214-109X(21)00255-2
7. SAGE Working Group on Typhoid Vaccines & the WHO Secretariat. *Background Paper to SAGE on Typhoid Vaccine Policy Recommendations*.
8. Antillón M, Warren JL, Crawford FW, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. *PLoS Neglected Tropical Diseases*. 2017;11(2):1-21. doi:10.1371/journal.pntd.0005376
9. Wang H, Naghavi M, Allen C, et al. Global, regional, and national life expectancy, all-cause mortality, and cause-specific mortality for 249 causes of death, 1980–2015: a systematic analysis for the Global Burden of Disease Study 2015. *The Lancet*. 2016;388(10053):1459-1544. doi:10.1016/S0140-6736(16)31012-1
10. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, et al. A study of typhoid fever in five Asian countries: Disease burden and implications for controls. *Bulletin of the World Health Organization*. 2008;86(4):260-268. doi:10.2471/BLT.06.039818
11. John A. Crump, Stephen P. Luby and EDM. The global burden of typhoid fever. Bull World Health Organ. *Bulletin of the World Health Organization*. 2004;82(5):346-353. doi:10.1590/S0042-96862004000500008
12. Azmatullah A, Qamar FN, Thaver D, Zaidi AKM, Bhutta ZA. Systematic review of the global epidemiology, clinical and laboratory profile of enteric fever. *Journal of Global Health*. 2015;5(2). doi:10.7189/jogh.05.020407
13. Marks F, von Kalckreuth V, Aaby P, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. *The Lancet Global Health*. 2017;5(3):e310-e323. doi:10.1016/S2214-109X(17)30022-0
14. Appiah GD, Chung A, Bentsi-Enchill AD, et al. Typhoid outbreaks, 1989-2018: Implications for prevention and control. *American Journal of Tropical Medicine and Hygiene*. 2020;102(6):1296-1305. doi:10.4269/ajtmh.19-0624
15. Wong VK, Baker S, Pickard DJ, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of Salmonella Typhi identifies inter-and intracontinental transmission events. *Nature Genetics*. 2015;47(6):632-639. doi:10.1038/ng.3281
16. Yousafzai MT, Qamar FN, Shakoor S, et al. Ceftriaxone-resistant Salmonella Typhi Outbreak in Hyderabad City of Sindh, Pakistan: High Time for the Introduction of Typhoid Conjugate Vaccine. *Clinical Infectious Diseases*. 2019;68(MIC):S16-S21. doi:10.1093/cid/ciy877
17. Jin C, Gibani MM, Moore M, et al. Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. *Lancet (London, England)*. 2017;390(10111):2472-2480. doi:10.1016/S0140-6736(17)32149-9
18. Khan MI, Franco-Paredes C, Sahastrabuddhe S, Ochiai RL, Mogasale V, Gessner BD. Barriers to typhoid fever vaccine access in endemic countries. *Research and Reports in Tropical Medicine*. 2017;Volume 8:37-44. doi:10.2147/rrtm.s97309
19. Lin FYC, Ho VA, Khiem HB, et al. The efficacy of a Salmonella Typhi Vi conjugate vaccine in two-to-five-year-old children. 2001;344(17):1263-1269.
20. Szu SC. Development of Vi conjugate - A new generation of typhoid vaccine. *Expert Review of Vaccines*. 2013;12(11):1273-1286. doi:10.1586/14760584.2013.845529
21. Crump JA, Sjölund-Karlsson M, Gordon MA, Parry CM. Epidemiology, clinical presentation, laboratory diagnosis, antimicrobial resistance, and antimicrobial management of invasive Salmonella infections. *Clinical Microbiology Reviews*. 2015;28(4):901-937. doi:10.1128/CMR.00002-15
22. Antillon M, Saad NJ, Baker S, Pollard AJ, Pitzer VE. The Relationship between Blood Sample Volume and Diagnostic Sensitivity of Blood Culture for Typhoid and Paratyphoid Fever: A Systematic Review and Meta-Analysis. *Journal of Infectious Diseases*. 2018;218(Suppl 4):S255-S267. doi:10.1093/infdis/jiy471
23. Parry CM, Wijedoru L, Arjyal A, Baker S. The utility of diagnostic tests for enteric fever in endemic locations. *Expert Review of Anti-Infective Therapy*. 2011;9(6):711-725. doi:10.1586/eri.11.47

24. Hefele L, Black AP, Tan T van, et al. An age-stratified serosurvey against purified Salmonella enterica serovar Typhi antigens in the Lao People´s Democratic Republic. *PLoS Neglected Tropical Diseases*. 2021;15(12). doi:10.1371/journal.pntd.0010017
25. Aiemjoy K, Seidman JC, Saha S, et al. Estimating typhoid incidence from community-based serosurveys: A multicohort study in Bangladesh, Nepal, Pakistan and Ghana. *medRxiv*. Published online 2021. doi:10.1101/2021.10.20.21265277
26. Uzzell CB, Troman CM, Rigby J, et al. Environmental surveillance for Salmonella Typhi as a tool to estimate the incidence of typhoid fever in low-income populations. *medRxiv*. Published online 2021. doi:10.1101/2021.05.21.21257547
27. WHO Expert Committee on Biological Standardization. *Annex 2 Recommendations to Assure the Quality, Safety and Efficacy of Typhoid Conjugate Vaccines Replacement of Annex 3 of WHO Technical Report Series, No. 987*.
28. Marchello CS, Hong CY, Crump JA. Global typhoid fever incidence: A systematic review and meta-analysis. *Clinical Infectious Diseases*. 2019;68:S105-S116. doi:10.1093/cid/ciy1094
29. Institute for Health Metrics and Evaluation Global Health Data Exchange. GBD Results Tool.
30. Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *The Lancet Infectious Diseases*. 2019;19(4):369-381. doi:10.1016/S1473-3099(18)30685-6
31. Antillón M, Warren JL, Crawford FW, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. Published online 2017. doi:10.1371/journal.pntd.0005376
32. Mogasale V, Maskery B, Ochiai RL, et al. Burden of typhoid fever in low-income and middle-income countries: A systematic, literature-based update with risk-factor adjustment. *The Lancet Global Health*. 2014;2(10):e570-e580. doi:10.1016/S2214-109X(14)70301-8
33. Kim JH, Mogasale V, Im J, Ramani E, Marks F. Updated estimates of typhoid fever burden in sub-Saharan Africa. *The Lancet Global Health*. 2017;5(10):e969. doi:10.1016/S2214-109X(17)30328-5
34. Garrett DO, Longley AT, Aiemjoy K, et al. *Incidence of Typhoid and Paratyphoid Fever in Bangladesh, Nepal, and Pakistan: Results of the Surveillance for Enteric Fever in Asia Project*. Vol 5. https://ssrn.com/abstract=3866551
35. Meiring JE, Shakya M, Khanam F, et al. Burden of enteric fever at three urban sites in Africa and Asia: a multicentre population-based study. *The Lancet Global Health*. 2021;9(12):e1688-e1696. doi:10.1016/S2214-109X(21)00370-3
36. Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively Drug-Resistant Typhoid — Are Conjugate Vaccines Arriving Just in Time? *New England Journal of Medicine*. 2018;379(16):1491-1493. doi:10.1056/nejmp1805927
37. Klemm EJ, Shakoor S, Page AJ, 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):1-10. doi:10.1128/mBio.00105-18
38. Qamar FN, Yousafzai MT, Khalid M, et al. Outbreak investigation of ceftriaxone-resistant Salmonella enterica serotype Typhi and its risk factors among the general population in Hyderabad, Pakistan: a matched case-control study. *The Lancet Infectious Diseases*. Published online 2018. doi:10.1016/S1473-3099(18)30483-3
39. Hooda Y, Saha S, Sajib MSI, et al. Emergence and molecular basis of azithromycin resistance in typhoidal Salmonella in Dhaka, Bangladesh. *bioRxiv*. Published online 2019:594531. doi:10.1101/594531
40. Iqbal, Junaid, Dehraj, Irum F., Carey, Megan E., Dyson, Zoe A., Garrett, Denise, Seidman, Jessica C., Kabir, Furqan, Saha, Senjuti, Baker, Stephen, Qamar FN. A Race against Time : Reduced Azithromycin Susceptibility in Salmonella enterica Serovar Typhi in Pakistan. *mSphere*. 2020;5(4):e00215-20.
41. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal . *JAC-Antimicrobial Resistance*. 2020;2(4). doi:10.1093/jacamr/dlaa109

42. Carey ME, Jain R, Yousuf M, et al. Spontaneous Emergence of Azithromycin Resistance in Independent Lineages of Salmonella Typhi in Northern India . *Clinical Infectious Diseases*. 2021;72(5):e120-127. doi:10.1093/cid/ciaa1773
43. Marchello CS, Carr SD, Crump JA. A systematic review on antimicrobial resistance among salmonella typhi worldwide. *American Journal of Tropical Medicine and Hygiene*. 2020;103(6):2518-2527. doi:10.4269/ajtmh.20-0258
44. Laghari GS, Hussain Z, Hussain SZM, Kumar H, Uddin SMM, Haq A. Antimicrobial Susceptibility Patterns of Salmonella Species in Southern Pakistan. *Cureus*. Published online April 4, 2019. doi:10.7759/cureus.4379
45. Hardjo Lugito NP, Cucunawangsih. Antimicrobial resistance of salmonella enterica serovars Typhi and paratyphi isolates from a general Hospital in Karawaci, Tangerang, Indonesia: A five-year review. *International Journal of Microbiology*. 2017;2017. doi:10.1155/2017/6215136
46. Murray CJ, Ikuta KS, Sharara F, et al. Global burden of bacterial antimicrobial resistance in 2019: a systematic analysis. *The Lancet*. 2022;399(10325):629-655. doi:10.1016/S0140-6736(21)02724-0
47. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global Salmonella Typhi genotyping framework. doi:10.1093/infdis/jiab414/6358992
48. Wong VK, Baker S, Connor TR, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. *Nature Communications*. 2016;7:1-11. doi:10.1038/ncomms12827
49. Pragasam AK, Pickard D, Wong V, et al. Phylogenetic Analysis Indicates a Longer Term Presence of the Globally Distributed H58 Haplotype of Salmonella Typhi in Southern India. 2020;(Xx):1-8. doi:10.1093/cid/ciz1112
50. Thanh DP, Karkey A, Dongol S, et al. A novel ciprofloxacin-resistant subclade of h58. Salmonella typhi is associated with fluoroquinolone treatment failure. *eLife*. 2016;5(MARCH2016):1-13. doi:10.7554/eLife.14003
51. Feasey NA, Gaskell K, Wong V, et al. Rapid Emergence of Multidrug Resistant, H58-Lineage Salmonella Typhi in Blantyre, Malawi. *PLoS Neglected Tropical Diseases*. 2015;9(4):1-13. doi:10.1371/journal.pntd.0003748
52. Mashe T, Leekitcharoenphon P, Mtapuri-Zinyowera S, et al. Salmonella enterica serovar Typhi H58 clone has been endemic in Zimbabwe from 2012 to 2019. *The Journal of antimicrobial chemotherapy*. 2021;76(5):1160-1167. doi:10.1093/jac/dkaa519
53. Typhi S, Esther da Silva K, Mohammad Tanmoy A, et al. The international and intercontinental spread and expansion of antimicrobial-resistant Salmonella Typhi. doi:10.1101/2021.09.03.21262852
54. Maes M, Dyson ZA, Higginson EE, et al. Multiple introductions of salmonella enterica serovar typhi H58 with reduced fluoroquinolone susceptibility into Chile. *Emerging Infectious Diseases*. 2020;26(11):2736-2740. doi:10.3201/eid2611.201676
55. Hooda Y, Sajib MSI, Rahman H, et al. Molecular mechanism of azithromycin resistance among typhoidal Salmonella stains in Bangladesh identified through passive pediatric surveillance. *PLoS neglected tropical diseases*. 2019;13(11):e0007868. doi:10.1371/journal.pntd.0007868
56. Duy PT, Dongol S, Giri A, et al. The emergence of azithromycin-resistant Salmonella Typhi in Nepal. *JAC-antimicrobial resistance*. 2020;2(4):dlaa109-dlaa109. doi:10.1093/jacamr/dlaa109
57. Laurens MB, Sirima SB, Rotrosen ET, et al. A Phase II, Randomized, Double-blind, Controlled Safety and Immunogenicity Trial of Typhoid Conjugate Vaccine in Children Under 2 Years of Age in Ouagadougou, Burkina Faso: A Methods Paper. *Clinical Infectious Diseases*. 2019;68(Supplement_2):S59-S66. doi:10.1093/cid/ciy1104
58. Date K, Shimpi R, Luby S, et al. Decision making and implementation of the first public sector introduction of typhoid conjugate vaccine-Navi Mumbai, India, 2018. *Clinical Infectious Diseases*. 2020;71:S172-S178. doi:10.1093/cid/ciaa597
59. Yousafzai MT, Karim S, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed Salmonella enterica serotype Typhi in an extensively drug-resistant

outbreak setting of Hyderabad, Pakistan: a cohort study. *The Lancet Global Health*. 2021;9(8):e1154-e1162. doi:10.1016/S2214-109X(21)00255-2
60. Batool R, Tahir Yousafzai M, Qureshi S, et al. Effectiveness of typhoid conjugate vaccine against culture-confirmed typhoid in a peri-urban setting in Karachi: A case-control study. *Vaccine*. 2021;39(40):5858-5865. doi:10.1016/j.vaccine.2021.08.051
61. Date KA, Harvey P, Bhatnagar P, et al. LB-5213 - Field Effectiveness of a Typhoid Conjugate Vaccine — Navi Mumbai (India), 2018-2020. In: *American Society of Tropical Medicine and Hygiene 2020 National Meeting*. ; 2020. Accessed March 15, 2022. https://www.abstractsonline.com/pp8/#!/9181/presentation/4739
62. Vadrevu KM, Raju D, Rani S, et al. Persisting antibody responses to Vi polysaccharide–tetanus toxoid conjugate (Typbar TCV®) vaccine up to 7 years following primary vaccination of children < 2 years of age with, or without, a booster vaccination. *Vaccine*. Published online 2021. doi:10.1016/j.vaccine.2021.07.073
63. Balaji V, Kapil A, Shastri J, et al. Longitudinal typhoid fever trends in India from 2000 to 2015. *American Journal of Tropical Medicine and Hygiene*. 2018;99(3):34-40. doi:10.4269/ajtmh.18-0139
64. Sur Di, Barkume C, Mukhopadhyay B, Date K, Ganguly NK, Garrett D. A Retrospective Review of Hospital-Based Data on Enteric Fever in India, 2014-2015. *Journal of Infectious Diseases*. 2018;218:S206-S213. doi:10.1093/infdis/jiy502
65. Sirima SB, Ouedraogo A, Barry N, et al. Safety and immunogenicity of Vi-typhoid conjugate vaccine co-administration with routine 9-month vaccination in Burkina Faso: A randomized controlled phase 2 trial. *International Journal of Infectious Diseases*. 2021;108:465-472. doi:10.1016/j.ijid.2021.05.061
66. Sirima SB, Ouedraogo A, Barry N, et al. Safety and immunogenicity of co-administration of meningococcal type A and measles–rubella vaccines with typhoid conjugate vaccine in children aged 15–23 months in Burkina Faso. *International Journal of Infectious Diseases*. 2021;102(September 2011):517-523. doi:10.1016/j.ijid.2020.10.103
67. Shakya M, Pant D, Hill J, et al. *Immunogenicity of Typhoid Conjugate Vaccine in Nepal: A Participant-Observer-Blind Phase III Randomized Controlled Trial (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses Meeting)*.; 2021.
68. Qadri F, Khanam F, Babu G, et al. *Immune Responses Induced in Children after Vaccination with Vi-TT Vaccine: Immunogenicity Data from a Cluster-Randomised Controlled Trial in Bangladesh (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses)*.; 2021.
69. Nampota-Nkomba N, Mbewe M, Nyirenda O, et al. *Safety and Long-Term Immunogenicity of a Novel Typhoid Conjugate Vaccine in Malawian Infants and Children (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses Meeting)*.; 2021.
70. Hoffman S, Leboa C, Date K, et al. *Impact of a Pediatric Typhoid Conjugate Vaccine Campaign in Navi Mumbai, India (Presented at 12th International Conference on Typhoid and Other Invasive Salmonelloses Meeting)*.; 2021.
71. Longley AT, Date K, Luby SP, et al. Evaluation of Vaccine Safety after the First Public Sector Introduction of Typhoid Conjugate Vaccine - Navi Mumbai, India, 2018. *Clinical Infectious Diseases*. 2021;73(4):E927-E933. doi:10.1093/cid/ciab059
72. Qamar FN, Yousafzai MT, Khaliq A, et al. Adverse events following immunization with typhoid conjugate vaccine in an outbreak setting in Hyderabad, Pakistan. *Vaccine*. 2020;38(19):3518-3523. doi:10.1016/j.vaccine.2020.03.028
73. World Health Organization. *Global Advisory Committee on Vaccine Safety, 5-6 December 2018*. Wkly Epidem Rec. 2019;94(4):45-52.
74. Medise BE, Soedjatmiko S, Rengganis I, et al. Six-month follow up of a randomized clinical trial-phase I study in Indonesian adults and children: Safety and immunogenicity of Salmonella typhi polysaccharide-diphtheria toxoid (VI-DT) conjugate vaccine. *PLoS ONE*. 2019;14(2). doi:10.1371/journal.pone.0211784
75. Medise BE, Soedjatmiko S, Gunardi H, et al. A novel Vi-diphtheria toxoid typhoid conjugate vaccine is safe and can induce immunogenicity in healthy Indonesian children 2–11 years: a phase II preliminary report. *BMC Pediatrics*. 2020;20(1). doi:10.1186/s12887-020-02375-4

76. Medise BE, Soedjatmiko S, Gunardi H, et al. One-month follow up of a randomized clinical trial-phase II study in 6 to <24 months old Indonesian subjects: Safety and immunogenicity of Vi-DT Typhoid Conjugate Vaccine. *International Journal of Infectious Diseases*. 2020;93:102-107. doi:10.1016/j.ijid.2020.01.045
77. Capeding MR, Teshome S, Saluja T, et al. Safety and immunogenicity of a Vi-DT typhoid conjugate vaccine: Phase I trial in Healthy Filipino adults and children. *Vaccine*. 2018;36(26):3794-3801. doi:10.1016/j.vaccine.2018.05.038
78. Capeding MR, Alberto E, Sil A, et al. Immunogenicity, safety and reactogenicity of a Phase II trial of Vi-DT typhoid conjugate vaccine in healthy Filipino infants and toddlers: A preliminary report. *Vaccine*. 2020;38(28):4476-4483. doi:10.1016/j.vaccine.2019.09.074
79. Capeding MR, Sil A, Tadesse BT, et al. Safety and immunogenicity of Vi-DT conjugate vaccine among 6-23-month-old children: Phase II, randomized, dose-scheduling, observer-blind Study. *EClinicalMedicine*. 2020;27. doi:10.1016/j.eclinm.2020.100540
80. Capeding MR, Tadesse BT, Sil A, et al. Immune persistence and response to booster dose of Vi-DT vaccine at 27.5 months post-first dose. *npj Vaccines*. 2022;7(1). doi:10.1038/s41541-022-00434-8
81. Saluja T, Giri BR, Chaudhary S, et al. Challenges and opportunities in setting up a phase III vaccine clinical trial in resource limited settings: Experience from Nepal. *Human Vaccines and Immunotherapeutics*. 2021;17(7):2149-2157. doi:10.1080/21645515.2020.1855955
82. RITABRATA KUNDU, AMBROSE KUMAR KANDULNA, UMA NAYAK, et al. Immunogenicity and Safety of Typhoid Conjugate Vaccine in Healthy Indian Subjects: A Randomized, Active-controlled, Comparative Clinical Trial. *Indian Pediatrics*. 2020;57:625-630.
83. Choi SK, Baik YO, Kim CW, et al. An open-label, comparative, single dose, clinical Phase I study to assess the safety and immunogenicity of typhoid conjugate vaccine (Vi-CRM197) in healthy Filipino adults. *Vaccine*. 2021;39(19):2620-2627. doi:10.1016/j.vaccine.2021.03.089
84. FELTP Placeholder.
85. Market Information for Access to Vaccines. *Global Market Study: Typhoid Vaccines*.; 2020. Accessed March 14, 2022. https://cdn.who.int/media/docs/default-source/immunization/mi4a/typhoid_vaccines-market_study_public_summary-november2020.pdf?sfvrsn=96993161_6&download=true
86. Jin C, Hill J, Gunn BM, et al. Vi-specific serological correlates of protection for typhoid fever. *Journal of Experimental Medicine*. 2020;218(2). doi:10.1084/JEM.20201116
87. Sirima SB, Ouedraogo A, Barry N, et al. Safety and immunogenicity of Vi-typhoid conjugate vaccine co-administration with routine 9-month vaccination in Burkina Faso: A randomized controlled phase 2 trial. *International Journal of Infectious Diseases*. 2021;108:465-472. doi:10.1016/j.ijid.2021.05.061
88. Mohan VK, Varanasi V, Singh A, et al. Safety and Immunogenicity of a Vi Polysaccharide–Tetanus Toxoid Conjugate Vaccine (Typbar-TCV) in Healthy Infants, Children, and Adults in Typhoid Endemic Areas: A Multicenter, 2-Cohort, Open-Label, Double-Blind, Randomized Controlled Phase 3 Study. *Clinical Infectious Diseases*. 2015;61(3):393-402. doi:10.1093/cid/civ295
89. Reddy R, Reddy B, Sarangi V, Reddy S, Ella R, Vadrevu KM. A multi-centre, post-marketing surveillance study of Vi polysaccharide–tetanus toxoid conjugate vaccine (Typbar TCV®) in India. *Human Vaccines and Immunotherapeutics*. Published online 2021. doi:10.1080/21645515.2021.1947761
90. Neuzil KM, Basnyat B, Clemens JD, et al. Early insights from clinical trials of typhoid conjugate vaccine. *Clinical Infectious Diseases*. 2020;71:S155-S159. doi:10.1093/cid/ciaa370
91. Lee EY, Park JY, Kim DR, et al. Comparison of anti-Vi IgG responses between two clinical studies of typhoid Vi conjugate vaccines (Vi-DT vs Vi-TT). *PLoS Neglected Tropical Diseases*. 2020;14(3). doi:10.1371/journal.pntd.0008171
92. Mejia N, Qamar F, Yousafzai MT, et al. Typhoid and Paratyphoid Cost of Illness in Pakistan: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S319-S335. doi:10.1093/cid/ciaa1336

93. Mejia N, Abimbola T, Andrews JR, et al. Typhoid and Paratyphoid Cost of Illness in Nepal: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S306-S318. doi:10.1093/cid/ciaa1335
94. Mejia N, Pallas SW, Saha S, et al. Typhoid and Paratyphoid Cost of Illness in Bangladesh: Patient and Health Facility Costs from the Surveillance for Enteric Fever in Asia Project II. *Clinical Infectious Diseases*. 2020;71:S293-S305. doi:10.1093/cid/ciaa1334

Appendix 10. Typhoid fever control in the 21st century: where are we now? (publication)

Typhoid fever control in the 21st century: where are we now?

Megan E. Carey[a,b], *Naina S. McCann*[c,d] *and Malick M. Gibani*[e]

Purpose of review
Momentum for achieving widespread control of typhoid fever has been growing over the past decade. Typhoid conjugate vaccines represent a potentially effective tool to reduce the burden of disease in the foreseeable future and new data have recently emerged to better frame their use-case.

Recent findings
We describe how antibiotic resistance continues to pose a major challenge in the treatment of typhoid fever, as exemplified by the emergence of azithromycin resistance and the spread of *Salmonella* Typhi strains resistant to third-generation cephalosporins. We review efficacy and effectiveness data for TCVs, which have been shown to have high-level efficacy (≥80%) against typhoid fever in diverse field settings. Data from randomized controlled trials and observational studies of TCVs are reviewed herein. Finally, we review data from multicountry blood culture surveillance studies that have provided granular insights into typhoid fever epidemiology. These data are becoming increasingly important as countries decide how best to introduce TCVs into routine immunization schedules and determine the optimal delivery strategy.

Summary
Continued advocacy is needed to address the ongoing challenge of typhoid fever to improve child health and tackle the rising challenge of antimicrobial resistance.

Keywords
antimicrobial resistance, typhoid fever, vaccines

INTRODUCTION

Typhoid fever remains a major public health challenge in low-resource settings. The nature of this challenge is highlighted by the persistent high-level morbidity and mortality across several regions, coupled with emergence of extensively drug-resistant (XDR) isolates of *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi). In many settings, treatment options are now severely limited, validating its status as a priority pathogen for antimicrobial resistance research.

Fortunately, the development, testing, and rollout of highly effective typhoid conjugate vaccines (TCVs) offer one effective tool to achieve meaningful disease control in the foreseeable future. In this review, we will highlight new research developments in typhoid fever epidemiology, diagnosis, treatment, and prevention since the publication of our previous review [1]. We will provide an overview of areas of ongoing study and identify knowledge gaps that need to be addressed to ensure that the global health community consolidates the gains made over the past decade.

BURDEN OF DISEASE

The past decade has seeen marked refinements in estimates of typhoid fever incidence, including from systematic reviews, modelled estimates, and population surveillance studies [2–4,5▪▪,6▪▪]. Most of these studies have demonstrated a trend towards declining global incidence over the past 20 years, albeit with sustained high rates of disease in several regions.

Investigators from the Institute for Health Metrics and Evaluation have updated modelled

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KEY POINTS

- Typhoid fever remains a major public health challenge in low-resource settings, and several recent surveillance studies have allowed for refined estimates of disease burden.
- The emergence of azithromycin-resistant and ESBL-producing strains of *Salmonella* Typhi presents a challenge to effective treatment.
- Typhoid conjugate vaccines (TCV) have demonstrated high-level efficacy and effectiveness in diverse field settings.
- Typhoid conjugate vaccines are being programmatically deployed in high-burden settings and offer the potential to reduce morbidity, mortality, and antimicrobial resistance.

estimates for *S*. Typhi, indicating that it was responsible for 9.24 million cases [95% confidence interval (CI) 5.94–14.13], 8.05 million (3.86–13.93) disability-adjusted life years (DALYs), and 110 029 (95% CI 52 810–191 205) deaths in 2019. This is compared with an estimated more than 20 million annual cases in 1990 [3]. The highest burden of disease is estimated to be in children 5–9 years of age, followed by 10–14-year-olds and 1–4-year-olds.

Several multicountry, standardized, population-based blood culture surveillance studies have improved our overall understanding of typhoid fever incidence [4,5▪▪,6▪▪]. The Surveillance for Enteric fever in Asia Project (SEAP) was a prospective blood culture surveillance study conducted at five hospitals in Bangladesh, Nepal, and Pakistan [6▪▪]. They employed a hybrid surveillance model, combining hospital-based blood culture surveillance and healthcare-utilization surveys, to estimate the burden of disease. High rates of disease were observed at all sites. After adjusting for blood culture sensitivity, probability of consent and blood sampling, probability of care-seeking at a study facility, and wealth and education multipliers, the authors presented adjusted estimated incidence of typhoid fever (adjusted) was as high as 1110 per 100 000 person-years in Dhaka, Bangladesh; 330 per 100 000 person years in Kathmandu, Nepal; 271 per 100 000 person years in Kavrepalanchok, Nepal, and 126–195 per 100 000 person years in Karachi, Pakistan.

The Strategic Typhoid Alliance across Africa and Asia (STRATAA) study employed multicomponent passive febrile illness surveillance study in three densely populated urban sites in Bangladesh, Nepal, and Malawi [5▪▪]. After adjusting for blood culture sensitivity, probability of receiving a blood culture and health-seeking behaviour, the authors presented adjusted estimates for typhoid fever of 1062 cases per 100 000 person-years of observation in Kathmandu, Nepal; 1135 cases per 100 000 person-years in Dhaka, Bangladesh and 444 cases per 100 000 person-years in Blantyre, Malawi. Burden of disease estimates were even higher when defined using serological surveillance.

Collectively, all studies to date have consistently demonstrated a high burden of typhoid fever in South and South-East Asia. New data suggested higher than previously thought incidence rates in parts of sub-Saharan Africa [5▪▪,7], which will be further refined by data from the upcoming Severe Typhoid Fever in Africa (SETA) study [4]. Accurate estimates of disease burden are becoming increasingly important given the advent of new control strategies such as TCVs, as countries decide how best to introduce TCVs into routine immunization schedules and determine the optimal delivery strategy.

IMPROVED DIAGNOSTICS FOR SURVEILLANCE

There is an ongoing need to develop and validate rapid, low-cost diagnostics for surveillance of typhoid fever. The current *de facto* gold-standard remains blood culture, which is routinely used in clinical practice (wherever available) and is recommended for routine surveillance of typhoid fever by the WHO. Blood culture diagnostics have several limitations, including: prolonged time-to-result; moderate sensitivity (40–60%, depending on prior antibiotic consumption and volume of blood drawn [8]), and the need for significant training and a consistent supply chain [9]. Novel, rapid diagnostics could facilitate appropriate antimicrobial prescribing practices as well as to improve local, regional, and global estimates of disease burden.

New approaches to serological surveillance using novel serological markers of infection and/or carriage (such as HlyE and CdtB) are being validated in multiple sites where blood culture surveillance is also ongoing and appear promising [10▪▪,11]. Approaches to validating standardized methods for detection of *S*. Typhi in environmental samples are also under evaluation in Blantyre, Malawi, and Vellore, India [12]. In the future, these approaches could potentially help fill regional data gaps, inform decision-making around TCV introduction, and could also be expanded to include surveillance of other epidemiologically relevant pathogens.

TREATMENT

Extended-spectrum cephalosporins are an important treatment option for enteric fever, particularly following the emergence of multidrug resistance (MDR) and fluoroquinolone resistance [13,14]. Since 2016, an outbreak of XDR *S.* Typhi has been identified in Pakistan, caused by an H58 clade harbouring MDR resistance, fluoroquinolone resistance, and extended-spectrum cephalosporin resistance, acquired through a plasmid encoding the $bla_{\text{CTX-M-15}}$ gene [15]. Over 15 000 XDR cases have been reported from Pakistan since then, with spread from the Sindh province to other areas in Pakistan [16]. Although XDR *S.* Typhi is not currently endemic in other countries, multiple countries have reported imported cases in travellers returning from Pakistan, highlighting the risk of further spread [17–25].

Third-generation cephalosporin resistance in *S.* Typhi isolates distinct from the XDR strain have also been reported. Case reports of *S.* Typhi encoding extended spectrum beta lactamase (ESBL) $bla_{\text{CTX-M-15}}$ have been reported from Democratic Republic of Congo and in travellers from Iraq [26,27]. Multiple reports from India describe ESBL isolates that carry plasmids, which encode $bla_{\text{SHV-12}}$, $bla_{\text{CMY-2}}$, $bla_{\text{ACC-1}}$, and $bla_{\text{DHA-1}}$ mutations [28▪]. Although cephalosporin resistance is not currently widespread (<5% of global strains), cases are increasing. The emergence of new, independent, mutations is concerning. The emergence of XDR *S.* Typhi can be considered an inflection point in typhoid fever control, providing an impetus to roll out TCVs more broadly to address the challenge of antimicrobial resistance [29▪▪,30].

Azithromycin is now the suggested treatment option for uncomplicated XDR or ESBL *S.* Typhi in endemic and nonendemic settings [31,32]. The emergence of azithromycin resistance is a major cause for concern. Resistant isolates have been reported in multiple settings, including Bangladesh [33], Pakistan [34], Nepal [35], India [36▪], Singapore [37], and Samoa [38]. Resistance is largely mediated by a point mutation in the *acrB* gene, which codes for an efflux pump. These mutations have appeared independently in multiple genetically distinct lineages and appear to be concentrated in areas with significant antimicrobial selection pressure where azithromycin is increasingly used as first-line treatment for typhoid and other blood stream infections. Programmatic distribution of azithromycin in some areas of the world, for example, mass administration for trachoma prevention and elimination, may further contribute to this, highlighting the need for ongoing genomic surveillance and monitoring in these settings and more broadly.

Carbapenems are now increasingly used to treat drug-resistant enteric fever. There are no randomized controlled trials evaluating the use of meropenem in enteric fever; however, observational data suggest it is effective as a treatment [39]. The use of carbapenems is limited by cost and the need to give them intravenously. Newer oral carbapenems such as tebipenem may be an effective option in the future, but further clinical trials are needed [40].

CARRIAGE

Chronic gallbladder carriage of *S.* Typhi remains poorly understood. Genomic studies of gallbladder isolates show that carriage isolates have comparable genotype distributions to circulating blood culture isolates in the same setting – including MDR strains [41▪,42]. Carriage isolates appear to have higher rates of mutations in membrane lipoproteins, transport proteins, surface antigens, and genes involved in polysaccharide synthesis [41▪,42]. A recent 3-year case–control study in an informal settlement of Nairobi, Kenya estimated a chronic carriage rate of 1.1% in children aged 16 years or less, highlighting a role for paediatric carriage in onward transmission in this setting [42]. There is limited available data to suggest treatment options for chronic carriage in an era of high fluroquinolone resistance [43]. A 28-day course of azithromycin can be used to treat fluoroquinolone-resistant carriers, although there is no trial evidence to assess this. Understanding how to diagnose and treat enteric fever chronic carriers remains a neglected area of research.

THE ERA OF TYHPOID CONJUGATE VACCINES

In 2017, SAGE issued updated policy recommendation for the use of typhoid vaccines in endemic countries. For the first time, this included a recommendation for programmatic use of TCVs in children aged under 2 years [44]. The SAGE recommendation was based primarily on safety and immunogenicity data [45,46▪], supported by evidence of efficacy in human challenge studies conducted in healthy adults [47], in the context of efficacy data from earlier generation TCVs [48]. The most studied TCV is a Vi polysaccharide-tetanus toxoid (Vi-TT) conjugate vaccine Typbar-TCV, manufactured by Bharat Biotech (Hyderabad, India). Typbar-TCV has been shown to be well tolerated and immunogenic on children as young as 6 months of age [46▪] and was prequalified in December 2017. TYPHIVEV – a Vi-CRM_{197} conjugate manufactured by Biological E (Pune, India) – has a comparable safety and immunogenicity profile to Typbar TCV [49] and received prequalification in December 2020.

Pivotal efficacy and effectiveness data for Typbar-TCV were generated by the Typhoid Vaccine Acceleration Consortium (TyVAC), led by the Center for Vaccine Development and Global Health at the University of Maryland School of Medicine, the Oxford Vaccine Group at the University of Oxford, and PATH. The TyVAC consortium evaluated Typbar-TCV in three large postlicensure efficacy and effectiveness studies – individually randomized trials in Malawi [50▪▪] and Nepal [51], alongside a cluster-randomized trial in Bangladesh [52▪▪]. Each trial had a unique design, but point estimates for efficacy against culture-confirmed typhoid fever were approximately 80% at 18–24 months in all study sites. High-level results are detailed in Table 1.

Previous studies have demonstrated some evidence for indirect protection conferred by Vi-polysaccharide vaccines [53]. The TyVAC investigators assessed whether Typbar-TCV conferred indirect protection in the Bangladesh cluster randomized trial [52▪▪]. They compared the incidence of typhoid fever among unvaccinated persons in Typbar-TCV clusters compared with unvaccinated persons in Japanese Encephalitis vaccine clusters. In this study, there was no evidence of significant indirect protection conferred by the typhoid conjugate vaccine. The authors noted that the study was not adequately powered to assess the anticipated level of herd protection (20%), and any indirect protection may have been diluted in the densely populated clusters. A cluster randomized trial of Typbar-TCV is currently underway in Asante-Akim, Ghana, which is anticipated to generate additional data on indirect protection in a different epidemiological context [54].

Several observational studies have since reinforced data on the effectiveness of TCVs (Table 1) [29▪▪,30,55,56]. The Navi Mumbai Municipal Corporation (NMMC) conducted the first public-sector TCV campaign with Typbar-TCV in 2018.[55] Vaccine effectiveness was estimated using a case–control design among age-eligible residents in urban health centres with typhoid fever, and age-matched and time-matched controls were randomly selected from a population-based household survey. Seven (17%) cases and 86 (51%) controls reported having received TCV, yielding an adjusted odds ratio of 0.184 (95% CI 0.074–0.46; $P = 0.0003$), equivalent to an effectiveness estimate of 81.6% (95% CI 54–92.6%).

Following an outbreak of XDR *S.* Typhi in Hyderabad, Pakistan, a reactive vaccination campaign using Typbar-TCV was conducted from February to December 2018, covering 207 000 children 6 months to 10 years of age [29▪▪]. A household census was conducted at baseline and active blood culture surveillance was established in hospitals, clinics, and laboratories. The vaccine effectiveness analysis included 24 407 children from the census registry and surveillance system, 13 436 of whom had received TCV. In this cohort study, Typbar-TCV was 55% effective (95% CI 52–57) against suspected typhoid fever, 95% (93–96%) effective against blood culture-confirmed typhoid fever and 97% (95–98%) effective against XDR *S.* Typhi in 6-month-olds to 10-year-olds.

A mass vaccination campaign using Typbar-TCV was also conducted in Lyari Town (an urban slum neighbourhood of Karachi, Pakistan) in response to the spread of XDR typhoid to this area. Effectiveness was evaluated using a matched case–control design [30]. Surveillance was conducted at three hospitals from August to December 2019, and children aged 6 months to 15 years presenting to a study facility with blood culture-confirmed *S.* Typhi were counted as cases. For each case, at least one age-matched afebrile facility control and two age-matched afebrile community controls were enrolled. Of the 82 confirmed typhoid cases, 8 (9.8%) had received TCV. Among the 163 community controls and 82 facility controls, 23.2 and 32.9% had received TCV, respectively. The age-adjusted and sex-adjusted TCV effectiveness was 72% (95% CI 34–88%).

Immunogenicity data for TCVs appear promising. A single dose of Typbar-TCV induces durable anti-Vi IgG above baseline out to 5 years [46▪]. The significance of anti-Vi IgG in conferring protection is yet to be determined, and work is ongoing to better define correlates of protection. The immunogenicity of Typbar-TCV is comparable across all study sites where efficacy has been demonstrated, although results may vary by local epidemiology owing to natural boosting. TyVAC investigators also conducted immunogenicity and co-administration studies in infants and toddlers in Burkina Faso. The available data have shown no evidence of TCV interference with multiple EPI vaccines, across diverse settings [57,58].

There is currently a comparatively robust TCV development pipeline. Four companies have programmes with a TCV candidate with phase III clinical trials ongoing or completed – BioTCV [Vi polysaccharide conjugated to diphtheria toxoid (Vi-DT), PT Biofarma, Indonesia], EuTYPH-C (Vi-CRM197, EuBiologics, South Korea) [59], SKYTyphoid (Vi-DT, SK Bioscience, South Korea) [60], and ZYVAC TCV (Vi-TT, Zydus Cadila, India, licensed in India). All four manufacturers are seeking WHO prequalification. This bodes well for future supply security as part of a healthy market framework. Licensure and prequalification of these vaccines will likely be based on safety and immunogenicity data that demonstrate noninferiority compared with Typbar-TCV. Additional

Table 1. overview of efficacy and effectiveness data for Typbar-TCV

Study site	Design	Control vaccine/ control group	Age	Number vaccinated	Duration follow-up	Vaccine efficacy or effectiveness (95% CI)[a]	Reference
Lalitpur, Nepal	Individually randomized	Meningococcal capsular group A conjugate vaccine	9 months to 16 years	20 019	24 months	79.1% (62.0–88.5)	[61]
Blantyre, Malawi	Individually randomized	Meningococcal capsular group A conjugate vaccine	9 months to 12 years	28 130	18–24 months	83.7% (68.1–91.6)	[50▪▪]
Dhaka, Bangladesh	Cluster randomized	SA 14–14-2 Japanese encephalitis (JE) vaccine	9 months to 16 years	67 395	24 months	85% (76.0–91.0)	[52▪▪]
Observational studies							
Navi Mumbai, India	Case–control (routine immunization)	Community controls	9 months to 14 years	160 000	15 months	80.2% (53.2–91.6)	[55]
Karachi, Pakistan	Case–control (outbreak response campaign)	Facility and community controls	6 months to 15 years	87 993	4 months	72% (34–88)	[30]
Hyderabad, Pakistan	Cohort study (outbreak response campaign)	Community controls	6 months to 10 years	207 000	18 months	95% (93–96)	[29▪▪]
Harare, Zimbabwe	Case–control (outbreak response)	Community controls Facility controls	6 months to 15 years	320 000	18 months	75% (1–94) 84% (57–94)[b]	[56]

[a]Blood culture-confirmed typhoid fever.
[b]Efficacy in 6 months to 45 year cohort compared with community controls was 67% (95% CI 33–83) Bangladesh [52▪▪] and individually-randomized trials in Malawi [50▪▪] and Nepal [51].

studies demonstrating efficacy or effectiveness against clinical disease may have an impact on country product preferences.

TCVs have been introduced into routine childhood immunization programmes in six countries to date. The first public sector TCV introduction was conducted at a subnational level in India in the Navi Mumbai Municipal Corporation and included an effectiveness and safety evaluation (Table 1). Pakistan was the first country to initiate a Gavi-supported national introduction in 2019, followed by Liberia (2021), Zimbabwe (2021), and Nepal (2022). Malawi also plans to introduce TCV with Gavi support in 2022. The Samoan government initiated a self-financed TCV introduction in 2021.

KNOWLEDGE GAPS AND FUTURE DIRECTIONS

Additional data are expected in the next 12–18 months that will help to inform TCV-deployment decisions. It is important to understand the duration of protection conferred by a single dose of a TCV and whether a booster dose is needed. As the TCV pipeline expands, it will be important to establish whether different TCV products can be used interchangeably as part of a primary dose with booster regimen, as countries may switch TCV products because of supply limitations, vaccine price, or availability of new data. Laying the groundwork to interrogate these questions now would seem logical rather than waiting until the need for a booster dose is definitively established, as setting up and conducting interchangeability studies will take time. Additionally, further studies are required to assess if efficacy varies depending on the initial age of administration and the background burden of disease, which may impact natural boosting.

Identification of a correlate of protection [62] could accelerate testing and postlicensure assessments of newer vaccines, which could create

additional demand for newer TCVs, but this may not be feasible in the short-term.

