THE MOLECULAR EPIDEMIOLOGY OF PAEDIATRIC ENTERIC FEVER IN NEPAL BETWEEN 2008 AND 2016, AND SOUTH INDIA BETWEEN 2016 AND 2017

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ABSTRACT

Enteric fever continues to affect people living in endemic settings substantially causing at least 20 million cases of febrile illnesses every year with 1% mortality[1]. Over the last decade there has been considerable debate surrounding the burden and disease profile of enteric fever in the paediatric population. This is partially due to the similarity of the clinical features of paediatric enteric fever to most other febrile illness seen in endemic settings.

The treatment of enteric fever is proving to be a challenge with the emergence of antimicrobial resistant strains, particularly the 4.3.1 genotype (H58 haplotype), which is spreading rapidly. Multi-drug resistant (MDR) enteric fever, defined as infection with typhoidal *Salmonellae* that exhibit a combined resistance to ampicillin, cotrimoxazole and chloramphenicol emerged in the 1990s and was mediated primarily *via* the 4.3.1 genotype population through the horizontal acquisition of antimicrobial resistance determinants. Subsequently, fluoroquinolones became the drug of choice and the treatment of enteric fever following which fluoroquinolone resistance emerged, again through the 4.3.1 genotype. However, these antimicrobial trends may not be uniform across endemic regions and an understanding of these differing patterns as well the temporal changes in these trends are important in planning treatment strategies.

In the short and medium term work needs to be focused on achieving the greatest benefits from the prudent use of the recently WHO pre-qualified Vi-TT conjugate vaccine candidate. Whilst the long term vision towards eradicating enteric fever needs to focus on better understanding the underlying the biology of this disease through the use of contemporary technologies while simultaneously improving infrastructure for the provision of clean water, adequate sanitation and hygiene.

This thesis aims to age-characterise the disease burden of typhoid fever in endemic regions of South and South-East Asia as well as the African continent. Following this, the molecular

epidemiology of enteric fever in two endemic settings in the Indian subcontinent is delineated with a keen focus on the 4.3.1 genotype (H58) population as well the phenotypic patterns and molecular determinants of antimicrobial resistance. This thesis finally systematically reviews the global trends of antimicrobial resistance of *S.* Typhi isolates over time both from a phenotypic and molecular perspective.

The key results from this thesis include; the age stratification of disease occurrence in endemic regions which showed a substantial proportion occurs in the youngest age group in both Africa and Asia, the uniform dominance of 4.3.1 genotypes conferring a high degree of fluoroquinolone resistance contrary to earlier suggestions of younger children being more susceptible to a broader range of infecting genotypes, the dissimilarities between the antimicrobial resistance carrying capabilities of lineage I and lineage II strains of the 4.3.1 genotype as well as novel AMR gene arrangements and finally the temporal trends of AMR in *S.* Typhi which were different between Asia an Africa. The high prevalence of lineage I strains in Africa and South-East Asia in contrast to the high prevalence of lineage II strains in the Indian subcontinent reflect the antimicrobial selection pressures as well the evolutionary characteristics of circulating pathogen populations in these regions.

The implications of the data reported in this thesis have implications for treatment and prevention strategies. For the first time in history an opportunity has risen to effectively vaccinate the youngest age group (0-4 years) from typhoid through the Vi-TT conjugate vaccine. As highlighted in this thesis the youngest age group (0-4 years) have a high disease occurrence in endemic areas as seen in a meta-analysis as well as through data from two endemic sites collated and reported in this thesis. The older age groups also suffer greatly from this disease calling for a broad based vaccine strategy. The implications for treatment of enteric fever are however more relevant in the immediate term which suggest that in endemic regions in Asia, fluoroquinolones have little role to play in treatment protocols while

fluoroquinolones are still relevant in the African setting. In Asia, reverting back to former first-line antimicrobials might be an option but the possibility of re-emergence of widespread resistance to these currently sensitive antimicrobials is very high exemplifying the ability of *S.* Typhi to adapt to changing antimicrobial pressures.

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The work described in this thesis is the result of a large body of work as well as a testament to the collaborative potential and ability of the Oxford Vaccine Group. The collaborating groups included the Patan Academy of Health Sciences, Nepal; Holt lab, University of Melbourne; the Wellcome Trust Sanger Institute (WTSI) and the St. John's Research Institute, Bengaluru, India.

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DECLARATION

The research presented in this thesis is original work with any contributions not made by the author clearly mentioned in the text. This thesis includes data generated from studies lead by the Oxford Vaccine Group in collaboration with the Patan Academy of Health Sciences, the St. John's Research Institute, the University of Melbourne and the Wellcome Trust Sanger Institute.

Exact nature of work done:

- Systematic reviews and meta-analysis

I was independently involved in the conceptualisation and design, study searches and inclusion of articles, data extraction, risk of bias assessment, data synthesis and analysis, creation of forest plots and associated figures.

- Microbiology methods

I performed the disc diffusion assays for antimicrobial sensitivity on all isolates reported in this thesis as well the DNA extraction and quantification from the aforementioned isolates

- Molecular analysis

I independently the phylogenetic analysis, identification of molecular determinants of antimicrobial resistance and genotyping

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Britto C, Dyson ZA, Duchene S, *et al.* Laboratory and molecular surveillance of paediatric typhoidal Salmonella in Nepal: Antimicrobial resistance and implications for vaccine policy. *PLoS Neglected Tropical Disease* 2018; 12: e0006408.

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Thomas R, Puranik P, Kalal B, Britto C, Kamalesh S, Rego S, Shet A. Five-year analysis of rickettsial fevers in children in South India: Clinical manifestations and complications. J Infect Dev Ctries, 2016 Jun 30;10(6):657-61

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"No half-heartedness and no worldly fear must turn us aside from following the light unflinchingly"
J.R.R. Tolkien

Chapter 1

Introduction

The epidemiological triad of agent, host and environment best describes most infectious disease models. New insights into enteric fever continue to be inferred using this simple model[2]. Enteric fever is caused by *Salmonella enterica* subspecies *enterica* serovars Typhi and Paratyphi A & B, which are Gram-negative bacilli, genetically monomorphic and human restricted[3]. Pseudogene formation and the acquisition of *Salmonella* Pathogenicity islands (SPIs) were critical features in the evolutionary history of this organism and were responsible for the convergence of serovars Typhi and Paratyphi to exclusively infect human hosts[4,5]. This invasive bacterial infection has been inflicting human populations for thousands of years as evidenced by the organism being isolated from ancient remains dating back to 430 BC[6,7]. Over 26 million people (age standardized) had a positive blood culture for enteric fever in 2010[8] with a disproportionately high attack rate in children and young adults[9]. These data might still be an under-estimate as the current diagnostics used in identifying enteric fever

lack reliability[10]. The human challenge model has enhanced our understanding of disease pathogenesis and susceptibility[11,12]. However, very many factors are still ambiguous which cloud our understanding of the transmission, aspects of paediatric enteric fever, role of chronic carriers and the correlates of protection of the disease.

Like other diseases transmitted *via* the faecal-oral route this disease flourishes in an environment of over-crowding, poor sanitation and contaminated water. The endemic nature of disease in affected areas suggests a long cycle of transmission *via* water and a short cycle of transmission *via* food handled by those who shed the organism as a result of acute or chronic disease. Asymptomatic human carriers who carry this organism in their gall bladder chronically act as reservoirs and continue to spread enteric fever to contacts[13]. "Typhoid Mary" and "Mr N the milker" are two such infamous examples of enteric fever carriers[14,15]. Though enteric fever has been a menace for centuries and its epidemiological triad seems straightforward, this disease continues to perplex epidemiologists and clinicians alike and lingers as a scourge on the developing world. The disease was described in the 19th century and significant progress in its understanding ensued, including its eradication from the Western world by simple means of hand washing and provision of clean water[16]. The antibiotic era consolidated efforts to eradicate this disease but the lack of clear strategies and indiscriminate antimicrobial use lead to further problems, culminating in the emergence of multi-drug resistant (MDR) strains. Socio-economic tribulations in most endemic areas, with limited access to clean water coupled with the emergence of antibiotic resistant strains makes vaccination the most effective short-term strategy for containment of this disease. Since 1897 when Wright and Semple described a typhoid vaccine that conferred a "bacterial proofness" on their subjects[17], the profile and ingenuity of vaccines against typhoid has evolved to enhance efficacy while reducing adverse reactions. Although typhoid vaccines have been extremely useful throughout the 20th century, particularly for army recruits and travellers,

their use in endemic settings was limited due to a plethora of factors. One of the most important limitations of the widely available polysaccharide vaccines is its restricted use in young children[18] who bear the brunt of the burden of enteric fever in endemic areas. The novel conjugate vaccine candidates, in theory, overcome these inadequacies but further epidemiological information regarding the age stratified disease burden as well as the molecular epidemiology of circulating pathogen strains is required to inform vaccine strategy.

1.1. Agent

1.1.1. Nomenclature, taxonomy and evolution

The family *Enterobacteriaceae*, encompasses several genera of bacteria including human pathogens within *Salmonella, Shigella, Escherichia, Klebsiella, Proteus, Enterobacter* and *Yersina.* From an evolutionary perspective, *Salmonella* diverged from *Escherichia* approximately 100 million years ago[19] and continue to share about 70% of their genes which are 90% identifiable at the amino acid level[20]. The genus *Salmonella* has two main species, which are differentiated by their evolutionary acquisition of *Salmonella* Pathogenicity islands (SPIs)[5] (**Figure 1.1.**). Although both species, *Salmonella enterica* (*S. enterica*) and *Salmonella bongori* (*S. bongori*) possess SPI1 which is absent in *Escherichia*[5,21], SPI2 is only present in *S. enterica* suggesting that a divergence from *S. bongori* occurred after the acquisition of SPI1 but before SPI2.

The species *S. enterica* is more relevant, as this species contains subspecies and serovars which cause disease in humans and other warm blooded vertebrates[5] and is divided into six subspecies namely *enterica, salamae, arizonae, diarizonae, houtenae* and *indica*[22]. These six subspecies include over 2500 serovars or serotypes, which have evolved owing to variation in genes required for host specificity and antigen biosynthesis[5,23]. Serovars are defined by their somatic and flagella antigens designated as O and H respectively, and written with antigenic formulae as: O antigens; H antigens (phase 1, phase 2)[22]. This scheme was developed by

Kauffmann-White[23] and is maintained and regularly updated by the WHO Collaborating Centres[24].

Salmonella enterica subspecies *enterica* contain most of the disease causing serovars including Typhi and Paratyphi[23] which are correctly written beginning with a capital letter and unitalicised[22]. They are formally designated as *Salmonella enterica* subspecies *enterica* serovar Typhi and *Salmonella enterica* subspecies *enterica* serovars Paratyphi A, B and C, they are often shortened to the form *S. enterica* serovar Typhi/Paratyphi , *S.* Typhi or simply referred to by the serovar name, Typhi. For brevity in this thesis, *S. enterica* subspecies *enterica* serovars Typhi and Paratyphi will be represented as *S.* Typhi and *S.* Paratyphi respectively.

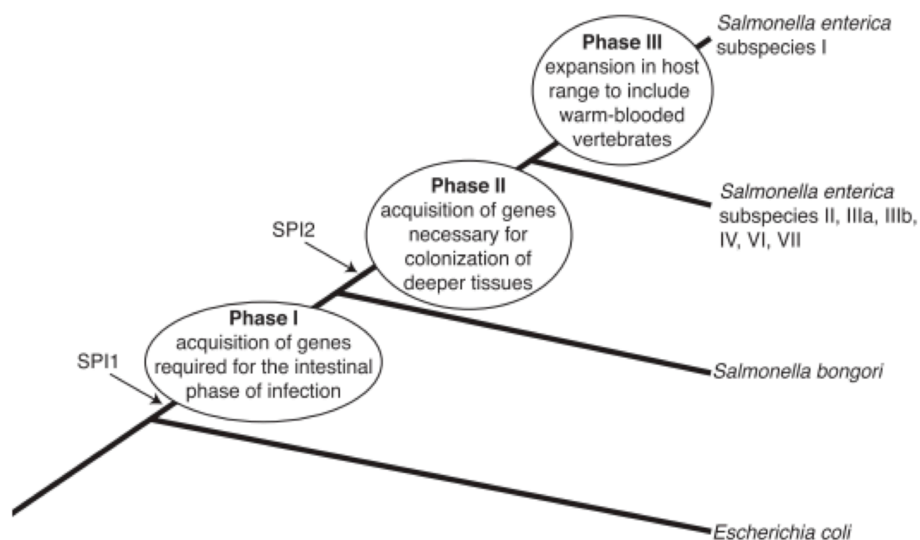


Figure 1.1: Evolutionary model of virulence in the genus *Salmonella* (Reproduced from Bäumler et al[5]). The acquisition of various pathogenicity islands corresponds to important evolutionary events in the *Enterobacteriaceae* family

1.1.2. Antigens and virulence factors

1.1.2.1. Surface structures and antigenic determinants

Like all Gram-negative bacteria, both *Salmonella enterica* serovars Typhi and Paratyphi possess a cell structure composed of an outer membrane, peptidoglycan layer (periplasm) and an inner membrane[25] **(Figure 1.2.)**. All three structural components have various pathogenic determinants, which are vital for the survival and virulence of the organism. Virulence proteins are encoded for by approximately 4% of the *Salmonella* genome[26].

The outer membrane (OM) is a highly dynamic lipid bilayer, which is protects the bacterial cell from the external environment and also serves as a semi-permeable structure. It contains the O-lipopolysaccharide (LPS) as one of its primary components in addition to a phospholipid bilayer and proteins (**Figure 1.2**). All the fatty acid chains present in LPS are saturated which significantly reduces the fluidity of the OM[27]. The lipopolysaccharide composition makes the LPS a unique entity as most other biological membranes are a glycerol-phospholipid structure[27]. This moiety is composed of three basic components: the outermost O-antigen polysaccharide region, which is in contact with the external environment; a core oligosaccharide region which is in turn connected to the third and an innermost hydrophobic lipid A region[27]. *In vitro* models suggests that hydrophobic probes diffuse poorly into the hydrophobic portion of LPS and permeate about 50 -100 times slower than compared with usual lipid bilayers[27]. The LPS thus serves as an efficient barrier against rapid penetration by lipophilic (hydrophobic) antibiotics and chemotherapeutic agents. The LPS has been used as a candidate in potential diagnostic tests for enteric fever as well as in vaccine technologies[28]. The surface LPS in encoded by the *wba* cluster of genes as well as genes (*wzx, wzy, wzz*), which code for the translocation across the inner membrane and the final polymerization into an oligosaccharide chain[29]. The *wba* cluster has a mosaic structure,

which is presumably the reason for the diversity in seen in over 50 *Salmonella* LPS antigens identified[30].

An O-antigen exopolysaccharide (extracellular polysaccharide), made up of O-units similar to those present in O-antigen LPS is also expressed by *Salmonella*[31]. The synthesis of this is governed by genes *yihU -yshA and yihV –yihW*, which are outside the *wba* cluster[31]. This polysaccharide is implicated in the formation of biofilm on gallstones, which subsequently determines long-term asymptomatic carriage in mammalian hosts[32].

Some of the outer membrane proteins (OMPs) may be linked to the underlying peptidoglycan layer, they may be porin or non-porin proteins and fulfill imperative functions such as solute and nutritive translocation as well as signal transduction[33,34]. OMPs such as Lpp1, Lpp2 and OmpX have been implicated in virulence[35] and some of these OMPs have been considered as possible antigens for vaccine candidates[34].

Outer membrane vesicles (OMVs) are unique to Gram-negative organisms and are bilayered, subcellular, protein coated vesicles of a size of 20 to 250 nm in diameter and have similar lipid compositions as the outer membranes of their donor cells[25,36]. These vesicles are extruded from the surface of the Gram negative bacterial cell and engulf some part of the underlying periplasm. They are therefore composed of OMPs, LPS and other periplasmic constituents, akin to a *S.* Typhi/ Paratyphi bacterium, albeit on a smaller scale and are therefore potent stimulators of the host immune system on one hand and potential vaccine candidates on the other[25,34–36].

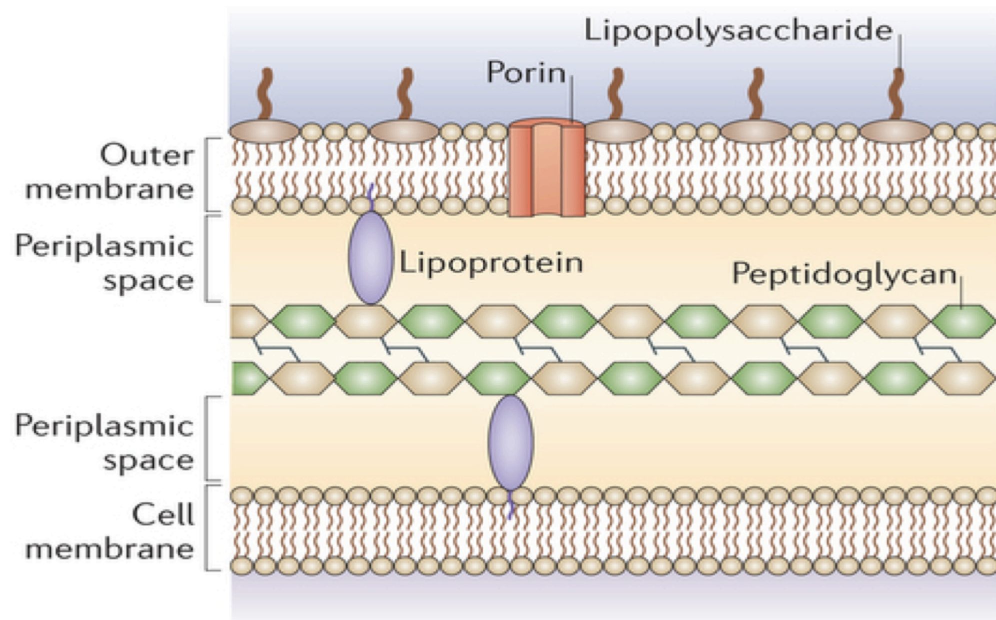


Figure 1.2: Structure of the Gram-Negative cell wall. (Reproduced from Brown et al[37]. Both *Salmonella enterica* serovars Typhi and Paratyphi possess a cell structure composed of an outer membrane, peptidoglycan layer (periplasm) and an inner membrane. All three structural components have various pathogenic determinants, which are vital for the survival and virulence of the organism.

Flagellae are highly specialized, energy efficient assemblies and serve as the prime locomotive structures. These structures are expressed on the cell surface of the *Salmonella enterica* serovars (but not *S. bongori*)[38]. A basal body lodged in the cell membrane which is attached to hook distally, which in turn is attached to polymerized units of the flagellin protein is what comprises its basic structure[39,40] (**Figure 1.3**). Numerous genes are involved in the expression of a flagellum[41], however two main genes *fliC* and *fljB* are expressed alternatively and constitute a process known as phase variation[42]. The phase switching occurs at a rate of $10^{-3} – 10^{-5}$ cell divisions[41,42] and is thought to be a mechanism of immune invasion as the flagella antigens are known to be potent inducers of the immune system and are even used in the Kauffmann-White serotyping scheme for *Salmonella*[23,43]. Each flagellum acts as a rotatory motor, propelling the bacterial cell using the electromotive gradient of protons or sodium ions across the cell's membrane as the energy source[43]. Interestingly, the generation of new

serovars are thought to be due the recombination between strains as well as between *fliC* and *fljB* resulting in variation in flagellin elements[38,42,44,45].