Understanding the impact of TCVs on AMR is also an important research need. There are several ways to potentially assess this – through measurements focused on pathogens in people (e.g. measuring the impact of TCV on incidence of infections caused by drug-resistant *S*. Typhi), or on antimicrobials (e.g. measuring the impact of TCV on antimicrobial consumption or use), or the environment [e.g. measuring impact of TCV introduction on prevalence of antimicrobial resistance genes (ARGs) in pooled environmental samples]. With the increasing prevalence and severity of AMR in circulating *S*. Typhi, particularly in South Asia, establishing the impact of TCV on AMR will be important in demonstrating the full public health value of TCVs, and guidance to countries for how best to measure this would be valuable.

CONCLUSION

In our previous review, we noted that several opportunities for achieving control of typhoid have been missed in the past [1]. The global health community is entering a new stage where we have high-level efficacy data for TCVs and a robust pipeline in development, allied with financial and regulatory support. Nevertheless, TCVs have been adopted in only small number of countries to date, and uptake has arguably been slower than anticipated in both Gavi and non-Gavi countries – driven in part by limited evidence of typhoid disease and economic burden in some countries, the COVID-19 pandemic, and other competing priorities. There is little room for complacency, and continued advocacy is needed to address the ongoing challenge of typhoid fever to improve child health and tackle the rising challenge of antimicrobial resistance.

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REFERENCES AND RECOMMENDED READING

Papers of particular interest, published within the annual period of review, have been highlighted as:
- ■ of special interest
- ■■ of outstanding interest

1. Gibani MM, Britto C, Pollard AJ. Typhoid and paratyphoid fever: a call to action. Curr Opin Infect Dis 2018; 31:440–448.
2. Marchello CS, Hong CY, Crump JA. Global typhoid fever incidence: a systematic review and meta-analysis. Clin Infect Dis 2019; 68:S105.
3. Stanaway JD, Parisi A, Sarkar K, *et al.* The global burden of nontyphoidal salmonella invasive disease: a systematic analysis for the Global Burden of Disease Study 2017. Lancet Infect Dis 2019; 19:1312–1324.
4. Carey ME, MacWright WR, Im J, *et al.* The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences. Clin Infect Dis 2020; 71:S102.
5. ■■ Meiring JE, Shakya M, Khanam F, *et al.* Burden of enteric fever at three urban sites in Africa and Asia: a multicentre population-based study. Lancet Glob Heal 2021; 9:e1688–e1696.

Population-based surveillance study providing crude and adjusted enteric fever incidence rates from predefined census populations in Bangladesh, Malawi, and Nepal.

6. ■■ Garrett DO, Longley AT, Aiemjoy K, *et al.* Incidence of typhoid and paratyphoid fever in Bangladesh, Nepal, and Pakistan: results of the Surveillance for Enteric Fever in Asia Project. Lancet Glob Heal 2022; 10:e978–e988.

Standardized, population-based blood culture surveillance study yielding age-specific crude and adjusted incidence rates of typhoid fever from sites in Bangladesh, Nepal, and Pakistan.

7. Marks F, von Kalckreuth V, Aaby P, *et al.* Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. Lancet Glob Heal 2017; 5:e310–e323.
8. Antillon M, Saad NJ, Baker S, *et al.* The relationship between blood sample volume and diagnostic sensitivity of blood culture for typhoid and paratyphoid fever: a systematic review and meta-analysis. J Infect Dis 2018; 218: S255–S267.
9. WHO. Typhoid: vaccine preventable diseases surveillance standards. 2018.
10. ■■ Aiemjoy K, Seidman JC, Saha S, *et al.* Estimating typhoid incidence from community-based serosurveys: a multicohort study. The Lancet Microbe 2022; 3:e578–e587.

Validation of novel serological approach to enteric fever surveillance conducted in sites with ongoing blood culture surveillance.

11. Hefele L, Black AP, Van Tan T, *et al.* An age-stratified serosurvey against purified Salmonella enterica serovar Typhi antigens in the Lao People's Democratic Republic. PLoS Negl Trop Dis 2021; 15:e0010017.
12. Uzzell CB, Troman CM, Rigby J, *et al.* Environmental surveillance for Salmonella Typhi as a tool to estimate the incidence of typhoid fever in low-income populations. medRxiv 2021. doi:10.1101/2021.05.21.21257547.
13. Frenck RW, Nakhla I, Sultan Y, *et al.* Azithromycin versus ceftriaxone for the treatment of uncomplicated typhoid fever in children. Clin Infect Dis 2000; 31:1134–1138.
14. Nair BT, Simalti AK, Sharma S. Study comparing ceftriaxone with azithromycin for the treatment of uncomplicated typhoid fever in children of India. Ann Trop Med Public Heal 2017; 10:205–210.
15. Klemm EJ, Shakoor S, Page AJ, *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:e00105-18.
16. Butt MH, Saleem A, Javed SO, *et al.* Rising XDR-typhoid fever cases in pakistan: are we heading back to the preantibiotic era? Front Public Heal 2022; 9:794868.
17. Herdman MT, Karo B, Dave J, *et al.* Increasingly limited options for the treatment of enteric fever in travellers returning to England, 2014-2019: a cross-sectional analytical study. J Med Microbiol 2021; 70:001359.
18. Eshaghi A, Zittermann S, Bharat A, *et al.* Importation of extensively drug-resistant Salmonella enterica Serovar Typhi cases in Ontario, Canada. Antimicrob Agents Chemother 2020; 64:e02581-19.
19. Procaccianti M, Motta A, Giordani S, *et al.* First case of typhoid fever due to extensively drug-resistant Salmonella enterica serovar typhi in Italy. Pathogens 2020; 9:151.
20. Howard-Jones A, Kesson AM, Outhred AC, Britton PN. First reported case of extensively drug-resistant typhoid in Australia. Med J Aust 2019; 211:86. e1–286.e1.
21. Hughes MJ, Birhane MG, Dorough L, *et al.* Extensively drug-resistant typhoid fever in the United States. Open Forum Infect Dis 2021; 47:153–154.
22. Nair S, Chattaway M, Langridge GC, *et al.* ESBL-producing strains isolated from imported cases of enteric fever in England and Wales reveal multiple chromosomal integrations of blaCTX-M-15 in XDR Salmonella Typhi. J Antimicrob Chemother 2021; 76:1459–1466.

23. Ingle DJ, Andersson P, Valcanis M, *et al.* Genomic epidemiology and antimicrobial resistance mechanisms of imported typhoid in Australia. Antimicrob Agents Chemother 2021; 65:e0120021.

24. François Watkins LK, Winstead A, Appiah GD, *et al.* Update on extensively drug-resistant Salmonella Serotype Typhi infections among travelers to or from Pakistan and report of ceftriaxone-resistant Salmonella Serotype Typhi infections among travelers to Iraq - United States. MMWR Morb Mortal Wkly Rep 2020; 69:618–622.

25. Wang Y, Lu D, Jin Y, *et al.* Extensively drug-resistant (XDR) Salmonella Typhi outbreak by waterborne infection — Beijing Municipality, China January–February 2022. China CDC Weekly 2022; 4:254–258.

26. Godbole G, McCann N, Jones SM, *et al.* Ceftriaxone-resistant Salmonella Typhi in a traveller returning from a mass gathering in Iraq. Lancet Infect Dis 2019; 19:467.

27. Phoba MF, Barbé B, Lunguya O, *et al.* Salmonella enterica serovar Typhi Producing CTX-M-15 Extended Spectrum β-Lactamase in the Democratic Republic of the Congo. Clin Infect Dis 2017; 65:1229–1231.

28. ■ Argimón S, Nagaraj G, Shamanna V, *et al.* Circulation of third-generation cephalosporin resistant Salmonella Typhi in Mumbai, India. Clin Infect Dis 2021; 74:2234–2237.
Emergence of ESBL *S.* Typhi in India, unrelated to XDR *S.* Typhi strain.

29. ■■ Yousafzai MT, Karim S, Qureshi S, *et al.* Effectiveness of typhoid conjugate vaccine against culture-confirmed Salmonella enterica serotype Typhi in an extensively drug-resistant outbreak setting of Hyderabad, Pakistan: a cohort study. Lancet Glob Heal 2021; 9:e1154–e1162.
Demonstration of effectiveness of TCV against *S.* Typhi and XDR *S.* Typhi following outbreak response TCV vaccination campaign.

30. Batool R, Tahir Yousafzai M, Qureshi S, *et al.* Effectiveness of typhoid conjugate vaccine against culture-confirmed typhoid in a peri-urban setting in Karachi: a case-control study. Vaccine 2021; 39:5858–5865.

31. Upadhyay R, Nadkar MY, Muruganathan A, *et al.* API recommendations for the management of typhoid fever. J Assoc Physicians India 2015; 63:77–96.

32. Nabarro LE, McCann N, Herdman MT, *et al.* British infection association guidelines for the diagnosis and management of enteric fever in England. J Infect 2022; 84:469–489.

33. Hooda Y, Sajib MSI, Rahman H, *et al.* Molecular mechanism of azithromycin resistance among typhoidal Salmonella strains in Bangladesh identified through passive pediatric surveillance. PLoS Negl Trop Dis 2019; 13: e0007868.

34. Iqbal J, Dehraj IF, Carey ME, *et al.* A race against time: reduced azithromycin susceptibility in Salmonella enterica Serovar Typhi in Pakistan. mSphere 2020; 5:e00215-20.

35. Duy PT, Dongol S, Giri A, *et al.* The emergence of azithromycin-resistant Salmonella Typhi in Nepal. JAC Antimicrobial Resist 2020; 2:dlaa109.

36. ■ Carey ME, Jain R, Yousuf M, *et al.* Spontaneous emergence of azithromycin resistance in independent lineages of Salmonella Typhi in Northern India. Clin Infect Dis 2021; 72:E120–E127.
Genomic investigation of azithromycin-resistant *S.* Typhi from South Asia, showing independent emergence in several lineages and locations.

37. Octavia S, Chew KL, Lin RTP, Teo JWP. Azithromycin-resistant Salmonella enterica Serovar Typhi AcrB-R717Q/L, Singapore. Emerg Infect Dis 2021; 27:624–627.

38. Sikorski MJ, Hazen TH, Desai SN, *et al.* Persistence of rare Salmonella Typhi genotypes susceptible to first-line antibiotics in the remote islands of Samoa. medRxiv 2022. doi:10.1101/2022.03.22.22271657.

39. Qureshi S, Naveed AB, Yousafzai MT, *et al.* Response of extensively drug resistant salmonella typhi to treatment with meropenem and azithromycin, in Pakistan. PLoS Negl Trop Dis 2020; 14:e0008682.

40. Mylona E, Voong Vinh P, Qureshi S, *et al.* Tebipenem as an oral alternative for the treatment of typhoid caused by XDR Salmonella Typhi. J Antimicrob Chemother 2021. doi:10.1093/jac/dkab326.

41. ■ Kariuki S, Dyson ZA, Mbae C, *et al.* Multiple introductions of multidrug-resistant typhoid associated with acute infection and asymptomatic carriage, Kenya. Elife 2021; 10:e67852.
Case–control study and genomic analysis in Nairobi providing evidence of carriers contributing to sustained community transmission of *S.* Typhi.

42. Duy PT, Thieu NTV, Nguyen TNT, *et al.* Gallbladder carriage generates genetic variation and genome degradation in Salmonella Typhi. PLOS Pathog 2020; 16:e1008998.

43. McCann N, Scott P, Parry C, Brown M. Antimicrobial agents for the treatment of enteric fever chronic carriage: a systematic review. medRxiv 2021; 17: e0272043.

44. WHO. Typhoid vaccines: WHO position paper – March 2018. Wkly Epidemiol Rec 2018; 93:477–500.

45. WHO: Weekly Epidemiological Record, 25 January 2019, vol. 94, 4 - Global Vaccine Safety meeting: safety of typhoid conjugate vaccine. Available at: https://www.who.int/publications/i/item/10665279829. [Accessed 29 May 2022]

46. ■ Vadrevu KM, Raju D, Rani S, *et al.* Persisting antibody responses to Vi polysaccharide-tetanus toxoid conjugate (Typbar TCV®) vaccine up to 7 years following primary vaccination of children. Vaccine 2021; 39:6682–6690.
Demonstration of duration of immune response following single dose of TCV and single dose plus booster 2 years after primary vaccination in Indian pediatric population.

47. Jin C, Gibani MM, Moore M, *et al.* Efficacy and immunogenicity of a Vi-tetanus toxoid conjugate vaccine in the prevention of typhoid fever using a controlled human infection model of Salmonella Typhi: a randomised controlled, phase 2b trial. Lancet 2017; 390:2472–2480.

48. Lin FY, Ho VA, Khiem HB, *et al.* The efficacy of a Salmonella typhi Vi conjugate vaccine in two-to-five-year-old children. N Engl J Med 2001; 344:1263–1269.

49. Thuluva S, Paradkar V, Matur R, *et al.* A multicenter, single-blind, randomized, phase-2/3 study to evaluate immunogenicity and safety of a single intramuscular dose of biological E's Vi-capsular polysaccharide-CRM 197 conjugate typhoid vaccine (TyphiBEV TM) in healthy infants, children, and adults in comparison with a licensed comparator. Hum Vaccin Immunother 2022; 18:2043103.

50. ■■ Patel PD, Patel P, Liang Y, *et al.* Safety and efficacy of a typhoid conjugate vaccine in Malawian Children. N Engl J Med 2021; 385:1104–1115.
Demonstration of safety and efficacy of a single dose of TCV in individually randomized trial in Malawi over 18–24-month follow-up.

51. Shakya M, Colin-Jones R, Theiss-Nyland K, *et al.* Phase 3 efficacy analysis of a typhoid conjugate vaccine trial in Nepal. N Engl J Med 2019; 381:2209–2218.

52. ■■ Qadri F, Khanam F, Liu X, *et al.* Protection by vaccination of children against typhoid fever with a Vi-tetanus toxoid conjugate vaccine in urban Bangladesh: a cluster-randomised trial. Lancet (London, England) 2021; 398:675–684.
Demonstration of safety, efficacy, and total vaccine protection conferred by a single dose of TCV in cluster-randomized trial in Bangladesh; study underpowered to measure herd protection.

53. Sur D, Ochiai RL, Bhattacharya SK, *et al.* A cluster-randomized effectiveness trial of Vi Typhoid vaccine in india. N Engl J Med 2009; 361:335–344.

54. Haselbeck AH, Tadesse BT, Park J, *et al.* Evaluation of typhoid conjugate vaccine effectiveness in Ghana (TyVEGHA) using a cluster-randomized controlled phase IV trial: trial design and population baseline Characteristics. Vaccines 2021; 9:281.

55. Date K, Shimpi R, Luby S, *et al.* Decision making and implementation of the first public sector introduction of typhoid conjugate vaccine—Navi Mumbai India. Clin Infect Dis 2020; 71:S172–S178.

56. Lightowler MS, Manangazira P, Nackers F, *et al.* Effectiveness of typhoid conjugate vaccine in Zimbabwe used in response to an outbreak among children and young adults: a matched case control study. Vaccine 2022; 40:4199–4210.

57. Sirima SB, Ouedraogo A, Barry N, *et al.* Safety and immunogenicity of Vi-typhoid conjugate vaccine co-administration with routine 9-month vaccination in Burkina Faso: a randomized controlled phase 2 trial. Int J Infect Dis 2021; 108:465–472.

58. Sirima SB, Ouedraogo A, Barry N, *et al.* Safety and immunogenicity of co-administration of meningococcal type A and measles-rubella vaccines with typhoid conjugate vaccine in children aged 15-23 months in Burkina Faso. Int J Infect Dis 2021; 102:517–523.

59. Choi SK, Baik YO, Kim CW, *et al.* An open-label, comparative, single dose, clinical Phase I study to assess the safety and immunogenicity of typhoid conjugate vaccine (Vi-CRM197) in healthy Filipino adults. Vaccine 2021; 39:2620–2627.

60. Kumar Rai G, Saluja T, Chaudhary S, Tamrakar D, *et al.* Safety and immunogenicity of the Vi-DT typhoid conjugate vaccine in healthy volunteers in Nepal: an observer-blind, active-controlled, randomised, noninferiority, phase 3 trial. Lancet Infect Dis 2022; 22:529–540.

61. Shakya M, Voysey M, Theiss-Nyland K, *et al.* Efficacy of typhoid conjugate vaccine in Nepal: final results of a phase 3, randomised, controlled trial. Lancet Glob Heal 2021; 9:e1561–e1568.

62. Jin C, Hill J, Gunn BM, *et al.* Vi-specific serological correlates of protection for typhoid fever. J Exp Med 2020; e20201116:218.

Appendix 11. Whole genome sequence analysis of *Salmonella* Typhi provides evidence of phylogenetic linkage between cases of typhoid fever in Santiago, Chile in the 1980s and 2010-2016 (publication)

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

Whole genome sequence analysis of *Salmonella* Typhi provides evidence of phylogenetic linkage between cases of typhoid fever in Santiago, Chile in the 1980s and 2010–2016

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Abstract

Typhoid fever epidemiology was investigated rigorously in Santiago, Chile during the 1980s, when *Salmonella enterica* serovar Typhi (*S*. Typhi) caused seasonal, hyperendemic disease. Targeted interventions reduced the annual typhoid incidence rates from 128–220 cases/10^5 population occurring between 1977–1984 to <8 cases/10^5 from 1992 onwards. As such, Santiago represents a contemporary example of the epidemiologic transition of an industrialized city from amplified hyperendemic typhoid fever to a period when typhoid is no longer endemic. We used whole genome sequencing (WGS) and phylogenetic analysis to compare the genotypes of *S*. Typhi cultured from acute cases of typhoid fever occurring in Santiago during the hyperendemic period of the 1980s (n = 74) versus the nonendemic 2010s (n = 80) when typhoid fever was rare. The genotype distribution between "historical" (1980s) isolates and "modern" (2011–2016) isolates was similar, with genotypes 3.5 and 2 comprising the majority of isolations, and 73/80 (91.3%) of modern isolates matching a genotype detected in the 1980s. Additionally, phylogenomically 'ancient' genotypes 1.1 and 1.2.1, uncommon in the global collections, were also detected in both eras, with a notable rise amongst the modern isolates. Thus, genotypes of *S*. Typhi causing acute illness in the modern nonendemic era match the genotypes circulating during the hyperendemic 1980s.

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The persistence of historical genotypes may be explained by chronic typhoid carriers originally infected during or before the 1980s.

Author summary

Studies of *Salmonella* Typhi (the cause of typhoid fever) rarely include isolates collected both before and after the interruption of hyperendemic transmission because this typically occurred decades before modern bacteria preservation methods. After substantial reduction in disease, it was assumed that sporadic cases and infrequent outbreaks were due to either chronic biliary carriers or importations, but this was difficult to characterize with low resolution bacterial typing methods. In Santiago, Chile, typhoid fever persisted at hyperendemic levels through the 1980s until organized control efforts in the 1980s and changes to wastewater policy in 1991 caused annual typhoid incidence to plummet.

In this study, we used whole genome sequencing (WGS) to investigate whether recent sporadic cases occurring in Santiago in the 2010s were genomically similar to *S*. Typhi circulating in the 1980s, or dissimilar, possibly representing importations of *S*. Typhi from outside of Chile. We found concordance amongst *S*. Typhi genotypes between the 1980s and 2010s, and differences from genotypes circulating in Southeast Asia and Africa where typhoid remains hyperendemic. Our findings suggest that a proportion of modern, rare typhoid cases in Santiago are autochthonous, and that chronic carriers or another unknown reservoir likely contribute. Broadly, our findings corroborate the epidemiologic importance of long-term reservoirs of typhoid fever decades after typhoid elimination.

Introduction

Typhoid fever is a systemic infection, caused by the bacterium *Salmonella enterica* subspecies *enterica* serovar Typhi (*S*. Typhi), which is spread through ingestion of food and water contaminated with human faeces containing *S*. Typhi. Typhoid fever in Santiago, Chile rose to hyperendemic levels in the mid-1970s with a striking annual incidence of 177–220 cases per 10^5 population per year from 1977–1983, partially declined during the years 1984–1991 (51–128 cases/10^5 per year), and finally plummeted below 8 cases/10^5 per year after 1991, reaching nonendemic levels of ~0.5/10^5 in 2012 [1]. During the hyperendemic years, the highest incidence rate was observed in school age children, and case numbers peaked in the rainless summer months [2]. To address this public health issue, in 1979 the Ministry of Health of Chile implemented a Typhoid Fever Control Program involving strategic preventive interventions including large-scale field trials of live oral typhoid vaccine Ty21a in >500,000 schoolchildren [3–6], and identification and treatment of chronic carriers among food handlers [7]. Collectively, these interventions led to a steady decline in typhoid cases between 1983 and 1990 [3–6,8].

Epidemiologic and environmental bacteriology studies in the 1980s undertaken by the Chilean Typhoid Fever Control Program also implicated the use of raw untreated sewage water for irrigation of vegetable crops during the rainless summer months as a key risk factor responsible for amplified transmission of *S*. Typhi in Santiago [8,9]. In April 1991, the government of Chile abruptly prohibited the practice of irrigating crops with untreated sewage wastewater in Santiago [1,8,10,11]. This action was taken following the explosive epidemics of El Tor cholera in 1991 that occurred in neighbouring countries to the North, including in Peru, beginning in late January [12,13], followed by Ecuador in February [13], and Colombia in March [13]. The pivotal stimulus

for the intervention was a small outbreak of cholera in Santiago, Chile, in April, 1991 [1,8,10,11]. Following imposition and strict enforcement of the prohibition of use of untreated sewage water for irrigation, the Santiago cholera epidemic quickly came to an end [10,11], and the incidence of typhoid dropped precipitously in subsequent years [1,8,10]. From 2000–2014, the annual incidence of confirmed typhoid fever in Santiago has ranged from 0.20–1.18 cases/10^5 population. The current rare sporadic cases of typhoid in Santiago include illnesses among: travellers who visit countries with high incidence rates of typhoid; immigrants from typhoid-endemic countries; and transmission from Chilean chronic carriers who were originally infected decades earlier (pre-1991) during the era of hyperendemic transmission.

Phylogenetic analysis of whole genome sequencing (WGS) data has become the gold standard for inferring relatedness between *S*. Typhi isolates [14]. WGS can also be used to assign genotypes, identify and characterize plasmids, compare gene content and homology, and identify molecular determinants of antibiotic resistance and virulence [15,16]. Whereas WGS data from many global isolates are available, few bacterial collections include isolates from both before and after the interruption of amplified hyperendemic typhoid fever. Also, WGS-derived information concerning the genotypes isolated from typhoid fever cases in South American countries is scarce. The majority of publicly available sequenced isolates come from hyperendemic regions in South Asia and East Africa where H58 (4.3.1) genotypes dominate [17].

Herein we describe a genomic analysis of *S*. Typhi isolates from Santiago, Chile from two distinct epidemiologic periods: the early 1980s (N = 74), when typhoid was hyperendemic, and from 2010–2016 (N = 80), a quarter century after the interruption of amplified transmission of *S*. Typhi in Santiago. We show that the genotypic composition of the population structure is similar between these two periods, even though the local epidemiology has changed drastically. In addition, we describe a potential carrier-related cluster of the "ancient" 1.1 clade, and the identification of a new variant of plasmid pHCM2 carried by 2 isolates of another ancient genotype (1.2.1).

Material and methods

Study design

In total, 74 "historical" isolates of *S*. Typhi from cases of acute typhoid fever in Santiago, Chile in the period 1981 to 1986, during the era of hyperendemic typhoid, were obtained from the culture collection of the Center for Vaccine Development and Global Health of the University of Maryland, Baltimore. "Modern" *S*. Typhi isolates (n = 80) were strains collected from acute cases of typhoid fever and referred to the Instituto de Salud Pública de Chile (ISP, Institute of Public Health) by clinical microbiology laboratories from 2011–2016; these referred strains were generally accompanied by only limited demographic and clinical information.

DNA extraction and sequencing

DNA was isolated from the 154 *S*. Typhi isolates using the Norgen DNA extraction kit as per the manufacturer's instructions. Genomic DNA was sequenced by Illumina HiSeq at the Wellcome Sanger Institute, generating 125 bp paired reads. Raw read data were deposited in the European Nucleotide Archive and individual accession numbers are listed in S1 Table.

Assembly, mapping, SNP-calling and genotype assignment

Raw Illumina reads were assembled using Velvet v1.2 via the Wellcome Sanger Institute automated analysis pipeline [18]. Sequenced reads were mapped and annotated, single nucleotide polymorphisms (SNPs) were called against the *S*. Typhi CT18 reference genome [19] using

SMALT v0.7.4 and subsequent variant calling was carried out as previously described [20]. Genotypes were assigned using GenoTyphi (v1.9.0) [14,16].

Phylogenetic analysis

Previously defined [21–23] recombinant regions such as prophages and plasmids were excluded manually, and any remaining recombinant regions were filtered using Gubbins (v2.5.0) [24]. The resultant core genome alignment was used to infer Maximum Likelihood (ML) phylogenies using RAxML (v8.2.8) [25], specifying a generalized time-reversible model and a gamma distribution to model site-specific rate variation (GTR+ Γ substitution model; GTRGAMMA in RAxML) with 100 bootstrap pseudoreplicates used to assess branch support. SNP distances for the core genome alignment of all the strains have also been calculated from this alignment using snp-dists package (available at: https://github.com/tseemann/snp-dists). Resulting phylogenies were visualized with iTOL [26] and are available for interactive inspection on Microreact [27] (https://microreact.org/project/j25cGgoVX8ixQCRstPVhgo-chile-1980-2010).

Molecular determination of AMR, plasmids and virulence genes

Raw read data were screened using SRST2 v0.2.0 [15] with the ARG-ANNOT [28], PlasmidFinder [29], and *Salmonella* Virulence Factor DB databases [30] to detect known and putative antimicrobial resistance (AMR) genes, known plasmid replicons, and known virulence-associated genes, respectively. Homology cutoffs of 90% nucleotide similarity were used for all SRST2 screens.

Plasmid pHCM2 sequences were assembled with Unicycler [31], and the circularised sequences extracted from *de bruijn* assembly graphs with Bandage (v0.8.1) [32]. These were then compared with the pHCM2 reference sequence (accession no: AL513384) using Artemis-ACT [33] and visualized with EasyFig [34].

Results

S. Typhi genotypes in Santiago, Chile

Genotypic analysis indicated that most of the isolates from Santiago in this study belonged to genotypes 2 (n = 43, 27.9%), 3.5 (n = 51, 33.1%) and 1.1 (n = 22, 14.3%) (Table 1). While these

Table 1. S. Typhi genotypes detected in isolates from acute clinical cases in Santiago, Chile during the hyperendemic 1980s versus the nonendemic 2010s.

	1983–1986	2011–2016	TOTAL
1.1	2 (2.7%)	20 (25%)	22 (14.3%)
1.2.1	1 (1.4%)	5 (6.3%)	6 (3.9%)
2	30 (40.5%)	13 (16.3%)	43 (27.9%)
2.0.2	11 (14.9%)	5 (6.3%)	16 (10.4%)
2.2	-	3 (3.8%)	3 (1.9%)
2.3.2	1 (1.4%)	1 (1.3%)	2 (1.3%)
2.3.3	1 (1.4%)	1 (1.3%)	2 (1.3%)
2.3.4	1 (1.4%)	-	1 (0.6%)
3.1	1 (1.4%)	-	1 (0.6%)
3.3	-	1 (1.3%)	1 (0.6%)
3.5	25 (33.8%)	26 (32.5%)	51 (33.1%)
4.1	1 (1.4%)	2 (2.5%)	3 (1.9%)
4.3.1.1	-	2 (2.5%)	2 (1.3%)
4.3.1.2	-	1 (1.3%)	1 (0.6%)
TOTAL	74	80	154

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three genotypes were the most prevalent, we also detected 11 additional distinct genotypes across the two time periods. These include three previously described importations of isolates belonging to H58 genotypes 4.3.1.1 and 4.3.1.2 [35].

The genomic population structure of *S*. Typhi in Santiago appeared highly consistent between the hyperendemic and post-hyperendemic eras (Fig 1). Both rare "ancient" genotypes 1.1 and 1.2.1, as well as more commonly found genotypes 2 and 3.5, were frequently present across both study periods. Despite several decades having passed since the cessation of the era of hyperendemic transmission, most of the post-2010 sporadic *S*. Typhi isolates shared bootstrap-supported phylogenetic clusters with the 1980 hyperendemic isolates. Only within genotype 1.1 did recent and historical sequences form independent clusters. The intermingling of historical and recent sequences suggests that the 2010s isolates originated from progenitors within the same pool of *S*. Typhi circulating in the 1980s.

Amongst the 151 non-H58 isolates, few antimicrobial resistance mechanisms were detected. One genotype 2.0.0 isolate carried a *tetB* gene, known to encode a mechanism of resistance to tetracyclines (albeit not an antibiotic of clinical relevance for treatment of typhoid fever). Two genotype 2.2 isolates and one genotype 4.1 isolate had single non-synonymous point mutations in the Quinolone Resistance Determining Region (QRDR) of genes *gyrA* and *gyrB*, associated with reduced susceptibility to fluoroquinolones [36]; in the late 1980s, ciprofloxacin was the antibiotic of choice for treating patients with typhoid fever. Only 17 candidate plasmids were detected across all the isolates, including a Colicin plasmid, three related to plasmid pHCM2 and 13 of incompatibility type IncX1 (Fig 1).

Global transmission dynamics

To better understand how this local population structure fits into the global context of *S*. Typhi, a maximum likelihood phylogenetic tree was generated using a total of 2,013 non-H58 *S*. Typhi genomes from approximately 70 countries (Fig 2). The *S*. Typhi isolates from Santiago of genotypes 3.5, 2, 1.1 and 1.2.1 clustered together and were genetically distinct from those sequenced previously from other countries. The other genotypes present in Santiago were mostly found on long branches, indicating significant differentiation from their closest relatives in the global context phylogeny.

"Ancient" *S*. Typhi clades endemic to Chile

Two of the clades common in our dataset, 1.1 and 1.2, are found closer to the root of the global *S*. Typhi tree and thus can be considered "ancient" clades, which are detected with very low global frequency. Of the 1.1 isolates (n = 22), two were from the 1980s collection (Fig 3). Only six other isolates, 0.3% of the global collection described by Wong et al [14], belonged to clade 1.1. Interestingly, a cluster of closely related genotype 1.1 *S*. Typhi isolates was detected in the years 2011 (1 isolate) and 2012 (19 isolates). These isolates also exhibit lower genetic variation (median SNP distance of 8) than those observed among the two 1980s 1.1 isolates (119 SNPs). The smallest SNP distance between a 1980s 1.1 isolate and a 2010s 1.1 isolate was 79 SNPs. This suggests the 2011–2012 isolates have a recent common ancestor.

We sought to determine if there was a specific virulence gene associated with the 2011–2012 genotype 1.1 cluster, using SRST2 with the *Salmonella* VFDB. Although there were no additional or missing virulence factor-associated genes detected, this screening did reveal that all of the genotype 1.1 isolates from 2011–2012 cluster harboured a SNP in the *ssrA* gene. The alignment of this gene revealed the same missense mutation (G to A at position 1648968 of CT18, causing a glycine to glutamic acid amino acid change) in the *ssrA* gene (*ssrA*-G639E), (Fig 3).

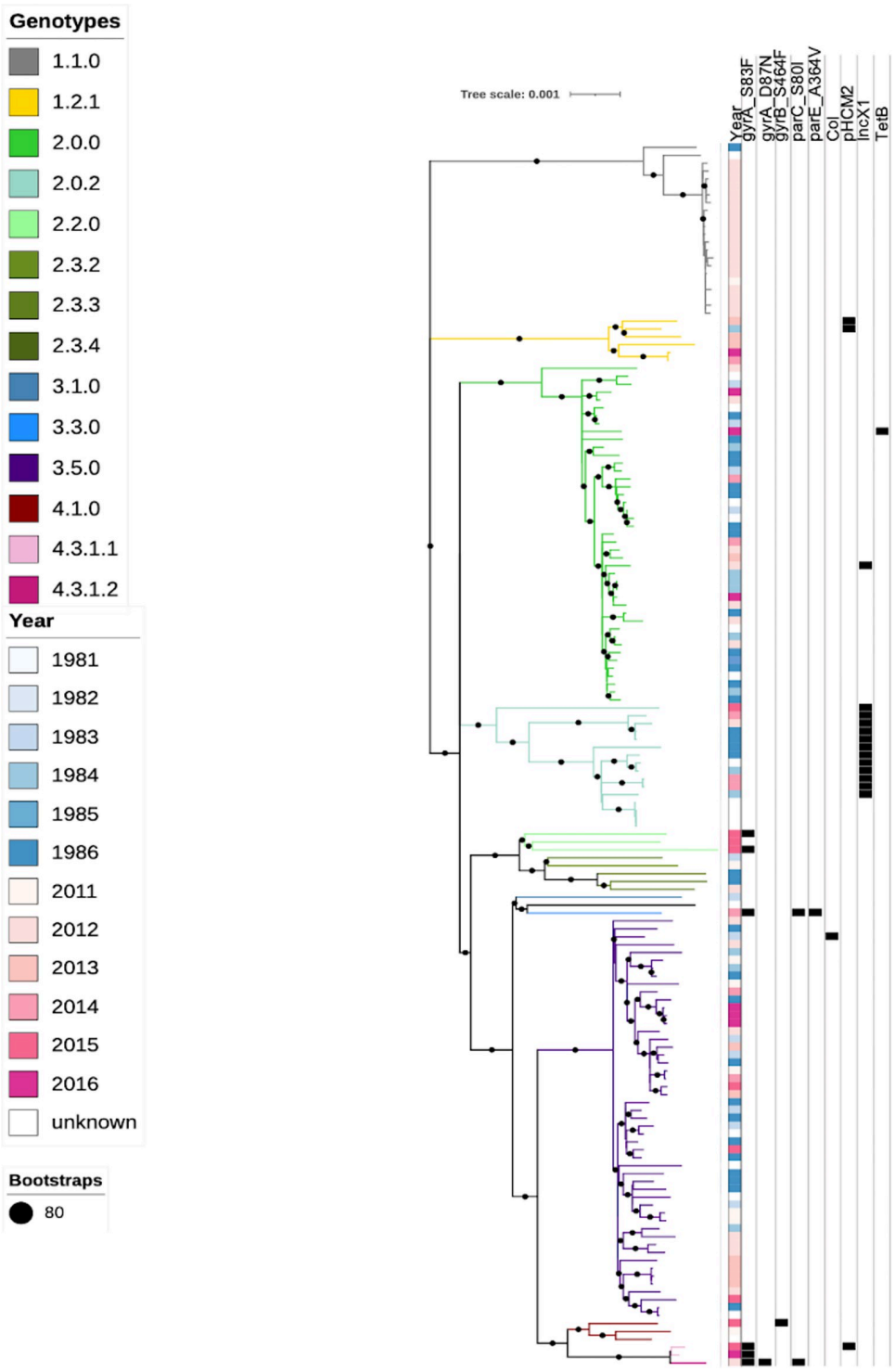

Fig 1. Genotypes and molecular determinates of AMR and plasmids in the Santiago collection. SNP based maximum likelihood core genome phylogeny indicating the genotypes of all the Santiago *S*. Typhi isolates is shown by the branch colour as per the inset legend. A diverse range of 14 genotypes were detected including 1.1, 1.2.1, 2, 2.0.2, 2.2, 2.3.2, 2.3.3, 2.3.4, 3.1, 3.3, 3.5, 4.1, 4.3.1.1, 4.3.1.2. Most of the isolates belonged to genotype 1.1, 2 and 3.5. Year of isolation, SNPs in the QRDR region associated with reduced susceptibility to fluoroquinolones as well as plasmids and detected resistance genes are shown in the columns adjacent to the tree. Bootstrap confidence of 80 or above is indicated with a black circle on the branch.

https://doi.org/10.1371/journal.pntd.0010178.g001

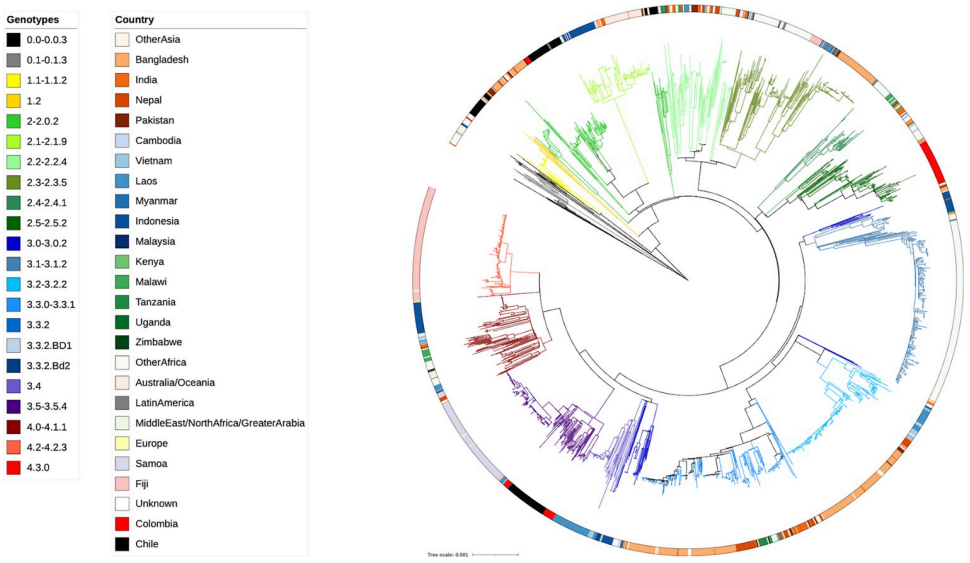

Fig 2. A global context maximum likelihood core genome phylogeny incorporating publicly available non-H58 and Santiago *S*. Typhi isolates. SNP based core genome maximum likelihood tree of 2013 global isolates, branches are coloured by genotype as per the inset legend; the ring indicates country of isolation as per the inset legend; Santiago isolates are highlighted in black and the Colombian isolates in red.

https://doi.org/10.1371/journal.pntd.0010178.g002

The other ancient clade present amongst the Santiago isolates was genotype 1.2.1. This was the only clade in our datasets (except from one recently imported H58 isolate) in which isolates harboured pHCM2 plasmids. pHCM2 plasmids are found in ~15% of *S*. Typhi and are

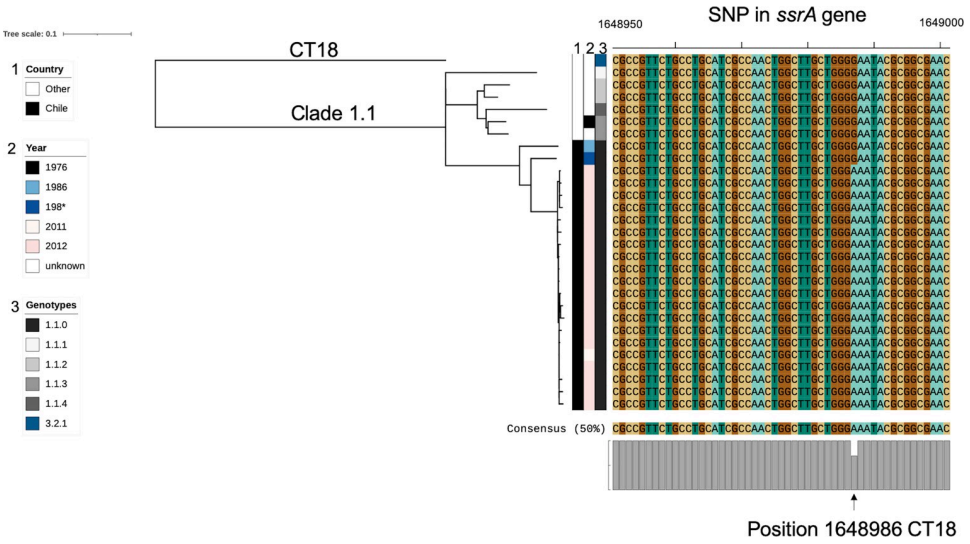

Fig 3. *S*. Typhi clade 1.1 phylogeny showing sequences carrying *ssrA* mutation (*ssrA*-G639E). Phylogenetic core genome analysis of all *S*. Typhi isolates of genotype 1.1 identified from the global collection. The majority of these isolates were collected in Santiago (black bar); two isolates belonged to the 1980s collection appearing ancestral to the 2010s collection. The twenty isolates from 2011–2012 showed an interesting cluster with a median SNP distance of 8 SNPs which is unlikely to have evolved among acute cases, where the mutation rate is calculated to be < 1 SNP/genome/year, in one year. Also shown is an alignment of a section of the *ssrA* gene, showing the conserved SNP at position 1648986 of the *S*. Typhi CT18 reference genome. * the precise isolation year of one of the 1980s isolates was unclear.

https://doi.org/10.1371/journal.pntd.0010178.g003

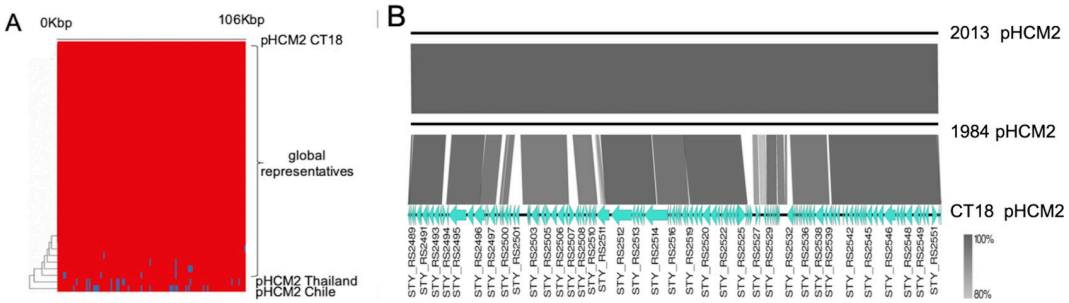

Fig 4. Genetic comparison of cryptic plasmid pHCM2-like sequences. Global representative collection of pHCM2 aligned to the reference pHCM2 from CT18. (A) Presence and absence of genes located on pHCM2. Red indicates presence of the genes located on the reference pHCM2 sequence from CT18, while blue indicates absence of the genes compared to the reference. Nucleotide BLAST comparison of the 2 Chilean assembled pHCM2-like plasmids (ERR271054, ERR4289185) against the reference pHCM2 plasmid from *S*. Typhi CT18 (accession no:AL513384) (B). Gene annotations are shown for the reference pHCM2 from CT18, with the shading intensity indicating percent nucleotide homology between the plasmid sequences.

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generally highly conserved (Fig 4A). To our knowledge, based on the data available in the *S*. Typhi public collection on Pathogenwatch: (https://pathogen.watch/collection/1y3cdsjq55hf-public-genomes- accessed Dec 2021), pHCM2 plasmids have not been detected in ancient clades 0.0 to 1.2 previously. Two pHCM2 plasmids were found among our isolates from genotype 1.2.1; one in a 1984 isolate (ERR2710544) and the other from 2013 (ERR4289185). These plasmids were highly similar to each other, but significantly different from the pHCM2 found on *S*. Typhi reference CT18 (accession no: AL513384), and from another novel pHCM2 found in a 1973 isolate from Thailand [37]. The pHCM2 sequences described here differed by >2000 SNPs (98.4% nucleotide identity, 89% coverage) from the reference (Fig 4).

Discussion

This study utilizes WGS analyses to compare *S*. Typhi isolates from patients who developed acute typhoid fever in Santiago during the modern era (i.e., 2011–2016, decades post-interruption of amplified typhoid transmission in 1991) with isolates from acute cases during the hyper-endemic era of the 1980s. Using phylogenetics and genome-wide screening, we compared the genotypes, AMR determinants and plasmid content of *S*. Typhi isolates between these two time periods. Whilst comparisons of isolates from the 1980's with isolates from 1990 (post vaccinating interventions-but pre-irrigation ban, when incidences were still high 47 cases /10^5 [1]) have been undertaken previously with pulse field gel electrophoresis (PFGE) [38], to our knowledge, this is the first WGS-based analysis comparing *S*. Typhi collected during a period of hyper-endemicity and decades after typhoid control.

The observation that the 1980s and 2010s collections of *S*. Typhi isolates do not cluster independently within our commonly found genotypes (2, 3.5 and 1.2.1) suggests that many of the sporadic cases detected in the 2010s share common ancestry with *S*. Typhi cases from the 1980s. These may, in turn, be linked to shedding of *S*. Typhi from chronic typhoid carriers. This is expected decades after hyper-endemic typhoid is controlled, as chronic carriers will continue to shed sporadically until they are treated or die out of the population [39].

Although there have been several detected introductions of the globally dominant H58 *S*. Typhi clade (4.3.1) into Santiago, as occurs with most industrialized countries in North America, Europe, and Australia, this was not a frequent genotype found in the sporadic modern Chilean cases. This is distinct from countries such as the United Kingdom where imported

cases of H58 *S*. Typhi have become common among travel-associated typhoid cases in recent years [40,41].

S. Typhi WGS data from a recent study in Colombia found genotypes 2, 2.5 and 3.5 to be the local endemic genotypes [42]. Genotype 2.5 was not detected among the Santiago isolates; however, genotype 3.5 and 2 were detected in Santiago, and were the nearest genomic neighbours of the Colombian isolates on the global tree. Even though the sequenced isolates from Colombia and Santiago, Chile both contained isolates from the 2010s and historical isolates, they clustered separately on the global tree, suggesting that there has not been obvious transmission between the two countries. More representative WGS data from other Central and South American countries are needed to determine if this is the case with other unsampled potentially endemic countries in the area.

One of the more interesting findings from this dataset was the cluster of 20 highly related strains of genotype 1.1 in 2011–2012. These isolates differed by a median of 8 SNPs in the core genome. For context, the average core genome mutation rate in *S*. Typhi is approximately 1 substitution/genome/year every year [21,43–45]. As such, it is unlikely that these isolates originated from a clonal source and sequentially acquired this genetic variation in <2 years. Conversely, these strains are clearly highly related and were all collected during a short time span. A potential explanation to account for this cluster is that it was an outbreak caused by bacteria shed from a long-term carrier. *S*. Typhi colonizing the gallbladder or other long-term physiologic niche of a chronic typhoid carrier may evolve and mutate over time into a heterogeneous, yet closely related, community of bacteria [46]. Thus, the closely related nature of these isolates occurring mainly over a single year and during a period when typhoid was no longer hyperendemic in Santiago could be explained by an outbreak instigated by a chronic carrier who was shedding multiple highly related strains. If this hypothetical carrier was working as a food handler, intermittently contaminating food or water consumed by subjects during 2011–12, this could result in the cluster of closely related isolates we observe. The cessation of cases could then be explained by a change of employment or residence, a cholecystectomy procedure, a course of antibiotics that concentrate in the gallbladder (e.g., ciprofloxacin) prescribed for another infection or surgery, or death of the hypothetical carrier at the end of 2012.

Notably, the only detectable difference in virulence factor-associated genes from this potential carrier-related 2011–2012 cluster is the *ssrA* missense mutation. The *ssrA/B* two component response regulator is a principle regulator of *Salmonella* pathogenicity island-2 (SPI-2) [47,48]. SPI-2 has been shown to be insignificant in *S*. Typhi macrophage survival capability and *S*. Typhi's infection efficiency of humanized mice [49,50]. The *ssrA* gene has been linked to biofilm formation in *Salmonella* and persistence on gallstones [51,52]. This observation may be worth additional investigation to investigate the role of SPI-2 and *ssrA* in Typhoid carriage.

Our analysis identified a novel variant of cryptic plasmid pHCM2 in two sequences from genotype 1.2.1. A pHCM2 type plasmid was present in the *S*. Typhi reference isolate CT18 from Vietnam [19] and the plasmid family was described in some detail [53]. Interestingly, any role for pHCM2 in *S*. Typhi biology remains elusive. We know that almost all of the pHCM2 plasmids in the global *S*. Typhi collection are highly conserved in terms of overall nucleotide diversity. However, the Chilean pHCM2 from clade 1.2.1 is substantially different from most other family members. The only other reported pHCM2 plasmid with a similar level of variation is harboured in a *S*. Typhi Thailand isolate from 1973 [37].

The main limitation of this study is the limited sample size of *S*. Typhi genomes from both the historical hyperendemic and modern nonendemic periods in Santiago, Chile. Although not all isolates were stored during the hyperendemic period more than 40 years ago, we incorporated all available stored isolates from acute cases. For the modern time period we used a

similar number of available isolates exclusively from acute cases to represent circulating infection. Among the approximately 7 million inhabitants in Santiago, Chile, few blood culture isolates from acute cases of typhoid fever were recorded in the 2010s, ($<0.5/10^5$ cases per year) and not all isolates are sent to the ISP; thus, only a limited number of *S*. Typhi isolates were available for sequencing. Nevertheless, the study design, based on *S*. Typhi causing acute disease between epidemiologically-distinct eras, lends support to our primary finding: the same genotypes circulating in the hyperendemic 1980s are found circulating in the modern 2010s period.

There are especially limited genomic data available for *S*. Typhi circulating in neighbouring countries, presenting another limitation of this study. Nevertheless, efforts to foster sequencing infrastructure and data generated in South America through the PAHO regional networks have accelerated during the Covid-19 pandemic. It is expected that whole genome sequencing of other pathogens, like *S*. Typhi, will follow in this region.

In conclusion, this genomics analysis suggests that asymptomatic typhoid carriers (or other unknown reservoirs) are likely responsible for continuing sporadic typhoid cases in Chile. The ancient clades present in the country have persisted. We recommend that all sporadic typhoid cases and clusters should be evaluated using epidemiological outbreak investigations and WGS as routine surveillance. This will help ensure the detection of any potential introduction or development of drug-resistant *S*. Typhi and may lead to the identification and treatment of carriers, which would further limit ongoing disease transmission.

Supporting information

S1 Table. S1 Table includes from left to right: Id, lane accession, __latitude, __longitude, genotype, Ampicillin resistance, Cephalexin resistance, Chloramphenicol resistance, Ciprofloxacin resistance, Nalidixic acid resistance, Sulfamethoxazole resistance, Trimethoprim resistance, Trimethoprim/Sulfamethoxazole resistance, Tetracycline resistance, Azithromycin resistance, Colistin resistance, Meropenem resistance, *gyrA*_D87N mutation, *gyrB*_S464F mutation, *parC*_S80I mutation, *perE*_A364V mutation, presence of *tetA* (B) gene, presence of *tetR* (B) gene, presence of pHCM2 plasmid (any variant), year of isolation, *ssrA*_G639E mutation, presence of pHCM2 Chile variant.
(CSV)

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References

1. Marco C, Delgado I, Vargas C, Munoz X, Bhutta ZA, Ferreccio C. Typhoid Fever in Chile 1969–2012: Analysis of an Epidemic and Its Control. Am J Trop Med Hyg. 2018; 99(3_Suppl):26–33. https://doi.org/10.4269/ajtmh.18-0125 PMID: 30047371
2. Levine MM BR, Ferreccio C, Clements ML, Lanata C, Sear S, Morris JG, et al. Interventions to control endemic typhoid fever: field studies in Santiago, Chile. Control and Eradication of Infectious Diseases, An International Symposium. 1985;Series 1:37–53.
3. Black RE, Levine MM, Ferreccio C, Clements ML, Lanata C, Rooney J, et al. Efficacy of one or two doses of Ty21a Salmonella typhi vaccine in enteric-coated capsules in a controlled field trial. Vaccine. 1990; 8(1):81–4. https://doi.org/10.1016/0264-410x(90)90183-m PMID: 2180234
4. Levine MM, Ferreccio C, Ortiz E, Cryz S. Comparison of enteric-coated capsules and liquid formulation of Ty21a typhoid vaccine in randomised controlled field trial. The Lancet. 1990; 336(8720):891–4. https://doi.org/10.1016/0140-6736(90)92266-k PMID: 1976928
5. Levine MM, Ferreccio C, Black RE, Germanier R. Large-scale field trial of Ty21a live oral typhoid vaccine in enteric-coated capsule formulation. Lancet. 1987; 1(8541):1049–52. https://doi.org/10.1016/s0140-6736(87)90480-6 PMID: 2883393
6. Ferreccio C, Levine MM, Rodriguez H, Contreras R. Comparative efficacy of two, three, or four doses of TY21a live oral typhoid vaccine in enteric-coated capsules: a field trial in an endemic area. J Infect Dis. 1989; 159(4):766–9. https://doi.org/10.1093/infdis/159.4.766 PMID: 2647863
7. Ferreccio C, Morris JG Jr., Valdivieso C, Prenzel I, Sotomayor V, Drusano GL, et al. Efficacy of ciprofloxacin in the treatment of chronic typhoid carriers. J Infect Dis. 1988; 157(6):1235–9. https://doi.org/10.1093/infdis/157.6.1235 PMID: 3373023
8. Gauld JS, Hu H, Klein DJ, Levine MM. Typhoid fever in Santiago, Chile: Insights from a mathematical model utilizing venerable archived data from a successful disease control program. PLoS Negl Trop Dis. 2018; 12(9):e0006759. https://doi.org/10.1371/journal.pntd.0006759 PMID: 30188904
9. Sears SD, Ferreccio C, Levine MM, Cordano AM, Monreal J, Black RE, et al. The use of Moore swabs for isolation of Salmonella typhi from irrigation water in Santiago, Chile. J Infect Dis. 1984; 149(4):640–2. https://doi.org/10.1093/infdis/149.4.640 PMID: 6373964
10. Levine M. South America: The return of cholera. The Lancet. 1991; 338(8758):45–6. https://doi.org/10.1016/0140-6736(91)90024-J
11. Medina E. [Epidemic of cholera in Chile; 1991]. Rev Med Chil. 1991; 119(8):943–56. PMID: 1844781
12. Swerdlow DL, Mintz ED, Rodriguez M, Tejada E, Ocampo C, Espejo L, et al. Waterborne transmission of epidemic cholera in Trujillo, Peru: lessons for a continent at risk. Lancet. 1992; 340(8810):28–33. https://doi.org/10.1016/0140-6736(92)92432-f PMID: 1351608
13. Tauxe RV, Blake PA. Epidemic cholera in Latin America. Jama. 1992; 267(10):1388–90. PMID: 1740864
14. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nature communications. 2016; 7 (1):1–11. https://doi.org/10.1038/ncomms12827 PMID: 27703135
15. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. 2014; 6(11):90. https://doi.org/10.1186/s13073-014-0090-6 PMID: 25422674
16. Dyson ZA, Holt KE. Five years of GenoTyphi: updates to the global Salmonella Typhi genotyping framework. The Journal of Infectious Diseases. 2021. https://doi.org/10.1093/infdis/jiab414 PMID: 34453548
17. Argimon S, Yeats CA, Goater RJ, Abudahab K, Taylor B, Underwood A, et al. A global resource for genomic predictions of antimicrobial resistance and surveillance of Salmonella Typhi at pathogenwatch. Nat Commun. 2021; 12(1):2879. https://doi.org/10.1038/s41467-021-23091-2 PMID: 34001879

18. Page AJ, De Silva N, Hunt M, Quail MA, Parkhill J, Harris SR, et al. Robust high-throughput prokaryote de novo assembly and improvement pipeline for Illumina data. Microb Genom. 2016; 2(8):e000083. https://doi.org/10.1099/mgen.0.000083 PMID: 28348874

19. 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

20. Kariuki S, Mbae C, Van Puyvelde S, Onsare R, Kavai S, Wairimu C, et al. High relatedness of invasive multi-drug resistant non-typhoidal Salmonella genotypes among patients and asymptomatic carriers in endemic informal settlements in Kenya. PLoS Negl Trop Dis. 2020; 14(8):e0008440. https://doi.org/10.1371/journal.pntd.0008440 PMID: 32745137

21. Britto CD, Dyson ZA, Duchene S, Carter MJ, Gurung M, Kelly DF, et al. Laboratory and molecular surveillance of paediatric typhoidal Salmonella in Nepal: Antimicrobial resistance and implications for vaccine policy. PLoS neglected tropical diseases. 2018; 12(4):e0006408. https://doi.org/10.1371/journal.pntd.0006408 PMID: 29684021

22. Rahman SIA, Dyson ZA, Klemm EJ, Khanam F, Holt KE, Chowdhury EK, et al. Population structure and antimicrobial resistance patterns of Salmonella Typhi isolates in urban Dhaka, Bangladesh from 2004 to 2016. PLoS neglected tropical diseases. 2020; 14(2):e0008036. https://doi.org/10.1371/journal.pntd.0008036 PMID: 32106221

23. Wong VK, Holt KE, Okoro C, Baker S, Pickard DJ, Marks F, et al. Molecular Surveillance Identifies Multiple Transmissions of Typhoid in West Africa. PLoS Negl Trop Dis. 2016; 10(9):e0004781. https://doi.org/10.1371/journal.pntd.0004781 PMID: 27657909

24. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 2015; 43(3):e15. https://doi.org/10.1093/nar/gku1196 PMID: 25414349

25. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22(21):2688–90. https://doi.org/10.1093/bioinformatics/btl446 PMID: 16928733

26. Letunic I, Bork P. Interactive Tree Of Life (iTOL): an online tool for phylogenetic tree display and annotation. Bioinformatics. 2007; 23(1):127–8. https://doi.org/10.1093/bioinformatics/btl529 PMID: 17050570

27. Argimón S, Abudahab K, Goater RJE, Fedosejev A, Bhai J, Glasner C, et al. Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. Microb Genom. 2016; 2(11):e000093. https://doi.org/10.1099/mgen.0.000093 PMID: 28348833

28. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother. 2014; 58(1):212–20. https://doi.org/10.1128/AAC.01310-13 PMID: 24145532

29. Carattoli A, Zankari E, Garcia-Fernandez A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014; 58(7):3895–903. https://doi.org/10.1128/AAC.02412-14 PMID: 24777092

30. Chen L, Yang J, Yu J, Yao Z, Sun L, Shen Y, et al. VFDB: a reference database for bacterial virulence factors. Nucleic Acids Res. 2005; 33(Database issue):D325–8. https://doi.org/10.1093/nar/gki008 PMID: 15608208

31. Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. PLoS Comput Biol. 2017; 13(6):e1005595. https://doi.org/10.1371/journal.pcbi.1005595 PMID: 28594827

32. Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novo genome assemblies. Bioinformatics. 2015; 31(20):3350–2. https://doi.org/10.1093/bioinformatics/btv383 PMID: 26099265

33. Carver TJ, Rutherford KM, Berriman M, Rajandream MA, Barrell BG, Parkhill J. ACT: the Artemis Comparison Tool. Bioinformatics. 2005; 21(16):3422–3. https://doi.org/10.1093/bioinformatics/bti553 PMID: 15976072

34. Sullivan MJ, Petty NK, Beatson SA. Easyfig: a genome comparison visualizer. Bioinformatics. 2011; 27(7):1009–10. https://doi.org/10.1093/bioinformatics/btr039 PMID: 21278367

35. Maes M, Dyson ZA, Higginson EE, Fernandez A, Araya P, Tennant SM, et al. Multiple Introductions of Salmonella enterica Serovar Typhi H58 with Reduced Fluoroquinolone Susceptibility into Chile. Emerg Infect Dis. 2020; 26(11):2736–40. https://doi.org/10.3201/eid2611.201676 PMID: 33079054

36. Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic resistance and typhoid. Clinical Infectious Diseases. 2019; 68(Supplement_2):S165–S70. https://doi.org/10.1093/cid/ciy1111 PMID: 30845331

37. Dyson ZA, Thanh DP, Bodhidatta L, Mason CJ, Srijan A, Rabaa MA, et al. Whole genome sequence analysis of Salmonella Typhi isolated in Thailand before and after the introduction of a national

immunization program. PLoS neglected tropical diseases. 2017; 11(1):e0005274. https://doi.org/10.1371/journal.pntd.0005274 PMID: 28060810

38. Fica AE, Prat-Miranda S, Fernandez-Ricci A, D'Ottone K, Cabello FC. Epidemic typhoid in Chile: analysis by molecular and conventional methods of Salmonella typhi strain diversity in epidemic (1977 and 1981) and nonepidemic (1990) years. J Clin Microbiol. 1996; 34(7):1701–7. https://doi.org/10.1128/JCM.34.7.1701-1707.1996 PMID: 8784573

39. Ames WR, Robins M. Age and Sex as Factors in the Development of the Typhoid Carrier State, and a Method for Estimating Carrier Prevalence. Am J Public Health Nations Health. 1943; 33(3):221–30. https://doi.org/10.2105/ajph.33.3.221 PMID: 18015749

40. Chattaway MA, Gentle A, Nair S, Tingley L, Day M, Mohamed I, et al. Phylogenomics and antimicrobial resistance of Salmonella Typhi and Paratyphi A, B and C in England, 2016–2019. Microb Genom. 2021; 7(8). https://doi.org/10.1099/mgen.0.000633 PMID: 34370659

41. 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. https://doi.org/10.1371/journal.pntd.0007620 PMID: 31513580

42. Guevara PD, Maes M, Thanh DP, Duarte C, Rodriguez EC, Montaño LA, et al. A genomic snapshot of Salmonella enterica serovar Typhi in Colombia. PLoS Negl Trop Dis. 2021; 15(9):e0009755. https://doi.org/10.1371/journal.pntd.0009755 PMID: 34529660

43. 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. Nature genetics. 2015; 47(6):632–9. https://doi.org/10.1038/ng.3281 PMID: 25961941

44. Dyson ZA, Malau E, Horwood PF, Ford R, Siba V, Yoannes M, et al. Whole genome sequence analysis of Salmonella Typhi in Papua New Guinea reveals an established population of genotype 2.1.7 sensitive to antimicrobials. medRxiv. 2021:2021.09.27.21264209.

45. Kariuki S, Dyson ZA, Mbae C, Ngetich R, Kavai SM, Wairimu C, et al. Multiple introductions of multidrug-resistant typhoid associated with acute infection and asymptomatic carriage, Kenya. Elife. 2021;10. https://doi.org/10.7554/eLife.67852 PMID: 34515028

46. Thanh Duy P, Thieu NTV, Nguyen Thi Nguyen T, Ngoc Dan Thanh H, Dongol S, Karkey A, et al. Gallbladder carriage generates genetic variation and genome degradation in Salmonella Typhi. PLoS Pathog. 2020; 16(10):e1008998. https://doi.org/10.1371/journal.ppat.1008998 PMID: 33085725

47. Johnson R, Mylona E, Frankel G. Typhoidal Salmonella: Distinctive virulence factors and pathogenesis. Cell Microbiol. 2018; 20(9):e12939. https://doi.org/10.1111/cmi.12939 PMID: 30030897

48. Murret-Labarthe C, Kerhoas M, Dufresne K, Daigle F. New Roles for Two-Component System Response Regulators of Salmonella enterica Serovar Typhi during Host Cell Interactions. Microorganisms. 2020; 8(5). https://doi.org/10.3390/microorganisms8050722 PMID: 32413972

49. Forest CG, Ferraro E, Sabbagh SC, Daigle F. Intracellular survival of Salmonella enterica serovar Typhi in human macrophages is independent of Salmonella pathogenicity island (SPI)-2. Microbiology (Reading). 2010; 156(Pt 12):3689–98. https://doi.org/10.1099/mic.0.041624-0 PMID: 20817644

50. Karlinsey JE, Stepien TA, Mayho M, Singletary LA, Bingham-Ramos LK, Brehm MA, et al. Genome-wide Analysis of Salmonella enterica serovar Typhi in Humanized Mice Reveals Key Virulence Features. Cell Host Microbe. 2019; 26(3):426–34.e6. https://doi.org/10.1016/j.chom.2019.08.001 PMID: 31447308

51. Desai SK, Winardhi RS, Periasamy S, Dykas MM, Jie Y, Kenney LJ. The horizontally-acquired response regulator SsrB drives a Salmonella lifestyle switch by relieving biofilm silencing. Elife. 2016;5. https://doi.org/10.7554/eLife.10747 PMID: 26880544

52. Marshall JM, Flechtner AD, La Perle KM, Gunn JS. Visualization of extracellular matrix components within sectioned Salmonella biofilms on the surface of human gallstones. PLoS One. 2014; 9(2):e89243. https://doi.org/10.1371/journal.pone.0089243 PMID: 24551241

53. Kidgell C, Pickard D, Wain J, James K, Diem Nga LT, Diep TS, et al. Characterisation and distribution of a cryptic Salmonella typhi plasmid pHCM2. Plasmid. 2002; 47(3):159–71. https://doi.org/10.1016/s0147-619x(02)00013-6 PMID: 12151231

Appendix 12. A genomic snapshot of *Salmonella enterica* serovar Typhi in Colombia (publication)

PLOS NEGLECTED TROPICAL DISEASES

RESEARCH ARTICLE

A genomic snapshot of *Salmonella enterica* serovar Typhi in Colombia

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Abstract

Little is known about the genetic diversity of *Salmonella enterica* serovar Typhi (*S*. Typhi) circulating in Latin America. It has been observed that typhoid fever is still endemic in this part of the world; however, a lack of standardized blood culture surveillance across Latin American makes estimating the true disease burden problematic. The Colombian National Health Service established a surveillance system for tracking bacterial pathogens, including *S*. Typhi, in 2006. Here, we characterized 77 representative Colombian *S*. Typhi isolates collected between 1997 and 2018 using pulse field gel electrophoresis (PFGE; the accepted genotyping method in Latin America) and whole genome sequencing (WGS). We found that the main *S*. Typhi clades circulating in Colombia were clades 2.5 and 3.5. Notably, the sequenced *S*. Typhi isolates from Colombia were closely related in a global phylogeny. Consequently, these data suggest that these are endemic clades circulating in Colombia. We found that AMR in *S*. Typhi in Colombia was uncommon, with a small subset of organisms exhibiting mutations associated with reduced susceptibility to fluoroquinolones. This is the first time that *S*. Typhi isolated from Colombia have been characterized by WGS, and after comparing these data with those generated using PFGE, we conclude that PFGE is unsuitable for tracking *S*. Typhi clones and mapping transmission. The genetic diversity of pathogens such as *S*. Typhi is limited in Latin America and should be targeted for future surveillance studies incorporating WGS.

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Author summary

Salmonella Typhi is the causative agent of typhoid fever, with between 9–13 million cases and 116,800 associated deaths annually. Typhoid fever is still a public health problem mainly in low and middle-income countries (LMICs), including in Latin America, which has a modelled incidence of up to 169 (32–642) cases per 100,000 person-years. Several international studies have aimed to fill data gaps regarding the global distribution and genetic landscape of typhoid; however, in spite of these efforts Latin America is still under-represented. The globally dominant lineages of *S*. Typhi (e.g., H58), which often carry multi-drug resistance (MDR) plasmids, decreased fluoroquinolone susceptibility, and now azithromycin resistance, are not detectable by the accepted method (PFGE) used to track outbreaks of typhoid in Latin America. We compared PFGE with whole genome sequence (WGS) and found it correlated poorly, resulting in the over clustering of cases. We additionally found that unlike in most endemic countries, *S*. Typhi in Colombia are highly antimicrobial susceptible and restricted to a limited number of genotypes that are not as commonly identified in other *S*. Typhi endemic countries. Our study provides the first enhanced insights into the molecular epidemiology of *S*. Typhi in Colombia, using WGS data for the first time to investigate the population structure in Colombia and identifying predominant circulating genotypes. Our work demonstrates that routine surveillance with the integration of WGS is necessary not only to improve disease burden estimates, but also to track the national and regional transmission dynamics of *S*. Typhi.

Introduction

Salmonella enterica serovar Typhi (*S*. Typhi) is the bacterial agent of typhoid fever. With between 9–13 million cases and 116,800 associated deaths annually, typhoid is still a public health problem in many low and middle-income countries (LMICs), particularly in South Asia and parts of sub-Saharan Africa[1,2]. Antimicrobial resistance (AMR) is a major issue, with multi-drug resistance (MDR; resistance to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) and fluoroquinolone resistance in genotype 4.3.1 (H58) organisms dominating the global genetic landscape [3,4]. The emergence of extensively-drug resistant (XDR; MDR and resistant to fluoroquinolones and third generation cephalosporins) in Pakistan and more recent reports of resistance to azithromycin in South Asia compound the problem [5,6]

Several international studies have aimed to fill data gaps regarding the global distribution of typhoid [7–10]. However, there have not been large multicenter population-based surveillance studies conducted in Latin America as there have been in sub-Saharan Africa and South Asia, nor is there routine blood culture surveillance, so this region represents a major data gap in global disease burden estimations [11–13]. The modelled incidence of typhoid in Latin America varies enormously, and estimates range from 1.0 (0.2–3.9) cases and 169 (32–642) cases per 100,000 person-years [8,14]. A lack of systematic surveillance also means that there are limited contemporary data on the circulating bacterial population, AMR profiles, and potential transmission dynamics within South America. However, a recent study revealed a large number of *S*. Typhi isolates with a high prevalence of decreased fluoroquinolone susceptibility in Colombia and El Salvador [15].

Pulsed Field Gel Electrophoresis (PFGE) is the conventional method for studying the genetic relationship between *S*. Typhi isolates in Latin America [16]. Using this method, we recently found that some *S*. Typhi isolates from Colombia shared indistinguishable PFGE patterns with organisms from Argentina, Chile, Perú, Venezuela, Brazil, and Guatemala,

indicative of the circulation of common "continental" genotypes [17,18]. However, PFGE has limited discriminatory power to support subtyping and cannot identify genotype 4.3.1, other emerging genotypes, or AMR genes. Whole genome sequencing (WGS) is the gold standard for the investigating population structures, transmission dynamics, and molecular mechanisms of AMR in *S*. Typhi. In 2015, a landmark global *S*. Typhi genotyping scheme was published, but comprised only 20 genome sequences originating from Latin America (Argentina, El Salvador, French Guiana, and Peru)[3]. Since then, there have been only three additional publications describing *S*. Typhi isolated in Latin America and characterized using WGS, generating a further 36 genome sequences [4,19,20]

In response to local concern regarding the increase of typhoid fever and the international threat of AMR, the National Surveillance System Public Health (SIVIGILA) of Colombia made typhoid fever a notifiable disease in 2006, requiring laboratory follow-up [21]. Here, we aimed to generate the first insights into the molecular epidemiology of typhoid in Colombia by performing AMR profiling and comparative genotyping using both PFGE and WGS on a cross-sectional collection of *S*. Typhi isolated in Colombia between 1997 and 2018.

Methods

Ethics statement

This study was conducted in accordance with the principles expressed in the Declaration of Helsinki. The clinical bacterial isolates were collected through the Colombian Laboratory National Surveillance System under the scientific, technical and administrative standard for health research established in Colombian resolution 8430 of 1993 of the Ministry of Health. Patient data were analysed anonymously; consequently, formal ethical approval for the study was not necessary.

Salmonella Typhi isolates

A total of 1,478 *S*. Typhi isolates were submitted to the surveillance program at the National Health Institute of Colombia between 1997 and 2018. These organisms were all associated with a reported typhoid and paratyphoid fever event and came from 22 of 32 Colombian departments and the Capital District of Colombia [21]. 1,077 (72.9%) of these isolates were successfully genotyped using the standard routine PFGE pipeline (S1 Fig) and 77 (5.2%) *S*. Typhi isolates were selected cross-sectionally for WGS (Fig 1 and S1 Table). Our aim was to generate a broad overview of circulating genotypes in Colombia and to identify genotype H58. Therefore, we included isolates from all years, from sampled departments, and a broad range of PFGE patterns, including at least one isolate of each mayor PFGE pattern and including the various AMR phenotypes. These isolates were both from outbreaks defined by the health authorities (n = 12) and sporadic cases (n = 65); 61 isolates originated from blood, 10 from stool, and six from other sources (3 bone marrow, 1 splenic abscess, 1 gluteus abscess, and 1 from a skin swab).

Bacterial identification and antimicrobial susceptibility testing

All isolates were identified using standard biochemical tests (Triple Sugar Iron Agar (TSI), Citrate, Urea, motility), the automated MicroScan, VITEK II system and the Kauffmann-White-Le Minor scheme to identify organisms suspected to be *S*. Typhi (Difco, United States) [22]

Antimicrobial susceptibility testing was performed using the Kirby-Bauer disk diffusion method against amoxicillin-clavulanic acid (AMC), chloramphenicol (CHL), nalidixic acid (NAL), tetracycline (TET), ampicillin (AMP), cefotaxime (CTX), ceftazidime (CAZ),

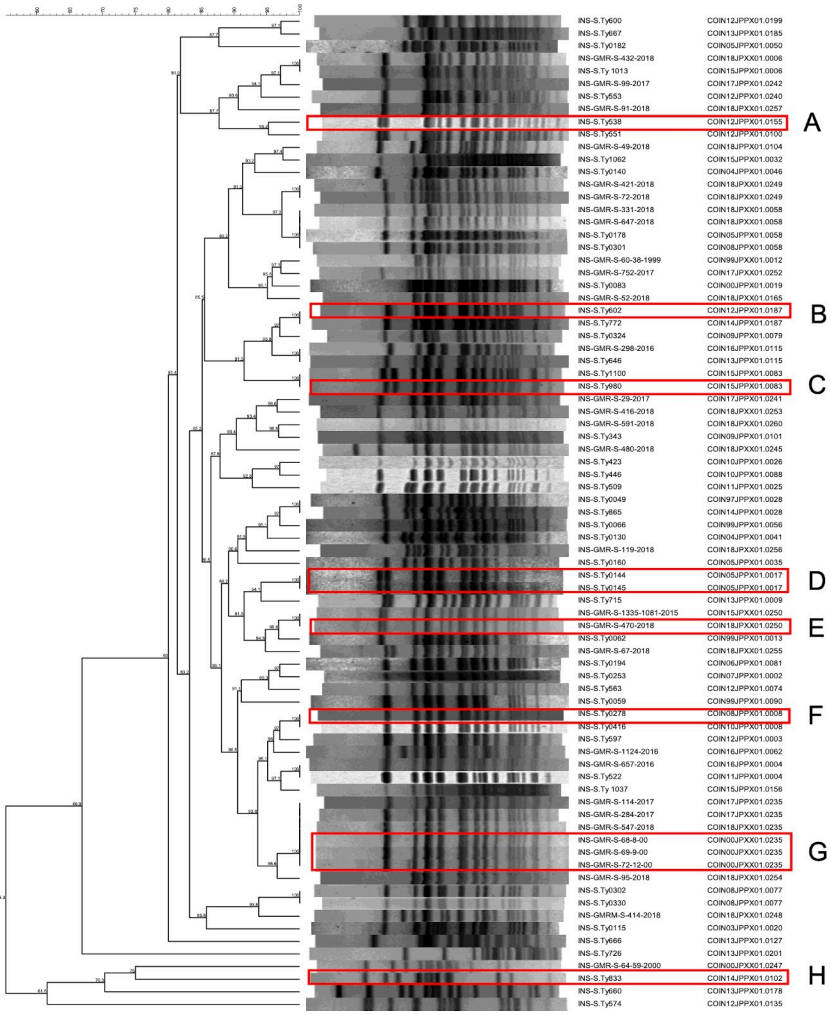

Fig 1. Colombian *Salmonella* Typhi isolates selected for whole genome sequencing. PFGE-*XbaI* dendrogram generated with Dice coefficient and UPGMA clustering method (tolerance and optimization 1.5%) of the 77 selected *S*. Typhi isolates with isolate identification and PFGE pattern code. The red boxes indicate isolates from epidemiological confirmed outbreaks (A-H).

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trimethoprim-sulfamethoxazole (SXT), and Meropenem in combination with the MIC-based methods using the MicroScansystems according to manufactures recommendations. Ciprofloxacin (CIP) susceptibility was determined by agar dilution assays according to the CLSI standards of 2019 [23] Extended-Spectrum Beta-Lactamase (ESBLs) activity mediated by bla_{SHV}, bla_{TEM}, and $bla_{\text{CTX-M}}$ genes was confirmed by PCR amplification [24].

Molecular subtyping by PFGE

All organisms were subtyped by PFGE following standardized PulseNet protocols. [16]. Briefly, genomic DNA were digested with *Xba*I (Promega, USA) and subjected to gel electrophoresis. PFGE patterns from the different runs were normalized by aligning the reference digestion pattern of *S*. Braenderup H9812. Bands were assessed visually and by a computerized program (Gelcompare 4.0 software (Applied Maths, Belgium). Parameters of tolerance and optimization were set to 1.5% and similarities calculated according to Dice coefficient. The Clustering dendrogram was based on the unweighted pair-group method using arithmetic

averages (UPGMA). The resulting *Xba*I patterns were compared with the local database and if indistinguishable (within this 1.5% tolerance) from an existing pattern the isolates was given the same PFGE code; if a unique pattern was deteremined a new PFGE code was assigned. All PFGE pattern codes were assigned following the PulseNet International guidelines for nomenclature, which includes 2 letters for the country or region, 3 letters for the serovar, 3 characters for the enzyme and 4 digits for the profile number (e.g. COINJPPX01.0001 for Colombia) [17]

Genome sequencing and SNP analysis

DNA was extracted using a Qiacube in combination with the Qiagen QIAamp DNA Mini Kit (Qiagen) at the Colombian National Health Institute (INS), following the manufacturer guidelines. DNA was quantified using a Qubit 2.0 fluorometer (Invitrogen) and 2μg of genomic DNA was subjected to indexed WGS by Illumina MiSeq platform to generate 100 bp paired end reads and 30x genome coverage. Genomic libraries were prepared with Nextera XT library prep Kit FC 121–1031. Raw Illumina reads were assembled using (Velvet v1.2) via an automated pipeline at the Wellcome Sanger Institute[25]. For preliminary analysis and global contextualization and for the detection of non-synonymous mutations in the Quinolone Resistance Determining Region (QRDR) of genes *gyrA*, *gyrB*, *parC*, and *are*, the assembled genomes were uploaded to PathogenWatch v3.2.2 (https://pathogen.watch/). Genotypes were assigned using GenoTyphi (https://github.com/katholt/genotyphi)

Sequenced reads and publicly available sequences were mapped and SNP called against the reference genome *S*. Typhi CT18 using the Sanger institute pipelines and following quality metrics as previously described [26]. Known recombinant regions such as prophage[4], were manually excluded, and any remaining recombinant regions were filtered using Gubbins (v1.4.10)[27]. The resultant core SNP alignment of 40,998 bp was used to infer Maximum Likelihood (ML) phylogenies using RAxML (v8.2.8)[28], specifying a generalized time-reversible model and a Gamma distribution to model site-specific rate variation (GTR+ Γ substitution model; GTRGAMMA in RAxML) with 100 bootstrap pseudoreplicates used to assess branch support. SNP distances for the core genome alignment of all the novel genome sequences were calculated from this alignment using snp-dists package (https://github.com/tseemann/snp-dists). SRST2 v0.2.0 [29] was used with the ARGannot [30] and PlasmidFinder [31] databases to detect the molecular determinants associated with AMR; standard cut-offs of >90% gene coverage and a minimum read dept of 5 were used. Maps drawn in inkscape v1.0.1 an open source scalable graphics editor.

Results

PFGE genotyping and isolate selection

PFGE is performed routinely for *S*. Typhi in Latin America; results are consolidated into the PulseNet Latin America and Caribbean Network database [16,17]. Organisms are given a unique PFGE code according to their genomic digestion pattern; 1,478 Colombian isolates were present in the national surveillance database at the initiation of this project. We selected 77 *S*. Typhi isolated between 1997 and 2018 to represent the broadest possible diversity (by PFGE; S1 Fig) for WGS. This collection comprised 60 unique PFGE profiles (Fig 1 and S1 Table), including the most commonly circulating restriction patterns in Colombia (e.g., COINXX.JPPX01. 0008-0083-0115)[18]. Twelve isolates also originated from eight outbreaks confirmed by the health authorities (A-H; 8, 4, 24, 9, 5, 2, 6, and 8 patients per outbreak respectively) (Figs 1 and S1); more than one isolate were included from two of these outbreaks (D and G). The selection was skewed towards more recent years based on number of available isolates and for AMR isolates [32].

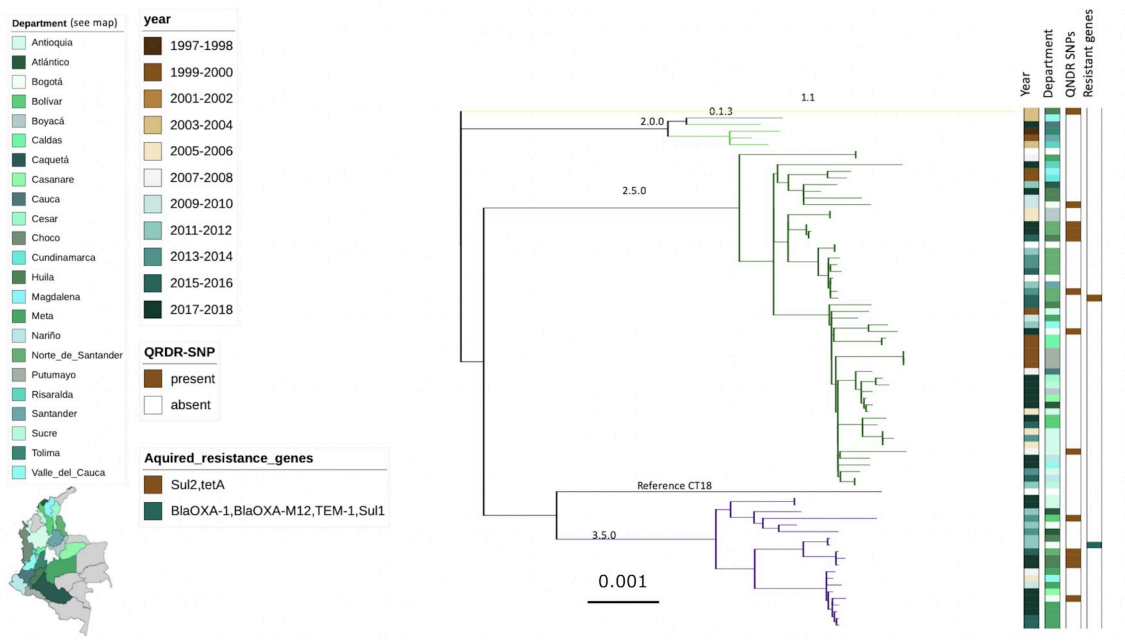

Fig 2. The phylogenetic structure of *Salmonella* Typhi in Colombia. SNP based RAxML generated Maximum Likelihood phylogenetic tree of 77 selected *Salmonella* Typhi isolates. Branches are coloured by genotype (numbers shown). Column 1 indicates year of isolation, column 2 indicates department (state) of isolation, column 3 indicates the presence (brown) of SNPs in the QNDR and column D indicates acquired AMR genes (see legend). The map was drawn by inkscape v1.0.1 an Open Source Scalable Vector Graphics Editor.

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AMR and population structure of Colombian *S*. Typhi

The 77 Colombian *S*. Typhi isolates were subjected to WGS and a phylogenetic tree was constructed from core genome SNPs (Fig 2). We found that genotypic variation in the population of Colombian *S*. Typhi was generally limited, with the majority of isolates restricted to two groups: major cluster 2 and 3. These clades could be further segregated into clades 2.5 (51/77; 66.2%), 3.5 (20/77; 24.9%), and 2 (4/77; 5.2%). In addition, we identified two isolates in major cluster 1; these organisms belonged to genotypes 0.1.3 and 1.1.

Notably, unlike a recent observation from Chile, we did not identify genotype 4.3.1 (H58) isolates in this set of Colombian sequences, despite being specifically enriched for organisms that exhibited resistance to antimicrobials. However, we did identify 14 organisms in genotypes 1.1, 2.5 and 3.5 that contained a single SNP in the QRDR region (Fig 2 and Table 1), resulting in reduced susceptibility to fluoroquinolones. Overall, and unlike contemporaneous *S*. Typhi collections from Africa and Asia, this collection contained a limited accumulation of acquired AMR genes. We identified one isolate carrying the *sul*2 and *tet*A genes associated with resistance to tetracycline and sulphonamides, respectively. We additionally detected one organism from Bogota, isolated in 2012, which carried $bla_{\text{CTX-M-12}}$, $bla_{\text{TEM-1}}$, $bla_{\text{OXA-15}}$, and *Sul*1, rendering it resistant to ampicillin, cephalosporins, and sulphamethoxazole (Fig 2 and Table 1).

Associations between PFGE and WGS

We next aimed to compare the PFGE patterns of the 77 Colombian *S*. Typhi with that of phylogenetic structure created by WGS. First, we found that the paired isolates from the outbreaks (D and G) were indistinguishable; these organisms had identical PFGEs patterns and displayed

Table 1. Colombian *Salmonella* Typhi isolates with antimicrobial resistance phenotypes and their associated genes and plasmids.