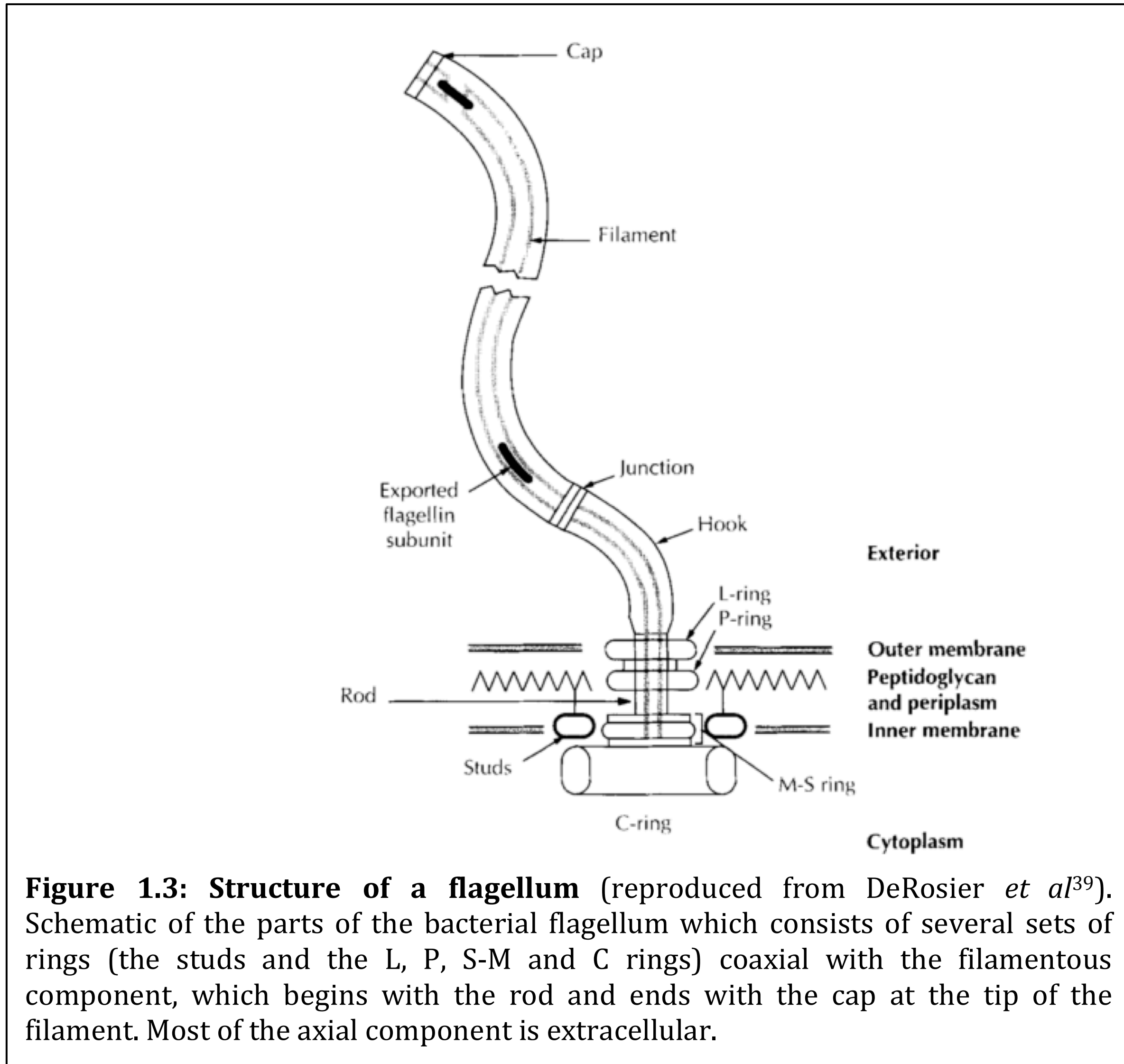


Figure 1.3: Structure of a flagellum (reproduced from DeRosier *et al*[39]). Schematic of the parts of the bacterial flagellum which consists of several sets of rings (the studs and the L, P, S-M and C rings) coaxial with the filamentous component, which begins with the rod and ends with the cap at the tip of the filament. Most of the axial component is extracellular.

Fimbriae are minute hair like appendages, emanating from the cell surface and are of prime importance in adhering to non-immune host cells[46,47]. Fimbral adhesins are assembled via three major gene regulatory pathways. The chaperone/usher dependent pathway is the most common and involves the biosynthesis of a chaperone that amalgamates fimbrial subunits as they enter the periplasmic space[47,48]. Once in the periplasmic space the chaperone bound complex is “ushered” across the outer membrane[46–50]. The other two pathways include a

nucleator-dependent assembly as well the pathway for type IV fimbriae[47,50]. *S.* Typhi isolates posses a unique combination of fimbrial operons which may be implicated in host specificity[50]. Variation in fimbrial genes dictates bacterial binding to host cells and has effects on bacterial colonization and pathogenicity[51]. Fimbrae might also be important from a diagnostic point of view *via* the putative fimbrial *staA* gene, particularly in differentiating acute typhoid from chronic carriers[52].

The Vi polysaccharide capsule is expressed by some wild type *S. enterica* serovars including *S.* Typhi but not *S.* Paratyphi[53]. The biosynthesis of the Vi capsule is governed by two well defined loci namely *viaA* and *viaB*[54]. These two loci are on separate regions on the chromosome and code for different capsular functions[54–56]. The *viaA* encoded genes can be identified on both capsulated and non-capsulated Salmonella strains whereas the *viaB* encoded genes are only found on the capsulated strains[55,56]. The *viaB* locus encode for the biosynthesis and export of the Vi antigen via the *tviA-tviE* and the *vexA-vexE* genes respectively[57] . A deletion in the *tviA* gene encoded within the *viaB* cluster can result in the loss of capsular expression in those strains previously expressing the capsule[54]. In vitro studies have shown that the level of Vi expression also changes in response to culture conditions, such as a change in sodium chloride[58]. The changes are regulated by a two component system of *OmpR* and *EnvZ* located on the *OmpB* operon[54,58]. Arguably, Vi is one of the most important antigens, as antibodies to it serve as serological marker of infection, but more importantly, the currently licensed parenteral typhoid vaccine and the novel conjugate vaccine both contain Vi as the antigenic stimulant[54,59,60]. The capsule is considered an important virulence factor and is known to be antiopsonic and antiphagocytic by increasing the level of resistance of the organism to oxidative killing[54].

1.1.2.2. *Salmonella* Pathogenicity islands (SPIs)

As the name suggests, these are identifiable chromosomal "islands" of virulence-associated genes within the *Salmonella* genome. An altered G+C content compared to the genome backbone, carriage of predictable virulence genes and a strong contribution to the organism's pathogenicity are some of the criteria used to designate SPIs[61]. These genes are an integral part of the virulence machinery and are regulated by a complex network of signals. These islands are intrinsically related to the evolutionary history of *Salmonella* as a genus (**Figure 1.1**) and provide insight into the pathogenesis, virulence and host restriction of various *Salmonella* infections.

Salmonella Pathogenicity Islands (SPIs) 1 and 2 are evolutionarily the most conserved and are probably the best described. Both SPIs 1 and 2 encode unique secretion systems that "inject" virulence associated effector proteins from the bacterial cell into the host cell[26]. These are designated as type III secretion systems (T3SS), which are of two types encoded by SPI1 and SPI2 respectively[26,62,63]. SPI1 encodes the T3SS while the bacterium comes in contact with epithelial cells in the host intestine initiating subsequent bacterial invasion[62,63]. This process is regulated via *hilA, hilC* and *hilD* in response to signals within the host intestine[64,65].

The T3SS system encoded by SPI2 found only in *S. enterica* subspecies is a more precisely controlled mechanism which occurs once the individual bacterium is within the *Salmonella*-containing vacuole (SVC) within the host cell[26]. The genes encoding the SPI2 T3SS are made up of two-component regulatory systems encompassing *OmpR-EnvZ* and SPI2 encoded *SsrA-B*[26].

SPI3 is 17-kb in size and varies in distribution and content among *Salmonella* subspecies indicating a multistep evolutionary acquisition and is thought to be responsible for survival within host macrophages[66]. SPI4 and SPI5 are found only within *S. enterica* subspecies *enterica* and are associated invasion and inflammation of the intestinal epithelium and is not

associated with systemic infections *per se*[67]. SPI6 and SPI10 encode certain fimbriae[68,69], SPI8 encodes two moieties that are toxic to other bacteria (bacteriocins) and SPI9 encodes a type 1 secretion system[68,69]. These SPIs are less conserved within the *Salmonella* species than SPIs 1-5[26,63,66,67,69].

In addition to being the largest pathogenicity island, SPI7 is vital for the expression of Vi antigen, *SopE* effector and type *IVB* pilli[70]. SPI7 is carried on most clinical isolates of *S*. Typhi and has been found on *S*. Paratyphi C, *Citrobacter freundii* and as well as on some serovar Dublin strains[70]. *S*. Typhi is thought to possess an unstable genome when compared to other serovars, which might be attributable to the acquisition of SPIs, particularly SPI7. Some clinical isolates loose SPI7 and subsequently demonstrate an enhanced invasiveness to epithelial cells[70]. The importance of the SPI7 in clinical manifestation of invasive Salmonellosis is therefore debatable, in addition to the aforementioned observations, *S*. Paratyphi A and B also cause enteric fever and do not possess SPI7[70]. Its important role in Vi expression assumes importance in understanding the host response to the Vi antigen, particularly since the majority of typhoid vaccines exploit this mechanism. However, this mechanism needs to be delineated further as it is known that Vi antibody is not uniformly protective and a correlate of protection has not been established yet.

SPIs 11-14 are present in a wide range of *S. enterica* serovars including Gallinarum and Choleraesuis. SPIs 16 and 17 were initially identified in the genome of *S*. Typhi but since been discovered in other serovars. The SPI15 has only been found in *S*. Typhi thus far[71]. The role of these SPIs are less well described but fulfill the criteria for pathogenicity islands[61].

1.1.2.3. Typhoid toxin

The typhoid toxin is one of the more recently discovered virulence factors and is thought to be mainly expressed by *Salmonella enterica* serovars Typhi and Paratyphi[72], although it has also been identified in the *bongori* species as well as the *arizonae* and *di arizonae* subspecies[73]. In

addition, it might provide insights into the host restriction of typhoidal *Salmonellae*[72,73]. The evolutionary basis for the acquisition of the typhoid toxin suggests that it has been horizontally acquired by different species and subspecies of *Salmonella* via multiple independent events[73].

The toxin consists of an A subunit and a B subunit indicating active and binding components respectively. Surprisingly, this type of toxin arrangement is usually characteristic of extracellular bacterial toxins[73]. This feature is thus peculiar to *S*. Typhi, which is primarily an intracellular pathogen and to compound our understanding further, it was found that the toxin is produced only intracellularly[72–74] and is activated only when all three components of its tripartite structure act synergistically in the extracellular environment. This mechanism is attributed to an evolutionary convergence of two separate toxins into the current tripartite structure, which is delivered extracellularly in an autocrine or paracrine fashion[73,74]. This tripartite exotoxin comprises of CdtB which is the cytolethal distending toxin B; PltA and PltB which are Pertussis like toxins A and B respectively[74]. Crystallographic studies of the toxin suggests that the five molecules PltB assemble with a molecule each of PltA and CdtB[75] forming a chimeric A_2B_5 complex[73,75]. The toxin is organized as a pyramidal structure with pentamer of PltB forming the base while PltA occupies the middle and CdtB forms the apex[73] (**Figure 1.4**)

The typhoid toxin primarily targets podocalyxin 1 and CD45 on epithelial cells as well as leucocytes and binds to specific glycans found on glycoproteins and gangliosides[73]. The binding of the toxin to gangliosides may indeed be the reason for neurological manifestations such as encephalopathy seen in some patients with enteric fever. The toxin exhibits a high affinity to human glycan residues and binds only weakly to other mammalian cells thus illustrating human host specificity[75]. The toxin is currently being studied in a human challenge model which is bound to enhance understanding of this virulence factor.

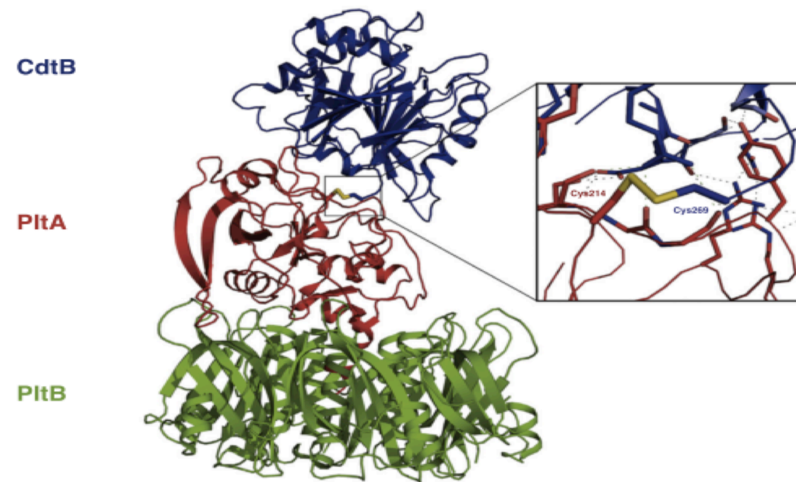


Figure 1.4: Structure of the typhoid toxin (adapted from Fowler *et al*[73]) where five molecules PltB assemble with a molecule each of PltA and CdtB forming a chimeric A_2B_5

1.1.3. Horizontal gene transfer

Genetic transfer between bacteria is an important function in the general virulence machinery. This process occurs *via* three mechanisms namely conjugation, transduction and transformation[76]. The DNA transferred from one bacterium to another can be integrated into the genome of the recipient by cytoplasmic proteins and enzymes[76]. This integration of foreign genetic material into the recipient chromosome is referred to as recombination[76]. Recombination is brought about by the *Rec* family of proteins, particularly *RecA* and *RecBCD.* These *Rec* proteins are mainly involved in DNA replication and repair but additionally serve as the initiators of horizontal gene transfer[76].

Transduction is the commonest form of gene transfer, which is carried out by bacteriophages (viruses). The phage occasionally incorporates bacterial DNA instead of phage DNA and then injects this into a new bacterium thus transferring DNA between bacteria[76]. In contrast, the vehicles for the conjugative process are plasmids, which are small, usually circular DNA molecules capable of replicating independent of the host chromosome. There are no known bacterial specific genes that regulate conjugation and the subsequent integration of the foreign DNA into the host chromosome is brought about by short transposable elements called insertion sequences[76]. In comparison to transduction and conjugation, transformation (also referred to as competence) refers to the uptake of genetic material from the environment. This process is governed by chromosomal genes and has great implications in genetic evolution of a bacterial species in various environments and may be important in conferring antibiotic resistance. The acquisition of novel genetic recombination comes at a cost and most often occurs at the expense of fitness[76].

Plasmids and conjugative resistance transposable elements can move between bacterial cells of the same or different genus. Plasmids are vehicles of unessential genes, which are ~100-400 kbp long in *S.* Typhi and Paratyphi A and are referred to as resistance plasmids if the

genes contained confer antimicrobial resistance[77]. Plasmids may be conjugative when they are capable of initiating conjugation of bacterial cells and thereby responsible for their own transmission, while mobilisable plasmids depend on conjugative plasmids to transfer their DNA from one bacterial cell to another[77]. The most commonly implicated antimicrobial resistance (AMR) plasmids found in *S*. Typhi are of the IncHI1 type[78–80]. The "Inc" refers to incompatibility which is defined as the inability of two related plasmids to be stably transmitted together[81]. Other incompatibility group plasmids such as IncY, IncN and Kpn3 are also known to be carried in a minority of *S*. Typhi populations[82].

Transposable elements are essentially mobile elements, which can be inserted into the DNA machinery of a cell thus changing its phenotypic expression[77]. Transposable elements that possess a resistance gene within its configuration are resistance transposons and confer phenotypic resistance to a corresponding antimicrobial. These elements possess the ability to "jump" from a plasmid into the bacterial genome as can be seen in *S*.Typhi isolates from South-Asia[83].

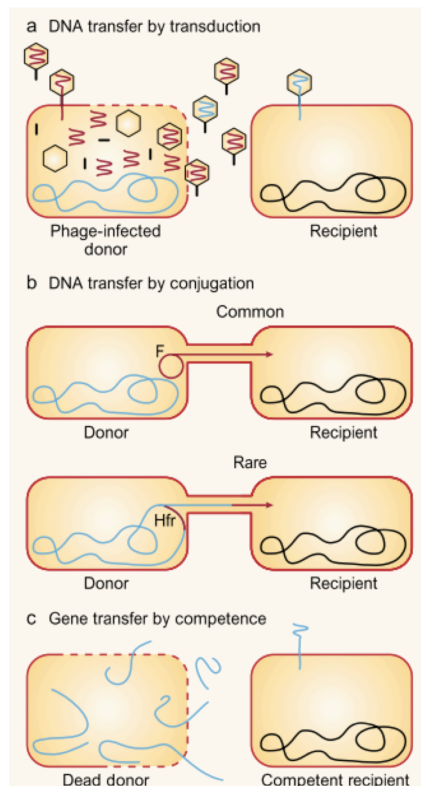


Figure 1.5: Types of horizontal gene transfer. (Adapted from Redfield, 1999[76]). a) Depicts the process of gene transfer *via* bacteriophages. b) Depicts the process of conjugation where gene transfer occurs *via* pilus formation and c) depicts gene transfer through competence which is basically the scavenging of genetic material.

1.1.3. Genome sequencing of *S.* Typhi and *S.* Paratyphi

The information garnered through sequencing pathogens continues to shed light on various features of host-pathogen interactions, transmission of disease, possible treatment strategies, novel diagnostic approaches and comprehensive preventive programmes. Mechanisms of antimicrobial resistance, in particular have been studied and understood to a large extent due to developmental strides in whole genome sequencing and high throughput technology. The possibility of studying pathogens in such high resolution offers a unique opportunity to enhance epidemiological understanding of the molecular structures of circulating strains as well as their evolution under certain environmental pressures.

1.1.3.1. Comparative genomics of *S.* Typhi and *S.* Paratyphi

The advent of high-throughput sequencing ushered in an era of high-resolution genomic information. The first genome of *S.* Typhi to be sequenced completely was the CT18 strain[84] which was isolated from the blood of a 9 year-old girl with typhoid in the Mekong Delta region of Vietnam[84]. Using this strain as the reference genome, comparisons can be made by mapping genomes of other strains to CT18. This approach allows insight into the expansion of globally dominant virulent strains as well as genetic relatedness within and between clonal populations. Insertion and deletions as well accumulation of pseudogenes and loss of function mutations can also be inferred *via* this approach.

Whole genome sequencing also stimulated new and improved techniques of genomic analysis, such as estimating gene content and expression with DNA microarray[85–87], variable number tandem repeats (VNTRs)[88–90] and identification of single nucleotide polymorphisms (SNPs)[83,91]. *S* Typhi and Paratyphi are genetically monomorphic pathogens[91,92] and are therefore not ideal candidates for multilocus enzyme electrophoresis[93] (MLEE) and multilocus sequence typing (MLST), where SNP analysis is most appropriate and informative in this regard[91].

1.1.3.2. Evolution of techniques adopted in studying the evolutionary history of *Salmonella* Typhi

Initial attempts to study the evolutionary history of *Salmonella* employed low resolution techniques such as MLEE[94] (multilocus enzyme electrophoresis), sequence information from housekeeping and virulence genes[95] (multilocus sequence typing) as well rRNA sequencing[96]. DNA microarray analysis was subsequently adopted[85], which like MLST, allowed for the distinction at the serovar level but did not possess the resolution required to differetiate within strains from a single serovar.

Roumagnac *et al*, through mutation discovery, investigated the evolutionary history of *S.*Typhi using 121 housekeeping genes, 50 genes encoding regulatory and pathogenic functions, as well as 29 pseudogenes[92].

These genes were derived from 105 globally representative strains of *S.* Typhi[92]. They identified 88 SNPs of which 4 represented recombination events with *S.* Typhimurium and 1 with *S.* Paratyphi[92]. H45 was probably the common ancestor and evolved via neutral genetic drift and the general appearance of the evolutionary tree suggests descent from H45 in multiple branches[92]. A new classification scheme (haplotype scheme) emerged in the process, with satisfactory resolution and which served as a helpful epidemiological tool. This scheme enhanced understanding of typhoid transmission and the pathogen response to antimicrobial pressure. A more recently developed genotyping scheme involved the use of 68 SNPs from almost 2000 globally representative *S.* Typhi isolates, obtained from over 60 countries around the world[97]. This genotyping technique offers a higher discriminatory index and is more informative than the haplotype scheme making it a supreme tool for epidemiological purposes.

1.1.3.3. Methods of studying the molecular structure of *S.* Typhi and Paratyphi populations

The population structure of *S.* Typhi is constantly subject to selective pressures. These pressures could be attributed to human host factors including gut mucosal immunity, antimicrobial use, vaccine implementation as well as environmental and ecological factors such as improvements in water and sanitation[98]. The phylodynamics of *S.* Typhi population structures have revealed important insights into the development of virulence, antigenic variation, drug resistance and fitness. In order to better understand these vital mechanisms a great amount of resolution is required which has resulted in rapid development of phylogenetic techniques and methods.