Organism id	Genotype	Resistance Kirby-Bauer (mm)*	MIC	Resistance profile	AMR Genes/QRDR mutations	Plasmid content
INS-S.Ty1062/1087-15	3.5	NAL(28),AMP(33), S(14)	AMP >16 CIP 0.008	AMP		
INS-S.Ty0330/256-08	2.5	NAL(27), AMP(16),S(12)	CIP 0.008	AMP(I)		
INS-S.Ty0115/36-03	2.0	NAL(28),SXT(6), TET(6), S (14)	CIP 0.008	SXT-TET		
INS-S.Ty551/52-12	3.5	NAL(24),AMP(6), CTX(8), TET(6), S(6)	CIP 0.008	AMP-CTX	*bla*CTX-M-12, *bla*OXA-15, *Sul*, *bla*TEM-*1*	IncL/M(pOXA-48) IncFIB (pHCM2)
INS-S.Ty980/524-15	2.5	NAL(22), TET(6), S(6) CTX(6), CTX-CLA(10) CAZ(17), CAZ-CLA(19)	AMP >16 CTX >32 CIP 0.016	AMP-CTX-TET-S	*TetA*(A), *Sul2*	ColRNAI
INS-S.Ty0130/69-04	1.1	NAL(6), S(14)	CIP 0.25	NAL-CIP(I)	*gyrA_S83F*	
INS-GMR-S-331-18	3.5	NAL(6),CIP(26)(I)		NAL-CIP(I)	*gyrA_D87Y*	
INS-S.Ty1100/1425-15	3.5	NAL(18)(I)	CIP 0.25	NAL(I)-CIP(I)	*gyrA_S83F*	
INS-S.Ty772/314-14	2.5	NAL(10), CIP(25), S(10)	CIP 0.032	NAL	*gyrA_D87V*	
INS-S.Ty423/316-10	2.5	NAL(6), S(14)	CIP 0.064	NAL	*gyrA_S83Y*	
INS-S.Ty666/329-13	3.5	NAL(6), S(11)	CIP 0.032	NAL	*gyrA_D87G*	
INS-S.Ty1013/755-15	2.5	NAL(6), S(13)	CIP 0.064	NAL	*gyrA_D87G*	
INS-GMR-S-752-17	2.5	NAL(6), CIP(25), S(12)	CIP 0.064	NAL	*gyrA_D87G*	
INS-GMR-S-91-18	3.5	NAL(6), CIP(24), S(14)	CIP 0.064	NAL	*gyrA_S83F*	
INS-GMR-S-480-18	2.5	NAL(6), CIP(26), S(13)	CIP 0.064	NAL	*gyrA_D87G*	
INS-GMR-S-432-18	3.5	NAL(6), CIP(23)	ND	NAL	*gyrA_S83F*	
INS-S.Ty0253/397-07	2.5	NAL(6), S(12)	CIP 0.064	NAL	*gyrA_D87N*	
INS-GMR-S-114-17	2.5	NAL(15),CIP(26), S(13)	CIP 0.064	NAL	*gyrB_S464Y*	
INS-S.Ty563/214-12	2.5	NAL(18), CIP(28)	CIP 0.032	NAL(I)	*gyrA_S83F*	
INS-S.Ty538/7-12	3.5	NAL(24), CIP(29), S(12)	CIP 0.008	Susceptible		IncFIB(pHCM2)

*Interpretation criteria according CLSI 2020

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no SNP differences in the WGS data (Figs 1 and 3). However, more generally, the PFGE restriction patterns and position in the dendrogram showed minimal concordance with their corresponding phylogenetic location from the WGS data (Fig 3). For example, three isolates from an outbreak (G) shared an identical PFGE restriction pattern (COINXX.JPPX01.0235). This association was encouraging, but on further investigation, an additional three *S*. Typhi isolates exhibited this same restriction profile. These three further isolates had no apparent epidemiological association with the specific outbreak, were from different geographical locations across Colombia, and were isolated several years after the outbreak (Fig 3). These isolates were determined to be >40 SNPs away in the phylogenetic tree from the isolates causing the

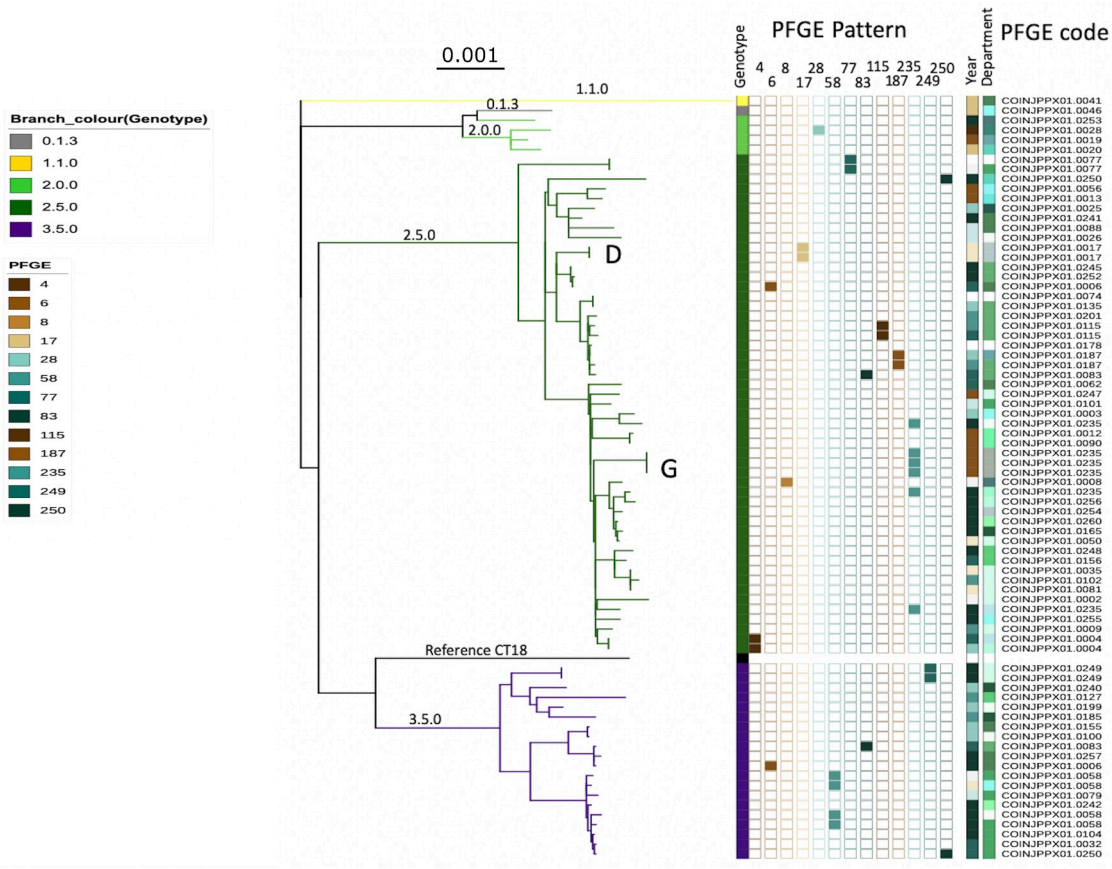

Fig 3. WGS and PFGE exhibit a limited correlation for *Salmonella* Typhi genotyping. The branches of the SNP based RAxML phylogenetic tree and the first column are coloured by genotype. The sequential columns highlight the PFGE patterns that were present more than one occasion. i.e., the bottom two isolates of clade 2.5 with PFGE code COINXX.JPPX01.0004 and the top two isolates of clade 3.5 with PFGE code COINXX.JPPX01.0249. All PFGE patterns are additionally listed under PFGE code (the PFGE pattern correspond to the last 4 digits of the PFGE code) for enhanced visibility only unique PFGE are listed by code and not highlighted. The year of isolation and department are coloured as in Fig 2. Letter D and G indicate the two outbreaks from which we sequenced more than one isolate, these correspond with outbreak strains D and G in Fig 1.

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outbreak. Lastly, we found a number of occasions where isolates within differing major WGS clades shared an identical PFGE digestion pattern. For example, isolates exhibiting the 0006, 0083, and 0250 PFGE patterns could be found in both clade 2.5 and clade 3.5 of the WGS-based phylogeny (Fig 3). As predicted, these data show that PGFE has limited discriminatory power to identify organisms that may or may not be closely genetically related, further supporting the transition to WGS for routine surveillance.

Colombian *S.* Typhi in a global context

To determine if the detected Colombian genotypes were more likely to be of Colombian origin or introduced from other continents, we placed these contemporaneous Colombian isolates into a global context with an international collection of *S.* Typhi genome sequences. We constructed a phylogenetic tree of 3,382 publicly available *S.* Typhi genome sequences with the 77 contemporaneous Colombian isolates; genotype 4.3.1 (H58) sequences were excluded as they were not identified in this collection (Fig 4). The Colombian organisms in clades 2.5 and 3.5 clustered alongside other Colombian organisms within their respective genotypes. The nearest

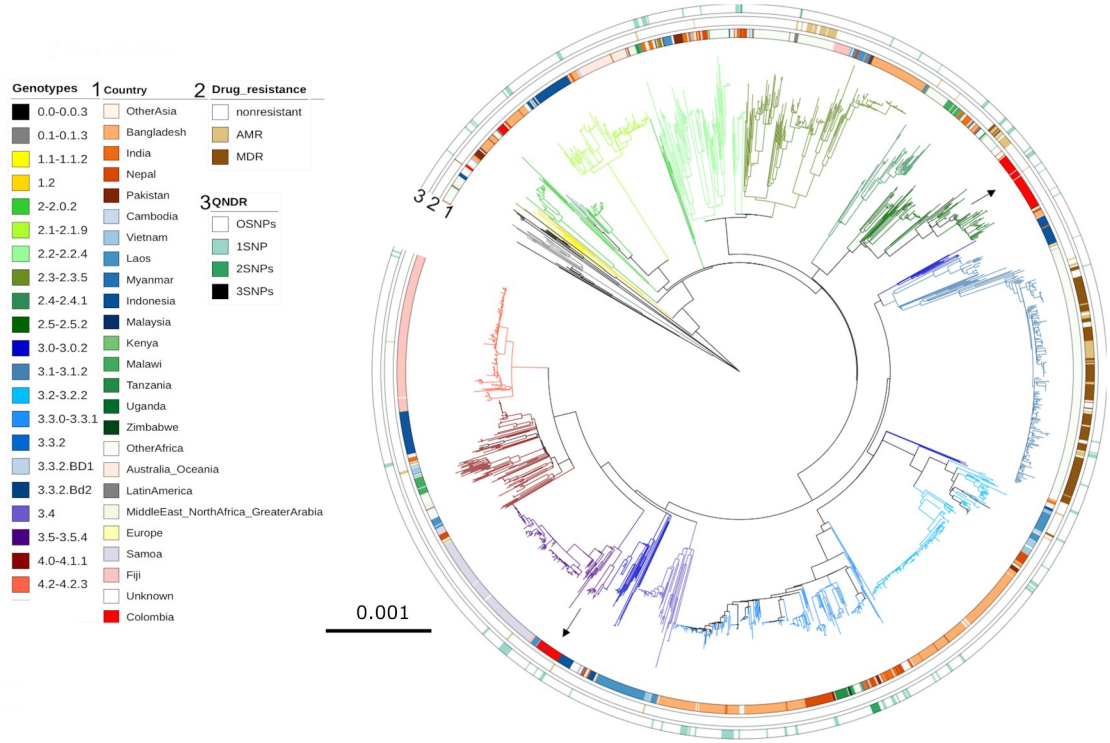

Fig 4. The phylogenetic location of Colombian *Salmonella* Typhi in a global context. SNP based RAxML based phylogenetic tree of the 77 Colombian isolates among 3,382 publicly available non-H58 *S*. Typhi genome sequences. Branches are coloured by genotype, inner ring depicts country of isolation, Asia in red shades, South East Asia in blue shades, Africa in green shades, Colombia in bright red and other Latin American countries in grey. The middle ring indicates AMR profile, any AMR is shown in light brown and MDR isolates are shown in dark brown. Finally, the outer ring shows number of QRDR SNPs with the colour intensity increases with increasing number of SNPs.

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neighbours to these organisms were isolated in India (10592_2_45, genotype 2.5) and Vietnam (10425_1_60, genotype 3.5) in 1997 and 1993, respectively. In the absence of further sampling, these data suggest that clade 2.5 and clade 3.5 are locally circulating genotypes in Colombia. Similarly, the presence of genotypes 1.1 and 0.1.3 in Colombia is indicative of limited circulation of overseas genotypes. Organisms belonging to genotypes 1.1 and 0.1.3 are considered ancient and presently uncommon on the international *S*. Typhi genotypic landscape and are historically associated with typhoid in Africa [3].

Discussion

Here, in this primary study of WGS data from *S*. Typhi circulating in Colombia, we show that the majority of organisms in the selected 20-year time span displayed limited genetic diversity, belonging mainly to two major clades: 3.5 and 2.5. The limited number of sequenced isolates from Latin America to date mainly belonged to primary cluster 2 and primary cluster 4, with genotype 2.0 in Argentina, Mexico, El Salvador, and Peru, genotype 2.3.2 in Argentina, El Salvador, and Mexico, genotype 4.1.0 in Brazil [19], and Argentina [3]. In the restricted number of isolates screened here, no genotype 4.3.1 (H58) isolates were found. Although samples were selected to present a maximal variation based on diverse PFGE patterns, included specifically outbreak and AMR related isolates, we cannot be certain H58 is not present in Colombia; however, we can surmise that this genotype is not as broadly distributed as in Asia and Africa. Before 2020, genotype 4.3.1 *S*. Typhi had not been detected in Latin America, but a recent

study identified three independent introductions of H58 into Chile [20]. In Chile, the spread of these isolates appears to have been contained; however, this observation highlights the need for sustained genomic surveillance to detect any additional introductions and potential increased circulation of genotype 4.3.1 [33].

A key observation is that the prevalence of AMR in Colombian *S*. Typhi appears to be significantly lower than that observed in South Asia or Africa. This study, despite being enriched for AMR isolates, indicates an exceptionally low background of AMR in *S*. Typhi, with only one isolate carrying a plasmid containing AMR genes (IncL/M; pOXA-48), with an additional cryptic no-AMR plasmid (IncFIB; pHCM2) also detected. The precise reason(s) for a lower prevalence of AMR in *S*. Typhi, in Colombia are unknown and requires additional investigation. We hypothesise that a lower prevalence of AMR *S*. Typhi, in comparison to Asia and Africa, may be related to antimicrobial access and global pathogen dynamics. Generally, antimicrobial is not better regulated in this region than other locations with a high density of LMICs in the past [34]. However, in the last decade many Latin American countries developed their own National Action Plans to combat AMR under the guidance of PAHO [35]. AMR in *S*. Typhi is not static, and the global trajectory of AMR is increasing; consequently, there is a constant threat of the importation of AMR organisms and sustained surveillance in Colombia remains crucial. These factors highlight the importance of global typhoid surveillance and not purely restricting observations to Africa and Southeast Asia.

We additionally aimed to assess the potential correlations and utility of PFGE for *S*. Typhi tracking across Latin America. We found that PFGE and SNP based phylogenetic do not correlate especially well. We found the same PFGE patterns in completely distinct primary clusters of the SNP based phylogeny. These observations again indicate that PFGE results in false clustering and is not appropriately sensitive for surveillance requiring high resolution delineation of local/regional population structure and dynamics of *S*. Typhi or for outbreak detection in Colombia. WGS is a more appropriate method and is therefore slowly being adopted as the gold standard for these purposes internationally. Lastly, we compared the Colombian isolates to publicly available non-H58 global isolates to determine whether Colombian organisms were imported. This global tree highlighted a lack of genomic information from Latin America. It was therefore impossible to determine whether observed cases are the result of introductions into Colombia from other Latin American countries or local endemic transmission. However, we found that even though the Colombian isolates were collected over 20-years, they formed their own clusters and were not closely related organisms from other locations. These observations suggest that the *S*. Typhi population structure in Colombia is likely driven by sustained endemic circulation of local genotypes.

This study has limitations, the main one being the small sample size of sequenced isolates. The need to select only a subset of samples meant we could have overlooked genotypes and the proportion of the detected genotypes may not be an accurate overview of the distribution. However, this study was aimed to assess *S*. Typhi genetic diversity in Colombia and we show that in spite of our diverse selection of organisms that 90% of the isolates belonged to two predominate clades. More thorough sequencing strategies are required to more accurately determine the distribution of genotypes.

This study provides an enhanced insight into the molecular epidemiology of *S*. Typhi in Colombia, constructing the pathogen population structure and identifying the predominant circulating genotypes. Our work demonstrates that routine surveillance with the integration of WGS is necessary not only to improve disease burden estimates, but also to track the national and regional transmission dynamics of *S*. Typhi and determine AMR profiles. These data will be pivotal to better estimate the burden of typhoid in the region, improve antimicrobial treatment practices and help policymakers to assess the need for typhoid conjugate vaccine

introduction. While the population of *S*. Typhi in Colombia appears isolated, the emergence and spread of AMR variants have been observed internationally [5,6,33]. Consequently, it is critical for improved control and prevention measures that we establish routine WGS surveillance in Colombia and other Latin American countries to strengthen surveillance and monitoring the continental spread of *S*. Typhi.

Supporting information

S1 Table. Profile of the organisms selected for sequencing.
(XLSX)

S1 Fig. PFGE clustering of Colombian *Salmonella* Typhi isolates. PFGE-*XbaI* dendrogram generated with Dice coefficient and UPGMA clustering method (tolerance and optimization 1,5%) of 1,077 isolates. The isolates showed 51.45% genetic similarity and represent 211 unique *Xba*I digestion patterns (as of June 2021). The grey dots indicate the isolates selected for WGS.
(PPTX)

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References

1. Stanaway JD, Reiner RC, Blacker BF, Goldberg EM, Khalil IA, Troeger CE, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. The Lancet Infectious Diseases. 2019; 19(4):369–81. https://doi.org/10.1016/S1473-3099(18)30685-6 PMID: 30792131
2. WHO. Typhoid vaccines: WHO position paper, March 2018–Recommendations. Vaccine. 2019; 37 (2):214–6 https://doi.org/10.1016/j.vaccine.2018.04.022 PMID: 29661581
3. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nature communications. 2016; 7 (1):1–11. https://doi.org/10.1038/ncomms12827 PMID: 27703135
4. 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 neglected tropical diseases. 2019; 13(9):e0007620. https://doi.org/10.1371/journal.pntd.0007620 PMID: 31513580
5. 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). https://doi.org/10.1128/mBio.00105-18 PMID: 29463654
6. Carey ME, Jain R, Yousuf M, Maes M, Dyson ZA, Thu TNH, et al. Spontaneous emergence of azithromycin resistance in independent lineages of Salmonella Typhi in Northern India. bioRxiv. 2020.
7. Ochiai RL, Acosta CJ, Danovaro-Holliday MC, Baiqing D, Bhattacharya SK, Agtini MD, et al. A study of typhoid fever in five Asian countries: disease burden and implications for controls. Bull World Health Organ. 2008; 86(4):260–8. https://doi.org/10.2471/blt.06.039818 PMID: 18438514
8. Darton TC, Meiring JE, Tonks S, Khan MA, Khanam F, Shakya M, et al. The STRATAA study protocol: a programme to assess the burden of enteric fever in Bangladesh, Malawi and Nepal using prospective population census, passive surveillance, serological studies and healthcare utilisation surveys. BMJ Open. 2017; 7(6):e016283. https://doi.org/10.1136/bmjopen-2017-016283 PMID: 28674145
9. Marks F, von Kalckreuth V, Aaby P, Adu-Sarkodie Y, El Tayeb MA, Ali M, et al. Incidence of invasive salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. Lancet Glob Health. 2017; 5(3):e310–e23. https://doi.org/10.1016/S2214-109X(17)30022-0 PMID: 28193398
10. Barkume C, Date K, Saha SK, Qamar FN, Sur D, Andrews JR, et al. Phase I of the Surveillance for Enteric Fever in Asia Project (SEAP): An Overview and Lessons Learned. J Infect Dis. 2018; 218 (suppl_4):S188–s94. https://doi.org/10.1093/infdis/jiy522 PMID: 30304505
11. Marco C, Delgado I, Vargas C, Muñoz X, Bhutta ZA, Ferreccio C. Typhoid Fever in Chile 1969–2012: Analysis of an Epidemic and Its Control. Am J Trop Med Hyg. 2018; 99(3_Suppl):26–33. https://doi.org/10.4269/ajtmh.18-0125 PMID: 30047371
12. Luby SP, Davis J, Brown RR, Gorelick SM, Wong THF. Broad approaches to cholera control in Asia: Water, sanitation and handwashing. Vaccine. 2020; 38 Suppl 1:A110–a7. https://doi.org/10.1016/j.vaccine.2019.07.084 PMID: 31383486
13. Carey ME, MacWright WR, Im J, Meiring JE, Gibani MM, Park SE, et al. The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) Population-based Enteric Fever Studies: A Review of Methodological Similarities and Differences. Clin Infect Dis. 2020; 71(Supplement_2):S102–s10. https://doi.org/10.1093/cid/ciaa367 PMID: 32725221
14. Antillón M, Warren JL, Crawford FW, Weinberger DM, Kürüm E, Pak GD, et al. The burden of typhoid fever in low- and middle-income countries: A meta-regression approach. PLoS Negl Trop Dis. 2017; 11 (2):e0005376. https://doi.org/10.1371/journal.pntd.0005376 PMID: 28241011
15. PAHO. Epidemiological Alert, Salmonella enterica serovar Typhi haplotype H58 2018. Available: http://www.paho.xn—orgpaho-qja6263e/WHO,2018
16. Campos J, Pichel M, Vaz TMI, Tavechio AT, Fernandes S, Muñoz N, et al. Building PulseNet Latin America and Caribbean Salmonella regional database: First conclusions of genetic subtypes of S. Typhi, S. Typhimurium and S. Enteritidis circulating in six countries of the region. Food Research International—FOOD RES INT. 2012; 45.
17. Chinen I, Campos J, Dorji T, Pérez Gutiérrez E. PulseNet Latin America and the Caribbean Network: Present and Future. Foodborne Pathog Dis. 2019; 16(7):489–97. https://doi.org/10.1089/fpd.2018.2587 PMID: 31081688
18. Diaz-Guevara P, Montaño LA, Duarte C, Zabaleta G, Maes M, Martinez Angarita JC, et al. Surveillance of Salmonella enterica serovar Typhi in Colombia, 2012–2015. PLoS Negl Trop Dis. 2020; 14(3): e0008040.18. https://doi.org/10.1371/journal.pntd.0008040 PMID: 32155148

19. Tiba-Casas MR, Sacchi CT, Gonçalves CR, Almeida EA, Soares FB, de Jesus Bertani AM, et al. Molecular analysis of clonally related Salmonella Typhi recovered from epidemiologically unrelated cases of typhoid fever, Brazil. Int J Infect Dis. 2019; 81:191–5. https://doi.org/10.1016/j.ijid.2019.02.009 PMID: 30849581

20. Maes M, Dyson ZA, Higginson EE, Fernandez A, Araya P, Tennant SM, et al. Multiple Introductions of Salmonella enterica Serovar Typhi H58 with Reduced Fluoroquinolone Susceptibility into Chile. Emerging infectious diseases. 2020; 26(11):2736. https://doi.org/10.3201/eid2611.201676 PMID: 33079054

21. Instituto Nacional de Salud. Protocolo de vigilancia en salud pública, fiebre tifoidea y paratifoidea. [Internet]. Available: http://www.ins.gov.co/buscador-eventos/Lineamientos/PRO Fiebre Tifoidea.pdf

22. Grimont P, Weill F-X. Antigenic formulae of the Salmonella servovars. WHO Collab Cent Ref Res Salmonella. 2008; 1–167. Available: http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089%5Cnpapers2://publication/uuid/CA3447A0-61BF-4D62-9181-C9BA78AF0312

23. CLSI. Performance Standards for Antimicrobial Susceptibility Testing Performance Standards for Antimicrobial Susceptibility Testing Suggested Citation. CLSI Doc M02-A11. 2018; 100–125. Available: https://clsi.org/media/2663/m100ed29_sample.pdf

24. Monstein HJ, Ostholm-Balkhed A, Nilsson MV, Nilsson M, Dornbusch K, Nilsson LE. Multiplex PCR amplification assay for the detection of blaSHV, blaTEM and blaCTX-M genes in Enterobacteriaceae. Apmis. 2007; 115(12):1400–8. https://doi.org/10.1111/j.1600-0463.2007.00722.x PMID: 18184411

25. Page AJ, De Silva N, Hunt M, Quail MA, Parkhill J, Harris SR, et al. Robust high-throughput prokaryote de novo assembly and improvement pipeline for Illumina data. Microb Genom. 2016; 2(8):e000083. https://doi.org/10.1099/mgen.0.000083 PMID: 28348874

26. Kariuki S, Mbae C, Van Puyvelde S, Onsare R, Kavai S, Wairimu C, et al. High relatedness of invasive multi-drug resistant non-typhoidal Salmonella genotypes among patients and asymptomatic carriers in endemic informal settlements in Kenya. PLoS Negl Trop Dis. 2020; 14(8):e0008440. https://doi.org/10.1371/journal.pntd.0008440 PMID: 32745137

27. Croucher NJ, Page AJ, Connor TR, Delaney AJ, Keane JA, Bentley SD, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. Nucleic Acids Res. 2015; 43(3):e15. https://doi.org/10.1093/nar/gku1196 PMID: 25414349

28. Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. Bioinformatics. 2006; 22(21):2688–90. https://doi.org/10.1093/bioinformatics/btl446 PMID: 16928733

29. Inouye M, Dashnow H, Raven LA, Schultz MB, Pope BJ, Tomita T, et al. SRST2: Rapid genomic surveillance for public health and hospital microbiology labs. Genome Med. 2014; 6(11):90. https://doi.org/10.1186/s13073-014-0090-6 PMID: 25422674

30. Gupta SK, Padmanabhan BR, Diene SM, Lopez-Rojas R, Kempf M, Landraud L, et al. ARG-ANNOT, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. Antimicrob Agents Chemother. 2014; 58(1):212–20. https://doi.org/10.1128/AAC.01310-13 PMID: 24145532

31. Carattoli A, Zankari E, García-Fernández A, Voldby Larsen M, Lund O, Villa L, et al. In silico detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014; 58(7):3895–903. https://doi.org/10.1128/AAC.02412-14 PMID: 24777092

32. Instituto Nacional de Salud. Salmonella spp.: "Colombia 1997–2018" Dirección Redes en Salud Pública. 2019.

33. 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. Nature genetics. 2015; 47(6):632–9. https://doi.org/10.1038/ng.3281 PMID: 25961941

34. Muñoz JS, Motoa G, Escandón-Vargas K, Bavestrello L, Quiros R, Hernandez C, et al. Current Antimicrobial Stewardship Practices in Latin America: Where Are we? Open Forum Infectious Diseases. 2015; 2(suppl_1).

35. Cox JA, Vlieghe E, Mendelson M, Wertheim H, Ndegwa L, Villegas MV, et al. Antibiotic stewardship in low- and middle-income countries: the same but different? Clin Microbiol Infect. 2017; 23(11):812–8. https://doi.org/10.1016/j.cmi.2017.07.010 PMID: 28712667

Appendix 13. The genomic epidemiology of multi-drug resistant invasive non-typhoidal *Salmonella* in selected sub-Saharan African countries (publication)

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The genomic epidemiology of multi-drug resistant invasive non-typhoidal *Salmonella* in selected sub-Saharan African countries

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ABSTRACT

Background Invasive non-typhoidal *Salmonella* (iNTS) is one of the leading causes of bacteraemia in sub-Saharan Africa. We aimed to provide a better understanding of the genetic characteristics and transmission patterns associated with multi-drug resistant (MDR) iNTS serovars across the continent.

Methods A total of 166 iNTS isolates collected from a multi-centre surveillance in 10 African countries (2010–2014) and a fever study in Ghana (2007–2009) were genome sequenced to investigate the geographical distribution, antimicrobial genetic determinants and population structure of iNTS serotypes–genotypes. Phylogenetic analyses were conducted in the context of the existing genomic frameworks for various iNTS serovars. Population-based incidence of MDR-iNTS disease was estimated in each study site.

Results *Salmonella* Typhimurium sequence-type (ST) 313 and *Salmonella* Enteritidis ST11 were predominant, and both exhibited high frequencies of MDR; *Salmonella* Dublin ST10 was identified in West Africa only. Mutations in the *gyrA* gene (fluoroquinolone resistance) were identified in *S.* Enteritidis and *S.* Typhimurium in Ghana; an ST313 isolate carrying $bla_{CTX\text{-}M\text{-}15}$ was found in Kenya. International transmission of MDR ST313 (lineage II) and MDR ST11 (West African clade) was observed between Ghana and neighbouring West African countries. The incidence of MDR-iNTS disease exceeded 100/100 000 person-years-of-observation in children aged <5 years in several West African countries.

Conclusions We identified the circulation of multiple MDR iNTS serovar STs in the sampled sub-Saharan African countries. Investment in the development and deployment of iNTS vaccines coupled with intensified antimicrobial resistance surveillance are essential to limit the impact of these pathogens in Africa.

Key questions

What is already known?

- ► Invasive non-typhoidal *Salmonella* (iNTS) disease is an emerging pathogen in sub-Saharan Africa.
- ► iNTS is now a leading cause of bacteraemia in sub-Saharan Africa.
- ► The disease is associated with specific sequence types of *S.* Enteritidis and *S.* Typhimurium.

BACKGROUND

The non-typhoidal members of *Salmonella enterica* are archetypal zoonotic pathogens typically associated with self-limiting diarrhoea in humans. However, certain non-typhoidal *Salmonella* serovars are also a recognised cause of invasive disease in specific geographical regions. Invasive non-typhoidal *Salmonella* (iNTS) is

Key questions

What are the new findings?

- iNTS disease is widespread across the sampled locations.
- *Salmonella* Typhimurium sequence-type (ST) 313 and *Salmonella* Enteritidis ST11 are the predominant pathogens.
- There is a high frequency of multi-drug resistant (MDR) phenotypes.
- The incidence of MDR-iNTS disease exceeded 100/100 000 person-years-of-observation in children aged <5 years in several West African countries.

What do the new findings imply?

- Sustained disease surveillance of MDR-iNTS organisms is essential.
- There is a need for acceleration of iNTS vaccines.
- We need a better understanding of the disease reservoir and human-to-human transmission.

most commonly observed in infants and young adults with HIV, malaria and malnutrition.[1] Annually, there are an estimated 3.4 million cases of iNTS globally, 20% of which are fatal.[2 3] The vast majority of iNTS disease is reported in sub-Saharan Africa, where annual incidence rates of 175–388 cases per 100 000 person-years and case fatality rates as high as 25% have been reported in young children.[4–6] A recent multi-centre study across 10 countries in sub-Saharan Africa identified iNTS as a major cause of bacteraemia in febrile patients, with incidence rates exceeding 100 cases per 100 000 person-years recorded in multiple sampling locations.[7]

Various *Salmonella* serovars have been associated with iNTS disease, including *S.* Isangi,[8] *S.* Concord,[9] *S.* Stanleyville and *S.* Dublin.[10] However, the majority of iNTS infections in sub-Saharan Africa can be attributed to *S.* Typhimurium and *S.* Enteritidis.[11 12] *Salmonella* Typhimurium iNTS in sub-Saharan Africa is largely associated with a multi-drug resistant (MDR; resistant to ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole) variant of ST313, which comprised two major lineages (I and II).[11] *Salmonella* Enteritidis account for approximately one-third of the iNTS cases reported in sub-Saharan Africa and are primarily associated with genotype ST11. There are three major clades of *S.* Enteritidis ST11 (Global epidemic, West African and Central/East African clades) co-circulating in this region, in which MDR phenotypes are common.[13]

Ampicillin, chloramphenicol and trimethoprim–sulfamethoxazole (co-trimoxazole) have traditionally served as first-line treatment for iNTS and typhoid fever in Africa.[14] The widespread use of these antimicrobials likely led to the emergence and spread of MDR iNTS organisms. Several sub-Saharan African countries have reported MDR iNTS, including Malawi,[15–17] Kenya,[18–20] Ghana,[21] Gambia,[5] Democratic Republic of the Congo (DRC),[22] Mozambique,[23 24] Tanzania,[25] Burkina Faso,[7] Guinea-Bissau[7] and Nigeria.[26] Consequently, alternative treatments, such as ciprofloxacin, azithromycin and ceftriaxone, are used increasingly to manage bloodstream infections. However, these drugs may be unavailable or costly, and resistance is emerging to these antimicrobial agents.[17] Concerningly, extensively drug-resistant (XDR) (MDR plus resistance to fluoroquinolones and third-generation cephalosporins) *S.* Typhimurium ST313 organisms have been reported in Kenya,[14 27] Malawi[28] and DRC.[29] These new resistance phenotypes pose a significant challenge for the control of iNTS disease.[14]

Here, we subjected a contemporaneous collection of iNTS organisms from multiple sites in sub-Saharan Africa to whole genome sequencing to investigate the phylogenetic distribution of these organisms and their corresponding sequence types (STs) and antimicrobial resistance (AMR) determinants. We also estimated the incidence rates of MDR iNTS disease in the sampling locations and performed phylogenetic analyses of *S.* Typhimurium ST313 and *S.* Enteritidis ST11 in a global context.

METHODS

Ethics approval and consent to participate

This research was conducted under the ethical principles of the Declaration of Helsinki. The IVI Institutional Review Board (IRB), the national ethical review committees in each participating country, and local research ethics committees approved the study protocol. All eligible patients meeting the study inclusion criteria were provided with a detailed explanation of the study purpose, and written informed consent was obtained prior to study enrolment. For children, written informed consent was obtained from parent or guardian.[30]

Study design and inclusion criteria

The majority of iNTS isolates (117/166) in this study originated from the Typhoid Fever Surveillance in Africa Program (TSAP),[30] conducted in 13 sites in 10 countries between 2010 and 2014. Febrile patients from all age groups (except in Ghana, where only children aged <15 years were enrolled) with a tympanic or axillary temperature of ≥38.0°C or ≥37.5°C, respectively, living in a defined study catchment area were eligible for recruitment. For inpatients, reported fever within 72 hours of admission was also necessary for inclusion. Written informed consent/assent was obtained. Clinical assessments of patients included history of illness, physical examination and clinical appraisal. Blood samples (5–10 mL for adults; 1–3 mL for children) were collected for microbiological testing and diagnosis. An additional 49 iNTS isolates were obtained from a febrile surveillance study conducted at the Presbyterian Hospital of Agogo in Ghana, between 2007 and 2009.[31]

Patient and public involvement statement

This TSAP study was performed under a single protocol with some site-specific and country-specific details; these details were developed with the study sites and local patient groups and communities attending the healthcare facilities. These data were essential for establishing the demographic framework of the sites and understanding how patients accessed healthcare and disease diagnosis. Therefore, these patient/community groups

Table 1 Distribution of iNTS serovars and genotypes circulating in the sampled countries in sub-Saharan Africa

Country (number of iNTS*) (a)	Serovars	Number of iNTS per serovar (b)	% of total number of iNTS per country (b)/(a)	Genotype (sequence type)	Number of iNTS per genotype (c)	% of total number of iNTS per country (c)/(a)
Burkina Faso (12)	Typhimurium	7	58	313	6	50
				19	1	8
	Enteritidis	4	33	11	4	33
	Dublin	1	8	10	1	8
Ghana (133)	Typhimurium	92	69	313	90	68
				19	2	2
	Enteritidis	20	15	11	18	14
				183	1	1
				2107	1	1
	Dublin	17	13	10	17	13
	Muenster	1	1	321	1	1
	Poona	1	1	308	1	1
	Stanleyville	1	1	339	1	1
	Virchow	1	1	359	1	1
Guinea-Bissau (9)	Typhimurium	5	56	313	2	22
				19	3	33
	Enteritidis	1	11	11	*1	11
	Choleraesuis	3	33	145	3	33
Kenya (1)	Typhimurium	1	100	313	1	100
Madagascar (4)	Typhimurium	1	25	19	*1	25
	Enteritidis	3	75	11	‡3	75
Senegal (2)	Typhimurium	1	50	19	*1	50
	Enteritidis	1	50	11	*1	50
South Africa (1)	Enteritidis	1	100	11	*1	100
Tanzania (4)	Typhimurium	3	75	19	3	75
	Unknown	1	25	2533	1	25

Total 8 countries† (166 iNTS isolates) (d)	Serovars	Number of iNTS per serovar (e)	% of total number of iNTS in all 8 countries (e)/(d)	Genotype (sequence type)	Number of iNTS per genotype (f)	% of total number of iNTS in all 8 countries (f)/(d)
	Typhimurium	110	66	ST313	99	60
				ST19	11	7
	Enteritidis	30	18	ST11	28	17
				Other STs‡	2	1
	Dublin	18	11	ST10	18	11
	Others	8	5	Other STs§	8	5

*iNTS: invasive non-typhoidal *Salmonella*.
†Total 8 countries: Burkina Faso, Ghana, Guinea-Bissau, Kenya, Madagascar, Senegal, South Africa and Tanzania presented in this table. No iNTS isolates were yielded in Sudan and Ethiopia.
‡Other sequence types of *S*. Enteritidis detected: 1 ST183 (isolate yielded from Ghana; age/sex unknown due to missing data) and 1 ST2107 (from Ghana; a 22-year-old woman); both non-MDR and no antimicrobial resistant genes detected.
§Sequence types of the other NTS serovars: 1 Muenster ST321 (yielded from Ghana; age/sex/year unknown due to missing data), 1 Poona ST308 (yielded from Ghana in 2008; age/sex unknown due to missing data), 1 Stanleyville ST339 (yielded from Ghana; age/sex/year unknown due to missing data), 1 Virchow ST359 (from Ghana; age/sex/year unknown due to missing data), 3 Choleraesui ST145 (two isolates yielded from 1-year-old female infants in 2010 and 2011 and 1 from a 3-year-old female infant in 2012; all from Guinea-Bissau), 1 unknown ST2533 from Tanzania.

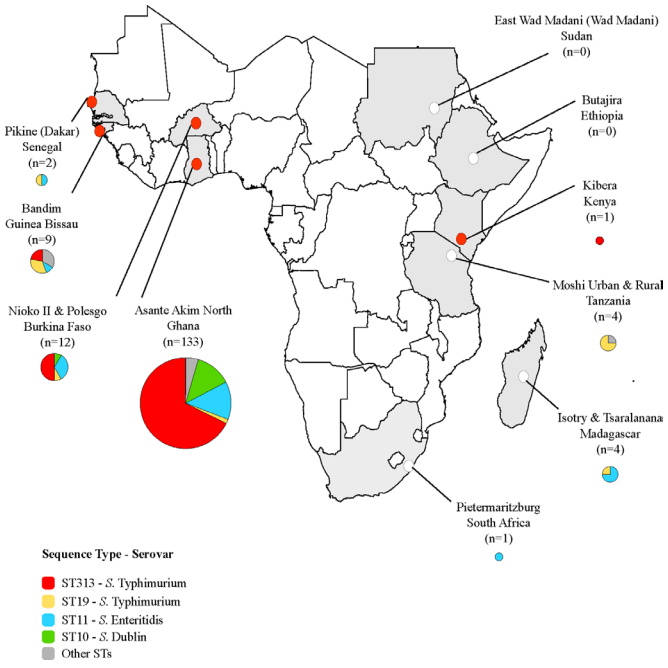

Figure 1 Geographical distribution of iNTS genotypes and serotypes in the sampled countries in sub-Saharan Africa. Different colours in the pie charts correspond to different sequence types and serovars of iNTS isolates in our study sites. The size of the pie charts corresponds to the numbers of isolates in each country. Countries coloured in grey highlight the study sites, while red and white circles indicate countries with and without MDR iNTS isolates, respectively.

informed the study design to address issues regarding healthcare access and practices. Local communities and patients acted as communicators in the local communities to encourage people to attend healthcare facilities when symptomatic. Data from the studies have been provided to study sites to inform the local community and patients of the study findings.

Bacterial isolates and antimicrobial susceptibility testing

Blood specimens were inoculated into an aerobic blood culture bottle and incubated in systems with automated growth detection (BACTEC Peds Plus Medium/BACTECT Plus Aerobic-F, BACTEC, Becton-Dickinson, New Jersey; or BacT/ALERT PF Paediatric FAN/BacT/ALERT FA FAN Aerobic, bioMerieux, Marcy l'Etoile, France). Blood cultures with bacterial growth were subcultured on blood and chocolate agar (Oxoid, Basingstoke, UK), and biochemical tests were conducted (API 20E; bioMerieux) to identify suspected *Salmonella* isolates.[30] Antimicrobial susceptibility testing was performed using agar diffusion tests according to the Clinical Laboratory and Standards Institute guidelines.[30]

Data sources and bacterial isolates

A total of 166 iNTS isolates were used for this investigation, which comprised 94 iNTS isolates from the TSAP study, an additional 23 iNTS isolates obtained from outside predefined TSAP study catchment areas and 49 iNTS isolates from other studies. In order to place these *S.* Typhimurium and *S.* Enteritidis isolates into a global phylogenetic context, the existing datasets were incorporated: 147 iNTS serotype Typhimurium ST313 isolates from seven countries (Malawi, Kenya, Mozambique, Uganda, DRC, Nigeria and Mali),[11] Nigeria and DRC,[12] Malawi,[28] Kenya,[14] Malawi[32] and 594 iNTS serotype Enteritidis ST11 isolates (selected from Feasey *et al*[13]; online supplemental table 1).

Whole genome sequencing

Genomic DNA was extracted from all *Salmonella* isolates using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA). Two micrograms of genomic DNA from each organism was subjected to indexed-tagged pair-end sequencing on an Illumina Hiseq 2000 platform (Illumina, CA, USA) at the Wellcome Sanger Institute to generate 100 bp paired-end reads. Data quality control was performed using in-house pipelines. Raw sequence data are available in the European Nucleotide Archive (Project number: ERP009684, ERP010763, ERP013866) (online supplemental table 2).

Single nucleotide polymorphism (SNP) calling and analyses

Raw Illumina reads were used to create multiple assemblies using Velvet V.1.2[33] with parameters optimised using VelvetOptimiser V.2.2.5,[34 35] and automated annotation was performed using PROKKA V.1.5.[36] Roary[37] was used to define the pan genome of 166 iNTS isolates with blastp percentage identity of 99% and a core definition of 99%. In total, 3450 core genes were identified (genes that were present in ≥99% strains) and 86 765 SNP sites were extracted from the core gene alignment using SNP-sites V.2.1.3.[35]

For *S.* Typhimurium ST313, raw Illumina reads of 99 isolates from this study and 147 *S.* Typhimurium ST313 from previous studies[11 12 29 33 38] were mapped to the reference sequence of *S.* Typhimurium strain SL1344 (accession: FQ312003.1), using SMALT V.0.7.4 (http://www.sanger.ac.uk/resources/software/smalt/). Candidate SNPs were called against the reference sequence using SAMtools[38] and filtered with a minimum mapping quality of 30 and minimum consensus base agreement of 75%. The allele at each locus in each isolate was determined by reference to the consensus base in that genome using SAMtools *mpileup* and removing low confidence alleles with consensus base quality ≤20, read depth ≤5 or a heterozygous base call. The repeat finding program in NUCmer V.3.1[39] was used to identify exact repetitive regions of ≥20 bp in length in the reference genome and SNPs called in these regions were excluded. SNPs called in phage sequences and recombinant regions identified using Gubbins[40] were further removed, resulting in a final set of 1960 chromosomal SNPs. The identification of SNPs for *S.* Enteritidis ST11 was performed following the same procedure as *S.* Typhimurium ST313. Briefly, the raw Illumina reads of 28 *S.* Enteritidis ST11 isolates from this study and 594 additional isolates from a global collection[13] were mapped to the reference sequence of *S.* Enteritidis strain P125109 (accession: NC_011294.1), using SMALT

Table 2 Distribution of MDR iNTS and *gyrA* mutation (fluoroquinolone resistance) in the sampled countries in sub-Saharan Africa

Serovars (number of isolates) n=166	MDR iNTS isolates per serovar*	MDR iNTS serovar per country (%, number)	MDR iNTS per genotype (%, number)
Typhimurium (n=110)	85% (94/110)	Burkina Faso (50, 6/12) Ghana (64, 85/133) Guinea-Bissau (22, 2/9) Kenya (100, 1/1)	ST313 (95, 94/99) ST19 (0, 0/11)
Enteritidis (n=30)	23% (7/30)	Burkina Faso (33, 4/12) Ghana (2, 2/133) Senegal (50, 1/2)	ST11 (25, 7/28)
Dublin (n=18)	6% (1/18)	Ghana (1, 1/133)	ST10 (6, 1/18)
Others (n=8)	0% (0/8)	n.a.	n.a.

Countries (number of iNTS)	Number of MDR iNTS (n=102)	% of MDR iNTS	*gyrA*
Burkina Faso (n=12)	10	10/12 (83)	0
Ghana (n=133)	88	88/133 (66)	S83Y (ST313, 2 isolates) D87G (ST11, 6 isolates) D87Y (ST11, 2 isolates) D87N (ST11, 3 isolates)
Guinea-Bissau (n=9)	2	2/9 (22)	0
Kenya (n=1)	1	1/1 (100)	0
Senegal (n=2)	1	1/2 (50)	0

*Multidrug resistance (MDR) definition used for the analysis presence of resistant genes for at least one agent in all three antimicrobial categories listed below (detected in this study): ampicillin (blaCTX_M, blaOXA, blaTEM), chloramphenicol (*catA1*), trimethoprim–sulfamethoxazole (sulfonamide (sul1, sul2) and trimethoprim (*dfrA, dfrA1, dfrA14, dfrA8*)).
†Refer to table 1 for the number of iNTS isolates per country used as a denominator to calculate the % of MDR per country in this table.
‡The 2 MDR iNTS isolates with *gyrA* mutation (fluoroquinolone resistance) were yielded from a 1-year-old female infant and a 10-month-old female infant in Agogo in 2011 (TSAP: Typhoid Fever Surveillance Program).
§*Spv* locus was detected in all MDR iNTS isolates.
n.a., not available.

followed by SNP calling and filtering as described previously, resulting in a final set of 25 121 SNPs.

Phylogenetic analyses

A maximum likelihood (ML) phylogenetic tree was constructed from the 86 765 SNP alignment of all 166 iNTS isolates using RAxML V.8.2.8 with a generalised time-reversible model and a Gamma distribution to model the site-specific rate variation (GTRGAMMA).[41] Clade support for this tree was assessed through a bootstrap analysis with 100 pseudo-replicates. To investigate the molecular epidemiology of the *S.* Typhimurium ST313 and *S.* Enteritidis ST11 isolates sequenced here in a global context, a ML tree was inferred from an alignment of 1960 SNPs for 246 *S.* Typhimurium ST313 (99 from this study and 147 from previous studies[11 12 29 33 38]) and an alignment of 25 121 SNPs for 622 *S.* Enteritidis ST11 isolates (28 from this study and 594 from a global collection[13]), using RAxML with GTRGAMMA model. Support for these phylogenetic trees was assessed through a 100 bootstrap pseudo-analysis. Tree annotation was visualised using ITOL.[42]

Antimicrobial resistance gene and plasmid analyses

From raw Illumina reads, Short Read Sequence Typing-SRST2[43] was used to identify acquired AMR genes and their precise alleles using the ARG-Annot database,[44] as well as plasmid replicons using the PlasmidFinder database.[45] Multilocus sequence typing (MLST) of all iNTS isolates was also determined using SRST2 together with the MLST database for *Salmonella enterica* downloaded from pubMLST (https://pubmlst.org/organisms/salmonella-spp).[46] *Salmonella* serotypes were identified using conventional serology as well as MLST-based approach[47] and SeqSero (genome-based approach)[48]; the final interpretation followed a consensus of MLST and SeqSero. Bandage[49] was used to investigate the assembled contigs carrying the AMR cassettes. Mutations in fluoroquinolone resistance genes (*gyrA, gyrB, parC, parE*) were identified using SeaView.[50]

Incidence analyses of MDR iNTS disease

Incidence rates of MDR iNTS were estimated for study sites in Burkina Faso, Ghana, Guinea-Bissau, Kenya and Senegal. Statistical methodology used previously to calculate the

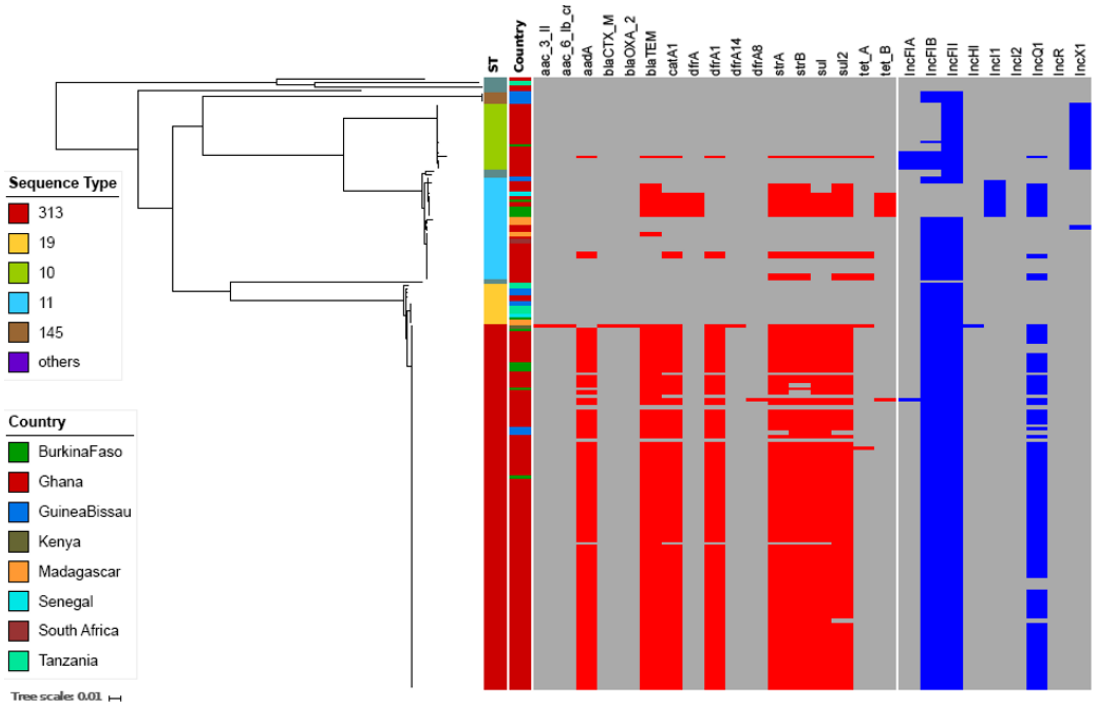

Figure 2 Antimicrobial resistance genes and plasmids associated with iNTS isolates circulating in sampled sub-Saharan African countries. Midpoint-rooted maximum likelihood phylogenetic tree based on the core genes of iNTS isolates sequenced in this study and their corresponding metadata. The first column shows the sequence types in different colours. The second column corresponds to the countries where our iNTS isolates were detected. The remaining columns exhibit a heatmap of detected AMR genes and plasmid replicons. The tree scale bar indicates the number of substitutions per variable site.

incidence of *S.* Typhi and iNTS disease in the TSAP study was used to calculate MDR iNTS incidence.[7 30 51] Demographic data from Health and Demographic Surveillance System (HDSS) sites were used to estimate population denominators where available. In non-HDSS sites, health-seeking behaviour as reported by randomly administered healthcare utilisation surveys were used to estimate population denominators for each study site. Crude incidence rates were adjusted to account for the proportion of surveyed individuals who reported seeking care for a febrile episode at a study facility to yield adjusted incidence rates. Adjusted incidence rates of MDR iNTS cases per 100 000 per person-years-of-observation (PYO) were estimated with 95% CIs using these adjustment factors and crude MDR iNTS case numbers. The previously established multi-country database (Microsoft Visual FoxPro 7.0, Redmond, Washington) for TSAP was used for the countries with MDR iNTS isolates.

RESULTS

Geographical distribution of iNTS serotypes and sequence types

The majority (66%; 110/166) of iNTS organisms in the sampled sub-Saharan African countries were *S.* Typhimurium, of which 90% (99/110) were ST313 and 10% (11/110) were ST19. *S.* Enteritidis accounted for 18% (30/166) of the isolates, composed of ST11 (93.3%; 28/30), ST183 (3.3%; 1/30) and ST2107 (3.3%; 1/30). *S.* Dublin (ST10) comprised a further 11% (18/166) of isolates. Several other serotype STs, including *S.* Choleraesuis ST145 (3/166), *S.* Muenster ST321 (1/166), *S.* Poona ST308 (1/166), *S.* Stanleyville ST339 (1/166) and *S.* Virchow ST359 (1/166), were also identified. *S.* Typhimurium ST313 was mostly limited to West Africa (table 1), whereas *S.* Enteritidis ST11 appeared to be pervasive in both West and Southern Africa. *S.* Dublin ST10 was identified in West Africa and *S.* Typhimurium ST19 was distributed across the continent.

Overall, 61% (102/166) of the iNTS organisms described here were MDR. These were isolated in Burkina Faso (83%; 10/12), Ghana (66%, 88/133), Guinea-Bissau (22%; 2/9), Kenya (100%; 1/1) and Senegal (50%; 1/2). *S.* Typhimurium exhibited the highest prevalence of MDR (85%; 94/110); 95% (94/99) of the ST313 isolates were MDR. In total, 23% (7/30) of *S.* Enteritidis and 6% (1/18) *S.* Dublin organisms were MDR; none of the *S.* Typhimurium ST19 were MDR (figure 1, table 2).

Phylogenetics and AMR of iNTS

A phylogenetic reconstruction of all iNTS isolates showed that the three major serovar STs—*S.* Typhimurium (ST313), *S.* Enteritidis (ST11) and *S.* Dublin (ST10)—formed independent clusters with dissimilar AMR gene profiles (figure 2). Almost all of the MDR *S.* Typhimurium ST313 (95%; 94/99) carried the Tn21 transposon-associated MDR-*loci* (*sulII-strAB-dfrA1-aadA1-sulI-cat-blaTEM*) on an IncF virulence-resistance plasmid (pSLT-BT).[11] A single MDR *S.* Typhimurium ST313 from Kenya additionally carried two copies of $bla_{\text{CTX-M-15}}$, conferring resistance to third-generation cephalosporins. One copy of $bla_{\text{CTX-M-15}}$ was located on the 300 kb IncHI2 plasmid, pKST313 (accession number: LN794248), and

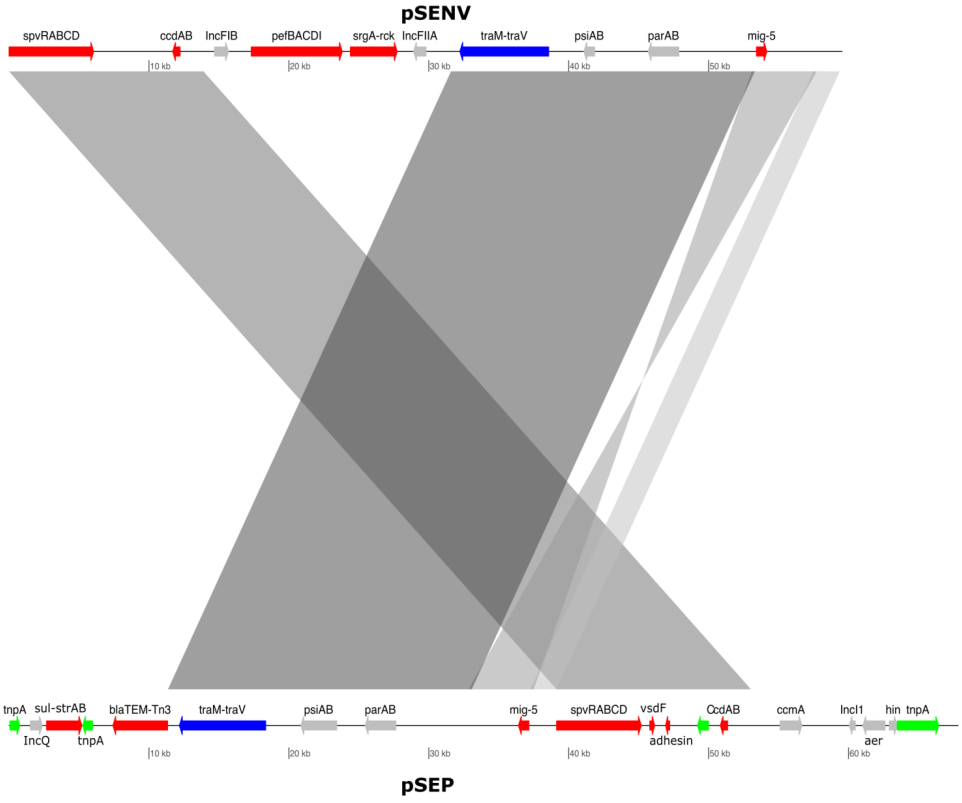

Figure 3 Novel IncI1 virulence-resistance plasmid (pSEP) in an *S*. Enteritidis ST11 isolate. Plasmid comparison analyses between the novel virulence-resistance IncI1 plasmid pSEP (bottom) and the reference virulence IncF plasmid pSENV (top). The grey blocks show the BLASTn comparison between the two plasmids using the bl2seq feature from the web-based BLAST. Some annotations are added for both plasmids. Red-coloured arrows are genes associated with virulence and AMR. Blue-coloured arrows are genes associated with conjugation. Grey-coloured arrows correspond to plasmid replication and stability. Green-coloured genes are associated with transposon elements.

the other on the chromosome disrupting the *ompD* locus. Reduced susceptibility to fluoroquinolones in *S.* Typhimurium ST313 was uncommon, with 2% (2/99) of Ghanaian ST313 isolates possessing a single mutation (S87Y) in *gyrA* (table 2).

The majority of *S.* Enteritidis ST11 (19/28; 68%) harboured the typical IncF virulence plasmid (60 kb), which was comparable with pSENV (accession number NC_019120.1, coverage 100%, identity 99%). The remaining *S.* Enteritidis ST11 (9/28; 32%) harboured a novel IncI1 virulence-resistance plasmid (pSEP, accession number: ERP121368) of approximately 68 kb (figure 3), of which 7/28 (25%) isolates (4 from Burkina Faso, 2 from Ghana, 1 from Senegal) carried the MDR-encoding Tn21-like transposon (*sulII-strAB-tetB-dfrA1-sulI-cat-blaTEM*), and 2/28 (7.1%) isolates carried a different AMR cassette (*TnpA-sulII-strAB-blaTEM-Tn3*). The novel IncI plasmid exhibited 60% homology to pSENV but did not harbour the IncF replicon, the *pefBACD* fimbriae-encoding operon, or the virulence-associated genes *srgA* and *rck* (figure 3). In addition, two non-MDR Ghanaian *S.* Enteritidis ST11 possessed an AMR cassette (*sul2-strAB-tetA*) carried on a small (11 kb) non-conjugative IncQ plasmid conferring resistance against sulfonamides, streptomycin and tetracyclines. This IncQ plasmid exhibited a similar genetic structure to pSTU288-2 from *S.* Typhimurium (accession number CP004059.1, coverage 98%, identity 99%). Two further non-MDR Ghanaian isolates carried a Tn21-mediated AMR cassette (*sulII-strAB-dfrA1-aadA1-sulI-cat*) on the virulence plasmid, and a single non-MDR isolate from Madagascar carried a bla_{TEM} Tn3 integrated into the virulence plasmid. Reduced susceptibility to fluoroquinolones was predicted by the sequences in 39% (11/28) of the *S.* Enteritidis-ST11, all of which originated from Ghana and displayed a variety of *gyrA* mutations (D87G: 6 isolates, D87N: 3 isolates, D87Y: 2 isolates) (table 2).

Phylogenetics of *S.* Typhimurium ST313 and *S.* Enteritidis ST11 isolates in global context

To investigate the population structures of *S.* Typhimurium ST313 and *S.* Enteritidis ST11 in a broader context, we constructed global phylogenies. All *S.* Typhimurium ST313 isolates from our study fell into lineage II (figure 4A). The single Kenyan ST313 isolate carrying two copies of $bla_{CTX\text{-}M\text{-}15}$ was part of the previously described clonal expansion of MDR ceftriaxone-resistant ST313 sub-lineage.[14] Notably, the Ghanaian ST313 isolates did not form a single cluster, but were associated with multiple clusters from Mali, Burkina Faso and Nigeria, indicating multiple introduction events (figure 4A).

A detailed phylogenetic investigation of *S.* Enteritidis ST11 demonstrated that 11/28 (40%) (Ghana: 5, Burkina Faso: 4, Senegal: 1, Guinea Bissau: 1) isolates fell into the

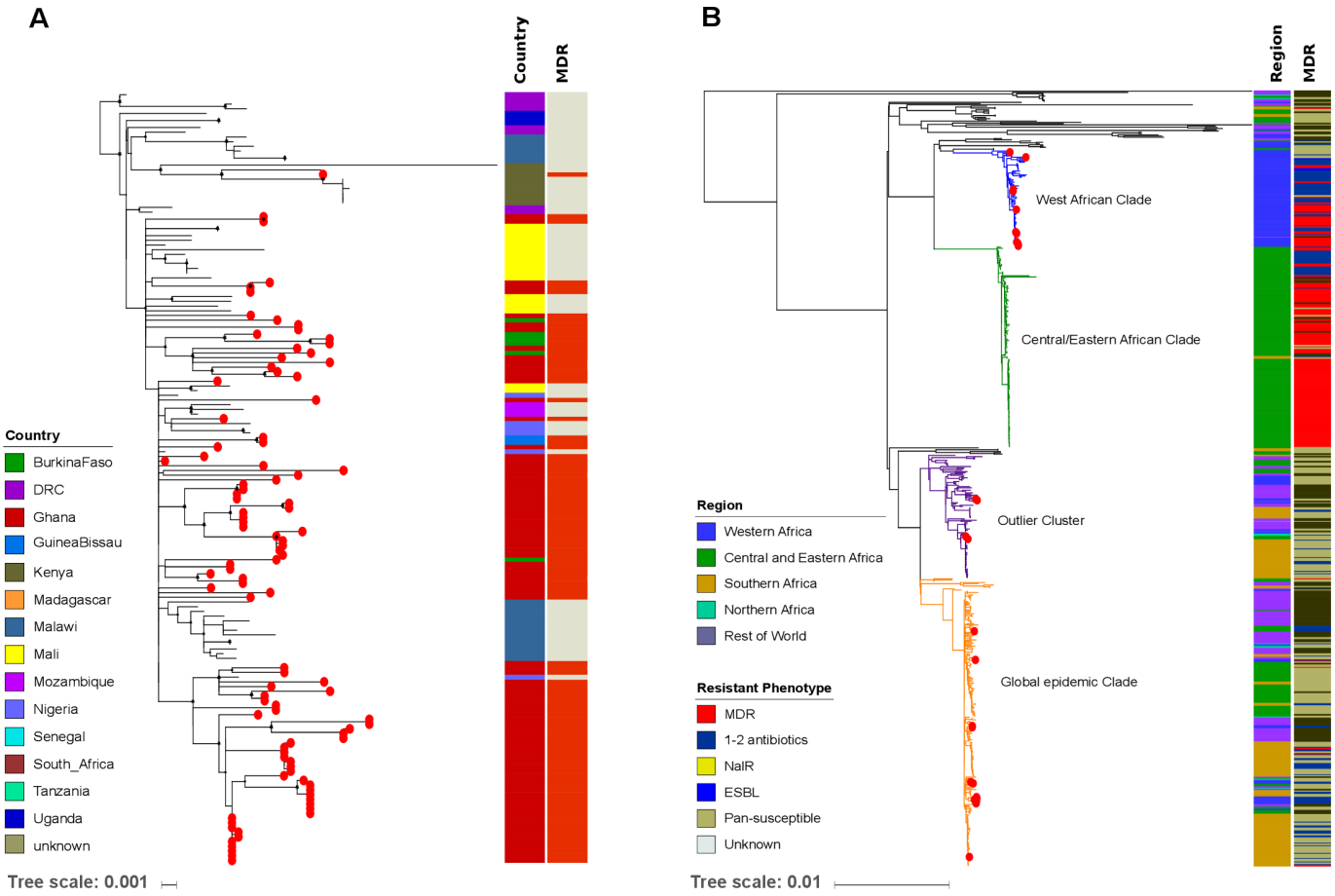

Figure 4 Phylogenetics of *S*. Typhimurium ST313 lineage II and *S*. Enteritidis ST11 in sub-Saharan Africa. (A) Maximum likelihood phylogenetic tree of *S*. Typhimurium-ST313 lineage II isolates from this study in the context of the sub-Saharan African continent; lineage I was pruned to enhance the visualisation of the tree. Red circles at the terminal leaves correspond to our study isolates. The first column shows different colour-coded countries from where all analysed isolates originate. The second column shows MDR and non-MDR isolates in red and grey, respectively. (B) Maximum likelihood phylogenetic tree of our *S*. Enteritidis-ST11 isolates in the global context of *S*. Enteritidis. Red circles at the terminal leaves correspond to our study isolates. The first and second columns show regions and resistant phenotypes in different colours. The tree scale bar indicates the number of substitutions per variable site.

West African clade.[13] The ST11 organisms within this West African clade displayed either MDR (seven isolates) or other non-MDR AMR phenotypes (two isolates). We found evidence that some Ghanaian isolates within this clade clustered alongside organisms from Burkina Faso and Mali, again suggesting international transmission. In addition, 13/28 (46%) of the *S*. Enteritidis ST11 (Ghana: 11, Madagascar: 1, South Africa: 1) belonged to the Global epidemic clade.[13] These isolates had phylogenetic links with their country-specific clusters, with the exception of two Ghanaian isolates that clustered with organisms from neighbouring Cameroon and Senegal. Lastly, 4/28 (14%) (2 from Ghana, 2 from Madagascar) of the ST11 isolates grouped within the outlier cluster (figure 4B).

Incidence of MDR iNTS disease in sub-Saharan Africa

We calculated the age-stratified incidence rates of MDR iNTS in previously described study catchment areas in Burkina Faso, Ghana, Guinea-Bissau and Kenya (table 3). The adjusted incidence of MDR iNTS disease exceeded 100/100 000 PYO in children <15 years of age in all West African countries: Burkina Faso (Nioko II, 274/100 000 PYO, 95% CI 185 to 406; Polesgo, 255/100 000 PYO, 95% CI 138 to 470), Ghana (Asante Akim North: Ghana-AAN, 414/100 000, 95% CI 333 to 515) and Guinea-Bissau (Bandim, 105/100 000, 95% CI 69 to 161). Among children <15 years, younger children (<2–4 years) exhibited the highest MDR iNTS incidence rates in both sites in Burkina Faso: 753/100 000 PYO (95% CI 460 to 1233) in Nioko II and 630/100 000 PYO (95% CI 288 to 1380) in Polesgo. In both settings in Burkina Faso, the incidence of MDR iNTS disease in the infant age group was slightly lower than in the group aged 2–4 years, but children <5 years old exhibited a high burden of MDR iNTS disease. In Ghana-AAN, infants aged <2 years had the highest incidence of MDR iNTS disease (1435/100 000; 95% CI 1110 to 1854) followed by children aged between 2 and 5 years (747/100 000; 95% CI 491 to 1135). Similarly, in Guinea-Bissau, infants <2 years old exhibited the highest incidence of MDR iNTS disease (291/100 000; 95% CI 176 to 482). The incidence rate of MDR iNTS in older age groups (≥15 years) was relatively

Table 3 Incidence estimates of MDR iNTS disease in sub-Saharan Africa

Country*	Age group in years	PYO estimation†				Recruitment proportion†	Genome-sequenced iNTS cases	Crude MDR iNTS cases	Crude MDR iNTS incidence per 100 000 PYO	Adjusted MDR iNTS cases	Adjusted MDR iNTS incidence per 100 000 PYO (95% CI)‡
		Proportion of catchment population visiting study facility in case of fever (95% CI)	Catchment population	Catchment population adjusted by health-seeking behaviour	PYO						
Burkina Faso											
Nioko II	0–1	81% (74 to 88)	2208	1788	2097	247/1297 (19)	2	1	48	5	251 (107 to 590)
	2–4	81% (75 to 86)	1823	1477	2097	235/1259 (19)	3	3	143	16	753 (460 to 1233)
	5–14	81% (78 to 84)	4295	3479	4889	228/889 (26)	2	1	20	4	79 (29 to 214)
	<15	n.a.	8326	6744	9083	n.a.	7	5	55	25	274 (185 to 406)
	≥15	81% (79 to 83)	9428	7637	10 676	208/759 (27)	1	1	9	4	35 (13 to 96)
	All	n.a.	17 754	14 381	19 759	n.a.	8	6	30	29	145 (100 to 209)
Polesgo	0–1	92% (86 to 99)	896	824	929	117/475 (25)	1	1	108	4	431 (162 to 1147)
	2–4	83% (76 to 89)	856	710	992	148/466 (32)	2	2	202	6	630 (288 to 1380)
	5–14	87% (83 to 91)	1734	1509	2104	252/510 (49)	0	0	0	0	0
	<15	n.a.	3486	3043	4025	n.a.	3	3	75	10	255 (138 to 470)
	≥15	87% (84 to 89)	4088	3557	4917	239/629 (38)	1	1	20	3	54 (16 to 179)
	All	n.a.	7574	6600	8942	n.a.	4	4	45	13	144 (83 to 249)
Ghana§											
AAN	0–1	16% (14 to 18)	11 222	1760	4080	41	88	24	588	59	1435 (1110 to 1854)
	2–4	16% (13 to 18)	8086	1268	2940	41	23	9	306	22	747 (491 to 1135)
	0–4	n.a.	n.a.	n.a.	n.a.	n.a.	111	33	n.a.	n.a.	n.a.
	5–14	16% (15 to 17)	34 439	5415	12 554	623/1657 (38)	6	6	48	16	126 (77 to 206)
	<15	n.a.	53 747	8443	19 574	n.a.	117	39	147	81	414 (333 to 515)
	≥15	n.a.	n.a.	n.a.	n.a.	n.a.	16	10	n.a.	n.a.	n.a.
	All_TSAP	n.a.	n.a.	n.a.	n.a.	n.a.	133	49	n.a.	n.a.	n.a.
	Non_TSAP¶	n.a.	n.a.	n.a.	n.a.	n.a.	49	39	n.a.	n.a.	n.a.
	All	n.a.	n.a.	n.a.	n.a	n.a.	182	88	n.a.	n.a.	n.a.
Guinea-Bissau											
Bandim	0–1	46% (39 to 54)	10 852	4992	5198	206/631 (33)	7	2	96	15	291 (176 to 482)
	2–4	43% (37 to 48)	7307	3142	3866	175/359 (49)	1	0	26	2	53 (13 to 208)
	5–14	42% (41 to 48)	19 905	8360	11 101	187/380 (49)	1	0	18	4	37 (14 to 97)
	<15	n.a.	38 064	16 494	20 165	n.a.	9	2	40	21	105 (69 to 161)
	≥15	45% (43 to 47)	62 694	28 212	37 109	105/163 (64%)	0	0	0	0	0
	All	n.a.	100 758	44 706	57 274	n.a.	9	2	14	21	37 (24 to 57)
Kenya											
Kibera	0–1	42% (38 to 47)	3467	1456	2031	99/99 (100)	0	0	0	0	0
	2–4	39% (36 to 43)	3070	1197	2039	312/312 (100)	0	0	0	0	0

Continued

Table 3 Continued

Country*	Age group in years	PYO estimation†				Recruitment proportion†	Genome-sequenced iNTS cases	Crude MDR iNTS cases	Crude MDR iNTS incidence per 100 000 PYO	Adjusted MDR iNTS cases	Adjusted MDR iNTS incidence per 100 000 PYO (95% CI)‡
		Proportion of catchment population visiting study facility in case of fever (95% CI)	Catchment population	Catchment population adjusted by health-seeking behaviour	PYO						
	5–14	43% (39 to 47)	7514	3231	5722	539/539 (100)	0	0	0	0	0
	<15	n.a.	14 051	5884	9792	n.a.	0	0	0	0	0
	≥15	35% (32 to 38)	15 263	5342	9228	301/301 (100)	1	1	11	1	11 (2 to 77)
	All	n.a.	29 314	11 227	19 020	n.a.	1	1	5	1	5 (1 to 37)
Senegal**											
Pikine	0–1	39% (32 to 46)	20 120	7837	11 194	n.a.	0	0	n.a.	n.a.	n.a.
	2–4	37% (33 to 41)	30 180	11 097	15 851	n.a.	1	1	n.a.	n.a.	n.a.
	5–14	31% (28 to 34)	96 152	29 807	42 577	n.a.	0	0	n.a.	n.a.	n.a.
	<15	n.a.	146 452	48 741	69 623	n.a.	1	1	n.a.	n.a.	n.a.
	≥15	30% (28 to 31)	195 726	58 718	83 874	n.a.	1	0	n.a.	n.a.	n.a.
	All	n.a.	342 178	107 459	153 496	n.a.	2	1	n.a.	n.a.	n.a.

*TSAP was performed in 10 countries, of which 8 countries (Burkina Faso, Ghana, Guinea-Bissau, Kenya, Madagascar, Senegal, South Africa and Tanzania in alphabetical order) exhibited positive iNTS isolates confirmed via whole genome sequencing. MDR iNTS were found in 5/8 countries presented in this table. No MDR iNTS were detected via whole genome sequencing from the isolates yielded from Madagascar, South Africa and Tanzania.
†PYO estimation methodologies have been published in detail in the TSAP typhoid burden paper (Marks *et al*, *Lancet Global Health*, 2017).
‡Adjusted MDR iNTS incidence per 100 000 PYO (95% CI): adjustments for case recruitment and error factors.
§Ghana samples included in this estimation of MDR iNTS are from the TSAP only.
¶Non-TSAP includes other fever surveillance performed in Agogo ('IsolateAgogo', 'FISA' and 'zTYSA'). Refer to the Methods section.
**Adjusted MDR incidence of iNTS per 100 000 PYO could not be estimated for the study site in Senegal due to unavailable data on the recruitment proportion. The one MDR iNTS patient confirmed and presented in this table (crude MDR iNTS case) was a male infant aged 17 months, infected in 2012 with *S.* Enteritidis ST11. The one non-MDR iNTS patient presented in this table (genome-sequenced iNTS case) was a male adult aged 66 years, infected in 2012 with *S.* Typhimurium.
n.a., not available; PYO, person-years-of-observation.

Table 4 All iNTS organisms analysed

	All iNTS isolates from TSAP† and non-TSAP projects‡ in Ghana (N=166)							
iNTS isolates analysed (N=945)	Burkina Faso	Ghana§	Guinea-Bissau	Kenya	Madagascar	Senegal	South Africa	Tanzania
	12	133	9	1	4	2	1	4
	4 (Polesgo) 8 (Nioko II)	84 (Asante Akim North) 49 (Agogo)¶ 36 ('IsolateAgogo') 5 ('FISA') 3 ('TYSA') 5 (unidentified)	6 (Simao Hospital) 2 (Bandim) 1 (Belem)	1 (Kibera)	3 (CHU Tsaralalana) 1 (Isotry)	1 (IPS) 1 (Dominique hospital)		2 (Moshi urban) 1 (Moshi rural) 1 (Moshi other)
	Published iNTS Typhimurium ST313 Lineage II isolates (N=102)**							
	DRC††	Kenya	Malawi	Mali	Mozambique	Nigeria	Uganda	
	8	9	55	18	3	6	3	
	Published iNTS Enteritidis ST11 isolates (N=677)‡‡							
	Western Africa	Central and Eastern Africa	Southern Africa	Northern Africa	Rest of the World			
	90	262	131	11	183			

*Total 945 iNTS organisms were analysed in this manuscript: 166 iNTS isolates from the TSAP and other Ghana projects; sequenced and published 102 iNTS serovar Typhimurium ST313 Lineage II isolates from seven countries (DRC/Kenya/Malawi/Mali/Mozambique/Nigeria/Uganda); and sequenced and published 594 (out of 677) iNTS serovar Enteritidis ST11 isolates (see online appendices for the metadata).
†TSAP: Typhoid Fever Surveillance Program.[7 30] Of the 10 countries under the TSAP, no iNTS was found in Sudan and Ethiopia. 4 and 1 iNTS strains were identified from Madagascar and South Africa, respectively, through the whole genome sequencing; no iNTS isolates were reported from Madagascar and South Africa based on the blood culture result. Few analysed iNTS isolates, which may have been detected outside the strictly predefined surveillance catchment area, are considered for the bacterial genomic analysis, but excluded from the incidence estimation of the multidrug resistant iNTS in respective sites in table 4. The 166 iNTS isolates included for the final genomic analyses are based on the screening of whole genome sequenced results.
‡Non-TSAP projects in Ghana include the 'Febrile Illnesses Surveillance in Africa (FISA)', 'Typhoid Surveillance in Africa (TYSA)' and 'IsolateAgogo' conducted in Agogo.
§Ghana: 133 iNTS isolates analysed in this manuscript include 84 iNTS detected through the TSAP in Asante Akim North and 49 iNTS from several non-TSAP projects conducted in Agogo.
¶49 (Agogo): 49 iNTS isolates detected from various non-TSAP projects in Agogo, Ghana as listed in this table.
**Total 102 iNTS Typhimurium ST313 Lineage II isolates, which had been collected in seven countries in sub-Saharan Africa and whole genome sequenced, were published and raw sequence data accessible at the Wellcome Trust Sanger Institute. Global collection of *S.* Typhimurium and additional *S.* Typhimurium from Malawi, Kenya, Mozambique, Uganda, DRC, Nigeria and Mali[11]; Nigeria and DRC[12]; Malawi[28]; Kenya (Kariuki *et al* 2015b); Malawi.[32] Whole genome sequenced 166 iNTS isolates from TSAP and non-TSAP projects in Ghana listed in this table included 99 iNTS Typhimurium ST313 Lineage II . This 99 ST313 Lineage II was combined with the raw sequence data of 102 ST313 Lineage II published isolates listed in this footnote. Total 201 ST313 Lineage II isolates were further analysed.
††DRC: Democratic Republic of Congo.
‡‡Raw sequence data of the published global collection of 594/677 *S.* Enteritidis isolated from human and animal samples (Feasey *et al* 2016) were accessible at the Wellcome Trust Sanger Institute. The associated metadata were publicly available online. These samples were collected and whole genome sequenced across the African continent and the world: Western Africa: Benin, Cameroon, Chad, Gabon, Guinea, Ivory Coast, Mali, Niger and Senegal. Central and Eastern Africa: Central African Republic, Comoros, Congo, Djibouti, Ethiopia, HPA, Kenya, Madagascar, Malawi, Mauritius, Mozambique, Rwanda and Uganda. Southern Africa: Republic of South Africa. Northern Africa: Algeria, Egypt, Mauritania, Morocco and Tunisia. Rest of the World: Angola, Argentina, Colombia, Germany, Italy, Japan, PTG, Ratin-manufactured rat poison, Slovakia, Spain, Thailand, UK, Uruguay, USA and unknown.

low, ranging between 0 (Guinea-Bissau) to 11 (Kenya) and 54 (Burkina Faso) per 100 000 PYO.

DISCUSSION

Our study shows that MDR iNTS is highly prevalent in several sub-Saharan African countries. Specifically, we found that MDR *S.* Typhimurium ST313 is the most common cause of iNTS disease, but that other iNTS serovars, principally *S.* Enteritidis ST11 and *S.* Dublin ST10 in West Africa, also constitute a major proportion of the disease burden.[52 53] Our phylogenetic analyses provide further evidence for the regional transmission of two MDR serovars/STs (*S.* Typhimurium ST313 and *S.* Enteritidis ST11) between Ghana and neighbouring countries Burkina Faso, Nigeria, Mali and Senegal. These transmission events highlight the need for intensified AMR surveillance, the coordination of AMR reporting, and sustained public health control measures between these and other African countries.

We calculated a particularly high incidence of MDR iNTS disease in the West African countries of Burkina Faso, Ghana (<5 years) and Guinea-Bissau (<2 years). The incidence rates

of MDR iNTS disease presented here generally correspond with the incidence of iNTS disease in other African countries.[7] Effective antimicrobial therapy is an essential component of iNTS management; however, the effectiveness of first-line treatments has been diminished due to the emergence and spread of MDR and XDR NTS strains.[13 29 54] Our data also depict the emergence of reduced fluoroquinolones susceptibility in both MDR ST313 and ST11 in Ghana, as well as the circulation of a ceftriaxone-resistant ST313 sublineage in Kenya. Invasive *Salmonella* with reduced susceptibility to ciprofloxacin have been reported in Burkina Faso,[7] Ghana,[7 21] Nigeria,[55] Senegal,[56] Mozambique,[23] the Congo,[29 57] Kenya[58] and South Africa.[59] This increasing trend in resistance against clinically important classes of antimicrobials in differing iNTS serovars across Africa is of major concern.[14] The growing use of ceftriaxone and ciprofloxacin for the treatment of febrile illnesses in Africa may lead to an increase in of MDR and XDR pathogens in this continent, which has already been observed across Asia in the last two decades.

Several limitations should be considered in interpreting and generalising these data beyond our study sites. While we are able to illustrate the magnitude of the problem of MDR iNTS in West Africa, there are relatively few genomic data points available from countries in East and Southern Africa. Previous studies showed high prevalence of MDR iNTS disease in Kenya[27 58] and a recent meta-analysis suggested that MDR iNTS has emerged across four regions of sub-Saharan Africa.[60] The generation and analysis of additional epidemiological and genomic iNTS data in Eastern/Southern Africa would help facilitate comparison of incidence rates and AMR profiles of iNTS-associated organisms between African regions. Further, the estimated incidence rates of MDR iNTS disease in our study should be interpreted with caution as the number of cases in some countries were relatively small. In parallel, the original study design may also have led to some underestimation of iNTS burden, as afebrile patients with other clinical symptoms associated with iNTS disease were not enrolled. As a result, the true incidence of MDR iNTS disease in some settings need to be further monitored with more systematic disease surveillance.

Despite the identified limitations, our study provides enhanced insights into the population structure and transmission dynamics of major MDR iNTS serovars in sub-Saharan Africa and identified countries with a high burden of MDR iNTS. There is an urgent need to expand clinical and genomic surveillance for pathogens causing bloodstream infections across continental Africa to improve our understanding of disease incidence and to monitor AMR trends. Such data can better inform antimicrobial stewardship to extend the life of existing antimicrobial therapies and prioritisation of preventative interventions including vaccines. The development and deployment of a safe, low-cost, highly efficacious multivalent vaccine should be prioritised for the management and prevention of iNTS disease in Africa, particularly in countries with high prevalence of MDR iNTS infections, as well as HIV, malaria and malnutrition.[61 62] Meanwhile, further investigations of household transmission dynamics and human and non-human reservoirs of infection are warranted to inform better iNTS control measures and, ultimately, optimal programmatic use of future vaccines.