Phage typing, utilizing over 100 WHO approved phage types[93], was formerly the classical test employed in grouping strains epidemiologically. It however failed to adequately discriminate between strains from geographically confined locations and did not provide information on direction of transmission[99]. While the acquisition of lysogenic phage confounded analysis, this method was technically difficult and could only be performed by reference laboratories[99,100]. Subsequently, insertion sequence typing via IS200 was a popular molecular typing technique. IS200, a 707-bp to 710-bp sequence, is widely distributed among conserved loci on the chromosomes of most *Salmonella* serotypes and is rarely translocated[101]. This technique was however, low in resolution and required expertise to perform[99]. Pulse field gel electrophoresis (PFGE) was technically easier to perform and allowed for a higher resolution when studying the differences within and between *Salmonella* populations. The level of discrimination was higher than that of phage typing and was fairly reliable in establishing clonal relationships among strains implicated in epidemics[100]. Ribosomal endonuclease analysis and ribosomal RNA gene restriction patterns (ribotyping) provided enhanced resolution[93]. Although the discriminatory indices of PFGE and ribotyping were comparable[99], cluster analysis of ribotyping came with the possibility of being subject to erroneous interpretation in the event of even a single genetic change. This occurred due to the relatively small number of bands generated during the process of ribotyping. A similar issue was not encountered with PFGE as a larger number of bands are produced which allowed for a more accurate cluster analysis[99]. This technique also required the preparation of rRNA probes, Southern blots as well as hybridization with radioactive or non-radioactive approaches which limited its use in laboratories without such facilities[100].

Sanger sequenced the complete genome of bacteriophage phi X174[102] in the late 1970's. Since then, new and improved techniques of pathogen genome sequencing have been developed using various platforms. Next generation sequencing (NGS) or high throughput sequencing

may either be in the form of sequencing by ligation (SOLiD, Complete Genomics) or sequencing by synthesis (Illumina, Qiagen, 454, Ion Torrent)[103]. The aforementioned platforms are all short read platforms (**Figure 1.6, Table 1.1**) and have been extremely useful in bringing down the cost and time of sequencing. A large number of short reads can be sequenced in a single run. Using flurophore labeled nucleotides and DNA polymerases, Illumina platforms offer an overall accuracy rate of >99.5%[104]. These platforms dominate the market share of NGS[103] and have been widely used to better understand *Salmonella* population structures with greater resolution. More recently, a new classification scheme has been developed using paired end Illumina reads from 1930 *S.* Typhi isolates, obtained from over 60 countries around the world[97]. Though short read sequencing has been a vital tool in enhancing genomic understanding, a few errors are encountered due to long repetitive elements, copy number alterations and structural variations within genomes[105–107]. These issues can be over come by long read sequencing which may be in the form of real time sequencing (Pacific Biosciences, Oxford Nanopore technologies) or synthetic long-read sequencing (10X Genomics)[103]. Long read sequencing techniques are however more expensive and computationally longer restricting its use to identifying novel genomic signatures or markers within a genome[103].

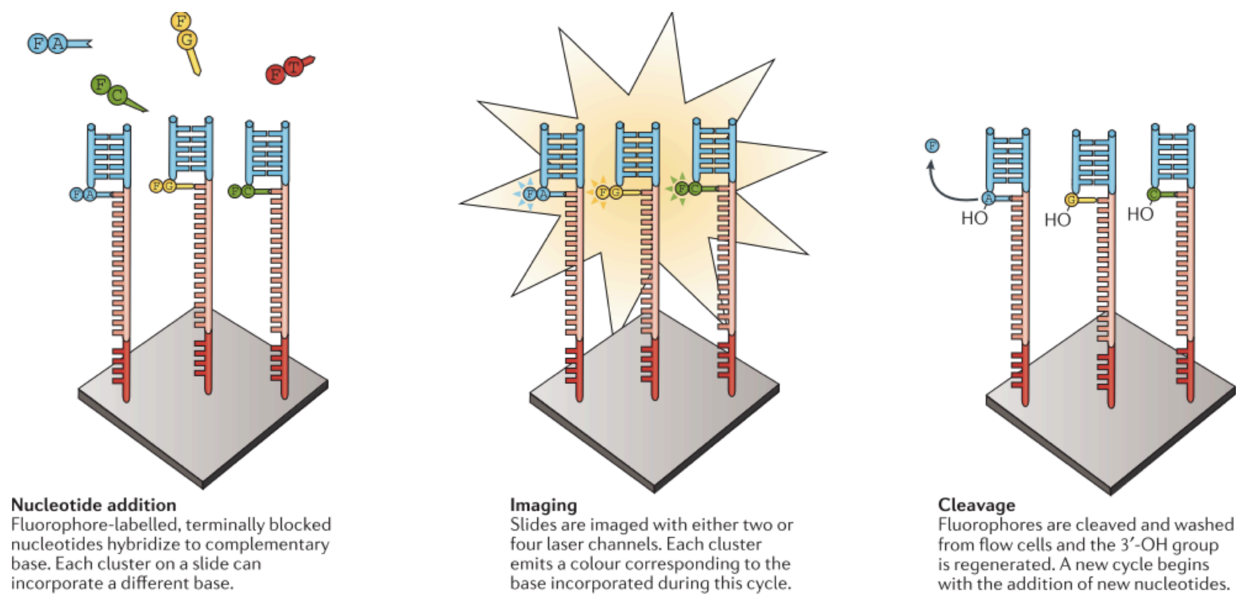


Figure 1.6: Illumina sequencing steps (reproduced from Goodwin *et al*[103]). A large number of short reads can be sequenced in a single run. Using flurophore labeled nucleotides (Step 1) and DNA polymerases, Illumina platforms offer an overall accuracy rate of >99.5%.

Table 1.1: Types of NGS platforms

Short Read Sequencing	
Sequencing by ligation	**Sequencing by synthesis**
SOLiD	Illumina
Complete Genomics	Qiagen
	454
	Ion Torrent
Long Read Sequencing	
Real time sequencing	**Synthetic long-read sequencing**
Pacific Biosciences	10X Genomics
Oxford Nanopore	

1.2. Host

1.2.1. Host Susceptibility

S. Typhi and Paratyphi must pass through the acidic environment in the stomach prior to gaining access to the intestinal lumen. The susceptibility to infection is inversely related to gastric acid secretion[108,109] and this finding was supported by observations from the human challenge model. In comparison to historical controls, the challenge dose required to establish infection in human volunteers was lower when sodium bicarbonate was co-administered with the challenge strain[110,111].

Host susceptibility at the genomic level, although incompletely delineated, may have an HLA basis. Genomic data from South and South-East Asia have identified variation at *HLA-DRB1* to be strongly associated with resistance to enteric fever.[112] Binding and entry of *S*. Typhi into intestinal epithelial cells in vitro is mediated by interaction between the bacterium and the CFTR protein and variation at this locus may contribute to host susceptibility to enteric fever.[113,114] There is currently no adequate consensus on the duration and extent of immunity conferred by a single episode of infection with *S.* Typhi and Paratyphi. Evidence from endemic settings suggests that serum bactericidal antibodies to *S*. Typhi increase with age owing to natural exposure possibly *via* contaminated ground water.[115] Limited insight exists, with regard to protection following a single episode of infection[116–118] with modelling data implying that multiple episodes of typhoid fever - predicted to be approximately 3 to 5 infection episodes - are required to confer functional immunity against future typhoid infection.[119]

1.2.2. Historical perspective of paediatric enteric fever

Historically, it was once thought that children were rarely afflicted with enteric fever and when contracted only incurred a mild infection[120]. Over time, this erroneous assumption was

challenged as it became evident that children and adolescents contributed significantly to enteric fever burden particularly in endemic regions.

The difference in opinion of paediatric enteric is one that goes back over half a century. Enteric fever was said to be infrequent below the age of 3 years[120–123]. Around the same period data highlighting the burden of disease in infants and preschool children surfaced[124,125]. In the recent past, robust data has emerged underlining the burden of disease in preschool children[126–131]. It has been suggested that the rates of enteric fever were once thought to be low due to the paucity of active surveillance, but recent data from active surveillance sites in Bangladesh, India and Indonesia reveal that the incidence rate of disease burden in the under 5 age group is higher than in other age groups[132,133]. A recent review further highlights the case fatality rate of children under 5 years of age is the highest[9]

For over a decade we have concentrated our immunization efforts towards school vaccination campaigns. This was in part due to the fact that the licensed vaccines were neither potent nor safe in younger children with the Vi polysaccharide vaccine incapable of inducing a strong antibody response below 2 years of age and the oral Ty21a vaccine available for use in only those over 5 years of age. The burden of enteric fever in younger children has recently been an area of debate, as data from endemic settings do not uniformly show a significant burden in young infants. We have long been awaiting a potent and safe vaccine for children under the age of 2 years. The Vi-TT conjugate vaccine was prequalified by the WHO recently and recommended by the WHO Strategic Advisory Group of Experts (SAGE) but the immunization strategy and target population need to be established in an evidence-based manner.

1.2.3. Clinical manifestations and complications of paediatric enteric fever

The clinical features of paediatric enteric fever overlap with a variety of infective etiologies in endemic settings[9,134] making clinical diagnosis problematic. Further, in blood culture

confirmed cases in children[135] rates of prior clinical suspicion of enteric fever may be as low as 4% at initial presentation. Clinical features, complications and outcomes differ between adults and children; even among children, differences exist between infants and older children as well as between children in Africa and Asia[9]. A recent systematic review of clinical and laboratory features of enteric fever, indicate that the risk of mortality from enteric fever is four times higher in children below the age of 5 years when compared with those over the age of 5 years[9].

1.2.3.1. Generalized systemic features

A typical febrile response occurs between 5 and 15 days after exposure to the organism as demonstrated in the challenge model in adult subjects[136]. A recently published systematic review reported that fever is a consistent feature of paediatric enteric fever, in 97-100% of cases[9], and often is the sole manifestation, not uncommonly presenting as a fever of unknown origin (FUO)[137] with inconclusive laboratory tests. Conversely, younger children with enteric fever may sometimes present with hypothermia[138]. The widespread and poorly regulated use of antibiotics and antipyretics in low- and middle-income settings (LMIC) settings not only confounds the clinical picture but also serves for the origin and propagation of antimicrobial resistant strains of *Salmonella* and other Gram-negative organisms. If untreated, the febrile response reaches its peak in the second week of illness in a characteristic "step ladder" pattern[139]. Chills and rigors are 4 times more common in adults than in children[9]. Relative bradycardia, a classical finding in enteric fever is identifiable in 11-30% of children[140–144] and appears to be 15 times more likely to occur in African children than the children in Asia[9].

Rose spots, mainly seen on the limbs and trunk of children may be identified in a narrow window, usually between day 5 and 7 of disease during acute enteric fever, however, the skin color of children in endemic settings and co-existence of other common skin pathologies makes it difficult to appreciate this finding[9]. Azmatullah's review of clinical features in

enteric fever reports a coated tongue in up-to 85% of children with enteric fever which is 3.5 times more common in children than adults[9].

1.2.3.2. Gastrointestinal features

Despite being the portal of entry, the manifestations of gastrointestinal (GI) infection in acute enteric fever are variable in children. Diarrhoea is seen over 2.5 times more commonly in enteric fever affected infants than in older children and adults[9,125,134,138]. A smaller proportion of children may have constipation, with some evidence indicating an association with disease relapse. This observation was mainly seen in children infected with MDR strains, according to data from a single centre study over a 15 year period[145]. GI bleeding and perforation, though rare have been known to occur in children and are 9 times higher in hospital based studies than community based settings[9] which possibly reflects a sampling bias of severe cases being referred to hospital from the community. Predictors of death due to perforation in children include older age, high temperature, post-operative anastomotic leak and faecal fistula[146,147]. A robust immune response coupled with well-primed Peyer's patches, are main requirements for these complications which increase with the increasing age of the child and is possibly why severe complications are seldom seen in the younger age groups[125,146,148]. Paralytic ileus is more commonly observed in infants and young children[149–151] being 7 times more likely in African children than their Asian counterparts according to Azmatullah et al[9]. Abdominal pain and nausea, which are commonly seen in adults are also seen in older children but are difficult to determine in young children.

1.2.3.3. Neurological features

The manifestation of seizures in enteric fever is more common in children than adults[125]. In susceptible children between 6 months and 5 years of age[152], the high temperatures associated with enteric fever could induce febrile seizures. The occurrence of febrile seizures may account for the higher seizure rate described in children. It is not clear whether *S.* Typhi has a

direct effect on the central nervous system (CNS) as the bacteria are seldom isolated from cerebrospinal fluid during lumbar puncture[153]. Nevertheless, the toxin released by the bacteria may elicit cortical irritation. The typhoid toxin binds exclusively to mammalian cell membranes, made up of glycoproteins containing Neu5Ac-terminated glycans[75], which are also found in gangliosides that are components of the neuronal cell membrane in the brain[154]. The toxin could therefore cause membrane depolarization by presumably interfering with the voltage gated sodium channels. In addition, a distinct frontal intermittent rhythmic delta activity (FIRDA) pattern on electroencephalogram (EEG) has been reported in *Salmonella* encephalopathy[155] which has been associated with the manifestation of seizures[156]. However, further work needs to be done to delineate the mechanism of typhoid toxin induced seizures at the cell membrane level. Secondary causes such as hyponatremia and hypoglycemia due to salt and water loss from the gut may also be contributing factors. Other neurologic complications, rarely encountered[134] but predominantly described in the paediatric age group include acute cerebellar ataxia, sinus thrombosis, meningism, cerebritis, pseudo tumour cerebri, encephalopathy, brain abscesses and Gullian-Barre syndrome[157–174]. Neuropsychiatric changes, delirium, insomnia and coma are also described in children but are more frequent in adults[125].

1.2.3.4. Hepatosplenic features

Tender hepatomegaly and splenomegaly are seen in up to 85% and 90% of paediatric enteric fever cases respectively[141,175]. Typhoid hepatitis or "hepatitis typhosa" is more frequently seen in younger children but assumes importance as it mimics acute viral hepatitis in the tropics and is likely to either be immunologically mediated or due to the direct effects of the typhoid toxin on the hepatocytes[176]. Acalculous cholecystitis has also been described in brief reports and occurs predominantly in younger children[9,149–151]. Hepatic and splenic abscesses with a subsequent splenic abscess rupture are more commonly encountered in younger children, the

immunosuppressed and those with haemoglobinopathies[151]. Splenic rupture, a devastating complication, though uncommon in children, is particularly associated with MDR infection,[177,178] although it is not clear whether this occurs due to treatment failure or virulence of the pathogen.

1.2.3.5. Cardiopulmonary features

Case reports and expert reviews describe cardiac complications such as myocarditis, endocarditis, pericarditis and pericardial effusion which are more common in older children and adults with risk factors such as congenital heart disease, rheumatic heart disease and valvular defects[151]. Respiratory symptoms may dominate the clinical picture of paediatric enteric fever with cough being the most common manifestation seen in up to 72% of cases[179]. A clinical picture of broncho-pneumonia, one of the respiratory complications of enteric fever, also occurs occasionally[149] and is twice as common in children than in adults[9]. A clinical picture of reactive airway disease with auscultatory rhonchi and occasional crepitations may be noted in younger children[144]. Risk factors for complications like pleural effusion, empyema and bronchopleural fistulas[151] though seldom reported in children may occur in those with previous respiratory infections, sickle cell anaemia and immunosuppression[151].

1.2.3.6. Haematological features

The transient pancytopenia seen in acute illness, may in part, be explained by seeding of *Salmonella* to the bone marrow[134]. A systematic review suggests that during an episode of enteric fever, children in Africa have a 5 times greater risk of severe anemia and a 15 times greater risk of thrombocytopenia than children in Asia[9]. These findings could be confounded by the high rates of malaria in Africa, while further evidence is required to confirm whether *S.*Typhi could independently have this effect and to what extent host genetics play a role. As a facultative intracellular Gram-negative organism, *Salmonella* induces apoptosis in the

macrophages that it infects contributing to the leucopenia[180]. Eosinopenia in particular is a significant indicator of disease severity[131] which may be seen in up to 70% of children with enteric fever and has been well documented in adult patients as well as in the human challenge model[136]. On the other hand and contrary to the leucopenia seen in adults, a peculiar finding in children is the relatively common occurrence of leucocytosis or the presence of a leucocyte count within the normal range[9,17]. In fact, leucocytosis is almost 3 times more likely to occur in children than in adults, while children under the age of 5 years are over 4 times more likely to have an elevated leucocyte count than older children. Butler observed a strong association of a leukemoid response with shigellosis[181] which was possibly induced by the lipopolysaccharide (LPS)[182] present in Gram-negative bacilli which might also be the case in enteric fever. Rare haematologic complications reported in enteric fever include disseminated intravascular coagulation (DIC), haemophagocytosis, bone marrow suppression and bone marrow granulomata all of which are more commonly described in adults than children[9,144,151].

1.2.3.7. Skeletal features

Skeletal manifestations in the form of *Salmonella* osteomyelitis and *Salmonella* arthritis are rare in haematologically normal children. However, non-typhoidal strains are more commonly implicated in *Salmonella* bone and joint infections, with the most commonly associated risk factor being sickle cell disease[151].

1.3. ENVIRONMENT AND TRANSMISSION

Social determinants of health play a key role in the endemicity of enteric fever, which thrives in settings of poverty and overcrowding and thus understandably affects the most impoverished and vulnerable populations of the world. Mathematical modelling suggests that, in endemic areas, contaminated food and water serves as the main source of infection in majority of cases while transmission *via* chronic carriers occurs less commonly[183].

The role of chronic carriers is still poorly understood, although there is modest evidence to suggest that these seeming well individuals are not the main sources of infection. *S.* Typhi strains from gall bladders of chronic carriers are often different from the circulating strains in that population[184]. The difficulty in identifying chronic carriers in a population is the biggest hurdle in understanding their role in disease transmission. Some mathematical models suggest that chronic carriers within a household are important in driving transmission in endemic areas[185] and this may be true particularly when food handlers are chronic carriers as shown in a recent study in Pakistan[186].

1.3.1. Delineating the dynamics of paediatric enteric fever transmission

With regard to paediatric enteric fever, the aspect of transmission assumes importance for a number of reasons. Firstly, young children are less likely to be exposed to contaminated sources of food and water[187] but this dynamic potentially changes when their caregivers are chronic carriers. Secondly, it is currently unknown whether children acquire enteric fever from adult cases or from other children. Thirdly, it is unclear which age group of children bears the heaviest burden of disease, which is an important factor in determining preventive interventions. It has however been postulated that young children (<3 years) are particularly important in this dynamic of transmission, as their immature immune systems are more susceptible to a wider range of *S.*Typhi haplotypes[128].

1.3.2. Risk factors associated with transmission

Recent mathematical modelling data from Nepal suggests that the migration of susceptible individuals into the more population dense areas of the country coupled with the emergence of antimicrobial resistance is responsible for the enhanced spread of enteric fever in the region[188]. While data from Bangladesh suggests that environmental factors such as proximity to water bodies, economic factors such as per-capita income, housing and literacy as well as season affect typhoid transmission[188]. A lower elevation has always been correlated with a

more severe risk of acquiring enteric fever in various endemic settings[188–190]. A single Ghanaian study suggested that enteric fever rates are 2-3 times more common among children in the less populous rural areas than urban settings[191]. From a global perspective, it is evident that H58 strains originated in Asia and subsequently spread to the rest of the world. This spread was enhanced by the development of resistance to multiple antimicrobials as well as increased fitness[83]. It is thus evident that the transmission of enteric fever is dependent on host, pathogen and environmental factors.

1.4. Laboratory diagnosis of paediatric enteric fever

The diagnosis of enteric fever in infants and young children poses unique challenges, which are highlighted in **Table 1.2**. These diagnostic challenges contribute to the lower reported rates of disease, which in turn have an impact on policy related decisions particularly with regard to target groups for vaccination.

The concentration of bacteria per unit volume of blood is higher in children when compared with adults[10], yet the confirmation of paediatric enteric fever in endemic settings, even those with well equipped blood culture facilities, may be only as high as 70%, still missing a significant proportion of children with the disease[192]. Culture negative children who were found to have evidence of enteric fever by PCR tend to be younger and with a shorter duration of admission[193]. However, the probability of a positive result is enhanced when a sufficient volume of blood is sampled, the lower limit being 3-5ml depending on the culture media and manufacturer instructions. This may be difficult to obtain particularly in younger children[127,194]. Although the amount of blood is a logical determinant of culture positivity, it is worth mentioning that there are studies reporting no association between low blood sample volumes and blood culture negativity in children with enteric fever[192,193]. This suggests that the low sensitivity of blood culture is multifactorial with others factors such as the use of

empirical antibiotics prior to blood culture, sampling after the first week of illness and the type of culture/enrichment medium playing important roles [10].

Bone marrow culture, though reliable, is impractical in most endemic settings mainly due to the discomfort and the expertise required to perform the procedure[10]. It has been shown that 15 ml of blood is required to match the yield of organisms obtained from 1 ml of bone marrow[195]. Microbiological cultures from stool and urine are seldom reliable for the diagnosis of acute illness in endemic settings[10]. Faecal culture sensitivity is low due to intermittent shedding and therefore repeated cultures need to be taken to enhance sensitivity[195]. Urine culture also has similar problems akin to faecal culture but has been proposed as a method to detect the Vi antigen[196]. Cultures from other sites such as a rose spots or duodenal string-capsule, though possible are impractical in children in LMICs particularly due to the difficulty in tolerating the duodenal string-capsule[197].