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REFERENCES

1 Morpeth SC, Ramadhani HO, Crump JA. Invasive non-typhi Salmonella disease in Africa. *Clin Infect Dis* 2009;49:606–11.
2 Feasey NA, Dougan G, Kingsley RA, *et al*. Invasive non-typhoidal Salmonella disease: an emerging and neglected tropical disease in Africa. *The Lancet* 2012;379:2489–99.
3 Ao TT, Feasey NA, Gordon MA, *et al*. Global burden of invasive nontyphoidal Salmonella disease, 2010(1). *Emerg Infect Dis* 2015;21:941–9.
4 Gordon MA. Invasive non-typhoidal Salmonella disease – epidemiology, pathogenesis and diagnosis. *Curr Opin Infect Dis* 2012;24:484–9.
5 Enwere G, Biney E, Cheung YB, *et al*. Epidemiologic and clinical characteristics of community-acquired invasive bacterial infections in children aged 2–29 months in the Gambia. *Pediatr Infect Dis J* 2006;25:700–5.
6 Berkley JA, Lowe BS, Mwangi I, *et al*. Bacteremia among children admitted to a rural hospital in Kenya. *N Engl J Med* 2005;352:39–47.
7 Marks F, von Kalckreuth V, Aaby P, *et al*. Incidence of invasive Salmonella disease in sub-Saharan Africa: a multicentre population-based surveillance study. *Lancet Glob Health* 2017;5:e310–23.
8 Feasey NA, Archer BN, Heyderman RS, *et al*. Typhoid fever and invasive nontyphoid salmonellosis, Malawi and South Africa. *Emerg Infect Dis* 2010;16:1448–51.
9 Beyene G, Nair S, Asrat D, *et al*. Multidrug resistant Salmonella Concord is a major cause of salmonellosis in children in Ethiopia. *J Infect Dev Ctries* 2011;5:023–33.
10 Tennant SM, Diallo S, Levy H, *et al*. Identification by PCR of non-typhoidal Salmonella enterica serovars associated with invasive infections among febrile patients in Mali. *PLoS Negl Trop Dis* 2010;4:e621.
11 Okoro CK, Kingsley RA, Connor TR, *et al*. Intracontinental spread of human invasive Salmonella typhimurium pathovariants in sub-Saharan Africa. *Nat Genet* 2012;44:1215–21. Available at http://www.nature.com/doifinder/10.1038/ng.2423
12 Leekitcharoenphon P, Friis C, Zankari E, *et al*. Genomics of an emerging clone of Salmonella serovar typhimurium ST313 from Nigeria and the Democratic Republic of Congo. *J Infect Dev Ctries* 2013;7:696–706.
13 Feasey NA, Hadfield J, Keddy KH, *et al*. Distinct Salmonella enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings. *Nat Genet* 2016;48:1211–7.
14 Kariuki S, Gordon MA, Feasey N, *et al*. Antimicrobial resistance and management of invasive Salmonella disease. *Vaccine* 2015;33:C21–9.
15 Musicha P, Cornick JE, Bar-Zeev N, *et al*. Trends in antimicrobial resistance in bloodstream infection isolates at a large urban hospital in Malawi (1998–2016): a surveillance study. *Lancet Infect Dis* 2017;17:1042–52.
16 Molyneux EM, Mankhambo LA, Phiri A, *et al*. The outcome of non-typhoidal Salmonella meningitis in Malawian children, 1997–2006. *Ann Trop Paediatr* 2009;29:13–22.
17 a GM, Graham SM, Walsh AL. Epidemics of invasive Salmonella enterica serovar enteritidis and S. enterica serovar typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis* 2008;46:963–9. Available at http://www.ncbi.nlm.nih.gov/pubmed/18444810
18 Kariuki S, Okoro C, Kiiru J, *et al*. Ceftriaxone-resistant Salmonella enterica serotype typhimurium sequence type 313 from Kenyan patients is associated with the *bla* $_{CTX-M-15}$gene on a novel IncHI2 Plasmid. *Antimicrob Agents Chemother* 2015;59:3133–9.
19 Oneko M, Kariuki S, Muturi-Kioi V, *et al*. Emergence of community-acquired, multidrug-resistant invasive nontyphoidal *Salmonella* disease in rural Western Kenya, 2009–2013. *Clin Infect Dis.* 2015;61:S310–6.
20 Akullian A, Montgomery JM, John-Stewart G, *et al*. Multi-drug resistant non-typhoidal Salmonella associated with invasive disease in Western Kenya. *PLoS Negl Trop Dis* 2018;12:e0006156.
21 Nielsen MV, Sarpong N, Krumkamp R, *et al*. Incidence and characteristics of bacteremia among children in rural Ghana. *PLoS One* 2012;7:e44063.
22 Kalonji LM, Post A, Phoba M-F, *et al*. Invasive *Salmonella* infections at multiple surveillance sites in the Democratic Republic of the Congo, 2011–2014. *Clin Infect Dis.* 2015;61:S346–53.
23 Mandomando Inácio, Jaintilal D, Pons MJ, *et al*. Antimicrobial susceptibility and mechanisms of resistance in *Shigella* and *Salmonella* isolates from children under five years of age with diarrhea in rural Mozambique. *Antimicrob Agents Chemother* 2009;53:2450–4.
24 Sigaúque B, Roca A, Mandomando I, *et al*. Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. *Pediatr Infect Dis J* 2009;28:108–13.
25 Blomberg B, Manji KP, Urassa WK, *et al*. Antimicrobial resistance predicts death in Tanzanian children with bloodstream infections: a prospective cohort study. *BMC Infect Dis* 2007;7:43.
26 Obaro SK, Hassan-Hanga F, Olateju EK, *et al*. Salmonella bacteremia among children in central and northwest Nigeria, 2008–2015. *Clin Infect Dis* 2015;61 Suppl 4:S325–31.
27 Kariuki S, Mbae C, Van Puyvelde S, *et al*. High relatedness of invasive multi-drug resistant non-typhoidal Salmonella genotypes among patients and asymptomatic carriers in endemic informal settlements in Kenya. *PLoS Negl Trop Dis* 2020;14:e0008440.
28 Feasey NA, Cain AK, Msefula CL, *et al*. Drug resistance in *Salmonella enterica* ser. typhimurium bloodstream infection, Malawi. *Emerg Infect Dis* 2014;20:1957–9.
29 Van Puyvelde S, Pickard D, Vandelannoote K, *et al*. An African Salmonella typhimurium ST313 sublineage with extensive drug-resistance and signatures of host adaptation. *Nat Commun* 2019;10:1–12. Available at.
30 von Kalckreuth V, Konings F, Aaby P, *et al*. The typhoid fever surveillance in Africa program (TSAP): clinical, diagnostic, and epidemiological methodologies. *Clin Infect Dis.* 2016;62:S9–16.
31 Frickmann H, Dekker D, Boahen K, *et al*. Increased detection of invasive enteropathogenic bacteria in pre-incubated blood culture materials by real-time PCR in comparison with automated incubation in sub-Saharan Africa. *Scand J Infect Dis* 2013;45:616–22.
32 Msefula CL, Kingsley RA, Gordon MA, *et al*. Genotypic homogeneity of multidrug resistant S. typhimurium infecting distinct adult and childhood susceptibility groups in Blantyre, Malawi. *PLoS One* 2012;7:e42085.

33 Zerbino DR, Birney E. Velvet: algorithms for de novo short read assembly using de Bruijn graphs. *Genome Res* 2008;18:821–9.
34 Gladman S, Seemann T, optimiser V. Available: https://github.com/Victorian-Bioinformatics-Consortium/VelvetOptimiser.git
35 Page AJ, Taylor B, Delaney AJ, *et al*. SNP-sites: rapid efficient extraction of SNPs from multi-FASTA alignments. *Microb Genom* 2016;2:e000056.
36 Seemann T. Prokka: rapid prokaryotic genome annotation. *Bioinformatics* 2014;30:2068–9.
37 Page AJ, Cummins CA, Hunt M, *et al*. Roary: rapid large-scale prokaryote pan genome analysis. *Bioinformatics* 2015;31:3691–3.
38 Li H, Handsaker B, Wysoker A, *et al*. The sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009;25:2078–9.
39 Kurtz S, Phillippy A, Delcher AL, *et al*. Versatile and open software for comparing large genomes. *Genome Biol* 2004;5:R12.
40 Croucher NJ, Page AJ, Connor TR, *et al*. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 2015;43:e15.
41 Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014;30:1312–3.
42 Letunic I, Bork P. Interactive tree of life (iTOL): an online tool for phylogenetic tree display and annotation. *Bioinformatics* 2007;23:127–8.
43 Inouye M, Dashnow H, Raven L-A, *et al*. SRST2: rapid genomic surveillance for public health and hospital microbiology labs. *Genome Med* 2014;6:90. Available at http://genomemedicine.com/content/6/11/90
44 Gupta SK, Padmanabhan BR, Diene SM, *et al*. ARG-annot, a new bioinformatic tool to discover antibiotic resistance genes in bacterial genomes. *Antimicrob Agents Chemother* 2014;58:212–20.
45 Carattoli A, Zankari E, García-Fernández A, *et al*. *In silico* detection and typing of plasmids using PlasmidFinder and plasmid multilocus sequence typing. *Antimicrob Agents Chemother* 2014;58:3895–903.
46 Jolley KA, Bray JE, Maiden MCJ. Open-access bacterial population genomics: BIGSdb software, the PubMLST.org website and their applications. *Wellcome Open Res* 2018;3:124.
47 Achtman M, Wain J, Weill F-X, *et al*. Multilocus sequence typing as a replacement for serotyping in Salmonella enterica. *PLoS Pathog* 2012;8:e1002776.
48 Zhang S, Yin Y, Jones MB, *et al*. Salmonella serotype determination utilizing high-throughput genome sequencing data. *J Clin Microbiol* 2015;53:1685–92.
49 Wick RR, Schultz MB, Zobel J, *et al*. Bandage: interactive visualization of de novo genome assemblies. *Bioinformatics* 2015;31:3350–2.
50 Gouy M, Guindon S, Gascuel O. SeaView version 4: a multiplatform graphical user interface for sequence alignment and phylogenetic tree building. *Mol Biol Evol* 2010;27:221–4.
51 Panzner U, Pak GD, Aaby P, *et al*. Utilization of healthcare in the typhoid fever surveillance in Africa program. *Clin Infect Dis.* 2016;62:S56–68.
52 Sow AG, Wane AA, Diallo MH, *et al*. Genotypic characterization of antibiotic-resistant Salmonella enteritidis isolates in Dakar, Senegal. *J Infect Dev Ctries* 2007;1:284–8.
53 Keddy KH, Takuva S, Musekiwa A, *et al*. An association between decreasing incidence of invasive non-typhoidal salmonellosis and increased use of antiretroviral therapy, Gauteng Province, South Africa, 2003–2013. *PLoS One* 2017;12:e0173091.
54 Kingsley RA, Msefula CL, Thomson NR, *et al*. Epidemic multiple drug resistant Salmonella typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Res* 2009;19:2279–87.
55 Akinyemi KO, Bamiro BS, Coker AO. Nigeria: incidence co-infection, patterns of antimicrobial resistance, and emergence of reduced susceptibility to fluoroquinolones. *J Heal Popul NUTR* 2007;25:351–8.
56 Harrois D, Breurec S, Seck A, *et al*. Prevalence and characterization of extended-spectrum β-lactamase-producing clinical Salmonella enterica isolates in Dakar, Senegal, from 1999 to 2009. *Clin Microbiol Infect* 2014;20:O109–16.
57 Lunguya O, Lejon V, Phoba M-F, *et al*. Antimicrobial resistance in invasive non-typhoid Salmonella from the Democratic Republic of the Congo: emergence of decreased fluoroquinolone susceptibility and extended-spectrum beta Lactamases. *PLoS Negl Trop Dis* 2013;7:e2103.
58 Kariuki S, Onsare RS. Epidemiology and genomics of invasive nontyphoidal Salmonella infections in Kenya. *Clin Infect Dis* 2015;61 Suppl 4:S317–24.
59 Keddy KH, Musekiwa A, Sooka A. Clinical and microbiological features of invasive nontyphoidal Salmonella associated with HIV-infected patients, Gauteng Province, South Africa. *Med* 2017;96:1–7.
60 Tack B, Vanaenrode J, Verbakel JY, *et al*. Invasive non-typhoidal Salmonella infections in sub-Saharan Africa: a systematic review on antimicrobial resistance and treatment. *BMC Med* 2020;18.
61 Piccini G, Montomoli E. Pathogenic signature of invasive non-typhoidal *Salmonella* in Africa: implications for vaccine development. *Hum Vaccin Immunother* 2020;16:2056–71.
62 Kariuki S, Owusu-Dabo E. Research on invasive nontyphoidal Salmonella disease and developments towards better understanding of epidemiology, management, and control strategies. *Clin Infect Dis* 2020;71:S127–9.

Appendix 14. The international and intercontinental spread and expansion of antimicrobial-resistant *Salmonella* Typhi: a genomic epidemiology study (publication)

The international and intercontinental spread and expansion of antimicrobial-resistant *Salmonella* Typhi: a genomic epidemiology study

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Summary

Background The emergence of increasingly antimicrobial-resistant *Salmonella enterica* serovar Typhi (*S* Typhi) threatens to undermine effective treatment and control. Understanding where antimicrobial resistance in *S* Typhi is emerging and spreading is crucial towards formulating effective control strategies.

Methods In this genomic epidemiology study, we sequenced the genomes of 3489 *S* Typhi strains isolated from prospective enteric fever surveillance studies in Nepal, Bangladesh, Pakistan, and India (between 2014 and 2019), and combined these with a global collection of 4169 *S* Typhi genome sequences isolated between 1905 and 2018 to investigate the temporal and geographical patterns of emergence and spread of antimicrobial-resistant *S* Typhi. We performed non-parametric phylodynamic analyses to characterise changes in the effective population size of fluoroquinolone-resistant, extensively drug-resistant (XDR), and azithromycin-resistant *S* Typhi over time. We inferred timed phylogenies for the major *S* Typhi sublineages and used ancestral state reconstruction methods to estimate the frequency and timing of international and intercontinental transfers.

Findings Our analysis revealed a declining trend of multidrug resistant typhoid in south Asia, except for Pakistan, where XDR *S* Typhi emerged in 2016 and rapidly replaced less-resistant strains. Mutations in the quinolone-resistance determining region (QRDR) of *S* Typhi have independently arisen and propagated on at least 94 occasions, nearly all occurring in south Asia. Strains with multiple QRDR mutations, including triple mutants with high-level fluoroquinolone resistance, have been increasing in frequency and displacing strains with fewer mutations. Strains containing *acrB* mutations, conferring azithromycin resistance, emerged in Bangladesh around 2013 and effective population size of these strains has been steadily increasing. We found evidence of frequent international (n=138) and intercontinental transfers (n=59) of antimicrobial-resistant *S* Typhi, followed by local expansion and replacement of drug-susceptible clades.

Interpretation Independent acquisition of plasmids and homoplastic mutations conferring antimicrobial resistance have occurred repeatedly in multiple lineages of *S* Typhi, predominantly arising in south Asia before spreading to other regions.

Funding Bill & Melinda Gates Foundation.

Introduction

Typhoid fever, the disease caused by *Salmonella enterica* serovar Typhi (*S* Typhi), remains a major public health concern worldwide,[1] causing 11 million cases and more than 100 000 deaths annually.[2,3] The highest incidence rates occur in south Asia, which contains 70% of the global disease burden, but substantial morbidity and mortality also occur in sub-Saharan Africa, southeast Asia, and Oceania.[4]

The effectiveness of antimicrobial therapy has been threatened by the emergence and expansion of antimicrobial-resistant strains. Multidrug-resistant variants, harbouring genes encoding resistance to ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole, first emerged in the 1970s; subsequently, a single lineage (4.3.1) associated with multidrug-resistance among the H58 haplotype became globally dominant.[5,6] Fluoroquinolones were initially effective against multidrug-resistant *S* Typhi and became the mainstay of therapy in the 1990s. However, by the 2010s, the majority of *S* Typhi in south Asia contained mutations in the quinolone resistance-determining regions (QRDR).[7,8] In 2016, a large outbreak of *S* Typhi containing plasmid-mediated resistance to third generation cephalosporins and fluoroquinolone, and chromosomally located genes encoding multidrug-resistance were identified in Pakistan and termed extensively drug-resistant (XDR).[9] In 2021 a single polymorphism in the AcrB efflux pump conferring

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See Online for appendix 1

For more on the **RedDog pipeline** see https://github.com/katholt/reddog

For more on **GenoTyphi** see https://github.com/katholt/genotyphi

For more on **The European Nucleotide Archive** see https://www.ebi.ac.uk/ena/browser/home

Research in context

Evidence before this study

We searched PubMed for relevant articles published in English from database inception to Oct 15, 2021, using the terms "*Salmonella* Typhi", "antimicrobial resistance", "whole genome sequencing", and "phylogeography analysis". Several studies have explored the phenotypic and genotypic diversity of *Salmonella enterica* serovar Typhi (S Typhi) isolates using whole genome sequencing, and most of them involved small number of isolates from multiple countries. Two studies have described phylogeographical analysis of dominant lineages and identified transfers from Asia to Africa and an ongoing, multidrug-resistance epidemic within Africa.

Added value of this study

This study represents the largest genome sequencing study of S Typhi to date, with 3489 newly sequenced isolates from prospective surveillance studies in four of the highest typhoid burden countries in the world: Bangladesh, Nepal, Pakistan, and India. We combined these data with 4169 previously sequenced strains to characterise the emergence and geographical spread of antimicrobial resistant S Typhi. We applied bacterial phylodynamic methods to investigate how antimicrobial resistance influenced the population size of S Typhi, including displacement of less-resistant strains. Dated phylogenetic reconstruction and phylogeographic analyses were performed to estimate the frequency and location of antimicrobial resistance acquisition, along with dates of international spread. Additionally, our analysis also describes the emergence and evolutionary history of non-H58 lineages, about which relatively little is known.

Implications of all the available evidence

The results indicate that south Asia continues to be a crucial hub for S Typhi antimicrobial resistance acquisition, and antimicrobial-resistant clones that emerge in this region have been regularly introduced across borders within the region and intercontinentally. Our analysis also suggests that multidrug-resistant strains are declining in most parts of south Asia but are being replaced with strains containing ceftriaxone resistance (extensively drug-resistant), high-level fluoroquinolone resistance, or azithromycin resistance, which are reversing declines in the effective population size of S Typhi. These findings of frequent international spread and expansion of antimicrobial-resistant S Typhi strains underscore the importance of viewing typhoid control strategies through a global rather than country-specific lens.

resistance to azithromycin was found to have independently arisen in multiple lineages of *S* Typhi, threatening the efficacy of all oral antimicrobials for typhoid treatment.[10]

Typhoid conjugate vaccines have proven effective for disease prevention, and WHO recommends introduction in countries with high burden of antimicrobial-resistant strains.[11] However, given the current trajectory of antimicrobial resistance in *S* Typhi, waiting until a high burden of antimicrobial resistance is present within a country to introduce typhoid vaccines might be ill-advised. Understanding the historical emergence, and geographical spread of antimicrobial-resistant *S* Typhi might yield insights into where resistant strains might spread and how quickly they will become dominant.

Here, we leveraged prospective, population-based typhoid surveillance studies from four of the highest burden countries in south Asia: Bangladesh, India, Nepal, and Pakistan. We sequenced 3489 *S* Typhi organisms isolated over a 6-year period, and these data were combined with a global collection of more than 4000 additional genomes to investigate the emergence and geographical spread of antimicrobial-resistant *S* Typhi over the past 3 decades.

Methods

Bacterial isolates

This study included *S* Typhi isolates obtained from the Surveillance for Enteric Fever in Asia Project (SEAP; Bangladesh, Nepal, and Pakistan; 2016–19), Etiologies of Acute Febrile Illness Study (Nepal; 2014–16), and Surveillance for Enteric Fever in India Project (SEFI; 2017–19). The study methodologies have been previously described.[12,13] Participants included children and adults presenting to study sites with febrile illness. These sites included five health facilities in Dhaka, Bangladesh, 18 facilities across 16 cities in India, 11 facilities across three cities in Nepal, and two hospitals and a university laboratory network in Karachi, Pakistan. Among 9945 blood culture-confirmed typhoid cases, we selected a country-stratified sample of 3489 isolates for sequencing based on random (SEAP) or convenience sampling (SEFI). Sampling details are available in appendix 1 (p 2).

Whole-genome sequencing and single-nucleotide polymorphism (SNP) analysis

Whole-genome sequencing was performed at the Wellcome Trust Sanger Institute (Hinxton, UK) using the Illumina HiSeq2500 platform (Illumina, San Diego, CA, USA), at a commercial Illumina service in Bangalore (India) and at the Wellcome Trust Research Laboratory in the Christian Medical College (Vellore, India) using the Illumina MiSeq platform (Illumina, San Diego, CA, USA). Paired-end reads were mapped to the *S* Typhi CT18 (AL513382) reference sequence using RedDog pipeline version V1beta.11. SNPs occurring in recombinant regions were detected by Gubbins version 2.4.1 and excluded.[14] SNP data were used to assign genotypes using GenoTyphi version 1.9.1. To provide global context, additional *S* Typhi genomes[9,15–24] were downloaded from The European Nucleotide Archive and

subjected to the same SNP calling and recombination filtering pipeline.

Phylogenetic analyses

Maximum likelihood phylogenetic trees were inferred from the SNP alignments using RAxML version 8.2.10.[25] A generalised time-reversible model and a gamma distribution was used to model site-specific rate variation with 100 bootstrap pseudoreplicates used to assess branch support for the phylogeny. We selected the tree with the highest likelihood score as the best tree.

Temporal and phylogeographical analysis

To investigate dates of emergence and geographical transfers, we inferred timed phylogenies. We used TempEst version 1.5 to assess temporal structure by conducting a regression of the root-to-tip branch distances of the tree as a function of the sampling time,[26] which was confirmed by a clustered permutation test using R package BactDating version 1.1.0.[27] For the non-H58 isolates, we estimated the best-fit models, tree topology, evolutionary rates, and phylogeography by using Bayesian Markov chain Monte Carlo method with BEAST2 version 2.6.2.[28] Separate trees were fit for the most common non-H58 lineages (2.3.3, 2.5, 3.2.2, and 3.3). Isolates from each lineage were selected based on temporal, geographical, and phylogenetic diversity, as described in appendix 1 (p 3).

For the BEAST analysis, a general time reversible with gamma distribution model was selected, and sampling times (tip dates) were defined as the year of isolation. We tested support for a strict clock for each lineage using the relaxed clock test in the treedater R package version 0.5.0, and the strict clock was rejected in each instance.[29] We therefore constructed time-phylogenies using coalescent exponential population priors with a relaxed clock (uncorrelated lognormal distribution).[5,24] Three independent runs were performed to ensure convergence. The effective sample sizes of the parameters were estimated to be more than 200 for all independent runs. Phylogeographical reconstruction was obtained by the continuous-time Markov chain process over discrete sampling locations.

For H58, we had 4761 isolates, which precluded temporal and phylogeographical analysis using BEAST due to computational constraints. To avoid significant down-sampling of isolates, we used the treedater R package[29] with an uncorrelated, relaxed molecular clock to estimate the timed phylogeny using all available H58 sequences, which yielded a time of the most recent common ancestor matching a root-to-tip based analysis using BactDating.[5] We reconstructed the ancestral state of nodes using the maximum parsimony approach with the Phangorn R package version 2.8.1, considering events with a location probability of more than 0·5 between connected nodes. For visualisation purposes, we selected a smaller subset of sequences to depict in a dated phylogenetic tree. For all phylogeographical analyses, we considered a geographical transfer when the most probable location between two connected nodes (or between a node and a tip) differed, and we considered the time window of transfer as the date range between the nodes (or between the node and tip). To investigate the geographical origin of H58 isolates, we evaluated the correlation between genetic diversity and geographical distances.[30] To obtain stable estimates of the pairwise distance distribution, we included all countries with at least 20 sequences. We created a geographical grid of coordinates representing potential origins of H58 and fit weighted linear regression models relating log pairwise SNP distance with log geographical distance. We considered the most probable origin as that which had the highest coefficient of determination (R^2). We fit separate models for Asia only and Asia and Africa, hypothesising that the relationship between diversity and distance might differ considering air travel across the Indian Ocean.

Non-parametric phylodynamic inference of effective population size

To evaluate the historical effective population size for H58 lineage strains, we used the time-stamped H58 tree to estimate the effective population size through time using the skygrowth package version 0.3.1. We compared the effective population sizes of antimicrobial resistant and sensitive populations within countries. Epidemic success of antimicrobial resistant populations was also measured by comparing time-scaled haplotypic densities (THD; appendix 1 pp 3–4).

For more on the **skygrowth package** see https://github.com/mrc-ide/skygrowth

Antimicrobial resistance associated gene detection and plasmid replicon analysis

Antimicrobial Resistance Identifier by Assembly (ARIBA) version 2.10.0 and the Comprehensive Antibiotic Resistance Database database version 1.1.8 were used to investigate antimicrobial resistance gene content. Point mutations in the QRDR of the DNA-gyrase *gyrA/B* and topoisomerase-IV *parC/E* genes, associated with reduced susceptibility to fluoroquinolones and quinolone resistance genes (*qnrS*), were also detected using ARIBA. Isolates were defined as multidrug-resistant if resistance genes were detected in the beta-lactam, trimethoprim–sulphonamide, and chloramphenicol classes. Plasmid replicons were identified using ARIBA and PlasmidFinder database.[30]

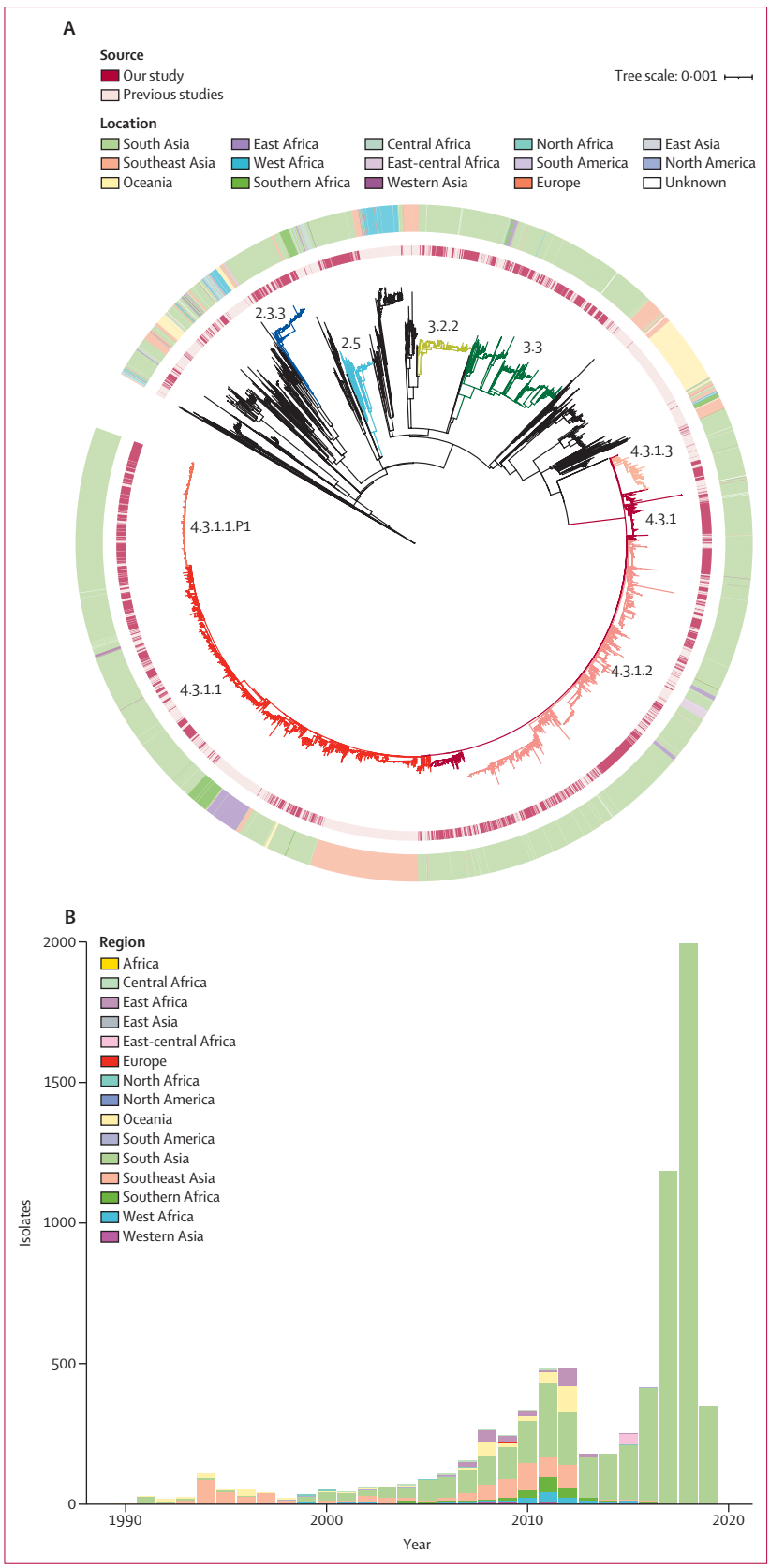
For more on **ARIBA** see https://github.com/suhrig/arriba

For more on the **Comprehensive Antibiotic Resistance Database database** see https://card.mcmaster.fca/home

Ethics statement

Ethical approvals were obtained from the Bangladesh Institute of Child Health Ethical Review Committee (01-02-2019), Christian Medical College Institutional Review Board (10393), Nepal Health Research Council (391/2018), Aga Khan University Hospital Ethics Committee and Pakistan National Ethics Committee (2019-0410-4188), Stanford University Institutional Review Board (39557),

For more on **Phangorn R package** see https://www.rdocumentation.org/packages/phangorn/versions/2.8.1



and US Centers for Disease Control and Prevention. Informed written consent was taken from adult participants and legal guardians of child participants.

Role of the funding source

The funder of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report.

Results

A total of 3489 *S* Typhi isolates collected between 2014 and 2019 were sequenced. Genotype analysis identified 29 distinct genotypes (appendix 1 p 5). Most isolates (2474 [70·9%]) belonged to genotype 4.3.1 (haplotype H58). We identified multiple, phylogenetically linked H58 sub-lineages shared across south Asia (appendix 1 p 6), most regularly between Bangladesh, Nepal, and India. Within the H58 isolates, 4.3.1.2 isolates formed distinct clades with intermingled isolates from India and Nepal, and 4.3.1.3 isolates, identified predominantly in Bangladesh, clustered with few isolates from India. By contrast, the H58 isolates from Pakistan largely clustered independently and was dominated by a monophyletic XDR clade (4.3.1.1.P1). Among non-H58 isolates, the most common subclades were 3.2.2 (190 [5·5%]), 3.3.2 (161 [4·6%]), 2.3.3 (140 [4·0%]), 2.5 (123 [3·5%]), and 3.3.1 (85 [2·4%]).

To provide additional context for the 3489 new South Asian genomes, and better understand temporal and spatial distribution of lineages, we constructed a phylogeny incorporating an additional 4169 *S* Typhi sequences from organisms isolated from 1905 to 2018 from more than 70 countries (figure 1). Overall, the new sequences clustered with previously sequenced south Asian isolates, generating a distinct geographical structure. Genotype 4.3.1 formed a large subclade. Primary clades 2, 3, and 4 were distributed across continents with few isolates outside these clades. Notably, four subclades (2.3.3, 2.5, 3.2.2, and 3.3) were dominant in south Asia, accounting for 1239 (75·7%) of the 1634 non-H58 organisms.

We classified isolates as multidrug-resistant if they simultaneously contained genes conferring resistance to ampicillin (bla_{TEM-1}), chloramphenicol (*catA1*), and trimethoprim–sulfamethoxazole (*dfrA7* plus *sul1* or *sul2*, or both). From 2000 onwards, we observed a declining trend in multidrug-resistant isolates in Bangladesh and India, a stable low proportion (30 [2·6%] of 1144) in Nepal, and an increasing proportion in Pakistan and

Figure 1: **Global phylogeny of *Salmonella* Typhi**
(A) Maximum likelihood tree of 7658 *S* Typhi isolates from the global collection. Branch colours indicate the lineages 2.3.3 (blue), 2.5 (turquoise), 3.2.2 (yellow), 3.3 (green), 4.3.1 (dark red), 4.3.1.1 (red), 4.3.1.1.P1 (orange), 4.3.1.2 (pink), 4.3.1.3 (salmon), and other non-H58 (black). The inner ring indicates the source. The outer ring indicates the region of isolation. The scale bar indicates nucleotide substitutions per site. (B) Temporal distribution of sequenced *S* Typhi isolates by region.

Africa (appendix 1 p 7). Acquired resistance genes that contribute to the multidrug-resistant phenotype were identified in 2047 (26·8%) of the 7657 isolates of the global collection (appendix 1 p 21); of these 2016 (98·4%) were H58 isolates and 31 (1·6%) were non-H58 isolates. Among these non-H58 multidrug-resistant isolates, resistance was almost entirely plasmid-mediated (30 [96·8%] of 31). By contrast, for H58 isolates we observed that plasmid-mediated resistance was persistent in the H58 isolates in the 1990s, but from 2000 onward was less frequent, with most multidrug-resistant isolates containing chromosomal insertions of drug-resistance genes (1516 [75·2%] of 2016).

By contrast to the temporal trends in multidrug resistance, there was a consistent rise in the proportion of global *S* Typhi that were fluoroquinolone non-susceptible, primarily associated with mutations in *gryA, gyrB, parC,* and *parE* (appendix 1 p 7). The largest increase occurred in Bangladesh, exceeding 77 (98·7%) of 78 in 2008, followed by India in 2008 (32 [96·9%] of 33), Nepal in 2012 (35 [97·2%] of 36), and Pakistan in 2016 (84 [96·5%] of 87). Fluoroquinolone non-susceptible *S* Typhi increased from two (22·2%) of nine in 2006 to 52 (71·2%) of 73 by 2011 in southeast Asia. In Africa, this increase occurred more recently, starting around 2010. Overall, we found that QRDR mutations were significantly more common in the H58 isolates (4353 [77·5%] 5613) compared with other lineages (1257 [22·5%] of 5613; p<0·0001). From 2010 onwards, an increasing number of isolates had multiple QRDR mutations; more than 10% of all isolates had three mutations (appendix 1 p 8). Among the novel genomes, 437 were triple mutants (appendix 1 p 22), which are associated with high-level resistance to fluoroquinolones.[20] Most (402 [92%] of 437) of these organisms occurred in H58 lineage II (4.3.1.2) in India and Nepal; the second most common (15 [3%] of 437) triple mutant genotype was 3.3, predominantly isolated in India. A comparison between genotypic and phenotypic resistance profile of all new isolates from south Asia are presented in appendix 1 (p 23).

Susceptibility to fluoroquinolones can be further reduced via plasmid-mediated acquisition of *qnr* genes. We identified *qnrS* in two non-H58 isolates and 686 H58 isolates that included genotype 4.3.1 (n=3), 4.3.1.1 (n=5), 4.3.1.P1 (n=550), and 4.3.1.3 (n=125). Most H58 isolates from Pakistan were XDR (4.3.1.P1) carrying the previously identified composite transposon containing bla_{TEM-1}, *catA1, dfrA7, sul1, sul2* inserted in the chromosome, and $bla_{CTX-M-15}$ and *qnrS* associated with an IncY plasmid.[9] Azithromycin resistance, conferred by *acrB* mutations (Arg717Gln and Arg717Leu), was identified in 54 isolates across eight different genotypes including 4.3.1 (n=1), 4.3.1.1 (n=31), 4.3.1.2 (n=5), 4.3.1.3 (n=2), and non-H58 isolates comprising, genotype 2.3.3 (n=2), 3.2.2 (n=9), 3.3.2 (n=3), and 3.5.4 (n=1).

To investigate how antimicrobial resistance has shaped the effective population size of *S* Typhi, we generated

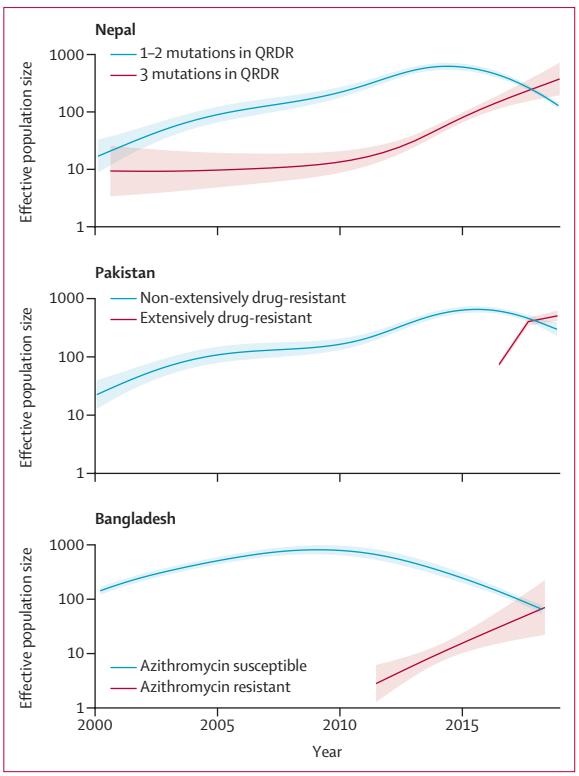


Figure 2: **The effective population size of H58 lineages strains according to antimicrobial resistance genotype in Nepal, Pakistan, and Bangladesh.** In Nepal, strains containing 1–2 mutations in the QRDR were compared with those containing three mutations. In Pakistan, XDR strains were compared with non-XDR strains. In Bangladesh, strains containing *acrB* mutations conferring azithromycin-resistance were compared with those not containing the mutations. Increased values indicate expanding effective population size. Light shading represents the 95% high probability density intervals of the estimates. QRDR=quinolone-resistance determining region. XDR=extensively drug-resistant.

timed phylogenies and modelled the effective population size of antimicrobial susceptible and resistant organisms over time. To minimise the effect of location and lineage, we focused on the largest haplotype (ie, H58) and performed analyses within countries, evaluating key antimicrobial resistance determinants. In Nepal, we found that the effective population size (N_e) of *S* Typhi containing one or two QRDR mutations rose steadily from 2000, beginning to decline from 2017, and triple mutants have steadily increased from 2010 (figure 2). In Pakistan, the N_e of non-XDR H58 *S* Typhi increased from 2000 until around 2015 and began to fall; XDR organisms emerged and have been rapidly growing in frequency since 2016, eclipsing the effective population of non-XDR organisms by 2018. In Bangladesh, the N_e of H58 had slowly declined from around 2010; however, azithromycin-resistant organisms emerged in 2013 with a corresponding increase in N_e. In all three settings, organisms with key antimicrobial resistance conferring mutations or genes appear to be replacing their susceptible (or, in the case of fluoroquinolones,

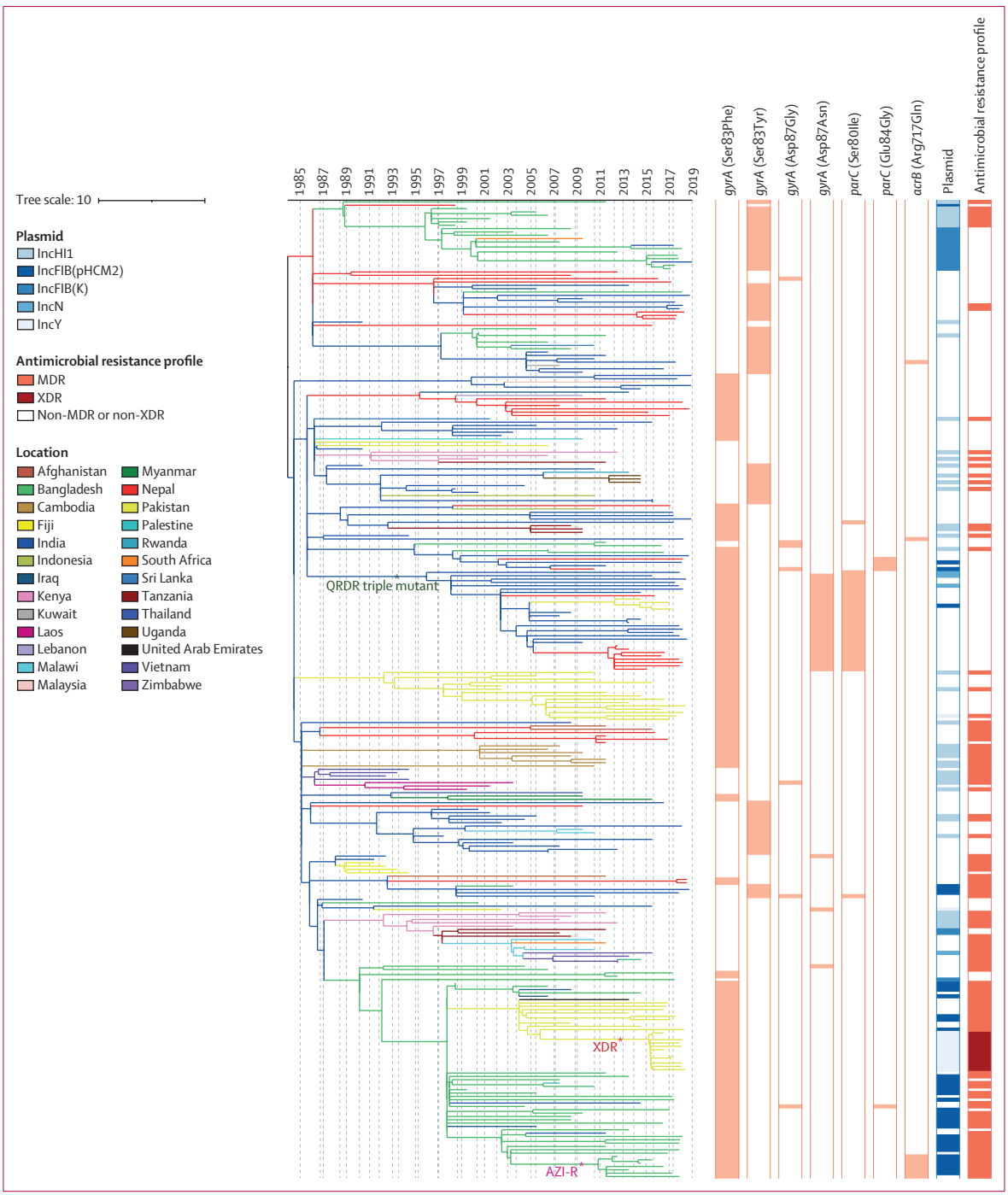


Figure 3: **Phylogeography and global expansion of genotype 4.3.1 (H58) *Salmonella* Typhi isolates**
Timed phylogenetic tree of genotype 4.3.1 *S* Typhi isolates. The branch lengths are scaled in years and are coloured according to the location of the most probable ancestor of descendant nodes. The scale bar indicates nucleotide substitutions per site. AZI-R=azithromycin resistant. MDR=multidrug resistant. QRDR=quinolone-resistance determining region. XDR=extensively drug-resistant.

less-resistant) counterparts. Additionally, we measured the epidemic success of antimicrobial resistant populations by comparing THD. We found that the THD success index was higher in QRDR triple mutant isolates than those containing 1–2 mutations ($p<0·0001$) or none ($p<0·0001$; appendix 1 p 9). We also found a significant positive association between THD and XDR strains ($p<0·0001$; appendix 1 p 10).