Serological tests for the diagnosis of enteric fever though not ideal, continue to be used in endemic settings. The low specificity of the Widal test in particular has been a matter of concern,[10] which may be explained in part by exposure to previous infections, subclinical infections or exposure to other *Enterobacteriaceae*. These factors may not come into play in young infants who have lower levels of exposure to contaminated food and water. A positive Widal test, even without a paired acute and convalescent sample may thus be significant in this age group. Rapid tests such as Typhidot (Malaysian Biodiagnostic Research SDN BHD, Kuala Lumpur, Malaysia), Typhidot-M, and TUBEX (IDL Biotech, Sollentuna, Sweden) are seen to be more reliable in children than in adults in endemic settings[10]. The recent Typhi/Paratyphi IgA assay which makes use of detection of antibodies in lymphocyte supernatant (ALS) shows promise with regard to the sensitivity and specificity particularly in young children with a negative blood culture, though it may be impractical in many settings[198].

Salmonella Typhi antigens, can be detected in the serum and urine of patients with typhoid fever, using co-agglutination or immunoelectrophoretic techniques[199–203] . Though sensitive, these tests have low specificities and are yet to be validated in large studies[10]. The detection of specific IgM antibodies using antigen microarray techniques has been promising in children and adults in endemic areas. The technique was understandably more specific in a non-endemic setting due to lower background seroreactivity[204], which is therefore likely to be a better diagnostic test in young children who will potentially have low background reactivity even in endemic areas[204]. Nucleic acid detection by polymerase chain reaction (PCR), though reliable is expensive and the duration of time to diagnosis[10] does not currently make it a point of care diagnostic method. Sensitivity and time to diagnosis may be further improved by using a combination of ox-bile for blood lysis and micrococcal nuclease for the removal of human DNA to enrich the yield of bacterial DNA[205] . The drawbacks of blood culture in children may be overcome by this method as a smaller volume of blood is required while pre-treatment with antibiotics and point of sampling in the course of illness do not affect the accuracy of results[10]. The quest to improve sensitivity with the use of nested primers also brings with it the possibility of false positive results due to non-specific amplification and contamination[10]. However, all these methods are hampered by blood volume: if there are no bacteria in the blood sample that is taken, they will not be detected by these assays.

Table 1.2 Characteristics of available diagnostics in endemic settings and paediatric challenges

Modality	**Diagnostic Test**	**Advantages**	**Disadvantages/Challenges**
Culture	Bone marrow culture [142,207,208]	• 100% specificity and high sensitivity • Subsequent antibiotic sensitivity testing possible • Unaffected by antibiotic use	• 2-5 days for results • Technical expertise • Exposure to sedation/anaesthesia • Highly invasive – issues with parental consent • Storage, transportation and culture media facilities
	Blood culture [10,205,209,210]	• 100 % specificity • Subsequent antibiotic sensitivity testing possible	• Variable (40-75%) sensitivity • Paediatric phlebotomist required for younger children • Suboptimal volume of blood • Issues with consent • Narrow window of bacteria in circulation • Positivity rates affected by prior antibiotic use • Storage, transportation and culture media facilities
Serology	Widal test (single)[10]	• Relatively quick • Inexpensive • Minimal expertise required	• Low sensitivity and specificity • No antibiotic sensitivity information
	Widal test (acute and convalescent)[10]	• Relatively quick • Inexpensive • Minimal expertise required • Sensitivity 63%	• Difficult to identify acute and convalescent • Lack of standardization of reagents
Rapid tests	Tubex [211–215]	• Relatively quick • Inexpensive • Minimal expertise required	• Variable (60-100%) Sensitivity and Specificity (58%-100%) • No antibiotic sensitivity information
	Typhidot [215–219]	• Relatively	• Variable (54%-100%) Specificity and

		quick • Inexpensive • Minimal expertise required	(20%-98%) Sensitivity
	Typhidot M [212,218,220,221]	• Relatively quick • Inexpensive • Minimal expertise required	• Variable (5%-93%) Sensitivity and Specificity (65%-93%)
Nucleic acid detection	PCR [10,222]	• 100% specificity	• Variable sensitivity (38%-100%) • Lack of standardization • Expensive • Time consuming • Laboratory training and equipment

1.5. Treatment

Antimicrobial therapy is the mainstay of treatment. As discussed above many cases are treated empirically because blood culture facilities are unavailable or as a result of the intrinsic difficulties in isolating the organism. Children with suspected enteric fever are usually commenced on empiric antibiotic therapy while the results of antibiotic sensitivity tests are awaited. Most studies pertaining to anti-microbial treatment of paediatric enteric fever came from the 1990s and these studies may not be as relevant today with the emergence of drug resistance. Nevertheless the previous studies do provide evidence of comparative efficacy and safety profiles of various antimicrobial drugs, which have been tabulated in **Table 1.3.** The emergence of antimicrobial resistance has brought with it unique challenges with the H58 clone displacing antibiotic sensitive strains[83,223] and spreading across East Africa and South Asia[83]. The acquisition of a *gyrA, parC* and *parE* mutation which confers resistance to fluoroquinolones has been identified in South, South-East Asia, Southern and

Eastern Africa [83,224]. Strains with resistance to cephalosporins have also been reported and show an increasing trend across South Asia[129].

1.5.1. First line antimicrobials

Chloramphenicol, ampicillin and trimethoprim(TMP)-sulfamethoxazole(SMZ) (cotrimoxazole) were traditionally used as first-line antibiotics for enteric fever. Their use has however been restricted owing to the emergence of drug resistant and MDR (resistance to all three first line antimicrobials) strains. Depending on drug sensitivities, trimethoprim - sulfamethoxazole, can be used in children at 4 mg TMP per kg and 20 mg SMZ per kg for 14 days[225]. Ampicillin and amoxicillin are used at a dose of upto 30 mg per kg per dose orally, i.m. or i.v., at three to four doses per day. No benefit has been reported to result from the addition of clavulanic acid to amoxicillin[226]. The use of first line antibiotics may still have utility in areas where they have been obsolete with sensitive strains currently emerging.

1.5.2. Fluoroquinolones

Fluoroquinolones represent another option for MDR *S.* Typhi and *S.* Paratyphi, owing to high intracellular concentrations of the drug and concentration within the gallbladder [227]. The available long term follow up data suggest that the risk of an untoward effect on growth and joint development is low and that musckuloskeletal side effects are reversible[228–233]. A decade ago short course ofloxacin was found to be effective in Vietnamese children[234] however, recent evidence from Nepal suggests that fluroquinolone resistance is widespread and parenteral ceftriaxone is better suited to sensitivity patterns of currently circulating strains[224]. Fluoroquinolone treatment might still be relevant in treating typhoid in Africa, as most strains are still fluoroquinolone sensitive.

1.5.3. Cephalosporins

Third-generation cephalosporins are commonly used in the empirical treatment of enteric fever particularly when the clinical syndrome is indistinguishable from other invasive

bacterial disease. These antimicrobials are also a valuable treatment option in the setting of MDR and fluoroquinolone resistance. Ceftriaxone has demonstrated efficacy in a number of clinical trials in doses between 30 and 70 mg/kg/day daily for between 3 and 14 days[235–240], a shortened regimen for 3 days demonstrated acceptable efficacy in children and adults in Nepal[239] but was associated with bacteriologic relapse among children in Pakistan[241]. The oral third generation cephalosporin cefixime has shown to be effective in children when given for 12 to 14 days[242] although two randomized controlled trials between cefixime and gatifloxacin suggest that cefixime was associated with a higher rate of disease relapse[243,244]. Cefpodoxime proxetil was found to have a similar efficacy to ceftriaxone in children and is a cheaper option[245] in LMICs. The reports of third generation cephalosporin resistance merit close monitoring of minimum inhibitory concentration (MIC) patterns in endemic settings[246]. There have been reports of cephalosporin resistant typhoid infection from India and Pakistan[247–250].

1.5.4. Azithromycin

At a single daily oral dose of 10 to 20mg/kg/day, for 5 to 7 days and excellent tissue penetration, azithromycin is a convenient treatment option. The drug achieves high intracellular concentrations within phagocytes up to 100 times more than serum which adds to the efficacy profile[251]. Azithromycin is proven to be more efficacious when compared with fluoroquinolones, cephalosporins and chloramphenicol[252–259] in terms of defervesence times, post-treatment faecal carriage and rates of relapse[253]. It represents a logical option for MDR and fluroquinolone resistant *S.* Typhi as resistance is currently rare, although isolates with increased azithromycin MICs and treatment failure have been reported [260–264].

In addition to antimicrobial therapy, general supportive care such as management of fluid and electrolyte balance, assessment and correction of the nutritional status as well as antipyretic treatment are important in remission and prevention of complications[265]. It should be

emphasized that the selection of antibiotics should be based on susceptibility of locally prevalent *Salmonella* strains. In endemic settings with known fluoroquinolone resistance, a first-generation antimicrobial (chloramphenicol or cotrimoxazole) or azithromycin may be started empirically until antibiotic sensitivity results are available, following which the appropriate antimicrobial may be continued.

1.5.5. Management of Complications

All children with enteric fever should be monitored for complications. Dexamethasone has been shown to reduce mortality in children with severe enteric fever with altered consciousness[266,267]. Mortality due to complications was reduced in children and adults when dexamethoasone (at a dose of 3mg/kg by slow IV infusion over 30 minutes, followed by 1mg/kg every six hours)[153] was administered along with the antimicrobial to which *S.*Typhi was sensitive.

Gastrointestinal bleeding in children with enteric fever should be closely monitored. The majority of cases of typhoid haemorrhage involves small vessels and are self-limiting, although life threatening haemorrhage can occur if large vessels are involved and may require additional intervention to control bleeding, including colonoscopy, surgery or administration of vasopressors[268–271].

A life threatening complication of enteric fever, intestinal perforation, should be managed with immediate fluid resuscitation, blood transfusion if required and immediate surgical intervention[272–274]. An 8 year experience of enteric fever perforations from a paediatric surgery referral unit in Africa found that primary closure or by-pass of the affected segment, in addition to thorough lavage of the peritoneal cavity had good rates of success[146]. Multiple perforations and a highly contaminated peritoneal cavity warrant segmental resection and primary anastomosis[146]. In the event of faecal fistula, which is a significant predictor of mortality, an ileostomy as a secondary procedure may be considered to avoid peritoneal

contamination[146]. Additional antibiotics, such as metronidazole, may be added to cover contaminating bacteria from the gut[275].

1.5.6. Rapidly emerging anti-microbial resistance

The adaptation of *S*. Typhi to diversifying pressures have been the norm throughout its evolutionary history[98]. Antimicrobials also serve as a selection pressure and constitute one of the most recent pressures to which *S*. Typhi has had to adapt to. The emergence of antimicrobial resistant *S.* Typhi is a global concern and is a testament to the success of bacterial adaptation[77]. The trend of antimicrobial resistance is alarming, as the production of new and innovative antimicrobials cannot keep pace with the mechanisms of anti-microbial resistance in the arsenal of *S.* Typhi.

1.5.6.1 Mechanisms of antimicrobial resistance

Bacterial populations adapt to antimicrobial pressure *via* DNA-modification strategies. The strategies may be by (1) mutations in the form of single nucleotide polymorphisms (SNPs) which cause subtle yet functional changes in genes already present in the genome or (2) acquisition of novel genetic material, which expands the genome[77]. *S.* Typhi and Paratyphi use both strategies to overcome antimicrobial pressure but favour one or the other strategy depending on the class of antimicrobial. The system enables the bacterial clone with the most successful adaptation strategy to proliferate and dominate the circulating population of strains thus confirming the Darwinian statement "survival of the fittest"[77]. The acquisition of novel genetic material alludes to horizontal gene transfer, which has been discussed above in 1.1.3.

1.5.6.2. Resistance to first line antibiotics

Resistance to the formerly used antimicrobials were conferred via IncHI1 plasmids, which often harbour the resistance genes responsible for MDR typhoid. Such resistance genes are cluster on composite transposons and include *catA, sul1, sul2, dfrA,* $bla_{TEM\text{-}1}$, *strA*, *strB, tetA,*

tetB, tetC and tetD. These MDR-associated genes have also been known to integrate within the chromosome of H58 *S*. Typhi in isolates from countries including India and Bangladesh[83].

1.5.6.3. Fluoroquinolone resistance

Quinolones and their subsequent fluorinated generations are a broad-spectrum class of antimicrobials, which act by inhibiting two key enzymes of bacterial DNA synthesis namely; DNA gyrase and topoisomerase IV. These enzymes are critical in the replication and transcription of the bacterial genetic material and work together closely. DNA gyrase consists of a tetramer of two 97kDa A subunits and two 90 kDa B subunits encoded by *gyrA* and *gyrB* genes respectively. The A subunits are mainly involved in DNA breakage and ligation, while the B subunits mainly exhibit ATPase activity. The topoisomerase IV unlinks newly replicated daughter chromosomes at the end of a replication cycle as well as to assist DNA gyrase in relaxing supercoils during the synthesis of newly formed DNA strands. Topoisomerase IV is also made up of two A subunits designated as ParC and two B subunits designated as ParE which are encoded by *parC* and *parE* genes respectively. A stable interaction of fluoroquinolones with the DNA gyrase-DNA complex is a prerequisite for DNA gyrase inhibition.

Resistance to fluoroquinolones occurs via chromosomal mutations in the aforementioned genes or in genes whose downstream effects reduce uptake of the drug or increase efflux. Cumulative mutations correspond to the degree of fluoroquinolone resistance, a single SNP in codon S83F of *gyrA* will produce a low level resistance (minimal inhibitory concentration (MIC) of ciprofloxacin of 0.125–0.25 mg/l) where as additional SNPs in *gyrA* (D87N) and *parC* (S80I) will confer a high level of ciprofloxacin resistance (MIC 8–64 mg/l) and is designated as the triple H58 mutant. SNPs in the D87V codon of *gyrA* as well as in the A364V codon of *parE* have also been observed in *S.* Typhi isolates from Nepal[276]. Another mechanism involves the horizontal acquisition of gene containing plasmids associated with

quinolone resistance. Plasmids containing genes such as *qnrB2*, *qnrB4* and *qnrS1* have been identified among a few *S.* Typhi isolates in South-Asia[277] and are expected to be a more common occurrence in future given the widespread use of fluoroquinolones in the region.

1.5.6.4. Cephalosporin resistance

Extended spectrum β lactamase (ESBL) producing *S*. Typhi isolates, which confer resistance to third-generation cephalosporins have been reported in India and Pakistan[247,248]. These isolates possess plasmids which contain ESBL genes[247,248].

1.5.6.5. Azithromycin resistance

Although currently rare, isolates with increased azithromycin MICs and treatment failure have been reported [260–264]. Azithromycin resistance is known to be mediated *via* the *ereA*, *msrD* and *msrA*[83]

Table 1.3 Studies evaluating antimicrobial safety and efficacy in the treatment of paediatric enteric fever

Author Year Country	Antimicrobial/s (/kg/day)	Sample size	Duration	Age range	Route of administration	Findings/ conclusions	Adverse events
Vinh 2005[234] Vietnam	**Ofloxacin** (10 mg/kg)	89 107	2 days vs 3 days	Not reported	Oral	No significant difference in fever clearance time or duration of hospitalization in children with uncomplicated enteric fever	None reported
Bhutta 2000[241] Pakistan	**Ceftriaxone** (65 mg)	29 28	7 days vs 14 days	2 – 11 years	Parenteral	14% of children in the short duration treatment arm had bacteriological relapse within 4 weeks as opposed to 0 % in the long duration arm	None reported
Shakur 2007[245] Bangladesh	**Cefpodoxime proxetil** (16 mg) vs **cefixime** (20 mg)	21 19	10 days	6 months – 12 years	Oral	Clinical efficacy similar in both groups. Cefpodoxime is effective, safe and cheaper	None
Acharya 1995[239] Nepal	**Ceftriaxone** (50 mg) vs **cholramphenicol**	8 8	3 days 14 days	Not reported	Parenteral	No difference in duration of clinical cure or onset of complications between the two groups.	None reported

	(60 mg)						
Parry 2007[278] Vietnam	**Ofloxacin** (20 mg) vs **azithromycin** (10 mg) vs **ofloxacin** (15 mg) + **azithromycin** (10mg)	Not reported	7 days	Not reported	Oral Oral Oral	Fever clearance time for patients treated with azithromycin was shorter than that for patients treated with ofloxacin + azithromycin and ofloxacin. A 7-day course of azithromycin may be used to successfully treat uncomplicated MDR typhoid fever with reduced susceptibility to fluoroquinolones.	Self limiting gastro intestinal side effects in each treatment arm but did not require discontinuation / interruption of therapy
Cao 1999[244] Vietnam	**Ofloxacin** (10 mg) vs **cefixime** (20 mg)	38	5 days 7 days	<15 years	Oral Oral	MDR enteric fever had 10 times more treatment failures in the cefixime group	None reported

		44					
Frenck 2000[258] Egypt	**Azithromycin** (10 mg) vs **ceftriaxone** (75 mg)	31 29	7 days	4 -17 years	Oral Parenteral	Oral azithromycin is safe, effective and convenient for uncomplicated paediatric enteric fever	No side effects
Frenck 2004[254] Egypt	**Azithromycin** (20 mg) vs **ceftriaxone** (75 mg)	32 36	5 days	3 -17 years	Oral Parenteral	A 5-day course of azithromycin was found to be an effective treatment for uncomplicated typhoid fever in children and adolescents.	No side effects
Girgis[279] 1995 Egypt	**Cefixime** (20-30 mg)	60	8 days	3 -16 years	Oral	Cefixime given as an 8 day course is safe and effective in the management of MDR typhoid in children	Nausea, vomiting (8%) and loose stools (6%)
Girgis 1995[280] Egypt	**Cefixime** (7.5 mg) vs **ceftriaxone** (50 –	50 43	14 days 5 days	Not reported	Oral Parenteral	All the 3 regimes were equally safe and efficacious. While ceftriaxone was more economical for in-patient care cefixime	Nausea, vomiting and abdominal pain in

	70 mg) vs **azetronam** (50 – 70 mg)	43	7 days		Parenteral	was better for out-patient care	some patients
Pandit 2007[243] Nepal	**Gatifloxacin** (10 mg) vs **cefixime** (20 mg)	Not reported	7 days	Not reported	Oral	Gatifloxacin was more efficacious in terms of defeversence time, treatment failure and relapse	Nausea and vomiting with gatifloxacin
Dolecek 2008[252] Vietnam	**Gatifloxacin** (10 mg) vs **azithromycin** (20 mg)	109 101	7 days	2 -15 years	Oral	Both antimicrobials are equally efficacious with gatifloxacin being one-third the cost of azithromycin	Self limiting gastrointestinal side effects
Arjyal 2011[281] Nepal	**Gatifloxacin** (10 mg) vs **chloramphenicol** (75 mg)	Not reported	7 days 14 days	Not reported	Oral	Gatifloxacin may be the preferred treatment because of its shorter treatment duration and fewer adverse effects	Vomiting (8%) and abdominal pain (2%)
Arjyal 2016[224] Nepal	**Gatifloxacin** (10mg) vs **ceftriaxone** (60mg)		7 days	2 -13 years	Oral Parenteral	Fluoroquinolones should no longer be used for treatment of enteric fever in Nepal*	None reported

*The data and safety monitoring board asked for the trial to be stopped on grounds supported by data on the changing susceptibility of *S.*Typhi

1.6. Prevention

Understanding the dynamics of enteric fever transmission to children is key in elucidating preventive measures, especially vaccination. The transmission of enteric fever may occur through the short cycle via household contacts, food and water or long cycle via the environment and water supply. In an effort to delineate the role of adult contacts in paediatric enteric fever transmission, it has been found that disease is more likely to be caused by genetically distinct strains, consistent with transmission through contaminated food and water rather than person-to-person transmission within the same household[184]. However, cluster-randomised trials with the Vi polysaccharide (ViPS) vaccine have indicated that mass vaccination in the population may confer an indirect protection to the 2-5 year old children in the population, by possibly reducing transmission from adults in the population[282,283].

1.6.1. Vaccines and immunization against typhoid

Typhoid vaccines have been studied through various implementation strategies across Asia[18]. School based campaigns as well as delivery strategies using the available health care structure have been effective in terms of coverage and cost-effectiveness[18]. Trials evaluating vaccine delivery strategies namely; community-based routine, community-based campaign and school-based have not shed light on long term effectiveness of these vaccines[18] although they have found to be effective in the immediate and short term[18]. Mathematical modelling can aid in the evaluation of different vaccination strategies and, importantly, help predict the expected impact resulting from the direct and indirect effects of vaccine introduction[185,284]. If appropriately parameterized *via* biological knowledge and tested against a range of data sets and epidemiological scenarios, mathematical modeling can be an effective tool for evaluating prevention strategies. The Ty21a vaccine with an efficacy between 33%-77% cannot be given to children under the age of 5 years as they are unable to swallow the capsule efficiently, the ViPS has an efficacy range between 55%-72% but is poorly immunogenic in children below

the age of 2 years due to the immature marginal zone of the spleen, further limiting the use of currently available vaccines. There has therefore been no dedicated approach to systematically target the pre-school age group, but that has the potential to change with the advent of typhoid conjugate vaccines (TCV) and their ability to be combined with vaccines in the Expanded Programme on Immunization (EPI)[59,60]. The novel protein conjugate vaccines (**Table 1.4**) which are T cell dependent, are immunogenic and safe in children including infants[60,285]. The TCVs have been prioritized by Gavi, the Vaccine Alliance but an ideal vaccination schedule and target population are still to be outlined.

Interestingly, there has been no attempt to study typhoid vaccines through a programmatic approach in Africa[18] presumably due to relatively low disease rates in the recent past. The identification of the H58 clone in Malawi, West Africa and Southern Africa[83] prompts the need for systematic use of vaccines to curtail the spread of enteric fever across other parts of the continent. The new TCVs offer a novel intervention against typhoid fever. However, the licensure of the two Indian vaccines was based on non-inferior immunogenicity rather than efficacy and questions regarding mechanisms and correlates of protection conferred by TCVs still remain. The quest for an immunogenic paratyphoid fever vaccine as is also relevant with LPS-conjugate paratyphoid vaccines in development[286], as the current TCVs do not offer protection against *S*. Paratyphi. Although immunogenicity data from Phase III trials of the TCV are promising, it should be remembered that vaccination alone cannot eradicate enteric fever and an evidence-based multi-pronged approach is warranted to effectively control this important cause of morbidity and death among children in some of the poorest countries in the world.

1.6.2. Protective effect of breastfeeding

Mucosal immunity in infancy can be modified by breastfeeding[287] which could protect infants in endemic areas. Breast milk, possessing an immunomodulatory effect has the

potential to enhance protection at the level of the gut mucosa resulting in subclinical gastrointestinal infections[288,287]. Limited evidence demonstrating the protective effects of breast milk in enteric fever exist[289] and though it may partly account for the lower rates of disease in infants (lack of exposure may be the most important), further work needs to be done to establish a proof of concept. While colostral and milk cells may be more active against *Salmonella* than blood neutrophils,[290] a higher prevalence of community acquired *Salmonella* infections in infants who were not breastfed was observed in a retrospective analysis by Akinyemi[289].

1.6.3. Provision of clean water and sanitation

The prevention and control of paediatric enteric fever requires a multi-pronged approach combining short, intermediate and long-term strategies. The prompt diagnosis and treatment of enteric fever, a short-term intervention offers control at an individual level. The development and implementation of vaccination strategies in high burden settings provides an intermediate approach, which could benefit children from infancy to adolescence. However, the optimal approach to break the transmission cycle as well as eliminate this human-restricted pathogen is via the implementation of access to safe water and improved sanitation, which would also reduce the burden of most other enteric pathogens

Table 1.4 Summary of Typhoid conjugate vaccines in the pipeline

Type	**Vi rEPA**		**Vi TT**		**Vi CRM_{197}**	**Vi DT**	**Vi conjugated to fusion protein PsaA-PdT**	**O:9-DT**
Protein conjugate	Non-toxic recombinant *Pseudomonas* aeruginosa exotoxin A (rEPA)		Tetanus toxoid (TT)		Non-toxic mutant of diphtheria toxin	Diphtheria toxoid (DT)	Species conserved pneumococcal antigen (SP1572) – penumolysoid (PdT)	O-specific polysaccharides of *S.*Typhi conjugated to diphtheria toxoid
Developer	National Institute of Health (NIH)	Lanzhou Institute of Biological research and product (China)	Typbar TCV ® (manufactured by Bharat Biotech)	PedaTyph™ (manu-factured by BIO MED Pvt. Ltd, India)	Biological E)	International Vaccine institute (South Korea)/ Shanta Biotech (India)	Harvard Medical school	International Vaccine institute
Settings tested	Vietnam		India, Human challenge model		India and Pakistan	-	-	-
Phase of trial	Phase 3		Phase 3		Phase 2	Phase 1	Preclinical	Preclinical
Licensure	Licensed in China		Licensed in India, WHO pre-qualified		-	-	-	-

Age- group tested	2-15 years	6 months - 45 years	6 months - 12 years	6 weeks - 5 years	-	-	-
Dose	22.5 ug of Vi and 22ug of rEPA in 0.5ml	25 ug of Vi	5 ug of Vi polysaccharide of S. typhi conjugated to 5 ug of TT.	25 mg of Vi antigen conjugated to CRM_{197} in 0.5 mL	-	-	-
	2-5 years, 2 doses >5 years, 1 dose	> 6m, 1 dose with a booster after 2 years	< 2 years, 2 doses with a booster at 2 years > 2 years, 1 dose	>6 weeks, 3 doses 9–59 months, 2 doses >59 months, 1 dose	-	-	-
Compatible with EPI	Unknown	Yes	Yes	Yes[291]	-	-	-

1.7. Thesis outline

This thesis aims to inform the field of enteric fever on the specifics of paediatric disease. The preliminary chapters provide an overview of the current knowledge of paediatric enteric fever regarding the age-stratified distribution and the particular aspects, which contribute to the under-diagnosis of this disease in children in endemic areas. The subsequent chapters adopt a more bottom-up approach using pathogen genomic data to inform prevention strategies and affect policy decisions. Pathogen genomic data from 198 *S.* Typhi and 64 *S*. Paratyphi A genomes from a single referral centre in Nepal over a period of 9 years are analysed along with AMR trends and molecular-clock analysis. Along with the molecular AMR data and age-specific burden of disease these data outline the sub-population of children with the greatest risk and offer advice on vaccine strategy with the novel Vi-conjugate typhoid vaccines. Chapter 6 describes cross-sectional data of the molecular structure of circulating typhoidal *Salmonella* strains (101 *S.* Typhi and 14 *S.* Paratyphi A) in Bangalore, India from a referral centre over a one-year period. These data are also linked with laboratory and clinical features and comparisons made between strains isolated from adults and children. These data sets from Nepal and India are then put into a worldwide context to inform inter-country and intra-country transmission dynamics in the Indian subcontinent as well as the development AMR. The thesis finally concludes with a systematic review of *S.* Typhi AMR trends globally to provide a background for policy makers to review the potential impact of Vi-conjugate vaccines in curtailing the further development of AMR in enteric fever.

1.8. Aims and objectives

The overarching aims of this thesis include:

- Delineating the age stratified distribution of paediatric enteric fever
- Describing the molecular epidemiology of paediatric enteric fever in two South-Asian sites
- Systematically reviewing the trends of antimicrobial resistance in typhoid fever globally.

The specific objectives were:

- To characterize the age distribution of paediatric enteric fever across Asia and Africa
- To characterize the in-patient disease trend of paediatric enteric fever since 2005 at Patan hospital, Nepal
- To describe the molecular epidemiology of paediatric enteric fever in Nepal and South India, and to put these strains into a global context.
- To study the antimicrobial resistance (AMR) patterns of paediatric enteric fever in Nepal and South India with a molecular focus.
- To use the information obtained from molecular characterisation of the *S.* Typhi and Paratyphi populations to aid in vaccine policy and sustainable treatment regimens.
- To compare the disease occurrence and pathogen genetics of enteric fever between adults and children admitted to St. John's Medical College Hospital, Bangalore, India
- To describe the temporal trends of antimicrobial resistance (AMR) of *S.* Typhi populations globally from a phenotypic and molecular perspective

"You wouldn't abandon ship in a storm just because you couldn't control the winds".
Thomas More

Chapter 2

General Methods

This chapter is a basic overview of the various methods employed in this thesis. Specific methodology employed for each chapter is detailed under the respective methods sections.

2.1. General methodology for systematic reviews and meta-analyses in this thesis.

2.1.1. Search strategy and inclusion

The preferred reporting items for systematic reviews and meta-analysis (PRISMA) guidelines were used as (illustrated below) in this thesis. The identification of eligible studies included searches through standard databases such as PubMed, MEDLINE, EMBASE, Scopus, Web of Science and Google scholar using, MeSH terms. Additionally, articles were also scoped using references from eligible articles.

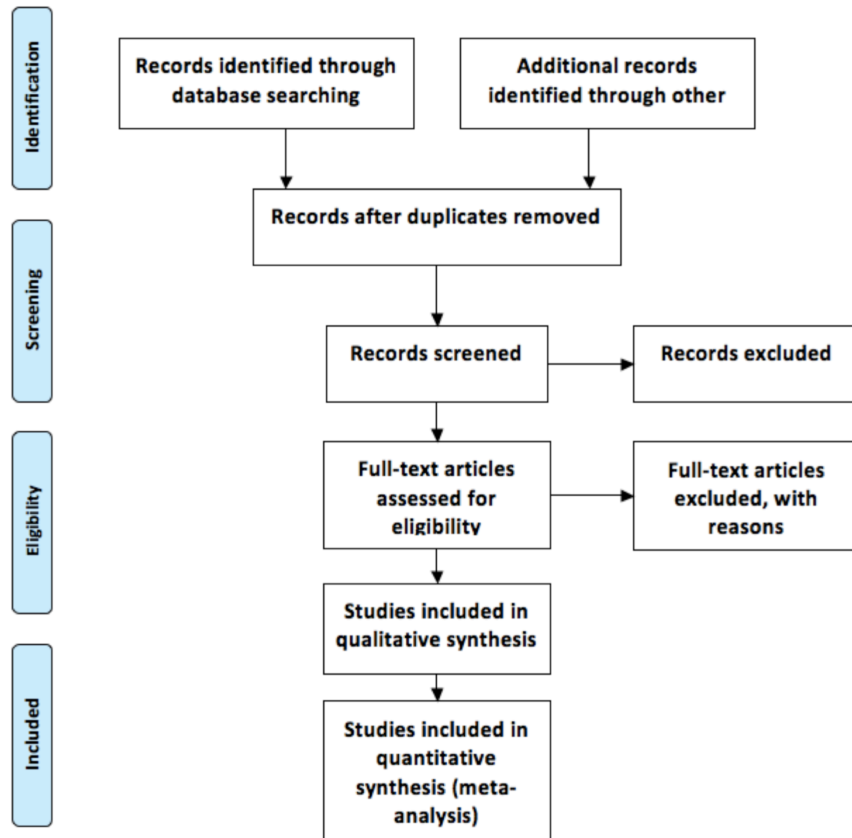


Figure 2.1: Overview of the methodology used for systematic reviews and meta-analyses. Figure adapted from Moher *et al* 2009

2.1.2 Data extraction

Data from individual studies were extracted under **study identifier, methodology** and **results.** Study-specific data extraction was done twice–overall for each objective in the respective systematic reviews.

2.1.3. Risk of bias

Inclusion criteria were used to establish study validity. Risk of bias (RoB) was assessed using two tools. The first classifies studies based low-, moderate- or high- risk of bias and is known

as the Quality In Prognosis Studies tool (QUIPS)[292]. The second is known as the Joanna Briggs Institute (JBI) tool[293] and reports RoB dichotomously. Parameters assessed for bias across the two tools included 1) Population description, i.e. whether community or hospital setting, 2) Study design, sample size and sampling techniques 3) Use of appropriate performance standards 4) the statistical analysis used for reporting summary measures.

2.2. General laboratory methods for isolates studied in this thesis

2.2.1. Blood culture processing

Aerobic blood culture bottles were used to culture 3-5 mL of blood, which were then incubated in a BD Bactec FX 40 incubator at 37°C for a maximum of 5 days. Turbid samples were then inoculated directly onto MacConkey agar and incubated for maximum of 5 days at 37°C to identify potential *S.* Typhi and *S*. Paratyphi A colonies. Candidate *S.* Typhi and *S.* Paratyphi A isolates were further subjected to standard biochemical tests for additional confirmation.

2.2.2. Typhoidal *Salmonella* identification

With confirmation of possible Salmonella growth (i.e. red or black colonies seen on XLD media or mauve colonies on chromogenic agar), further biochemical and serological identification steps are performed in accordance with standard procedures. One colony was picked for subculture and mixed with 5mL normal saline prior to inoculating in Columbia blood (for serological testing) and MacConkey agar (to ensure homogeneity and purity) plates.

Serological testing was done using 'O' and 'H' antigen specific sera and following the Kauffmann-White scheme by slide agglutination the following method was adopted. Briefly, colonies were selected and emulsified in normal saline before an equal volume (2µL) of antisera was added and mixed. Positive agglutination was identified by the formation of grainy white particles within 20-seconds of antisera addition, visible against a dark

background. When required, unmasking of the O antigen (by the Vi capsule) was performed by boiling a dense solution for 30-minutes before repeating the agglutination procedure. All agglutinations were controlled for using a negative reaction.

2.2.3. Antibiotic susceptibility testing

The sensitivities of these typhoidal *Salmonella* isolates were assessed according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. Frozen isolates were defrosted and plated on Nutrient agar and incubated at 37.5°C. Colonies from these over night cultures were then emulsified in 3ml of peptone water until turbidity was comparable with a McFarland 0.5 standard. A bacterial lawn was then created by dipping a swab into the suspension and streaking it across Muller Hinton agar plates (Oxoid, Basingstoke, United Kingdom). Antimicrobial discs (Oxoid, Basingstoke, United Kingdom) were then placed on the plate and was subsequently incubated at 37.5°C. Zone edges were read from the front of the plate with the lid removed and in reflected light and with *E. coli* ATCC 25922 breakpoints used as for quality control. The CLSI breakpoints contained in 27th edition (published in January 2017) were used to interpret susceptibility zone diameters[294]. Isolates displaying sensitivity to the tested antimicrobials as per the cut-off values in the CLSI guidelines were designated as susceptible and those that were intermediate (I) or resistant (R) to the tested antimicrobials were designated as resistant.

2.2.4. Pathogen DNA extraction and sequencing

DNA was extracted using the Wizard Genomic DNA Extraction Kit on site (Promega, Wisconsin, USA), according to manufacturers instructions. A single colony was picked and incubated in liquid broth in 3 ml Bijou tubes at 38° C overnight. 1 ml of the overnight culture was then added to a 1.5 ml microcentrifuge tube. The liquid culture was then centrifuged and at 13,000-16,000 x *g* to pellet the cells. Nuclei lysis solution (600ul) was then added, incubated at 80°C for 5 minutes, then cooled to room temperature and finally 3 ul of RNase

solution was added. Protein precipitate solution was then added and incubated on ice for 5 minutes. Following this the solution was then centrifuged and the resultant supernatant containing DNA was then purified using isopropanol and ethanol. The purified DNA was suspended in a rehydration solution and then quantified using Qubit™ 4 Fluorometer. It was then stored at 0° C and subsequently shipped to the Wellcome Trust Sanger Institute. Genomic DNA was then subjected to indexed whole genome sequencing on an Illumina Hiseq 2500 platform at the Wellcome Trust Sanger Institute to generate paired-end reads of 100-150 bp in length.

2.3. General Bioinformatic pipeline for pathogen genomic analysis in this thesis

2.3.1. Mapping of reads

For analysis of SNPs in the genomes of *S*. Typhi and *S*. Paratyphi A, the RedDog (V1beta.10.3) mapping pipeline, available at https://github.com/katholt/RedDog was employed to map the sequenced Illumina reads to the reference genome sequence of strain CT18[84] (accession AL515582) and *S*. Paratyphi A strain AKU_12601[295] (accession FM200053) respectively. RedDog uses Bowtie (v2.2.9)[296] to map reads to the reference sequence and SAMtools (v1.3.1)[297] to identify SNPs with phred quality scores above 30. It filters out those supported by <5 reads or with >2.5 times the average read depth (representing putative repeated sequences), or with ambiguous consensus base calls. For each SNP that passed these criteria in any one isolate, consensus base calls for the SNP locus were extracted from all genomes (ambiguous base calls and those with phred quality scores less than 20 were treated as unknowns and represented with a gap character).

2.3.2. Genotyping

These SNPs were used to assign isolates to previously defined lineages according to an extended *S*. Typhi genotyping framework[97] (code available at https://github.com/katholt/genotyphi). This framework is based on 68 SNPs that can

assign each individual *S.* Typhi one of four primary clusters, which is in turn subdivided into 16 clades and 49 further subclades. Each genotype thus has three digits separated by periods and each digit represents the cluster, clade and subclade respectively. For example, an isolated that is genotyped as 4.3.1 indicates that belongs to cluster 4, clade 1, subclade 1. The scheme has a higher discriminatory power than the haplotyping scheme described by Roumagnac *et al*[92].

An illustration of this scheme in comparison with the scheme described by Roumagnac *et al*[92] is depicted below .

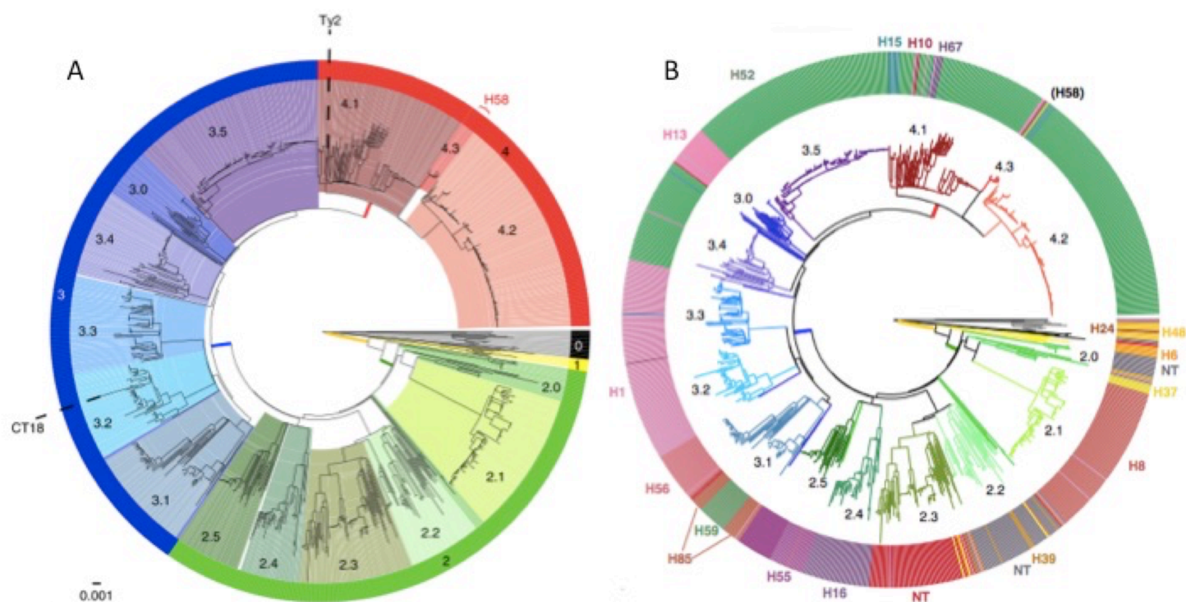


Figure 2.2. The genotyping framework. Panel A represents the novel genotyping framework illustrating the clusters and clades by which genotyping occurs. Panel B represents the comparison between the older haplotyping scheme (outer ring) and the novel genotyping framework (inner circular tree coloured by genotype clusters. Figure adapted from Wong *et al* 2016

2.3.3. Phylogenetic analysis

For phylogenetic analyses, SNPs with confident homozygous allele calls (i.e. phred score >20) in >95% of these genomes (representing a 'soft' core genome of common *S.* Typhi sequences) were concatenated to produce an alignment of alleles at variant sites. SNPs called in phage regions, repetitive sequences or in recombinant regions identified using Gubbins (v2.0.0)[298] were excluded, resulting in a final set of SNPs identified in an alignment length equivalent to the lengths of the reference genomes. SNP alleles from *S*. Paratyphi A strain AKU_12601[295] (accession FM200053) were also included as an outgroup to root the tree in case of the *S.* Typhi isolates and the CT18 strain was used to root the *S.* Paratyphi A tree. Maximum likelihood (ML) phylogenetic trees were inferred from SNP alignments using RAxML (v8.1.23)[299], with the 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. The resulting trees were visualized using Microreact[300] and the R package ggtree[301] .