Using country of sampling as a discrete trait, we generated dated phylogenies to reconstruct the

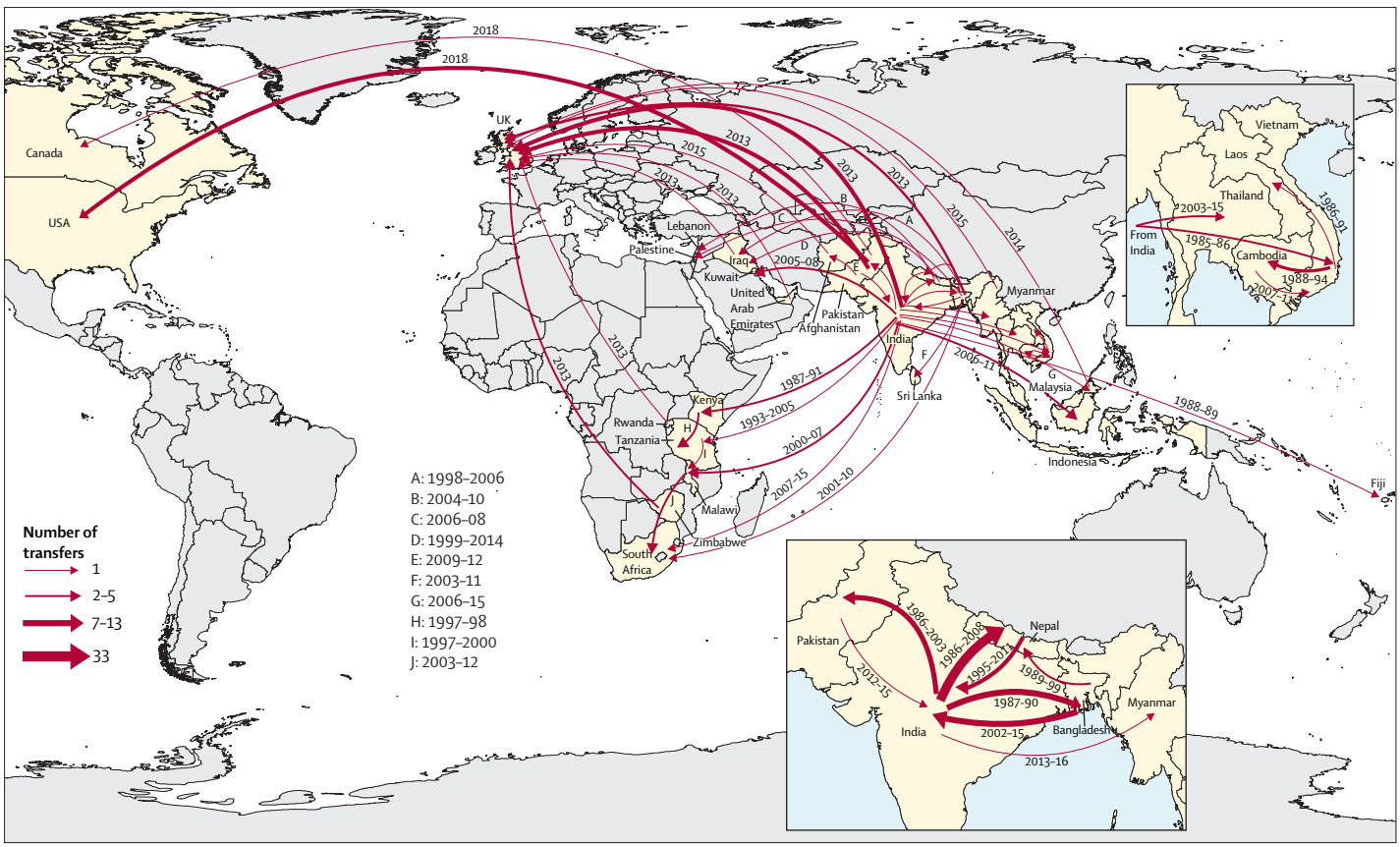


Figure 4: **Geographical transfers within lineage 4.3.1 (H58) inferred from ancestral state reconstruction of the timed phylogenetic tree**
The size of each arrow is scaled to the estimated number of transfers between the countries. Dates indicate the estimated first transfer between each pair of countries.

evolutionary history and geographical spread of H58 lineage and the four common non-H58 genotypes. Phylogeographical reconstruction of H58 isolates estimated that the time of most recent common ancestor of all contemporary H58 strains existed around 37 years ago (1984). The distribution of isolates and tree topology are consistent with at least 138 international transfer events, including multiple introductions within south Asia and dissemination from south Asia into southeast Asia and Africa, as well as many travel-related cases identified in the UK and USA (figure 3; figure 4). We also predicted that ciprofloxacin-resistant triple mutant isolates most probably originated in India around 1996 and were introduced into Pakistan between 2005 and 2013 and into Nepal on at least three occasions (2003–15). We identified frequent transmissions of international transfer of multidrug-resistant isolates (n=33), with multiple introductions from south Asia to southeast Asia and Africa followed by local expansion.

Our models assessing pairwise diversity in H58 isolates and geographical distance identified India as the most probable origin, in both the model including Asian and African isolates and the model restricted to Asian isolates. We found moderate support for a log-linear relationship between pairwise diversity and distance in the model including Asian and African isolates (R^2=0·72) but strong correlation (R^2=0·90) in the model of diffusion within Asia (appendix 1 pp 11–12).

The major non-H58 clades also acquired antimicrobial resistance loci and spread within and from south Asia. Genotype 2.3.3 circulated predominantly in Bangladesh but spread to Pakistan and India within the past 30 years (appendix 1 pp 13–14). Genotype 2.5, which might have circulated in India for more than 100 years (appendix 1 pp 15–16), has been transferred to sub-Saharan Africa and Nepal multiple times, including two instances with strains containing QRDR mutations since 2015. Genotype 3.2.2 organisms originating from Bangladesh were observed in south Asia only. We observed a single instance of transfer from Bangladesh to Nepal and ongoing local expansion. By contrast, we found that these organisms have been regularly transferred from Bangladesh to India (appendix 1 pp 17–18). Transfer events included at least four recent introductions of fluoroquinolone non-susceptible organisms between 2006 and 2017. The most recent common ancestor of genotype 3.3 was estimated to have been from India more than 200 years ago (appendix 1 pp 19–20), but moved extensively across south Asia, establishing large subclades in Bangladesh and Nepal, before progressing to sub-Saharan Africa, the Middle East,

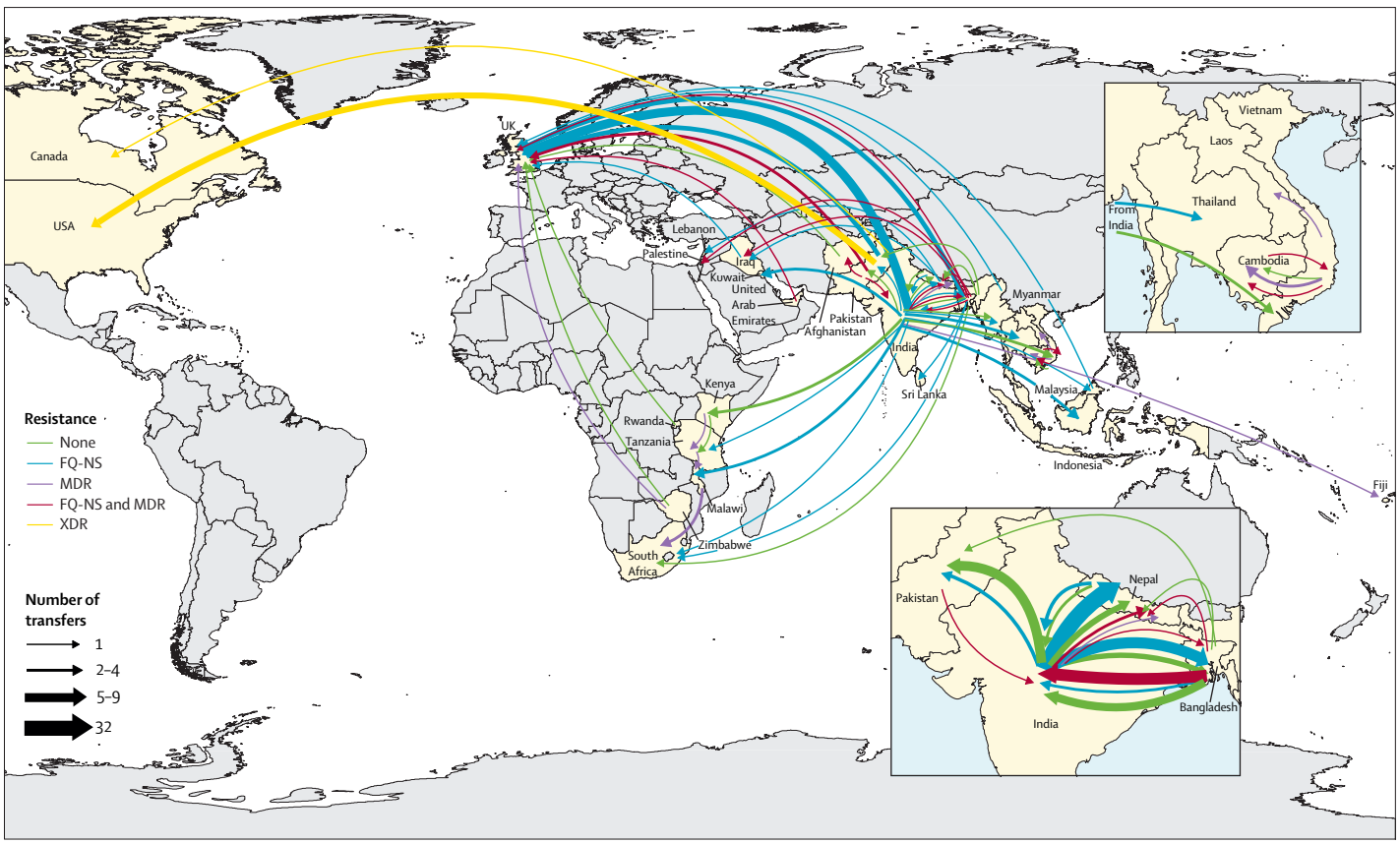


Figure 5: **Major geographical transfers from 1990 onwards within the non-H58 and H58 lineages, inferred from the phylogenetic trees**
The size of each arrow indicates the relative number of probable transfers between the countries. Arrow colours indicate antimicrobial resistance pattern. FQ-NS=Fluoroquinolone non-susceptible. MDR=multidrug resistant. XDR=extensively drug resistant.

and southeast Asia. Genotype 3.3 organisms with QRDR mutations have moved from India to Nepal on multiple occasions.

Overall, our analysis identified evidence for at least 197 introduction events between countries, of which 138 were intracontinental and 59 were intercontinental (figure 5). The most common international transmission events were within south Asia and from south Asia to southeast Asia, east Africa, and southern Africa. We estimated that resistance-conferring mutations to fluoroquinolones (n=94) or azithromycin (n=7) have independently emerged on at least 101 separate occasions within the past 30 years, mostly in south Asia (n=94), and occasionally arising in southeast Asia, Africa, and South America. Additionally, isolates carrying QRDR mutations were transferred between countries on at least 119 independent occasions.

Discussion

This analysis of *S* Typhi genome sequences reveals that acquisition of antimicrobial resistance through plasmids or homoplastic mutations has occurred frequently across multiple lineages and been accompanied by expansion and international spread of antimicrobial-resistant *S* Typhi clones. We identified numerous international and intercontinental transfers of *S* Typhi over the past 30 years, with the majority associated with antimicrobial resistance. Once introduced to a new setting, antimicrobial-resistant *S* Typhi became quickly fixed, as broadly exemplified with fluoroquinolone non-susceptible clades in multiple countries and XDR *S* Typhi in Pakistan. This rapid emergence, spread, and fixation of antimicrobial resistance suggests that making decisions regarding typhoid conjugate vaccine introduction based on current antimicrobial resistance data might miss a crucial window for prevention. Specifically, we found that south Asia continues to be an important hub for the generation of antimicrobial resistance and that the clones emerging here regularly move internationally, underscoring the need for resources to support typhoid control in this region.

Our ancestral state reconstruction and analysis of diversity loss over distance both identified the Indian subcontinent as an origin for most *S* Typhi lineages. Our analysis of diversity and distance from the hypothetical geographical origin (in India) found support for a log-linear relationship between diversity

and distance within Asia, although model fit declined when incorporating samples from Africa, probably due to the role of air travel in the spread of *S* Typhi from south Asia to east Africa. The spread of *S* Typhi within and from south Asia might be linked to migration patterns, both between countries in south Asia and to other regions such as east Africa and southeast Asia, where *S* Typhi was able to spread due to poor water and sanitation infrastructure. The relationship between the Indian diaspora and spread of H58 strains has been previously recognised.[31]

A 2015 study investigated the emergence and global spread of the dominant H58 lineage in *S* Typhi, and a subsequent analysis examined the spread of H58 and 3.1.1 in sub-Saharan Africa.[5,24] Our findings affirm and extend these findings with a much larger set of sequences, characterising the evolutionary history and phylogeography of H58 and four major non-H58 sublineages. Leveraging advances in maximum likelihood-based timed phylogenetic reconstruction enabled us to incorporate far more sequences in the temporal and phylogeographical analysis (eg, 4761 strains compared with 114 strains in the study by Wong and colleagues[5]). This analysis in turn provided a higher resolution window into timing and location of antimicrobial resistance emergence, as well as the geographical spread of *S* Typhi. Furthermore, the application of phylodynamic methods enabled quantification of the effects of antimicrobial resistance emergence on the population size of the dominant *S* Typhi lineage, providing new evidence that antimicrobial resistance facilitates its spread and, in some contexts, reversed trends of declining *S* Typhi populations.

Our data are consistent with studies suggesting that multidrug-resistant *S* Typhi (strains resistant to the classical first line drugs) is now generally on the decline in south Asia.[32–34] The decline of multidrug-resistant *S* Typhi in Asia has been accompanied by a decrease in the proportion of isolates carrying IncHI1 plasmids (except for Pakistan, where the multidrug-resistant decline reversed amid the emergence of the XDR lineage). In our study, multidrug resistance was principally associated with H58 carrying chromosomally integrated antimicrobial resistance genes. The integration of antimicrobial resistance genes into the *S* Typhi chromosome remains a concern, as it provides a mechanism for stable vertical transmission of multidrug-resistant phenotypes.[5,35] By contrast to south Asia, multidrug-resistant typhoid associated with H58 and non-H58 isolates appears to be increasing in parts of Africa, with outbreaks being reported in the past decade.[24,36] QRDR mutations have independently arisen frequently in all *S* Typhi lineages. Nearly all sustained clones containing QRDR mutations appear to have arisen in south Asia, and many have spread regionally and globally. Notably, our analysis revealed that highly fluoroquinolone-resistant *S* Typhi triple mutants have recently emerged in six different genotypes. Our phylogeographical analysis suggests that these clones most probably originated in India and disseminated to neighbouring countries including Nepal and Pakistan.

The emergence and spread of resistance to third-generation cephalosporins and azithromycin in the past decade further complicates typhoid fever treatment.[8,9] By 2019, within 3 years of its first recognition, the XDR genotype (4.3.1.1.P1) became the dominant genotype in Pakistan. At present, all XDR *S* Typhi strains identified have been susceptible to azithromycin and carbapenems.[37] Concerningly, azithromycin-resistant *S* Typhi have recently been reported in Bangladesh, India, Pakistan, Nepal, and Singapore,[10,38,39] arising from mutations in *acrB*. These mutations have arisen independently multiple times in distinct lineages.[10] To date, XDR *S* Typhi isolates containing mutations in *acrB* have not yet been identified. Such organisms would preclude effective treatment with established oral antimicrobials, which could lead to increased hospitalisation rates and potentially greater morbidity and mortality.

Our findings should be interpreted within the context of the limitations of the available data. Although this analysis included the largest collection of novel *S* Typhi genomes to date, there remains underrepresentation of sequences from several regions, including disproportionately small numbers from many countries in sub-Saharan Africa and Oceania where typhoid is endemic. This underrepresentation constrains the inference of timed phylogenies and ancestral state reconstruction; more sequences from these regions are needed to improve our understanding of timing and patterns of spread. Even in countries with more dense sampling, most isolates were derived from a small number of surveillance sites and might not be representative of the distribution of circulating strains. Because *S* Typhi genomes only cover a fraction of all typhoid fever cases, phylogenies are highly incomplete; our estimates for antimicrobial resistance-conferring homoplastic mutations and international transfers represent lower bounds might substantially underestimate their true frequency. These circumstances highlight the need for expanding genomic surveillance to provide a more comprehensive window into the emergence, expansion, and spread of antimicrobial-resistant organisms.

This study highlights the emergence and geographical spread of antimicrobial-resistant *S* Typhi, with evidence of frequent international exportation. This observation underscores the importance of approaching typhoid fever control and antimicrobial resistance as a global rather than local problem. The recent emergence of XDR and azithromycin-resistant *S* Typhi creates greater urgency for rapidly expanding prevention measures, including use of typhoid conjugate vaccines in typhoid-endemic countries. Such measures are needed in countries where antimicrobial resistance prevalence among *S* Typhi isolates

is currently high, but given the propensity for international spread, should not be restricted to such settings.

Contributors

JRA, SKS, SS, DOG, FNQ, JJ, and GK designed the study and oversaw data collection. KEdS, AMT, AKP, JI, MSIS, and JRA analysed data; YH and SS assisted with design and interpretation of the analysis. KEdS and JRA had full access to all the data in the study and verified the data. KES and JRA wrote the first draft of the manuscript, and all authors reviewed the manuscript critically for content and approved the decision to submit for publication.

Declaration of interests

IB has consulted for BlueDot, a social benefit corporation that tracks the spread of emerging infectious diseases. SPL reports travel fees from the Bill & Melinda Gates Foundation. ZAD is a coordinator and founding member of the Global Typhoid Genomics Consortium and reports grants from EU Horizon 2020 for typhoid research outside the submitted work. All other authors declare no competing interests.

Data sharing

Illumina sequence data was submitted to the European Nucleotide Archive. Sequence data from 4169 *S* Typhi strains from previous studies were also included for global context raw sequence data for these isolates are available in European Nucleotide Archive. Details and individual accession numbers of sequence data included in our analysis have been included in appendix 2 and appendix 3.

See **Online** for appendix 2 and 3

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Editorial note: The Lancet Group takes a neutral position with respect to territorial claims in published maps and institutional affiliations.

References

1 Dougan G, Baker S. *Salmonella enterica* serovar Typhi and the pathogenesis of typhoid fever. *Annu Rev Microbiol* 2014; **68:** 317–36.
2 Antillón M, Warren JL, Crawford FW, et al. The burden of typhoid fever in low- and middle-income countries: a meta-regression approach. *PLoS Negl Trop Dis* 2017; **11:** e0005376.
3 Stanaway JD, Reiner RC, Blacker BF, et al. The global burden of typhoid and paratyphoid fevers: a systematic analysis for the Global Burden of Disease Study 2017. *Lancet Infect Dis* 2019; **19:** 369–81.
4 Mogasale V, Maskery B, Ochiai RL, et al. Burden of typhoid fever in low-income and middle-income countries: a systematic, literature-based update with risk-factor adjustment. *Lancet Glob Health* 2014; **2:** e570–80.
5 Wong VK, Baker S, Pickard DJ, et al. Phylogeographical analysis of the dominant multidrug-resistant H58 clade of *Salmonella* Typhi identifies inter- and intracontinental transmission events. *Nat Genet* 2015; **47:** 632–39.
6 Dyson ZA, Klemm EJ, Palmer S, Dougan G. Antibiotic resistance and typhoid. *Clin Infect Dis* 2019; **68** (suppl 2): S165–70.
7 Andrews JR, Qamar FN, Charles RC, Ryan ET. Extensively drug-resistant typhoid - are conjugate vaccines arriving just in time? *N Engl J Med* 2018; **379:** 1493–95.
8 Hooda Y, Sajib MSI, Rahman H, et al. Molecular mechanism of azithromycin resistance among typhoidal *Salmonella* strains in Bangladesh identified through passive pediatric surveillance. *PLoS Negl Trop Dis* 2019; **13:** e0007868.
9 Klemm EJ, Shakoor S, Page AJ, 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:** e00105-18.
10 Sajib MSI, Tanmoy AM, Hooda Y, et al. Tracking the emergence of azithromycin resistance in multiple genotypes of typhoidal *salmonella*. *MBio* 2021; **12:** 1–12.
11 World Health Organization. Typhoid vaccines: WHO position paper, March 2018 – Recommendations. *Vaccine* 2019; **37:** 214–16.
12 Carey ME, MacWright WR, Im J, et al. The Surveillance for Enteric Fever in Asia Project (SEAP), Severe Typhoid Fever Surveillance in Africa (SETA), Surveillance of Enteric Fever in India (SEFI), and Strategic Typhoid Alliance Across Africa and Asia (STRATAA) population-based enteric fever studies: a review of methodological similarities and differences. *Clin Infect Dis* 2020; **71** (suppl 2): S102–10.
13 Andrews JR, Vaidya K, Bern C, et al. High rates of enteric fever diagnosis and lower burden of culture-confirmed disease in peri-urban and rural Nepal. *J Infect Dis* 2018; **218** (suppl 4): S214–21.
14 Croucher NJ, Page AJ, Connor TR, et al. Rapid phylogenetic analysis of large samples of recombinant bacterial whole genome sequences using Gubbins. *Nucleic Acids Res* 2015; **43:** e15.
15 Rahman SIA, Dyson ZA, Klemm EJ, et al. Population structure and antimicrobial resistance patterns of *Salmonella* Typhi isolates in urban Dhaka, Bangladesh from 2004 to 2016. *PLoS Negl Trop Dis* 2020; **14:** e0008036.
16 Ingle DJ, Nair S, Hartman H, et al. Informal genomic surveillance of regional distribution of *Salmonella* Typhi genotypes and antimicrobial resistance via returning travellers. *PLoS Negl Trop Dis* 2019; **13:** e0007620.
17 Tanmoy AM, Westeel E, De Bruyne K, et al. *Salmonella enterica* serovar typhi in Bangladesh: exploration of genomic diversity and antimicrobial resistance. *MBio* 2018; **9:** e02112-18.
18 Britto CD, Dyson ZA, Mathias S, et al. Persistent circulation of a fluoroquinolone-resistant *Salmonella enterica* Typhi clone in the Indian subcontinent. *J Antimicrob Chemother* 2020; **75:** 337–41.
19 Pragasam AK, Pickard D, Wong V, et al. Phylogenetic analysis indicates a longer term presence of the globally distributed H58 haplotype of *Salmonella* Typhi in southern India. *Clin Infect Dis* 2020; **71:** 1856–63.
20 Pham Thanh D, Karkey A, Dongol S, et al. A novel ciprofloxacin-resistant subclade of H58 *Salmonella* Typhi is associated with fluoroquinolone treatment failure. *eLife* 2016; **5:** e14003.
21 Britto CD, Dyson ZA, Duchene S, et al. Laboratory and molecular surveillance of paediatric typhoidal *Salmonella* in Nepal: antimicrobial resistance and implications for vaccine policy. *PLoS Negl Trop Dis* 2018; **12:** e0006408.
22 Dyson ZA, Thanh DP, Bodhidatta L, et al. Whole genome sequence analysis of *Salmonella* Typhi isolated in Thailand before and after the Introduction of a National Immunization Program. *PLoS Negl Trop Dis* 2017; **11:** e0005274.
23 Wong VK, Baker S, Connor TR, et al. An extended genotyping framework for *Salmonella enterica* serovar Typhi, the cause of human typhoid. *Nat Commun* 2016; **7:** 12827.
24 Park SE, Pham DT, Boinett C, et al. The phylogeography and incidence of multi-drug resistant typhoid fever in sub-Saharan Africa. *Nat Commun* 2018; **9:** 5094.
25 Stamatakis A. RAxML version 8: a tool for phylogenetic analysis and post-analysis of large phylogenies. *Bioinformatics* 2014; **30:** 1312–13.
26 Rambaut A, Lam TT, Max Carvalho L, Pybus OG. Exploring the temporal structure of heterochronous sequences using TempEst (formerly Path-O-Gen). *Virus Evol* 2016; **2:** vew007.
27 Didelot X, Croucher NJ, Bentley SD, Harris SR, Wilson DJ. Bayesian inference of ancestral dates on bacterial phylogenetic trees. *Nucleic Acids Res* 2018; **46:** e134–134.
28 Drummond AJ, Rambaut A. BEAST: Bayesian evolutionary analysis by sampling trees. *BMC Evol Biol* 2007; **7:** 214.
29 Volz EM, Frost SDW. Scalable relaxed clock phylogenetic dating. *Virus Evol* 2017; **3:** vex025.
30 Tanabe K, Mita T, Jombart T, et al. *Plasmodium falciparum* accompanied the human expansion out of Africa. *Curr Biol* 2010; **20:** 1283–89.
31 Wirth T. Massive lineage replacements and cryptic outbreaks of *Salmonella* Typhi in eastern and southern Africa. *Nat Genet* 2015; **47:** 565–67.
32 Carter AS, Luby SP, Garrett DO. Introducing Typhoid conjugate vaccine in south Asia: lessons from the Surveillance for Enteric Fever in Asia Project. *Clin Infect Dis* 2020; **71** (suppl 3): S191–95.
33 Balaji V, Kapil A, Shastri J, et al. Longitudinal Typhoid fever trends in India from 2000 to 2015. *Am J Trop Med Hyg* 2018; **99** (suppl 3): 34–40.
34 Qamar FN, Yousafzai MT, Dehraj IF, et al. Antimicrobial resistance in Typhoidal *Salmonella*: Surveillance for Enteric Fever in Asia Project, 2016-2019. *Clin Infect Dis* 2020; **71** (suppl 3): S276–84.
35 Holt KE, Baker S, Weill FX, et al. *Shigella sonnei* genome sequencing and phylogenetic analysis indicate recent global dissemination from Europe. *Nat Genet* 2012; **44:** 1056–59.

36 Wong VK, Holt KE, Okoro C, et al. Molecular surveillance identifies multiple transmissions of Typhoid in west Africa. *PLoS Negl Trop Dis* 2016; **10:** e0004781.
37 Health Alert Network C. Extensively drug-resistant *Salmonella* Typhi infections among U.S. residents without international travel. *HAN Arch* 2021; **CDCHAN-00439:** 0–5.
38 Octavia S, Chew KL, Lin RT, Teo JW. Azithromycin-resistant *Salmonella enterica* serovar Typhi AcrB-R717Q/L, Singapore. *Emerg Infect Dis J* 2021; **27:** 624.
39 Carey ME, Jain R, Yousuf M, et al. Spontaneous emergence of azithromycin resistance in independent lineages of *Salmonella* Typhi in northern India. *Clin Infect Dis* 2021; **72:** e120–27.

Appendix 15. Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi (publication)

PLOS PATHOGENS

RESEARCH ARTICLE

Gallbladder carriage generates genetic variation and genome degradation in *Salmonella* Typhi

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Data Availability Statement: The raw sequence data generated from this study are available in the European Nucleotide Archive (ENA) under the accession numbers described in S1 Table.

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Abstract

Despite recent advances in typhoid fever control, asymptomatic carriage of *Salmonella* Typhi in the gallbladder remains poorly understood. Aiming to understand if *S*. Typhi becomes genetically adapted for long-term colonisation in the gallbladder, we performed whole genome sequencing on a collection of *S*. Typhi isolated from the gallbladders of typhoid carriers. These sequences were compared to contemporaneously sampled sequences from organisms isolated from the blood of acute patients within the same population. We found that *S*. Typhi carriage was not restricted to any particular genotype or conformation of antimicrobial resistance genes, but was largely reflective of *S*. Typhi circulating in the general population. However, gallbladder isolates showed a higher genetic variability than acute isolates, with median pairwise SNP distances of 21 and 13 SNPs ($p = 2.8x10^{-9}$), respectively. Within gallbladder isolates of the predominant H58 genotype, variation was associated with a higher prevalence of nonsense mutations. Notably, gallbladder isolates displayed a higher frequency of non-synonymous mutations in genes encoding hypothetical proteins, membrane lipoproteins, transport/binding proteins, surface antigens, and carbohydrate degradation. Specifically, we identified several gallbladder-specific non-synonymous mutations involved in LPS synthesis and modification, with some isolates lacking the Vi capsular polysaccharide vaccine target due to the 134Kb deletion of SPI-7. *S*. Typhi is under strong selective pressure in the human gallbladder, which may be reflected phylogenetically by long terminal branches that may distinguish organisms from chronic and acute infections. Our work shows that selective pressures asserted by the hostile environment of the human gallbladder generate new antigenic variants and raises questions regarding the role of carriage in the epidemiology of typhoid fever.

manuscript; and decision to submit the manuscript for publication.

Competing interests: The authors have declared that no competing interests exist.

Author summary

Salmonella Typhi is the bacterium that causes typhoid. *Salmonella* Typhi is infamous for being able to be carried in the gallbladder, with Typhoid Mary being the best-known example of a typhoid carrier. Despite having new tools for typhoid control, we have made little progress in understanding this disease process. Aiming to understand if *Salmonella* Typhi is adapted for long-term survival in the gallbladder, we sequenced the genomes of 24 *Salmonella* Typhi isolated from the gallbladders of typhoid carriers. We compared these genomes to *Salmonella* Typhi from acute typhoid patients within the same population. The carriage of *Salmonella* Typhi was not restricted to any specific genotype or resistance to antibiotics, but reflective of the organisms causing acute disease. However, gallbladder isolates had higher genetic variability than acute isolates, with a higher frequency of mutations changing the amino acid sequences of hypothetical proteins, membrane lipoproteins, transport/binding proteins, surface antigens, and carbohydrate degradation. We identified several gallbladder-specific mutations involved in polysaccharide synthesis on the bacterial surface. Our work shows that selective pressures asserted by the hostile environment of the human gallbladder generates genetic variation, which is not observed in acute isolates, raising questions regarding the role of carriage in the epidemiology of typhoid.

Introduction

Typhoid fever, a life-threatening systemic infection caused predominantly by the bacterium *Salmonella enterica* serovar Typhi (*S*. Typhi), remains a significant public health problem in resource-poor settings including parts of Asia and Africa [1]. The disease is contracted via ingestion of contaminated food or water or through contact with individuals excreting the organism [2]. The majority of typhoid patients fully recover with appropriate treatment; however, some individuals can become asymptomatic carriers and shed infectious bacteria in their faeces for an ill-defined period of time. Asymptomatic carriage of *S*. Typhi has been recognized as a public health threat for more than a century, with infamous typhoid carriers like Mary Mallon, a cook in New York, and Mr N, a milker in England, identified in the early part of the 20th century [3,4].

Typhoid carriage can be differentiated into three categories depending on the duration of shedding: convalescent (three weeks to three months), temporary (three to twelve months), and chronic (more than one year) [5]. In endemic regions, an estimated 2–5 percent of acute typhoid patients become chronic carriers, meaning that they continue to intermittently shed the bacteria indefinitely after apparent clinical resolution [3,5]. Consequently, chronic carriers are widely believed to be an ecological niche that facilitates the transmission and persistence of typhoid in human populations [6]. *S*. Typhi is a human-restricted pathogen, meaning that the disease may be theoretically eliminated locally by reducing transmission through targeted treatment, improved sanitation, and mass vaccination. Consequently, understanding the role of chronic carriers in disease transmission, and detecting them prospectively, may accelerate disease elimination.

Despite substantive gains in understanding the biology of typhoid, we have generated limited new insights into typhoid carriage in recent decades. Data from murine models of *Salmonella* carriage and human clinical investigations have determined that the gallbladder is a key permissive niche for long-term bacterial persistence [7–13]. Various epidemiological investigations have shown that gallstones and gallbladder damage may facilitate typhoid carriage [9,13–17], and that *Salmonella* preferentially attach to and form biofilms on cholesterol-rich

gallstones [7,11,13,18,19]. *S*. Typhi carriage isolates have been previously genetically compared to isolates from acute infections, with the aim of identifying signatures associated with carriage [20–23]. However, these studies were unable to infer how carriage isolates directly relate to those causing acute disease.

It is apparent we need a better understanding of the role of the typhoid carrier and associated organisms to generate new approaches to the management of such individuals in endemic locations. Although it is widely accepted that that *S*. Typhi carriage plays a key role in the transmission of typhoid in endemic settings, it is unknown if carriage organisms are somehow adapted for long-term colonisation. Aiming to address this question, we performed whole genome sequencing and detailed genetic analyses of *S*. Typhi isolated from the gallbladders of typhoid carriers in Kathmandu. We compared these data to the sequences of contemporaneous organisms isolated from the blood of acutely infected patients in the same community over the same time period. Our data show that whilst carriage isolates are reflective of the general *S*. Typhi population circulating in the community, selective pressures during gallbladder carriage induce increased genetic variation and genomic degradation.

Results

The phylogenetic relationships between acute and gallbladder S. Typhi isolates

Between June 2007 and October 2010, we conducted a *Salmonella* carriage study in Kathmandu[13]. Patients undergoing cholecystectomy for acute or chronic cholecystitis were enrolled; bile and stool samples from 1,377 individuals were collected and subjected to microbiological examination. Twenty-four *S*. Typhi were isolated from bile samples taken from these patients and designated as gallbladder isolates. Ninety-six *S*. Typhi isolates recovered from patients with acute typhoid fever living in the same population over the same time period were used for comparison [24] (denoted as acute isolates) (S1 Table). A phylogenetic analysis of these 120 *S*. Typhi isolates demonstrated that subclade 4.3.1 (H58) was the dominant genotype, constituting 62.5% (15/24) of all gallbladder isolates and 65.6% (63/96) of all acute isolates. The second most common genotype was 3.3.0 (H1), accounting for 12.5% (3/24) and 14.6% (14/96) of all gallbladder and acute isolates, respectively.

We identified a significant degree of genetic diversity within this collection of acute and carriage organisms, with multiple less-common genotypes co-circulating, included various clades (4.1, 3.1 and 2.2), subclades (3.2.2, 3.0.1, 2.2.2 and 2.2.1), and organisms within primary cluster 2 (Fig 1). The less common genotypes from the gallbladder fell within subclade 3.2.2 (8.3%; 2/24), 2.2.2 (4.2%; 1/24), clade 2.2 (8.3%; 2/24) and primary cluster 2 (4.2%; 1/24). Overall, gallbladder isolates were not significantly associated with subclade 4.3.1 in comparison with other genotypes (15/24 versus 9/24, $p = 0.083$; Chi-squared test). These initial observations indicate that *S*. Typhi carriage was not restricted to any particular *S*. Typhi genotype; instead, the genotype distribution among gallbladder isolates generally reflected a phylogenetic structure similar to that of the acute *S*. Typhi infections circulating in the community.

Antimicrobial susceptibility

We speculated resistance to key antimicrobials may facilitate the development of carriage. However, we found that the *S*. Typhi gallbladder isolates did not carry any acquired AMR genes. However, chromosomal mutations associated with reduced susceptibility to fluoroquinolones were common. These fluoroquinolone resistance-associated mutations within the gallbladder organisms were more commonly observed in subclade 4.3.1 than in non-subclade 4.3.1 (73%

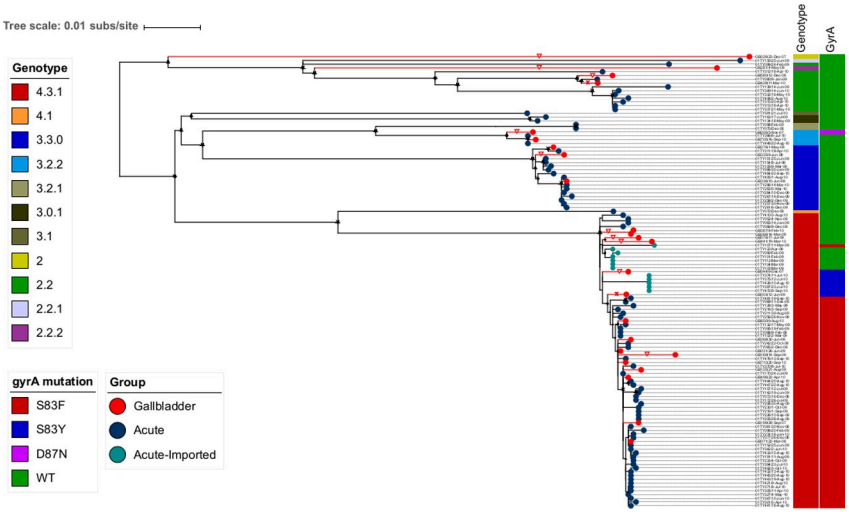

Fig 1. The phylogenetic structure of gallbladder and acute *S*. Typhi isolates collected between 2007 and 2010. Rooted maximum likelihood tree (*S*. Paratyphi A used as an outgroup to root the tree and pruned for visualization) based on core-genome SNPs of 120 *S*. Typhi isolates with the corresponding metadata: genotype, *gyrA* mutation. Gallbladder and acute isolates are shown as red and dark circles at the terminal nodes, respectively. Acute isolates originating from importation are also highlighted by turquoise circles at the terminal nodes. Terminal branches leading to gallbladder isolates are highlighted in red. Red triangles show gallbladder isolates associated with unusually long terminal branches.

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(11/15) versus 11% (1/9), $p = 0.01$; Chi-squared test). We compared *gyrA* mutation profiles between acute and gallbladder isolates within subclade 4.3.1; 76.2% (48/63) of acute isolates and 60% (9/15) of gallbladder isolates carried the S83F mutation, 7.9% (5/63) of acute isolates and 13.3% (2/15) of gallbladder isolates carried the S83Y mutation, and 15.9% (10/63) of acute isolates and 26.7% (4/15) of gallbladder isolates had no *gyrA* mutation. Consequently, there was no significant difference ($p = 0.327$; Chi-squared test) in the presence of fluoroquinolone resistance-associated mutations between acute and gallbladder isolates within subclade 4.3.1.

Phylogenetic signatures of long-term Salmonella Typhi carriage

Despite the acute and gallbladder *S*. Typhi isolates generally clustering within the same genotypes across the phylogeny, we observed that a substantial proportion of the gallbladder isolates had higher genetic variability, as illustrated by their placement at the tips of long terminal branches within the phylogeny (Fig 1). The median pairwise SNP distance of gallbladder isolates within subclade 4.3.1 was 21 SNPs (IQR: 12–24), which was significantly greater than that of the corresponding acute isolates (13 SNPs (IQR: 8–19 SNPs) ($p = 2.8\text{x}10^{-9}$, Wilcoxon rank-sum test) (S2 Fig). Similarly, the median pairwise SNP distance of gallbladder isolates within subclade 3.3.0 (20 SNPs, IQR: 13–22 SNPs) was higher than that of acute isolates (13 SNPs, IQR: 4–15 SNPs) ($p = 0.26$, Wilcoxon rank-sum test).

We mapped the contemporary acute and gallbladder *S*. Typhi sequences onto the global *S*. Typhi phylogeny, which indicated that the majority of these Nepalese acute and gallbladder *S*. Typhi isolates fell within known domestic genotypes, with limited evidence of importation from other countries (S1 Fig). This observation suggests that the long terminal branches associated with gallbladder isolates were unlikely to be driven by the importation of these organisms from alternative countries.

We next estimated and plotted the nearest phylogenetic distances (NPDs) between each taxon and its nearest neighbour on the subclade 4.3.1 tree versus the year of isolation, the age

of the individual from whom the organism was isolated, and the *gyrA* mutation profile. We hypothesized that the annual distribution of NPDs of *S*. Typhi acute isolates would represent the phylogenetic diversity (mutation accumulation) occurring annually via acute disease transmission and would be comparable over multiple years. Alternatively, we considered that *S*. Typhi in the gallbladder may develop characteristic adaptive mutations facilitating long-term persistence, causing them to gradually become increasingly distinct from contemporaneous acute isolates, leading to greater phylogenetic distances relative to their neighbours. In addition, given that all acute subclade 4.3.1 isolates here exhibited a *gyrA* mutation, the gallbladder subclade 4.3.1 isolates without a *gyrA* mutation were more likely to have colonized the gallbladder prior to nalidixic acid resistance becoming commonplace.

Our analyses showed that the average (±SD) NPD per year of acute subclade 4.3.1 isolates was comparable; specifically, 0.00163 (± 0.00202) substitutions/site (~3.6 (± 4.4) SNPs) in 2008; 0.00110 (± 0.00229) substitutions/site (~2.4 (± 5) SNPs) in 2009, and 0.00144 (± 0.00238) substitutions/site (~3.2 (± 5.2) SNPs) in 2010. The majority of the subclade 4.3.1 gallbladder isolates (8/10) for which NPDs fell within the annual NPD distribution of acute subclade 4.3.1 isolates were associated with comparable terminal branch lengths and had a *gyrA* S83F mutation. Based on these findings, we surmised that gallbladder colonization with these isolates was likely to have occurred relatively recently in these individuals. Notable exceptions were two gallbladder isolates (GB266 and GB387) that did not possess a *gyrA* mutation and were associated with long terminal branches but had low NPDs as they clustered closely within the main phylogeny (Figs 1 and 2). Further, our data showed that all subclade 4.3.1 gallbladder isolates exhibiting abnormally high NPDs were associated with long terminal branches, suggestive of chronic carriage (Fig 2). In particular, two subclade 4.3.1 gallbladder isolates (GB76 and GB441) lacked *gyrA* mutations, two others (GB003 and GB044) had *gyrA* S83Y mutations, and the remaining one (GB169) exhibited *gyrA* mutation S83F. With respect to the age distribution, typhoid carriers were significantly older (median age 36 years, range: 20–67) than patients with acute illness (median age 16 years, range: 0–31) ($p = 3.8\text{x}10^{-8}$, Wilcoxon rank-sum test). The gallbladder

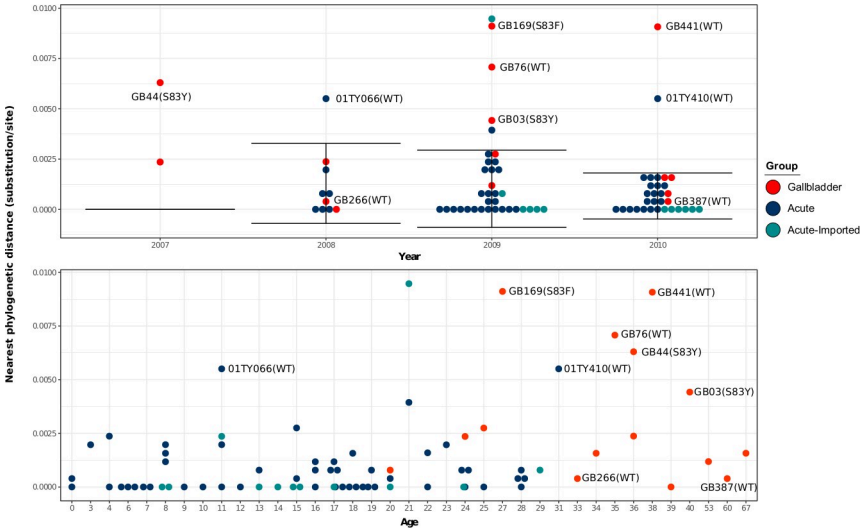

Fig 2. The Distribution of nearest phylogenetic distances of gallbladder and acute H58 isolates over the study period. Each circle represents the phylogenetic distance from each isolate to its nearest neighbour on the phylogenetic tree. The error bar represents the average phylogenetic distance to the nearest neighbour (± standard deviation) for acute H58 isolates. Gallbladder and acute isolates estimated to have originated from chronic carriers are labelled with their corresponding strain names.