2.3.4. Detection of AMR determinants

The Illumina reads of the genomes of individual isolates were mapped using the mapping based allele typer SRST2[302] to detect plasmid replicons as well as acquired AMR genes and determine their precise alleles, by comparison to the ARG-Annot[303] and ResFinder[304] databases (for AMR genes) and PlasmidFinder[303] (for plasmid replicons). Where AMR genes were observed without evidence of a known resistance plasmid, raw read data were assembled *de novo* with SPAdes (v3.7.1)[305] and Unicycler (v0.3.0b)[306] and examined visually using the assembly graph viewer Bandage (0.8.1)[307] to inspect the composition and insertion sites of resistance-associated transposons. These putative transposon sequences were annotated using Prokka (v1.11)[307] followed by manual curation, and visualized using the R package *genoPlotR*[307]

SNPs in the QRDR of *gyrA, gyrB, parC* and *parE* genes, which are associated with reduced susceptibility to fluoroquinolones in *S.* Typhi, *S*. Paratyphi A and other species[276], were extracted from the whole genome SNP alignments using Reddog (V1beta.10.3) mapping pipeline, available at https://github.com/katholt/RedDog. A schematic depiction of the bioinformatic pipeline is illustrated below.

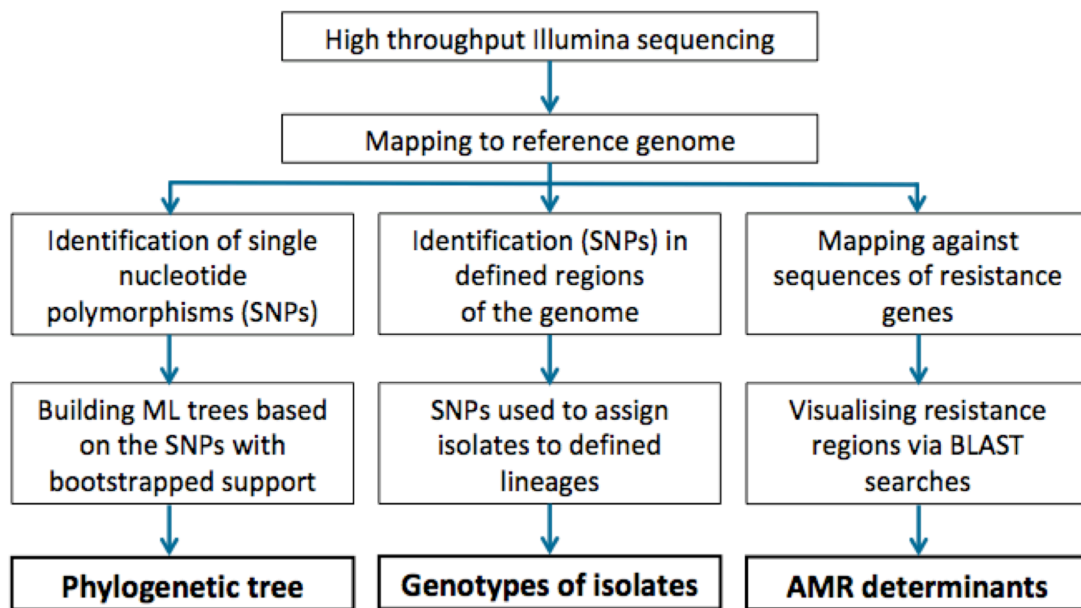


Figure 2.3. Overview of the bioinformatic pipeline used to analyse typhoidal *Salmonellae* genomes in this thesis.

2.3.5. Nucleotide sequence and read data accession numbers

Raw sequence data have been deposited in the European Nucleotide Archive under project PRJEB14050; and individual accession numbers are listed in **Appendix 1**

"Pray as though everything depended on God. Work as though everything depended on you."
-St. Augustine

Chapter 3

Age-stratified disease occurrence of paediatric enteric fever across Africa and Asia

3.1. Introduction:

The majority of existing epidemiological evidence of the burden of enteric fever has been obtained from studies with predominantly adult cases. There are conflicting opinions about the rate of disease in young children, especially infants[2-7], even though a substantial burden of disease is suffered by young children in endemic regions[9,132].

3.1.1. Conflicting opinions surrounding paediatric enteric fever

Differences in opinion about the burden of enteric fever in children have been apparent in the literature for almost half a century. Reports from the 1970s, 1980s and 1990s indicate that typhoid was infrequent below the age of 3 years[120–123], but contrasting studies highlighting

the burden of disease in infants and preschool children also surfaced around the same period[124,125].

3.1.2. Recent epidemiological evidence

Data, including corroborative affirmation from active surveillance over the last decade, highlight the burden of disease in preschool children[126–129,131,133,308], with over 50% of burden in the under 5 year age group[129,309]. This trend is however not uniform across sites in Asia or Africa. Most enteric fever study designs capture the disease burden in the population as a whole. Though this is an efficient approach, subtle yet important characteristics of paediatric enteric fever such as clinical features of undifferentiated febrile illness as well as the procedure and reliability of diagnostic tests, differ from adult disease, which could lead to an underestimate of disease burden in the paediatric population in surveillance studies.

3.1.3. Rationale for the study

Paediatric enteric fever is a major global health concern, particularly in resource-limited settings due to increasing antimicrobial resistance (AMR), increased frequency of epidemics and only moderate efficacy of the Ty21a and ViPS vaccines. There is still significant controversy as to which age group is most affected by enteric fever, profoundly hampering infection control and treatment strategies. The novel typhoid conjugate vaccine gives hope for an effective vaccination strategy useful in children due to its marked immunogenicity even in infants, compared to the plain ViPS and live attenuated oral Ty21a vaccine[18]. However, the existing epidemiological knowledge of paediatric enteric fever does not inform us adequately with regard to the most efficacious vaccine strategy and the target population.

In order to answer some of the pressing issues of paediatric enteric fever this systematic review and meta-analysis was conducted. Studies reporting blood culture positive paediatric enteric fever cases were included to compare the relative proportion of children with enteric fever in the <5 years, 5-9 years and 10-14 years age groups in Asia and Africa.

3.2. Methods

3.2.1. Study design and objectives

This chapter aims to determine the occurrence of paediatric enteric across Asia and Africa through a systematic review and meta-analysis of existing data. Characterizing the age-distribution of paediatric enteric fever in Africa and Asia to inform immunization strategy was a secondary objective.

3.2.2. Search strategy

Studies were identified from PubMed, Embase, Global health, Scopus and Web of Science using the search terms “typhoid" OR "enteric fever” AND epidemiology AND (child* OR pediatrics OR paed*), “Typhoid fever” OR “paratyphoid fever” OR “salmonella infections”, “Typhoid fever” AND “Diagnosis” AND “Child”, “Signs and symptoms” AND “typhoid fever” AND “child”

3.2.3. Screening and eligibility

Studies conducted in Africa or Asia, which reported blood culture positive enteric fever cases in an age stratified manner and were published in English between 1 Jan 2000 and Dec 31, 2015 were included. Articles were also retrieved from references of studies pertaining to enteric fever and from personal archives.

Of 1347 articles identified, 999 were reviewed for inclusion. Selection criteria also included discernment about the quality of studies with respect to sample size and bias, and their relevance for Paediatricians and Infectious Disease specialists. Seventy-seven studies were identified, of which 32 were excluded as they were published before 2000 while 8 were excluded as they were conducted outside Asia and Africa. Another 7 and 6 were excluded, as they did not present data in stratified age groups, which were required for analysis and did not use blood culture as the method for diagnosis respectively. The meta-analysis included 24 studies of which 6 were community based and 18 were hospital based. Risk of bias

assessment was done using the Quality in Prospective Studies (QUIPS) tool (**Appendix 2**).

3.2.4. Data items, summary measures and bias

The proportion of paediatric enteric fever cases was determined for each age-category (<5 years, 5-9 years and 10-14 years), with the denominator being the number of cases aged 0 to 14 years. To remove the possibility of bias due to different age-distributions in the population for different countries, the proportion of paediatric enteric fever cases in each age category were standardized based on age-specific population proportions for the relevant country[310]. African and Asian studies were meta-analysed separately (**Figures 3.2-3.5**). Sensitivity analyses were conducted which excluded small studies of less than 50 participants. All meta-analyses were conducted using RevMan version 5.3[311] with random effects models[312].

3.3 Results

The screening, eligibility and exclusion of studies are illustrated in the PRISM flow diagram in **Figure 3.1**. There were 16 studies from Asia[128,129,133,135,309,313–323] and 8 from Africa[130,308,324–329] included in the initial meta-analyses of which 5 were excluded from the sensitivity analysis[128,135,327–329]

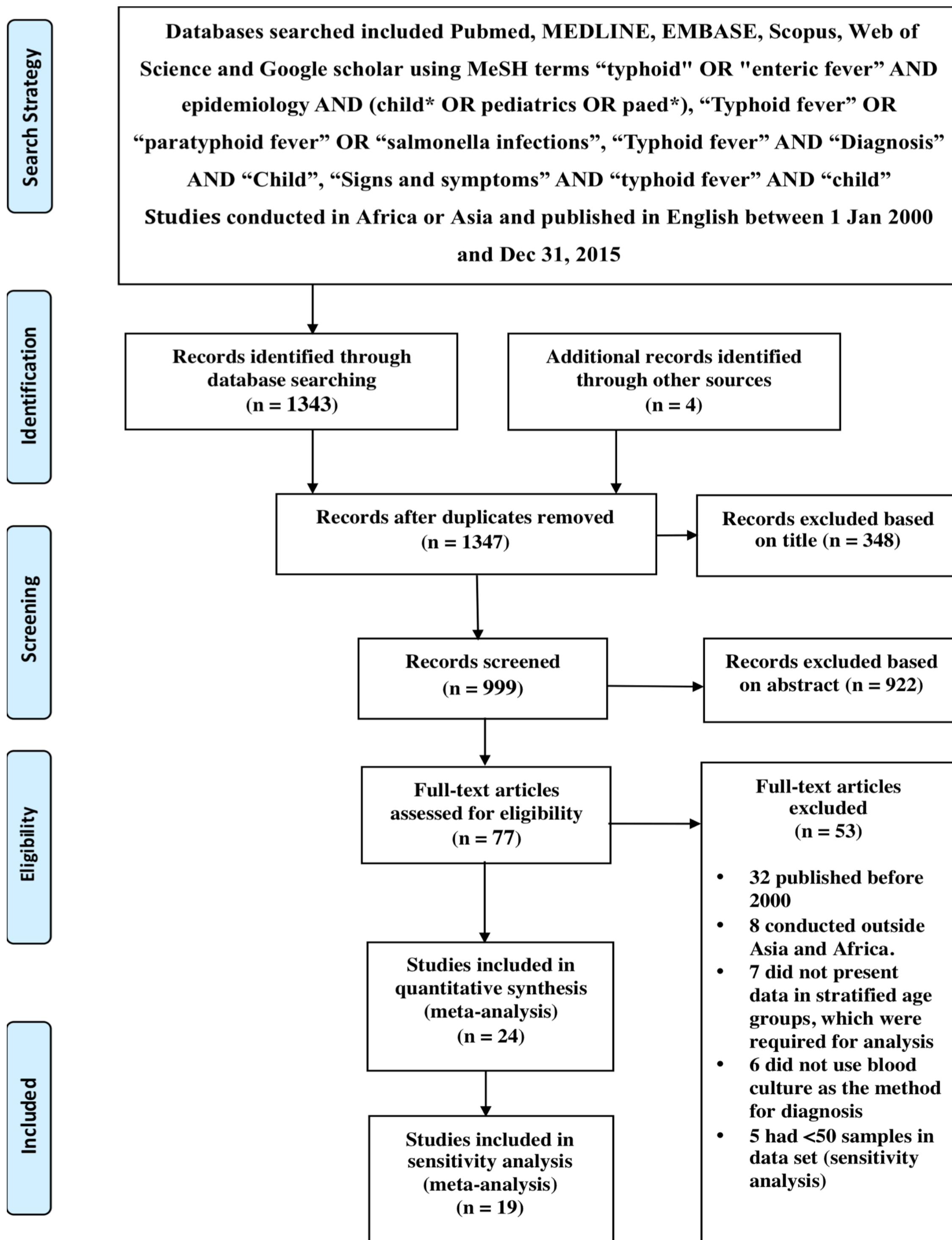


Figure 3.1: Search strategy and characteristics of included studies in the meta-analysis

3.3.1. Synthesis of results and sensitivity analysis

The data was analysed separately for the Asian and African studies in an attempt to characterize disease occurrence by region as well as to ascertain salient epidemiological features specific to each region.

3.3.1.1. Disease occurrence in Africa

In studies conducted in Africa, the smallest proportion (27%) of cases was seen in the 10-15 years age group, followed by the <5 years age group (30%) and the 5–9 years age group (43%) (**Figure 3.2**). Owing to the heterogeneity associated with study estimates, a sensitivity analysis was performed. This was done by including only those studies that had at least 50 cases. On applying this directive, the burden of enteric fever in children increased with age. Overall the smallest proportion of cases (24%) was observed in the <5 years age group, followed by 36% in those 5-9 years of age, and 41% in the older age group. Substantial heterogeneity existed between studies in the youngest and oldest age groups. Estimates in those under 5 years of age ranged from 14% to 29%, compared with 30% to 44% in the 5-9 years group, and 28% to 52% in the 10-14 years group. Thus, even in the presence of statistical heterogeneity, it would appear that younger children form a smaller part of the burden of paediatric enteric fever in Africa (**Figure 3.3**).

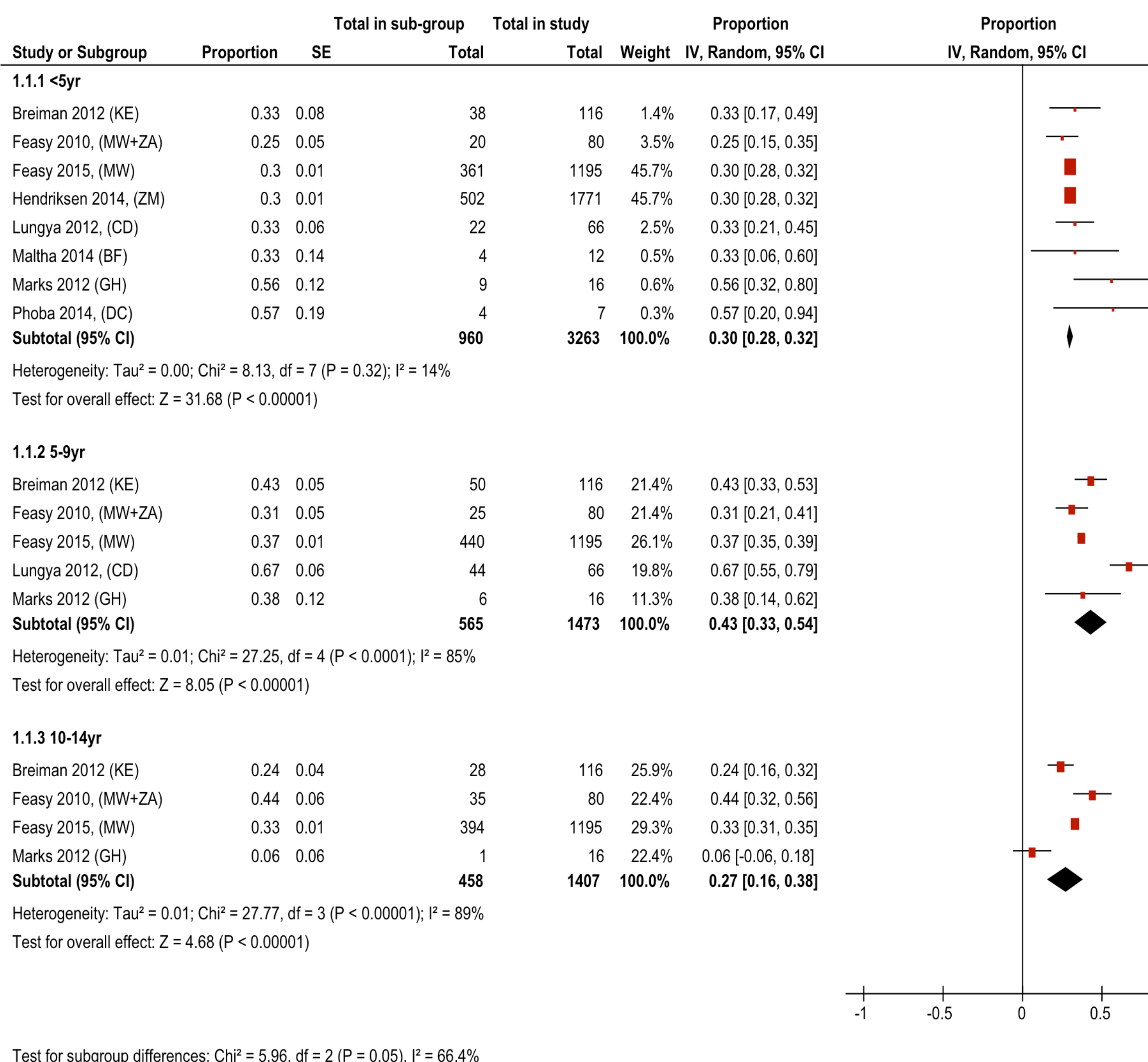


Figure 3.2: Meta-analysis comparing the age stratified prevalence of paediatric enteric fever cases in Africa (adapted from Britto *et al*, 2017). The smallest proportion (27%) of cases was seen in the 10-14 years age group, followed by the <5 years age group (30%) and the 5–9 years age group (43%)

Studies in the meta-analysis are identified based on author name, year of publication with country codes in parenthesis (country codes used are those supplied by the International Standardization Organisation). Studies with 50 or fewer cases were excluded.

SE: standard error; IV: inverse variance; Proportion: (Number of children in age group / number of children in the study aged 0–14 years). Proportions are standardized

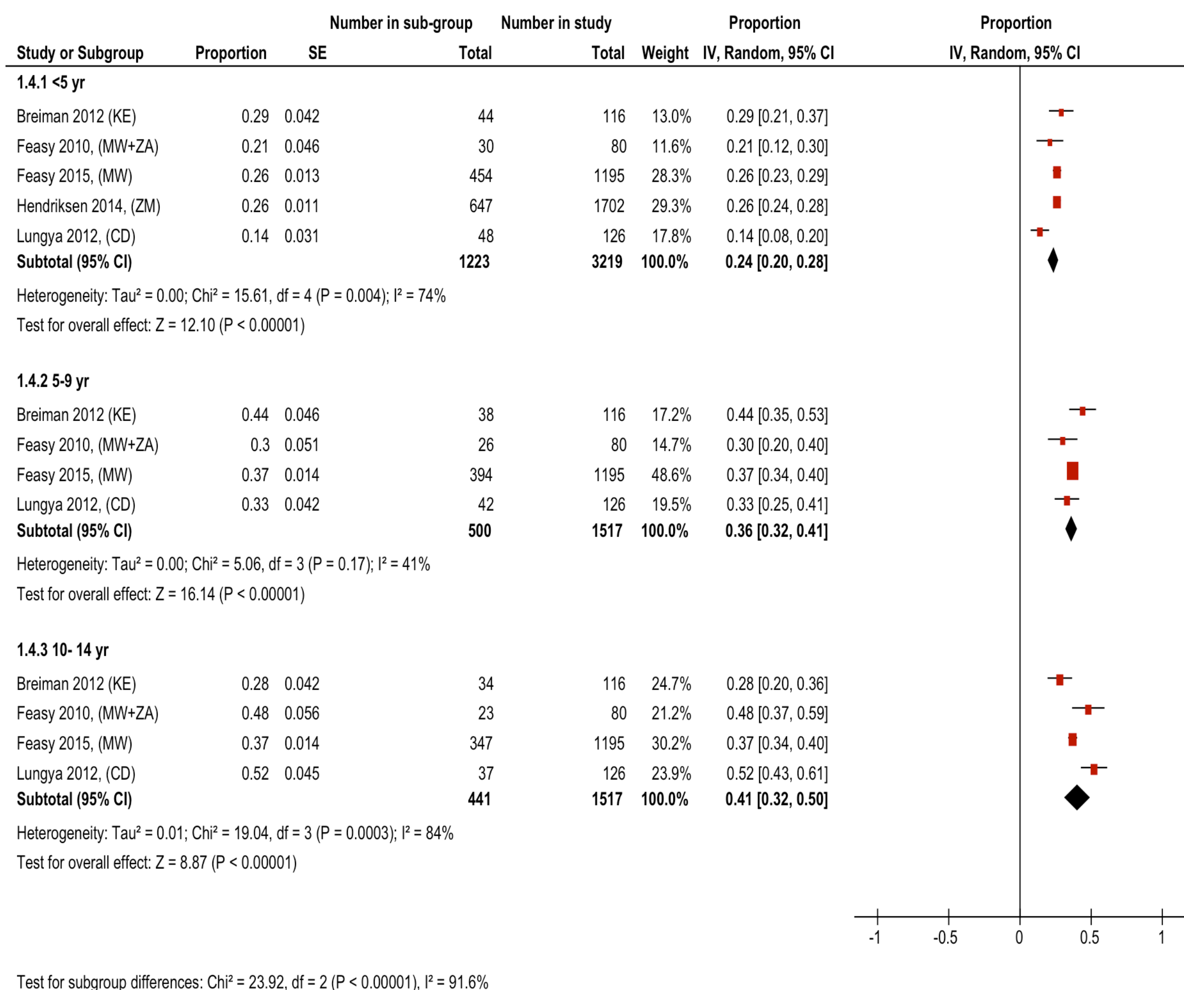


Figure 3.3: Sensitivity analysis of the age stratified prevalence of paediatric enteric fever cases in Africa (adapted from Britto *et al*, 2017). The <5 year age group account for the lowest disease occurrence

3.3.1.2. Disease occurrence in Asia

In comparison with Africa, the Asian studies also revealed that the youngest children had the least disease occurrence (32%) but unlike in Africa the 5-9 years age group accounted for the highest occurrence (40%) followed by the 10-14 years age group (36%) (**Figure 3.4**). Sensitivity analysis using the same precedent as for the African studies revealed a marginal difference in overall occurrence of disease in all age groups with the <5 years age group accounting for 30% of disease occurrence which was still the lowest among the three age groups while the 5-9 years and 10-14 years age groups accounted for 45% and 37% of the disease occurrence respectively (**Figure 3.5**). However, estimates for all age groups showed substantial heterogeneity among the Asian studies, which was most notable in those less than 5 years of age, making comparisons between summary estimates in each age group problematic. The proportion of cases in each study in the <5 years age group ranged from 5% to 73%. Of the 14 studies included in the analysis of the under 5 age group, 3 studies[313,316,321] estimated that 15% or less of the burden of disease was in this age group, whereas 3 studies[129,309,322] estimated that more than half the burden of paediatric disease was in this age group.