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isolates thought to have originated from chronic carriers (based on above data) were obtained from individuals between aged between 27 and 40 years, which was older than the majority of the sampled acute typhoid patients; however, there was no significant difference in age distribution between those estimated to be recent and chronic carriers (Fig 2).

The genetic traits of Salmonella Typhi gallbladder isolates

To identify potentially adaptive mutations associated with typhoid carriage, all nonsynonymous SNPs (NSs) occurring exclusively within the *S*. Typhi gallbladder genome sequences were identified and grouped by their predicted or known function. A corresponding analysis was performed for all NSs in the acute *S*. Typhi isolates. A total of 228 gallbladder-specific NSs (212 missense and 16 nonsense mutations) and 469 acute-specific NSs (437 missense and 32 nonsense mutations) were identified. In general, there was no significant difference ($p = 0.924$; Chi-square test) in the proportion of nonsense mutations out of total specific NSs in the gallbladder versus the acute isolates across all genotypes. However, among subclade 4.3.1 isolates, the proportion of nonsense mutations out of total specific NSs was significantly higher for gallbladder isolates than for acute isolates (10/60 compared to 2/67, Fisher exact test, $p = 0.01$). These data suggest that gene degradation resulting from nonsense mutations was more common in the subclade 4.3.1 gallbladder isolates compared to the subclade 4.3.1 acute isolates.

Inactivated genes in the gallbladder isolates included genes involved in the synthesis of peptidoglycan (*pbpC*), vitamin B12 receptor (*btuB*), general stress response regulator (*rpoS*), a laterally acquired protein in SPI-7 (STY4562), membrane transport protein (STY3932), central metabolism (STY0230, *ggt*), hypothetical proteins (STY0929 and STY4178), and osmotically inducible lipoprotein E precursor (*osmE*) (Table 1).

Overall, the gallbladder- and acute-specific NSs across all genotypes could be grouped into 78 functional categories. The highest prevalence of these NSs was found in genes encoding hypothetical proteins, membrane lipoproteins, unknown functions, transport/binding proteins, SPI-7, general regulatory functions, surface polysaccharides and antigens, carbohydrate degradation, and DNA replication/modification. The proportions of NSs in SPI-7, surface polysaccharides and antigens, pathogenicity, cell envelope, anaerobic respiration, fatty acid biosynthesis and transport/binding proteins were all higher in gallbladder than acute isolates (Fig 3). Notably, the data showed that the proportion of NSs in the *viaB* operon (encoding the Vi antigen, target of the typhoid conjugate vaccine (TCV)) was significantly higher in gallbladder isolates compared to the acute isolates across all genotypes (9/228 compared to 7/469, Chi-squared test, $p = 0.04$). Similar results were obtained when considering only *S*. Typhi isolates belonging to subclade 4.3.1, with gallbladder isolates having more specific NSs in the *viaB* operon than the acute isolates (5/60 compared to 1/67, Fisher's exact test, $p = 0.09$). Additionally, we identified two gallbladder isolates (GB428 and GB003) that had lost the Vi capsular polysaccharide due to the deletion of the entire 134kb SPI-7 region.

Positive selection associated with typhoid carriage

Finally, we investigated signatures of positive selection by identifying analogous genetic variation detected in different gallbladder isolates. Among the gallbladder specific NSs, a number of different mutations were present in the same gene or the same biological pathway in at least two phylogenetically unlinked gallbladder isolates. For example, within the *viaB* operon, there were two NSs at codon 137 and 462 in the *tviE* gene (isolates GB580 and GB026) and six NSs in codons 166, 504, 506, 508, 665 and 752 in the *tviD* gene (isolates GB005, GB026, GB076, GB125 and GB281, respectively). Both genes facilitate the polymerization and translocation of the Vi capsule [25]. Convergent NSs were also observed in the *rpoS* gene (sigma factor sigma-

Table 1. Nonsense mutations and their predicted functions in gallbladder isolates.

Position in CT18	S/NS	Gene	Product	Functional class	GB005	GB026	GB044	GB076	GB125	GB169	GB199	GB266	GB281	GB335	GB368	GB387	GB441	GB580	GB705
239853.. 241370	STOP	STY0230	Deoxyguanosine-triphosphate trophosphohydrolase	Central intermediary metabolism							E496*								
378398.. 378796	STOP	STY0368	probable secreted protein	membrane lipoprotein									C46*						
598006.. 600618	STOP	fimD	outer membrane usher protein FimD precursor	surface structure														Q386*	
1457973.. 1458758	STOP	STY1502	probable secreted protein	membrane lipoprotein										W162*					
complement (1721748.. 1722089)	STOP	STY1802	osmotically inducible lipoprotein E precursor	unknown						Q99*									
complement (2513933.. 2514808)	STOP	STY2679	sulphate transport system permease protein CysW	transport anion		W175*													
complement (2629668.. 2631983)	STOP	pbpC	pencillin binding protein 1C	murein sacculus peptidoglycan			Q246*												
complement (2915077.. 2916069)	STOP	rpoS	RNA polymerase sigma subunit RpoS	broad regulatory function					W247*										
complement (3601247.. 3603091)	STOP	STY3744	vitamin B12 receptor protein	cell envelope											W33*				
3795271.. 3796734	STOP	STY3932	putative membrane transport protein	transport/ binding protein					Q15*										
complement (4037181.. 4038752)	STOP	STY4178	Conserved hypothetical protein	hypothetical protein											W413*				
4123472.. 4125214	STOP	ggt	gamma-glutamyltranspeptidase precursor	thioredoxin											Q105*				
4307686.. 4308996	STOP	STY4438	putative exported protein	membrane lipoprotein														W184*	
4442121.. 4444211	STOP	STY4562	hypothetical protein	SPI-7								W234*				W234*			
4593908.. 4595071	STOP	yjfC	conserved hypothetical protein	hypothetical protein		W9*													

* mutations generating a stop codon

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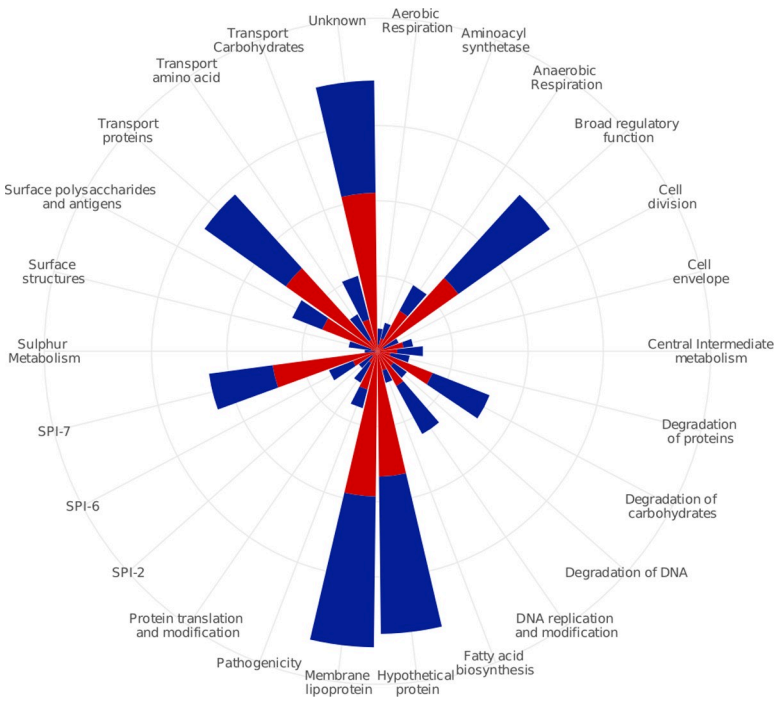

Fig 3. Functional classes of *Salmonella* Typhi genes associated with the highest prevalence of gallbladder-specific nonsynonymous SNPs versus acute-specific nonsynonymous SNPs. Functional classes are shown on the outermost circle. Four circles from the middle represent 5–10–15–20 percent of the cumulative percentage of functional classes. Red and blue blocks are representatives of gallbladder and acute isolates, respectively.

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38) of isolates GB125 (nonsense mutation at codon 247) and GB705 (NSs at codon 94 and 250), which may impact general stress response and nutrient starvation. A further example was NSs at codon 59 and 230 in the *degS* gene (serine protease) (isolates GB005 and GB169). *DegS* is a component of the DegS-DegU two-component regulation system involved in expression of several degradative enzymes for salt stress responses and growth-limiting conditions. Additionally, three isolates (GB005, GB026 and GB705) each possessed an NS (codons 335, 406 and 946, respectively) in STY1242 (*ptsG*—glucose-specific PTS system IIBC component). PtsG enzyme is a component of the glucose-specific phosphotransferase system and plays a role in phosphorylation and translocation of glucose across the bacterial membrane, and is induced in carbon-limited conditions [26]. NSs in several other genes were observed in >2 carriage isolates, including STY0429 (*SbcC*—exonuclease), STY0661 (*dmsC*—molybdopterin containing oxidoreductase membrane anchor subunit), STY1447 (putative ribulose-5-phosphate 3-epimerase) and STY2760 (*ratA*—putative exported protein) (Table 2).

Evidence of selective pressure on lipopolysaccharide

With respect to convergent mutations within the same biological pathways, there were a number of gallbladder-specific NSs involved in LPS O-antigen synthesis and modification; for example, an NS in the *rfc* gene (regulator of O-antigen polymerization) in isolate GB441, an NS in STY2629 (LPS modification acyltransferase) in isolate GB335, two NSs in *rfbE* (CDP-tyvelose-2-epimerase) and *rfaG* genes (LPS core biosynthesis protein) in isolate GB281, and three NSs in the *rfbK* (phosphomannomutase), *manB* (phosphomannomutase), and *rfaD* genes (ADP-L-Glycero-D-mannoheptose-6-epimease) in isolate GB026. *RfbK* and *manB* are both related to GDP-mannose synthesis for the LPS, and *rfaD* is an enzyme that catalyzes the

Table 2. Nonsynonymous mutations associated with positive selection in gallbladder isolates.

Position in CT18	S/NS	Gene	Product	Functional class	GB005	GB026	GB044	GB076	GB125	GB169	GB199	GB266	GB281	GB335	GB368	GB387	GB441	GB580	GB705
complement (437771..440875)	NS	STY0429	exonuclease sbcC	degradation of DNA				R394H					L646P						
661366..662133	NS	STY0661	molybdopterin-containing oxidoreductase	unknown		E5K	V9M												
1196033..1197466	NS	STY1242	PTS, glucose specific IIBC component	transport carbohydrate	A112T	H136R													G316S
complement (1399142..1399774)	NS	STY1447	ribulose-5-phosphate 3 epimerase	unknown		E164K							I101V						
complement (2220042..2221727)	NS	STY2389	two-component system sensor kinase	broad regulatory function									P11L	V49A					
complement (2331373..2334009)	NS	STY2499	DNA gyrase submit A	DNA replication and function	D97N								L824F						
complement (2915077..2916069)	NS	rpoS	RNA polymerase sigma subunit RpoS	broad regulatory function			Q246*		W247*										T94P, E250V
complement (2915077..2916069)	NS	degS	serine protease	degradation of proteins	E230K					V59A									
complement (4516537..4518273)	NS	tviE	glycosyl transferases	SPI-7		A462T												H137Y	
complement (4519050..4521545)	NS	tviD	Vi polysaccharide biosynthesis protein	SPI-7	F166L	P752Q, V508I		G506C	R665H				Q504L						

* mutations generating a stop codon

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conversion of ADP-D-glycerol-D-mannoheptose to ADP-L-glycerol-D-mannoheptose, a precursor for the synthesis of inner-core LPS.

Discussion

As stakeholders consider introduction of TCV into their national immunization programmes, research into the role of chronic carriers in bacterial persistence and disease transmission is needed to forecast the feasibility of typhoid elimination and to inform appropriate public health measures. However, epidemiological investigations of typhoid carriage are challenging, given that this population is difficult to identify and follow. Currently, the environmental factors driving the evolution of *S*. Typhi within the gallbladder are poorly understood and little is known about the adaptive mechanisms that may promote long-term survival. Our relatively small study is the largest genomic investigation of *S*. Typhi gallbladder carriage in a typhoid endemic setting and allowed us to provide unprecedented insight into the genetic and phylogenetic signatures associated with typhoid carriage.

Our data demonstrated, contrary to previous suggestions [27], that carriage of typhoid in the gallbladder was not restricted to any particular genotype and was associated a diverse range of bacterial genotypes, which largely mirrored the genetic structure of the bacterial population causing acute disease in Nepal. Further, typhoid carriage was not confined to specific AMR phenotypes, signifying that carriage is not associated with treatment failure with specific antimicrobials interacting with corresponding AMR profiles. However, by comparing the pairwise SNP distances between gallbladder and acute isolates within the same genotype, we found that gallbladder isolates displayed significantly greater genetic diversity compared to acute isolates, which suggests that long-term exposure to the gallbladder environment results in different accumulated adaptive mutations over time than would be generated in acute isolates. Our phylogenetic reconstruction of *S*. Typhi revealed that a number of gallbladder isolates were located at the tips of atypically long terminal branches, signifying that chronic carriage isolates develop distinct phylogenetic signatures which could be potentially utilized for the identification of organisms arising from chronic carriers. Further investigating this phenomenon, we found that the annual distribution of NPDs of acute isolates, which likely reflects mutation accumulation in the natural environment, was highly comparable across years and could be exploited to disaggregate recent carriers from longer-term carriers. If carriers are relevant for sustaining transmission in endemic area, then we predict that they would play an increasingly important role as acute transmission declines. For example, if environmental transmission decreases through chlorination or acute transmission is controlled through immunisation, transmission via person-to-person transmission via chronic carriers would become proportionally more prevalent. Therefore, the annual NPD distribution could be used to measure the impact of typhoid vaccination on disease transmission dynamics in locations introducing new disease control measures.

The role of chronic carriage in disease transmission represents one of the most long-standing questions in typhoid fever. Though typhoid carriers have been widely considered as an important source of infection, their exact contribution to transmission in endemic areas is not well understood. Previous molecular epidemiological studies in endemic regions highlighted an abundance of long-cycle environmental transmission in these settings, with a wide diversity of co-circulating bacterial genotypes isolated from acute typhoid patients [28–31], suggesting that person-to-person transmission makes a minimal contribution to new typhoid cases in an endemic area. Here, few gallbladder isolates clustered in close proximity or were directly linked with acute isolates and could generally be identified by their placement on long terminal branches. This increase in genetic diversity and the lack of known *S*. Typhi virulence factors

(SPI-7) in some carriage isolates, which was not reciprocated in the acute isolates, call into question the role of carriers in disease transmission. Notably, none of the pre-surgical stool cultures from these patients undergoing cholecystectomy were positive for *S*. Typhi. However, the infectivity and transmission fitness of gallbladder isolates must be investigated further, as we cannot rule out the possibility that gallbladder carriage of *S*. Typhi can become a more important reservoir of infection when environmental transmission is successfully reduced. Further, the fact that gallbladder isolates display greater genetic variation than acute isolates implies that the gallbladder may act as an important ecological niche for generating novel genotypes.

By identifying NS mutations occurring specifically in gallbladder isolates and classifying them into predicted functional classes for comparison with those of acute isolates, we found that gene degradation by nonsense mutations was significantly higher in gallbladder isolates compared to acute isolates within subclade 4.3.1. The effects of gene inactivation on phenotype, fitness and adaptation of carriage isolates inside the gallbladder are currently unknown. Further investigation of this phenomenon is necessary, as gene inactivation has been shown to be an important molecular mechanism in human adaptation in the evolutionary history of *S*. Typhi [32,33].

We additionally found evidence for the enrichment of NSs in genes encoding the Vi polysaccharide capsule in gallbladder isolates. The Vi antigen is immunogenic and anti-Vi antibody gradually wanes in acute typhoid patients after recovery, but can be detected in plasma from chronic carriers [34,35]. Data from sero-surveillance studies for chronic carriage have commonly reported elevated anti-Vi antibodies in healthy individuals, which could be associated with carriage or repeated infection [36,37]. Immunofluorescent staining of biofilms produced by *S*. Typhi on the surface of human gallstones demonstrated an abundance of Vi capsule on the surface of the colonising bacteria, suggesting that *S*. Typhi expresses Vi during colonisation of the apical surface of the gallbladder [19]. The increased frequency of nonsynonymous mutations in the viaB operon (*tviB*, *tviD* and *tviE*) of gallbladder isolates, combined with high anti-Vi antibody titres in plasma [38] suggest that *S*. Typhi residing in the gallbladder are under sustained immune pressure. The observation that two gallbladder isolates lacked genes encoding proteins for Vi capsule biosynthesis again suggests that these were subject to selective pressure and that the loss of Vi may be an adaptive mechanism for long-term survival. The generation of Vi-negative *S*. Typhi may also call into question the possibility of their proliferation following mass immunization with TCV.

Identifying genes under selection among gallbladder isolates is crucial for understanding the evolutionary forces and bacterial adaptation to the gallbladder environment during carriage. Signatures of positive selection were detected in a number of genes containing differing gallbladder-specific NS mutations in at least two phylogenetically unlinked gallbladder isolates. Many of these genes are associated with gene regulation under stress and virulence gene expression. For example, the global regulatory gene *rpoS* is responsible for general stress responses and nutrient starvation, and regulates biofilm formation, colonization of Peyer's patches, persistence in the spleen and the synthesis of Vi [39–41]. The *degS* gene is involved in salt stress responses and growth-limiting conditions; STY1242 (*ptsG*—glucose-specific PTS system IIBC component) is activated during carbon starvation. These observations suggest that *S*. Typhi is exposed to a range of stressors within the gallbladder. Furthermore, genes responsible for LPS biosynthesis were also enriched for NS mutations. LPS is the major component of the outer membrane of Gram-negative bacteria and represents one of the main factors contributing to bile salt resistance [42,43]. LPS is also a key structural component of the biofilm extracellular matrix forming on human gallstones [19,44]. The enrichment of NS mutations in genes involved in LPS biosynthesis and modification leads to structural changes in LPS, which we predict will enhance bile resistance and biofilm formation.

This study has its limitations. The number of gallbladder and acute isolates was relatively small and thus impacts interpretation of the mechanisms underlying the phylogenetic distances between some of the gallbladder isolates and their nearest neighbours. Specifically, our ability to infer associations within uncommon genotypes was limited. Additionally, the identified phylogenetic signature inferred to be associated with carriage was not observed for all gallbladder isolates, due to an underrepresentation in the acutely infected population. Additionally, it was impossible to determine the duration of carriage to confirm our findings, as most typhoid carriers from our study did not recall a history of typhoid [13]. However, our data suggest that the potential duration of carriage within our gallbladder isolates was variable, thus leading to variable terminal branch lengths. Despite these limitations, our study is unique and opens up new possibilities for evaluating associations between gallbladder-specific genetic variation and phenotypic differences to better understand the biology of this infectious disease paradox.

Conclusions

We conclude that typhoid carriage is not associated with any specific genotype nor driven by AMR phenotypes. However, we show that long-term gallbladder carriage generates genetic variation and results in atypically long phylogenetic branch lengths that could be used to distinguish carriage from acute infection. Further, gene degradation via nonsense mutation is enriched in gallbladder isolates in subclade 4.3.1, potentially reflecting selection for survival in the hostile gallbladder environment. The genetic diversity identified here calls into question the role of typhoid carriers in transmission and suggests that carriage may generate novel genotypes. It remains important that we further investigate the epidemiology, genomics, biology and public health impacts of carriage in parallel to the deployment of disease control and elimination efforts. The role of carriers may become increasingly important as we move toward eradication.

Methods

Ethics approval and consent to participate

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the institutional ethical review boards of Patan Hospital, The Nepal Health Research Council and The Oxford University Tropical Research Ethics Committee (OXTREC, Reference number: 2108). All enrollees were required to provide written informed consent for the collection and storage of all samples and subsequent data analysis. In the case of those under 18 years of age, a parent or guardian was asked to provide written informed consent. Consent for publication was incorporated as a component of entrance into the study.

Sampling

Between June 2007 and October 2010, we conducted a *Salmonella* carriage study at Patan Hospital in Kathmandu[13]. In brief, patients residing in the Kathmandu Valley undergoing cholecystectomy for acute or chronic cholecystitis were enrolled; bile and stool samples from these patients were subjected to microbiological examination. *S*. Typhi were isolated from bile samples taken from these patients (referred to as gallbladder isolates). Additionally, 96 randomly selected *S*. Typhi isolates recovered from the blood of patients with acute typhoid fever residing in the same catchment population within the Kathmandu valley were used for a comparison [24] (referred to as acute isolates) (S1 Table).

Bacterial isolation and antimicrobial susceptibility testing

Bile and stool were collected from all cholecystectomy patients for culture. Bile was inoculated into equal volumes of Selenite F broth and Peptone broth and incubated at 37˚C overnight. Broth was subcultured onto MacConkey agar and Xylene Lysine Deoxycholate (XLD) agar. After overnight incubation at 37˚C, the plates were examined for the growth of Gram-negative bacteria and colonies were identified by API20E (bioMerieux, France). *S*. Typhi/*S*. Paratyphi A were confirmed by slide agglutination using 02, 09, and Vi antisera (Murex Biotech, Biotech, England).

For the acute isolates, 5–10 ml of blood was taken from all patients with a clinical suspicion of typhoid fever and inoculated into media containing tryptone soya broth and sodium polyanethol sulphonate (up to 25mL). Blood culture bottles were incubated for up to seven days, with blind sub-cultures at 24 hours, 48 hours, and 7 days, or when the broth became cloudy on sheep blood, chocolate, and MacConkey agar. Presumptive *Salmonella* colonies were identified as above.

Antimicrobial susceptibility testing was performed by the modified Bauer-Kirby disc diffusion method with zone size interpretation based on CLSI guidelines [45]. Etests® were used to determine MICs following the manufacturer's recommendations (bioMérieux, France). Ciprofloxacin MICs were used to categorise *S*. Typhi isolates as susceptible ($\leq$0.06 μg/mL), intermediate (0.12–0.5 μg/mL) and resistant ($\geq$1 μg/mL) following CLSI guidelines [45].

Vi agglutination assay

Two gallbladder isolates of *S*. Typhi (GB003 and GB428) that lacked the Vi polysaccharide biosynthesis (*viaB*) operon were grown on LB agar plates supplemented with increasing concentrations (1mM, 85mM and 170mM) of NaCl. Vi agglutinations were performed on microscope slides by mixing 10μl of single colony suspensions with 50μl of Vi antisera (Murex Biotech, Biotech, England). Agglutination was recorded after gently agitating the slide for 1 minute. Two gallbladder isolates of *S*. Typhi (GB125 and GB169) containing the *viaB* operon were used as controls.

Whole genome sequencing and SNP analyses

Total genomic DNA from acute and gallbladder *S*. Typhi isolates was extracted using the Wizard Genomic DNA Extraction Kit (Promega, Wisconsin, USA) (S1 Table). 50ng of genomic DNA was subjected to library preparation using the Nextera DNA library prep kit; whole genome sequencing (WGS) was performed on an Illumina MiSeq platform following the manufacturer's recommendations to generate 250bp paired end reads.

Single nucleotide polymorphisms (SNPs) were called using previously described methods [46]. Briefly, all reads were mapped to the reference sequence of *S*. Typhi strain CT18 (Accession no: AL513382), plasmid pHCM1 (AL513383) and pHCM2 (AL513384) using SMALT (version 0.7.4). Candidate SNPs were called against the reference sequence using SAMtools and filtered with a minimal mapping quality of 30 and a quality ratio cut-off of 0.75. The allele at each locus in each isolate was determined by reference to the consensus base in that genome. This process was performed using *samtools mpileup* and by removing low confidence alleles with consensus base quality $\leq$20, read depth $\leq$5 or heterozygous base calls. SNPs in phage regions, repetitive sequences or recombinant regions were excluded, [47,48] which resulted in a final set of 2,186 chromosomal SNPs. SNPs were subsequently annotated using the parseSNPTable.py script in the RedDog pipeline (https://github.com/katholt/RedDog). From the identified SNPs in *S*. Typhi genomes, a subset of 68 were used to assign *S*. Typhi isolates to previously defined lineages according to the existing extended *S*. Typhi genotyping framework [49].

To identify the potential function of genes containing key SNPs, we investigated the known or predicted functions of the identified genes. We identified SNPs occurring exclusively in either acute or gallbladder isolates and genes containing these SNPs were grouped by their predicted or known function based on the *S*. Typhi functional classification scheme developed by the Sanger Institute (www.sanger.ac.uk) using the genome annotation of *S*. Typhi CT18 [50].

The antimicrobial resistance (AMR) gene and plasmid contents of *S*. Typhi isolates were determined using a local assembly approach with ARIBA (Antimicrobial Resistance Identifier by Assembly) [51]. Resfinder [52] and Plasmidfinder [53] were used as reference databases of antimicrobial resistance genes and plasmid replicons, respectively.

Phylogenetic analyses and pairwise SNP distance

A maximum likelihood phylogenetic tree was reconstructed from the SNP alignment of 120 *S*. Typhi isolates (an *S*. Paratyphi A isolate was included as an outgroup) using RAxML (version 8.2.8) with the generalized time-reversible model and a Gamma distribution to model the site-specific rate variation (GTR+Γ). Support for the maximum likelihood (ML) tree was assessed via bootstrap analysis with 1,000 pseudoreplicates. Pairwise phylogenetic distances depicting the phylogenetic branch length separating each pair of taxa within subclade 4.3.1 (H58) were estimated using the function *cophenetic* in the ape package (v4.1) in R (v3.3.2). Phylogenetic distances between each taxon and its nearest neighbour on the phylogenetic tree of subclade 4.3.1 were plotted using ggplot2. To investigate the phylogenetic structure of acute and gallbladder *S*. Typhi isolates from Nepal in the global context, a second maximum likelihood tree was inferred from a separate alignment of 23438 SNPs identified from 120 Nepali *S*. Typhi along with 1820 globally representative *S*. Typhi described previously [54]. A *S*. Paratyphi A isolate was included as an outgroup to root the tree. Support for this ML tree was assessed via 100 bootstrap replicates.

Pairwise genetic distances (the difference in the number of SNPs) within and between acute and gallbladder *S*. Typhi isolates were estimated from the SNP alignment using the ape (v4.1) and adegenet (v2.0.1) packages in R (v3.3.2). Pairwise SNP distances were extracted and plotted using the function *pairDistPlot* in the adegenet package. The Wilcoxon rank-sum test was used for testing the difference in the average pairwise SNP distances between groups.

The raw sequence data generated from this study are available in the European Nucleotide Archive (ENA) under the accession numbers described in S1 Table.

Supporting information

S1 Table. Gallbladder and acute *Salmonella* Typhi isolates and their associated metadata.
(XLS)

S1 Fig. Phylogenetic structure of acute and gallbladder *Salmonella* Typhi isolates from Nepal in the global context.
(TIF)

S2 Fig. Distribution of pairwise SNP distances within and between gallbladder (GB) and acute isolates belonging to subclades 4.3.1 (H58) and 3.3.0 (H1).
(TIF)

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References

1. Crump JA, Luby SP, Luby SP, Mintz ED, Mintz ED. The global burden of typhoid fever. Bull World Health Organ. 2004; 82: 346–53. PMID: 15298225
2. Schwartz E. Typhoid and Paratyphoid Fever. Trop Dis Travel. 2010; 366: 144–153.
3. Ledingham JCG. Mr N the milker, and Dr Koch ' s concept of the healthy carrier. Lancet. 1999; 353: 1354–1356. https://doi.org/10.1016/S0140-6736(98)11315-6 PMID: 10218549
4. Carrier of typhoid fevver-1912. i.
5. Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ, Minh HC, et al. Typhoid fever. N Engl J Med. 2002; 347: 1770–1782. https://doi.org/10.1056/NEJMra020201 PMID: 12456854
6. Gonzalez-Escobedo G, Marshall JM, Gunn JS. Chronic and acute infection of the gall bladder by Salmonella Typhi: understanding the carrier state. https://doi.org/10.1038/nrmicro2490 PMID: 21113180
7. Prouty AM, Schwesinger WH, Gunn JS. Biofilm formation and interaction with the surfaces of gallstones by Salmonella spp. Infect Immun. 2002; 70: 2640–2649. https://doi.org/10.1128/iai.70.5.2640-2649.2002 PMID: 11953406
8. Basnyat B, Baker S. Typhoid carriage in the gallbladder. The Lancet. 2015. p. 1074. https://doi.org/10.1016/S1474-4422(15)00256-2 PMID: 26466776
9. Schiøler H, Christiansen ED, Høybye G, Rasmussen SN, Greibe J. Biliary calculi in chronic Salmonella carriers and healthy controls: a controlled study. Scand J Infect Dis. 1983; 15: 17–9. https://doi.org/10.3109/inf.1983.15.issue-1.04 PMID: 6405479
10. Marshall JM, Flechtner AD, La Perle KM, Gunn JS. Visualization of extracellular matrix components within sectioned Salmonella biofilms on the surface of human gallstones. PLoS One. 2014;9. https://doi.org/10.1371/journal.pone.0089243 PMID: 24551241
11. Crawford RW, Rosales-Reyes R, Ramirez-Aguilar M d. l. L, Chapa-Azuela O, Alpuche-Aranda C, Gunn JS. Gallstones play a significant role in Salmonella spp. gallbladder colonization and carriage. Proc Natl Acad Sci. 2010; 107: 4353–4358. https://doi.org/10.1073/pnas.1000862107 PMID: 20176950
12. Gonzalez-Escobedo G, Gunn JS. Gallbladder epithelium as a niche for chronic salmonella carriage. Infect Immun. 2013; 81: 2920–2930. https://doi.org/10.1128/IAI.00258-13 PMID: 23732169
13. Dongol S, Thompson CN, Clare S, Nga TVT, Duy PT, Karkey A, et al. The Microbiological and Clinical Characteristics of Invasive Salmonella in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal. PLoS One. 2012;7. https://doi.org/10.1371/journal.pone.0047342 PMID: 23077595
14. Levine MM, Black RE, Lanata C. Precise estimation of the numbers of chronic carriers of Salmonella typhi in Santiago, Chile, an endemic area. J Infect Dis. 1982; 146: 724–6. https://doi.org/10.1093/infdis/146.6.724 PMID: 7142746

15. Mian MF, Pek EA, Chenoweth MJ, Coombes BK, Ashkar AA. Humanized mice for Salmonella typhi infection: new tools for an old problem. Virulence. 2011; 2: 248–252. https://doi.org/10.4161/viru.2.3.16133 PMID: 21623167

16. Mateen MA, Saleem S, Rao PC, Reddy PS, Reddy DN. Ultrasound in the diagnosis of Typhoid fever. Indian J Pediatr. 2006; 73: 681–685. https://doi.org/10.1007/BF02898444 PMID: 16936362

17. Mathur R, Oh H, Zhang D, Park SG, Seo J, Koblansky A, et al. A mouse model of salmonella typhi infection. Cell. 2012; 151: 590–602. https://doi.org/10.1016/j.cell.2012.08.042 PMID: 23101627

18. Crawford RW, Gibson DL, Kay WW, Gunn JS. Identification of a bile-induced exopolysaccharide required for salmonella biofilm formation on gallstone surfaces. Infect Immun. 2008; 76: 5341–5349. https://doi.org/10.1128/IAI.00786-08 PMID: 18794278

19. Marshall JM, Flechtner AD, La Perle KM, Gunn JS, Heuser J. Visualization of Extracellular Matrix Components within Sectioned Salmonella Biofilms on the Surface of Human Gallstones. Bengoechea JA, editor. PLoS One. 2014; 9: e89243. https://doi.org/10.1371/journal.pone.0089243 PMID: 24551241

20. Yap KP, Gan HM, Teh CSJ, Baddam R, Chai LC, Kumar N, et al. Genome sequence and comparative pathogenomics analysis of a salmonella enterica serovar typhi strain associated with a typhoid carrier in Malaysia. Journal of Bacteriology. 2012. pp. 5970–5971. https://doi.org/10.1128/JB.01416-12 PMID: 23045488

21. Baddam R, Kumar N, Shaik S, Lankapalli AK, Ahmed N. Genome dynamics and evolution of Salmonella Typhi strains from the typhoid-endemic zones. Sci Rep. 2014;4. https://doi.org/10.1038/srep07457 PMID: 25504040

22. Ong SY, Pratap CB, Wan X, Hou S, Rahman AYA, Saito JA, et al. The Genomic Blueprint of Salmonella enterica subspecies enterica serovar Typhi P-stx-12. Stand Genomic Sci. 2013; 7: 483–496. https://doi.org/10.4056/sigs.3286690 PMID: 24019994

23. Baddam R, Kumar N, Shaik S, Suma T, Ngoi ST, Thong K-L, et al. Genome sequencing and analysis of Salmonella enterica serovar Typhi strain CR0063 representing a carrier individual during an outbreak of typhoid fever in Kelantan, Malaysia. Gut Pathog. 2012; 4: 20. https://doi.org/10.1186/1757-4749-4-20 PMID: 23234298

24. Koirala S, Basnyat B, Arjyal A, Shilpakar O, Shrestha K, Shrestha R, et al. Gatifloxacin versus ofloxacin for the treatment of uncomplicated enteric fever in Nepal: an open-label, randomized, controlled trial. PLoS Negl Trop Dis. 2013; 7: e2523. https://doi.org/10.1371/journal.pntd.0002523 PMID: 24282626

25. Virlogeux I, Waxin H, Ecobichon C, Popoff MY. Role of the viaB locus in synthesis, transport and expression of Salmonella typhi Vi antigen. Microbiology. 1995; 141: 3039–3047. https://doi.org/10.1099/13500872-141-12-3039 PMID: 8574397

26. Steinsiek S, Bettenbrock K. Glucose transport in Escherichia coli mutant strains with defects in sugar transport systems. J Bacteriol. 2012; 194: 5897–908. https://doi.org/10.1128/JB.01502-12 PMID: 22923596

27. Hatta M, Pastoor R, Scheelbeek PFD, Sultan AR, Dwiyanti R, Labeda I, et al. Multi-locus variable-number tandem repeat profiling of Salmonella enterica serovar Typhi isolates from blood cultures and gallbladder specimens from Makassar, South-Sulawesi, Indonesia. PLoS One. 2011;6. https://doi.org/10.1371/journal.pone.0024983 PMID: 21949819

28. Baker S, Holt K, Van De Vosse E, Roumagnac P, Whitehead S, King E, et al. High-throughput genotyping of Salmonella enterica serovar Typhi allowing geographical assignment of haplotypes and pathotypes within an urban district of Jakarta, Indonesia. J Clin Microbiol. 2008; 46: 1741–1746. https://doi.org/10.1128/JCM.02249-07 PMID: 18322069

29. Holt KE, Baker S, Dongol S, Basnyat B, Adhikari N, Thorson S, et al. High-throughput bacterial SNP typing identifies distinct clusters of Salmonella Typhi causing typhoid in Nepalese children. BMC Infect Dis. 2010; 10: 144. https://doi.org/10.1186/1471-2334-10-144 PMID: 20509974

30. Baker S, Holt KE, Clements ACA, 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: 110008. https://doi.org/10.1098/rsob.110008 PMID: 22645647

31. Holt KE, Dolecek C, Chau TT, Duy PT, La TTP, Hoang NVM, et al. Temporal Fluctuation of Multidrug Resistant Salmonella Typhi Haplotypes in the Mekong River Delta Region of Vietnam. PLoS Negl Trop Dis. 2011; 5: e929. https://doi.org/10.1371/journal.pntd.0000929 PMID: 21245916

32. Holt KE, Thomson NR, Wain J, Langridge GC, Hasan R, Bhutta Z a, et al. Pseudogene accumulation in the evolutionary histories of Salmonella enterica serovars Paratyphi A and Typhi. BMC Genomics. 2009; 10: 36. https://doi.org/10.1186/1471-2164-10-36 PMID: 19159446

33. McClelland M, Sanderson KE, Clifton SW, Latreille P, Porwollik S, Sabo A, et al. Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of Salmonella enterica that cause typhoid. Nat Genet. 2004; 36: 1268–1274. https://doi.org/10.1038/ng1470 PMID: 15531882

34. Felix A. Detection of chronic typhoid carriers by agglutination tests. Lancet. 1938; 232: 738–741. https://doi.org/10.1016/S0140-6736(00)40980-3

35. Nolan CM, White PC, Feeley JC, Brown SL, Hambie EA, Wong KH. Vi serology in the detection of typhoid carriers. Lancet. 1981; 317: 583–585. https://doi.org/10.1016/S0140-6736(81)92033-X

36. House D, Ho VA, Diep TS, Chinh NT, Bay P V., Vinh H, et al. Antibodies to the Vi capsule of Salmonella Typhi in the serum of typhoid patients and healthy control subjects from a typhoid endemic region. J Infect Dev Ctries. 2008; 2: 308–312. https://doi.org/10.3855/jidc.227 PMID: 19741294

37. Gupta A, My Thanh NT, Olsen SJ, Sivapalasingam S, My Trinh TT, Phuong Lan NT, et al. Evaluation of community-based serologic screening for identification of chronic Salmonella Typhi carriers in Vietnam. Int J Infect Dis. 2006; 10: 309–314. https://doi.org/10.1016/j.ijid.2005.06.005 PMID: 16412678

38. Lanata CF, Ristori C, Jimenez L, Garcia J, Levine MM, Black RE, et al. Vi Serology in detection of chronic salmonella typhi carriers in an endemic area. Lancet. 1983; 322: 441–443. https://doi.org/10.1016/S0140-6736(83)90401-4

39. Coynault C, Robbe-Saule V, Norel F. Virulence and vaccine potential of Salmonella typhimurium mutants deficient in the expression of the RpoS (σ(S)) regulon. Mol Microbiol. 1996; 22: 149–160. https://doi.org/10.1111/j.1365-2958.1996.tb02664.x PMID: 8899717

40. Nickerson CA, Curtiss R. Role of sigma factor RpoS in initial stages of Salmonella typhimurium infection. Infect Immun. 1997; 65: 1814–1823. https://doi.org/10.1128/IAI.65.5.1814-1823.1997 PMID: 9125566

41. Santander J, Wanda S-Y, Nickerson CA, Curtiss R, III. Role of RpoS in fine-tuning the synthesis of Vi capsular polysaccharide in Salmonella enterica serotype Typhi. Infect Immun. 2007; 75: 1382–92. https://doi.org/10.1128/IAI.00888-06 PMID: 17178790

42. Prouty AM, Van Velkinburgh JC, Gunn JS. Salmonella enterica serovar typhimurium resistance to bile: Identification and characterization of the tolQRA cluster. J Bacteriol. 2002; 184: 1270–1276. https://doi.org/10.1128/jb.184.5.1270-1276.2002 PMID: 11844755

43. Gunn JS. Mechanisms of bacterial resistance and response to bile. Microbes Infect. 2000; 2: 907–913. https://doi.org/10.1016/s1286-4579(00)00392-0 PMID: 10962274

44. Kim SH, Wei CI. Molecular characterization of biofilm formation and attachment of Salmonella enterica serovar typhimurium DT104 on food contact surfaces. J Food Prot. 2009; 72: 1841–1847. https://doi.org/10.4315/0362-028x-72.9.1841 PMID: 19777884

45. CLSI. Performance Standards for Antimicrobial Susceptibility Testing. Informational Supplement. CLSI Document M100-S25. 2014. https://doi.org/10.1186/1476-0711-9-23

46. Thanh DP, Karkey A, Dongol S, Thi NH, Thompson CN, Rabaa MA, et al. A novel ciprofloxacin-resistant subclade of h58. Salmonella typhi is associated with fluoroquinolone treatment failure. Elife. 2016;5. https://doi.org/10.7554/eLife.14003 PMID: 26974227

47. Roumagnac P, Weill F-X, Dolecek C, Baker S, Brisse S, Chinh NT, et al. Evolutionary history of Salmonella typhi. Science. 2006; 314: 1301–1304. https://doi.org/10.1126/science.1134933 PMID: 17124322

48. Holt KE, Parkhill J, Mazzoni CJ, Roumagnac P, Weill F-X, Goodhead I, et al. High-throughput sequencing provides insights into genome variation and evolution in Salmonella Typhi. Nat Genet. 2008; 40: 987–993. https://doi.org/10.1038/ng.195 PMID: 18660809

49. Wong VK, Baker S, Connor TR, Pickard D, Page AJ, Dave J, et al. An extended genotyping framework for Salmonella enterica serovar Typhi, the cause of human typhoid. Nat Commun. 2016; 7: 12827. https://doi.org/10.1038/ncomms12827 PMID: 27703135

50. 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: 848–852. https://doi.org/10.1038/35101607 PMID: 11677608

51. Hunt M, Mather AE, Sánchez-Busó L, Page AJ, Parkhill J, Keane JA, et al. ARIBA: rapid antimicrobial resistance genotyping directly from sequencing reads. bioRxiv. 2017; 1–21. https://doi.org/10.1101/118000

52. Zankari E, Hasman H, Cosentino S, Vestergaard M, Rasmussen S, Lund O, et al. Identification of acquired antimicrobial resistance genes. J Antimicrob Chemother. 2012; 67: 2640–2644. https://doi.org/10.1093/jac/dks261 PMID: 22782487

53. Carattoli A, Zankari E, Garciá-Fernández A, Larsen MV, Lund O, Villa L, et al. In Silico detection and typing of plasmids using plasmidfinder and plasmid multilocus sequence typing. Antimicrob Agents Chemother. 2014; 58: 3895–3903. https://doi.org/10.1128/AAC.02412-14 PMID: 24777092

54. 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: 632–9. https://doi.org/10.1038/ng.3281 PMID: 25961941