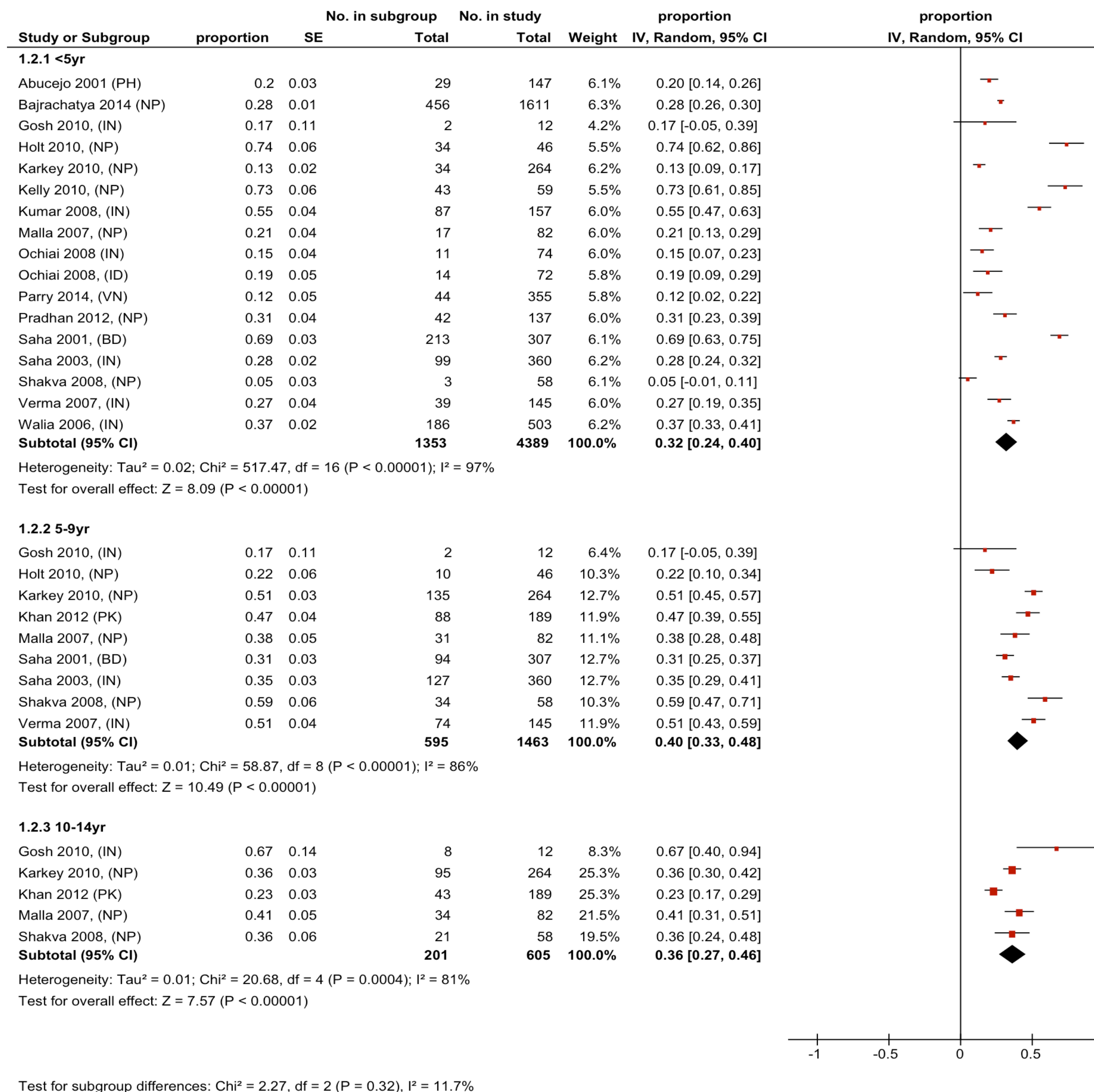


Figure 3.4: Meta-analysis of all studies comparing the age stratified occurrence of paediatric enteric fever cases in Asia. (adapted from Britto *et al*, 2017) The youngest children had the least disease occurrence (32%), the 5-9 years age group accounted for the highest occurrence (40%) followed by the 10-14 years age group (36%).

Studies in the meta-analysis are identified based on author name, year of publication with country codes in parenthesis (country codes used are those supplied by the International Standardization Organisation). Studies with 50 or fewer cases were excluded.

SE: standard error; IV: inverse variance; Proportion: (Number of children in age group / number of children in the study aged 0–14 years). Proportions are standardized according to population age-distributions for the three age categories. The analysis was done using random effects model.

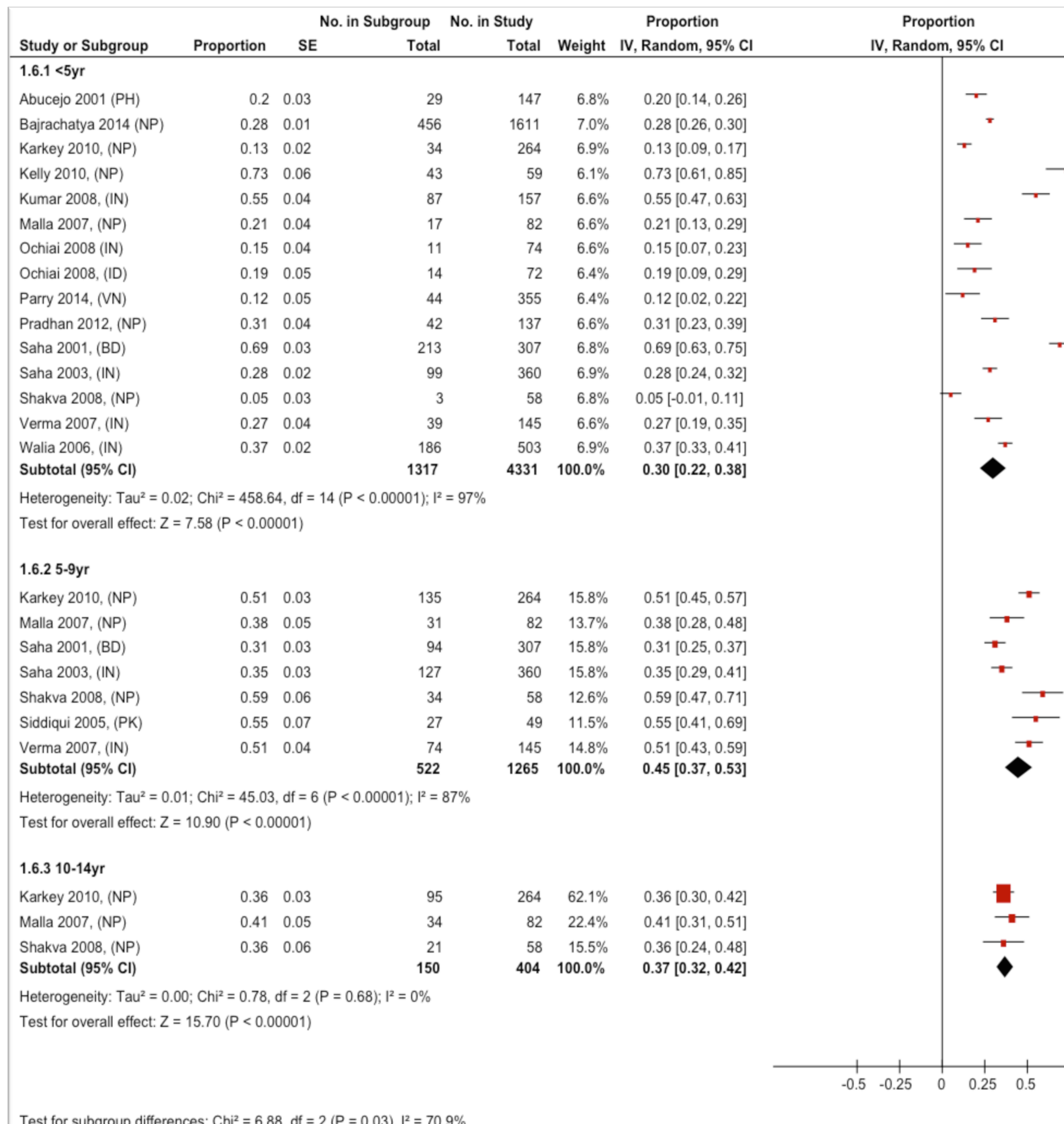
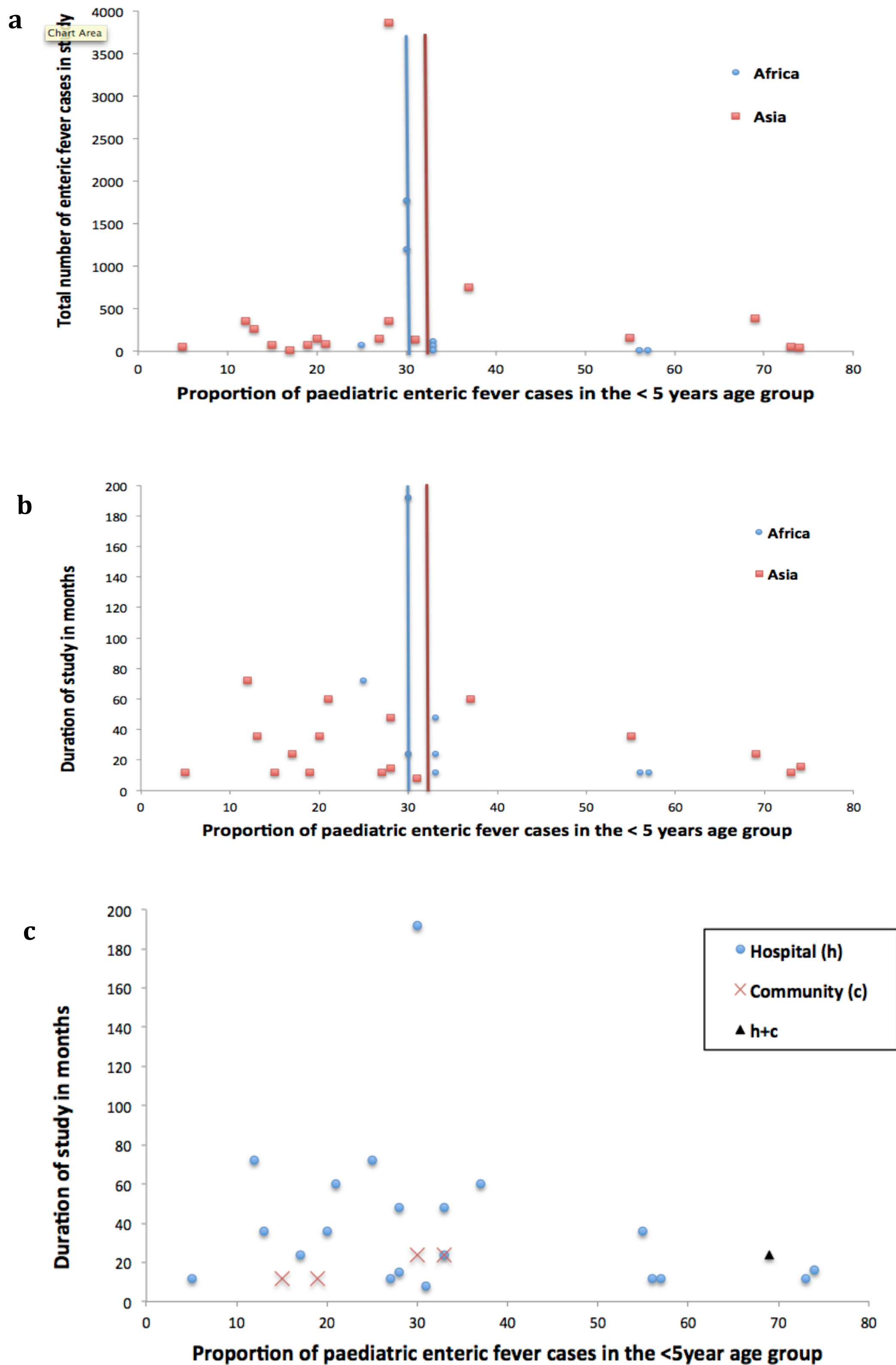


Figure 3.5: Sensitivity analysis of the age stratified prevalence of paediatric enteric fever cases in Asia (adapted from Britto *et al*, 2017). The <5 year age group account for the lowest disease occurrence

3.3.2. Examining sources of heterogeneity

These vastly contradictory findings illustrate the difficulty of diagnosis of enteric fever in children and thus the lack of expert consensus on this topic. Sources of variability in diagnosing younger children could be related to the high numbers of other non-specific illnesses in this age group, difficulty in obtaining adequate volumes of blood for culture, lower rates of exposure and protective effect of breast-feeding. Other potential sources of heterogeneity include study design, the duration of follow up, diagnostics used, and the possible use of vaccines. We investigated graphically, the possible study specific sources of heterogeneity in the < 5 years age group, including prospective or retrospective data collection, community or hospital settings, and duration of follow up and study size. No strong associations were observed; however there appeared to be a greater degree of variability in smaller studies than larger ones (**Figures 3.6 a-c**).



Figures 3.6 a-c (adapted from Britto *et al*, 2017): a) Plot between duration of study in months and proportion of paediatric enteric fever cases in the < 5 year age group. **b)** Plot between total number of enteric fever cases in studies and proportion of paediatric enteric fever cases in the < 5 year age group. **c)** Association between duration of study and disease proportion in <5 years age group between hospital based and community-based studies. Vertical lines indicate the mean proportion of paediatric enteric fever in the <5 year age group in Africa (30) and Asia (32) respectively

Table 3.1: Summary of included studies in the meta-analysis

	Author (year)	Country	Study design		Study Setting	Period of Data Collection	Sample size
African Studies							
1.	Breiman (2012)[324]	Kenya	Prospective	Population based surveillance	Community based	2007-2009	116
2.	Feasy (2015)[308]	Malawi	Prospective	Passive surveillance	Hospital based	1998-2014	1195
3.	Hendriksen (2014)[325]	Zambia	Retrospective	Cross-sectional study during an outbreak	Community based	2010-2012	1771
4.	Lungya (2012)[326]	Democratic republic of Congo	Prospective	Laboratory surveillance study	Hospital based (7 centres)	2007-2011	66
5.	Feasy (2010)[130]	Malawi and South Africa	Retrospective (Malawi) Laboratory Surveillance (South Africa)	Hospital based	Hospital based	1998-2004 (Malawi) 2003-2004 (South Africa)	80
6.	Maltha (2014)[327]	Burkina Faso	Prospective	Invasive bacterial surveillance	Hospital based	2008-2009	12
7.	Marks (2010)[328]	Ghana	Prospective		Hospital based	2007-2008	16
8.	Phoba (2014)[329]	Democratic republic of Congo	Prospective	Active surveillance	Hospital based	2011-2012	11
Asian Studies							
1	Abucejo (2001)[319]	Philippines	Prospective	Laboratory surveillance	Hospital based	1994-1997	147
2	Bajrachatya (2014)[320]	Nepal	Retrospective	Retrospective chart review	Hospital based (11 centres)	2008-2012	3857

3	Gosh (2010)[135]	India	Prospective	Passive surveillance	Hospital based	2003-2005	12
4	Holt (2010)[128]	Nepal	Prospective	Invasive bacterial (passive) surveillance	Hospital based	2005-2006	46
5	Karkey (2010)[321]	Nepal	Retrospective	Invasive bacterial (passive) surveillance	Hospital based	2005-2008	264
6	Kelly (2010)[322]	Nepal	Retrospective	Invasive bacterial (passive) surveillance	Hospital based	2005-2006	59
7	Kumar (2008)[129]	India	Prospective	Passive surveillance	Hospital based	1999, 2002, 2005	157
8	Malla (2007)[323]	Nepal	Retrospective	Chart review	Hospital based	2000-2005	82
9	Ochiai (2008)[133]	India	Prospective	Active Surveillance?	Community based	2003-2004	74
	Ochiai (2008)[133]	Indonesia	Prospective	Active Surveillance?	Community based	2003-2004	72
10	Parry (2014)[313]	Vietnam	Retrospective	Chart review	Hospital based (2 centres)	1993-1995, 1997-1999	355
11	Pradhan (2012)[314]	Nepal	Prospective	Invasive bacterial surveillance	Hospital based	April-August 2006, December-February 2007	137
12	Saha (2001)[309]	Bangladesh	Prospective	Passive surveillance	Community + hospital based	1998-1999	391
13	Saha (2003)[315]	India	Prospective	Passive surveillance	Hospital based	1990-2002	360
14	Shakva (2008)[316]	Nepal	Prospective	Passive surveillance	Hospital based	2006-2007	58
15	Verma (2007)[317]	India	Prospective	Passive surveillance	Hospital based	2004-2005	145
16	Walia (2006)[318]	India	Retrospective	Chart review	Hospital based	1999-2003	750

3.4. Discussion

3.4.1. Paediatric enteric fever in Asia:

The findings of this chapter highlight the general trends of paediatric enteric fever where much of our current understanding comes from surveillance sites in South and South-East Asia,[60,285,330–332] where accumulating evidence indicates a substantial burden of paediatric enteric fever. Settings with an incidence of >100/100,000 cases/year are considered high-incidence settings while those between 10-100/100,000 and < 10/100,000 cases/year are medium and low incidence settings respectively[1]. Some sites in Asia record incidence rates as high as 573/100,000 /year[133] and many other sites are mostly high incidence settings[132,133,333,334] . Disease rates in the control arm of cluster-randomized trials can also provide estimates of disease burden among children in the population under survey. Data from Karachi, evaluating the effectiveness of the ViPS vaccine, revealed incidence rates in the control arm of 230/100,000 and 190/100,000 person-years in the 2-4 year and 5-16 year age groups respectively, while data from a ViPS vaccine trial in Kolkata reported a burden of 354/100,000 person-years and 167/100,000 person years in the 2-4 year and 5-16 year age groups respectively [282,283]. It is likely that the presence of disease and intensity of transmission varies geographically, both within and between countries, which complicates development of control strategies. Risk factors for enteric fever identified in studies in endemic areas include residence in urban slums, the wet months of the year, lack of soap for hand washing and overcrowding in households[128,132,335].

School aged children over the age of 5 years have consistently been reported to have high rates of enteric fever, with incidence rates up to 4 times higher than adult populations[133]. Nevertheless, it is becoming increasingly evident that pre-school children are also significantly affected, with a surveillance site in Bangladesh reporting an 8.9 times higher likelihood of pre-school children acquiring enteric fever than older children and adults[127]. It

should, however, be borne in mind that this discrepancy in disease burden may be due to the implementation of school-based vaccination programmes in some areas which is reflected in the lower disease burden among school age children in some reports[132]. In addition to suffering increased morbidity, data from Nepal suggest that younger children may be important in maintaining transmission, as they appear susceptible to a broader range of haplotypes than are older children, including MDR haplotypes such as some H58 clones [9,128,138] which acquired resistance to ampicillin, chloramphenicol, and co-trimoxazole via a large conjugative incHI1 pST6 plasmid[80]. The emergence of multi-drug resistance has an important bearing on paediatric enteric fever treatment with defervescence periods being more prolonged in MDR infected children, particularly younger children who suffer increased morbidity and mortality[9,138].

Although paratyphoid fever in the paediatric populations is thought to occur mainly in older children and accounts for a lower proportion of enteric fever cases in South Asia[336], there are important areas of concern with this specific organism. Incidence estimates of *S*. Paratyphi A infection in children are similar to those seen in adults ranging between 51 and 76 cases/100,000 person years. *S*. Paratyphi B and C are rare and only seen sporadically[336]. Interestingly, some evidence suggests that a relatively higher burden of paratyphoid fever may follow ViPS vaccine introduction [336]. Evidence of cross protection with the available typhoid vaccines to *S*. Paratyphi B has been demonstrated with the Ty21a vaccine but, there is no strong evidence with respect to *S*. Paratyphi A cross protection[336,337]. China, particularly the East region, accounts for the highest burden of paratyphoid fever, while data from India demonstrated considerable heterogeneity between regions with hospital based data indicating higher proportions of paratyphoid from the West and the South when compared with the North and the East[336]. Rates from Pakistan seemed to mirror the pattern seen in North and East India[336]. It is currently difficult to assess whether the burden of enteric fever in Asia in

increasing or decreasing as there are no repeat studies done in areas of prior disease estimation.

3.4.2. Paediatric enteric fever in Africa

The pattern of disease and epidemiological features of paediatric *Salmonella* infections are different between the endemic regions of Africa and Asia. In Asia, *S*. Paratyphi is of increasing importance as the etiological agent of enteric fever and there is an insignificant burden of invasive non-typhoidal *Salmonella* (iNTS), unlike the situation in Africa, where iNTS and *S.* Typhi dominate. The emergence of MDR strains of *S.* Typhi, in particular the H58 clone has resulted in a sharp increase in disease burden in East Africa[29–39]. Disease rates have increased five-fold over the last 3 years in Malawi primarily due to the arrival of MDR strains[130]. Longitudinal studies from Malawi and the Democratic Republic of Congo highlight a similar distribution of disease burden among children in the 0-4 year, 5-9 year and 10-14 year age groups[308,40,326] while contributing to over 70% of cases in the community[308]. *S*.Typhi strains with reduced susceptibility towards ciprofloxacin are highly prevalent in Kenya but are not yet uniformly present across the rest of sub-Saharan Africa[349]. Recent collaborative efforts and investment in laboratory infrastructure[350] have supported improved invasive bacterial disease surveillance[326,327,351,352] resulting in better estimates of invasive *Salmonella* disease through the Typhoid Fever Surveillance in Africa Program (TSAP)[353],particularly amongst the paediatric population. However, most of these surveillance data capture inpatient burden, which potentially reflects severe disease. A large proportion of outpatient cases may thus go unobserved and this is particularly relevant in enteric fever where paediatric outpatients are 66% more likely to have *S*.Typhi in the blood stream than adult outpatients in sub-Saharan Africa[352]. This trend might also be true in Asian settings as seen with one study in Kathmandu, Nepal, where 10% of children with fever

attending a walk-in clinic (i.e. not requiring hospital admission) had blood cultures positive for *S.* Typhi and 3% for *S*. Paratyphi A during the rainy season[314].

Studies evaluating risk factors for transmission in urban informal settlements[324,351] ("slums") report that enteric fever incidence is ten times higher among children in the 2-9 year age group compared with their counterparts in rural areas[324]. Conversely, data from Ghana suggest that enteric fever rates are 2-3 times higher among children in the less populous rural than urban settings[191]. Over the last two decades most of sub-Saharan Africa has borne a heavy burden of iNTS which appears to have enhanced transmission in a setting of severe malaria, HIV and malnutrition, with a bimodal mortality in young infants and middle-aged immune compromised adults[350,308]. There is a gradual change with the emergence of antibiotic resistant typhoidal *Salmonella* providing an increasing relative burden, in the absence of a vaccine strategy across the continent.

3.5. Conclusions

The burden of enteric fever in children is difficult to measure, particularly with respect to which age group bears the brunt of the burden. Some studies report an equal occurrence among preschool and older children while others report a significantly higher burden in the preschool age group. It should be noted that school based vaccination programmes have been implemented erratically in South Asia, which may account for varied disease prevalence in the school-aged children in Asia.

"The things we pray for, good Lord, give us grace to labour for"
- Thomas More

Chapter 4

The molecular structure of *S.* Typhi strains isolated from Nepali children over a 9-year period

4.1. Introduction

The highly conserved and clonal nature of the *S.* Typhi population necessitates the use of whole genome sequencing to satisfactorily discriminate between lineages among locally circulating pathogen populations.

4.1.1. Molecular structure of Nepali strains prior to this study

Single nucleotide polymorphism (SNP) genotyping of *S.* Typhi isolated in a study of paediatric enteric fever cases at Patan Hospital in Kathmandu, Nepal between 2005 and 2007 suggested that, among children treated as inpatients, those aged ≤4 years were susceptible to

a wider range of haplotypes probably attributable to immune naivety[128]. The most common haplotype was H58 (70%), followed by H42 (19%)[128]. Another study of adults and children at Patan Hospital from 2005 to 2009 found that typhoid fever was mainly caused by H58 lineage II (61%) or other H58 (3%), or H42 (15%)[354]. More recently, whole genome sequencing (WGS) was applied to study *S.* Typhi isolates collected during a randomized controlled trial of gatifloxacin vs ceftriaxone for treatment of blood culture confirmed enteric fever at Patan Hospital between 2011 and 2014, and found the H58 population of strains continued to dominate the circulating *S.* Typhi population (83%)[276].

4.1.2. Global dominance of H58

A 2016 publication by Wong and colleagues highlighted the global dominance of H58 strains (4.3.1 genotype) and suggested that this clonal population is responsible for the widespread AMR related to enteric fever[83]. This genotype is widely prevalent across endemic settings of South and South East Asia as well as Southern and Eastern Africa. It is unknown whether children and adults are affected by different circulating strains or whether there are certain transmission niches among certain age groups.

4.1.3. Rationale for the study

This study was conducted with the aim of identifying the molecular structure of circulating *S.* Typhi strains isolated from Nepali children between 2008 and 2016. In addition, these strains were also placed in a regional and worldwide context to gauge genetic relatedness and delineate patterns of intra- and inter-region transmission.

4.2. Methods

4.2.1. Ethics statement

Ethical approval was obtained from the Oxford Tropical Research Ethics Committee (OxTREC) as well as local institutional approval from the Nepal Health Research Council (R31579/CN007).

4.2.2. Study Setting

Nepal is a low income[355], landlocked Himalayan nation with an under-five year old mortality rate of 35.8 per 1000 live births as of 2015[356]. Kathmandu Valley, the main urban centre of Nepal, has three districts and a population of 2.5 million[357] (average population density: 2,372/km^2) of which 31% are between 0-14 years under age[358]. Over the course of the study, the Patan Academy of Health Sciences (Patan Hospital) was one of only two large hospitals in Kathmandu Valley with referral and paediatric intensive care services. Patan Hospital accepts patients from all over the Valley. Annually the paediatric department cares for over 50,000 outpatients (21% of all hospital outpatient attendances) and accepts approximately 2,700 inpatient admissions. Only 10% of the patients reside outside Kathmandu Valley.

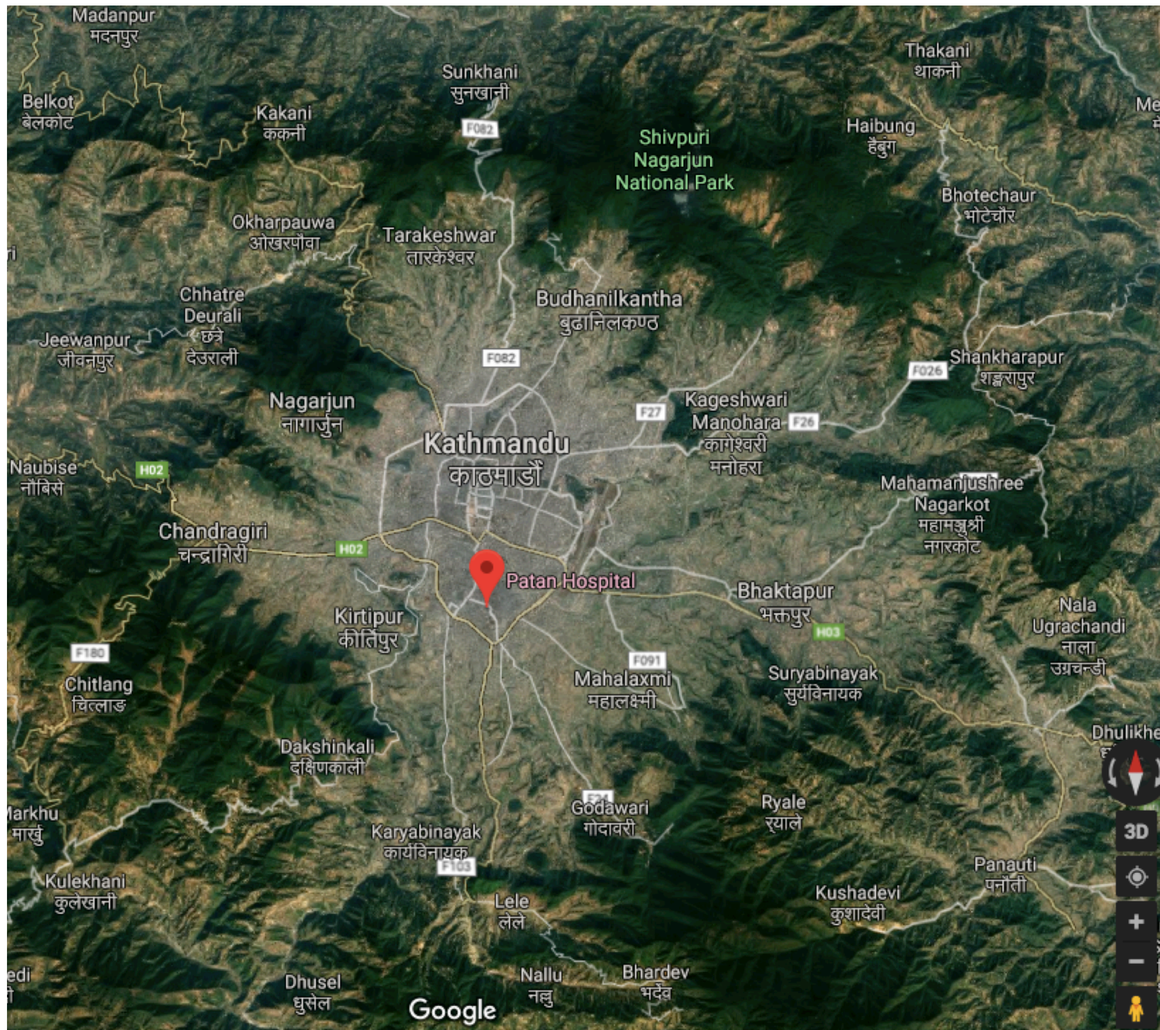


Figure 4.1: The location of Patan Hospital in the Kathmandu valley. This satellite image shows the landscape of the region where the densely populated Kathmandu valley is surrounded by the sparsely populated mountainous terrain.

4.2.3. Surveillance of culture confirmed enteric fever amongst inpatients

Febrile children under 14 years of age, attending PAHS with clinical suspicion of invasive bacterial disease between January 2008 and December 2016 were included in an invasive bacterial disease surveillance database after informed consent from the parents as described previously[359]. Inclusion criteria were: clinical presentation indicating an invasive bacterial infection requiring inpatient care with intravenous antibiotics. Blood culture was conducted as described below. Of the patients included in the database, all those that had blood cultures positive for *S*. Typhi or *S*. Paratyphi A were included in the present study, along with relevant demographic data. A random collection of 67 *S*. Typhi isolates and 17 *S*. Paratyphi A isolates were selected for whole genome sequencing; these represent isolates associated with the severe spectrum of paediatric enteric fever presenting to the hospital.

4.2.4. Isolates collected from outpatients

Children with milder clinical presentations who are usually treated with oral antibiotics as outpatients were not included in the invasive bacterial disease database; however they are subjected to the same microbiological diagnostic procedures as inpatients (as detailed below). A total of 1283 *S*. Typhi and 926 *S*. Paratyphi A isolates from paediatric outpatients were stored between 2008 and 2016; every 10th *S*. Typhi isolate and every 5th *S*. Paratyphi A isolate were included in this current study, representing isolates associated with milder presentation of paediatric enteric fever at the hospital.

4.2.5. Mapping of sequences, production of a WGS alignment and SNP analysis

For analysis of SNPs in *S*. Typhi, the bioinformatic pipeline elaborated in **section 3 of chapter 2** was used and SNPs identified through the pipeline were concatenated to produce an alignment of alleles at 233,527 variant sites resulting in a final set of 2,187 SNPs identified in an alignment length of 4,809,037 bp for the 198 novel Nepali *S*. Typhi isolates.

4.2.6. Regional and global context

To provide regional context, genome data from: (i) a published study of mainly Nepali adults[276] (n=95) between 2011 and 2014, (ii) a global *S*. Typhi genome collection[97] (n=1,221) from 66 countries spanning over 30 years; were subjected to SNP calling and genotyping, resulting in an alignment of 12,216 SNPs for a total of 1,514 isolates. An additional analysis of 261 H58 strains (genotype 4.3.1) from Nepal was carried out in the same manner, resulting in an alignment of 631 SNPs.

4.2.7. Phylogenetic analysis

These analyses were carried out as per the methodology entailed in the pipeline in **section 2.3.3 of chapter 2**. For visualization purposes, *S*. Typhi isolates representing 'outbreaks' (defined as members of the same monophyletic clade, isolated from the same study location in the same year) were manually thinned to a single representative.

4.2.8. Temporal analysis

To investigate the temporal signal and emergence dates of antimicrobial resistance determinants for Nepali *S*. Typhi 4.3.1, we used several methods. First, we used TempEst (v1.5)[360] to assess temporal structure (i.e. whether the data have clocklike behaviour) by conducting a regression of the root-to-tip branch distances of the Nepal H58/4.3.1 ML tree as a function of the sampling time, using the heuristic residual mean squared method with the best-fitting root selected. The resultant data were then visualized in R[361]. To estimate divergence times we analysed the sequence data in BEAST2 (v2.4.7)[362]. We used both constant-coalescent population size and Bayesian skyline tree priors, in combination with either a strict molecular clock model or a relaxed (uncorrelated lognormal distribution) clock model to identify the model that best fits the data. For the BEAST2 analysis the GTR+Γ substitution model was selected, and the sampling times (tip dates) were defined as the year of isolation to calibrate the molecular clock. For all model and tree prior combinations, a

chain length of 100,000,000 steps sampling every 5000 steps was used[363]. The relaxed (uncorrelated lognormal) clock model, which allows evolutionary rates to vary among branches of the tree together with the skyline demographic model, proved to be the best fit for the data. To assess the signal of these Bayesian estimates we conducted a date-randomization test whereby sampling times were assigned randomly to the sequences, and the analysis re-run 20 times[363,364]. These randomization tests were conducted with the same 'best fit' models (uncorrelated lognormal clock and skyline demographic). This test suggested that the data display 'strong' temporal structure[363].

For the final analysis reported here, 5 independent runs conducted with a chain length of 600,000,000 states, sampling every 300,000 iterations, were combined using LogCombiner (v2.4.7)[362] following removal of the first 10% of steps from each as burn-in. Maximum-clade credibility (MCC) trees were generated with 'keep target heights' specified for node heights using TreeAnnotator (v2.4.7)[362,365]. The effective sample sizes from the combined runs were estimated to be >200 for all reported parameters.

4.3. Results

4.3.1. High occurrence of paediatric enteric fever over the period of surveillance

Blood cultures were performed on 11,430 children with a suspected invasive bacterial infection and requiring inpatient care with intravenous antibiotics and supportive care. Of these, 129 had blood cultures positive for the enteric fever agents *S*. Typhi (n=102, 79%) or *S*. Paratyphi A (n=27, 21%). Relevant patient characteristics are reported in **Table 4.3.1**. Most cases of culture-confirmed enteric fever (n=83, 64%) occurred between the hot and rainy months of May and October. However, a substantial proportion (36%) of cases also occurred in colder months, indicating perennial transmission. Children under 5 years of age accounted for 45% of the disease burden among inpatients, with children under 2 years of age

accounting for 18% (**Table 4.1**). Clinical suspicion of enteric fever at presentation was significantly lower amongst children under 2 years with culture-confirmed infection (13% vs. 52%, p=0.0005 using Fisher's exact test; (**Table 4.1**), highlighting the undifferentiated febrile nature of the disease even in an endemic setting such as Nepal.

Table 4.1: Hospital based (inpatient) paediatric enteric fever surveillance

Total blood cultures performed	11430			
Total number of significant cultures	1048 (9.2%)			
Total number of enteric fever pathogens *S.* Typhi *S.* Paratyphi A	129 (1.1%) 102 (0.9%) 27 (0.2%)			
Age stratified characteristics of blood-culture positive enteric fever patients				
	Age groups			
	<2 years	**2-4 years**	**5-9 years**	**10-14 years**
Number	23	35	39	31
Median age (years)	1.2	3.3	6.8	11.8
Male (%)	16 (70%)	23 (66%)	24 (62%)	18 (58%)
Median temperature at admission (C^o) (range)	37.2 (36.7-38.9)	38.3 (36.5-39.9)	38.9 (36.1-40.5)	37.2 (36.5-39.2)
Median duration of admission (days) (range)	6 (2-23)	6.5 (1-19)	8 (2-36)	7.5 (3-20)
Enteric fever suspicion on admission (%)	3 (13%)	17 (49%)	19 (49%)	19 (61%)

4.3.2. Phylogenetic structure of paediatric isolates from Nepal

The genomes of *S*. Typhi isolated from inpatient surveillance (n=67) and a random selection of isolates from outpatients (n=131) were sequenced and subjected to SNP genotyping and phylogenomic analysis. The resulting phylogeny (**Figure 4.2 A**) revealed the presence of 8 distinct genotypes, each corresponding to a different subclade including 2.0.0 (N=1, 0.5%) 2.2.0 (N=10, 5%), 2.3.4 (N=2, 1%), 3.2.2 (N=6, 3%), 3.3.0 (N=19, 9.6%), 3.3.1 (N=3, 1.5%), 4.1.0 (N=3, 1.5%), and 4.3.1 (N=154, 77.8%).

A) Phylogenetic tree in this study

B) Heterogeneity in pathogen molecular structure over time

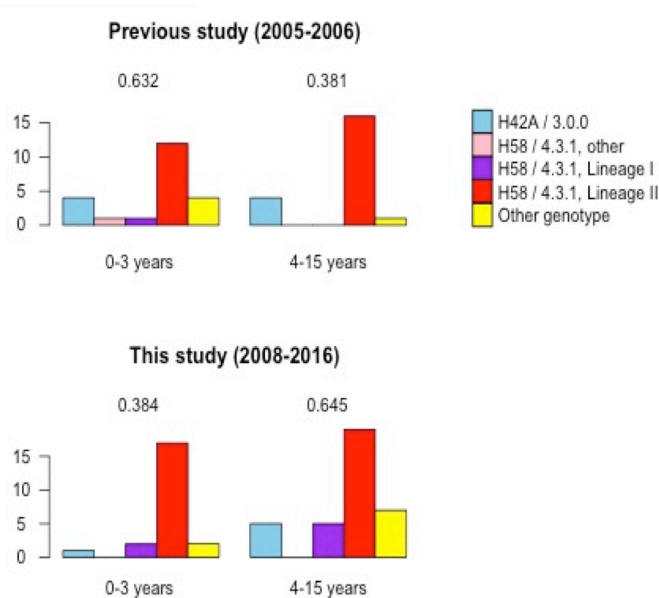


Figure 4.2: The molecular structure of *S.* Typhi strains isolated from children in Nepal between 2008-2016.

Panel A represents the phylogenetic tree and characterisation of isolates between 2008-2016 (this study). Branch colours indicate the genotypes which are labeled at each branch; branch lengths are indicative of the estimated number of substitutions rate per variable site; the tree was outgroup rooted using *S.* Paratyphi A strain AKU_12601. Panel B depicts the comparison in the heterogeneity of strains as indicated by the Simpson's index above each graph. The previous study refers to Holt *et al* 2010 which analysed the heterogeneity of strains in the same setting between 2005-2007. The initial hypothesis from the 2005-2007 strains that younger age groups were more susceptible to a wide range of genotypes is contradicted by this study. The H58 Lineage strains continue to dominate the circulating population.

There was no association between genotype and treatment status (outpatient vs. inpatient), period of isolation (**Figure 4.3 A**) or patient age (**Figure 4.3 B**).

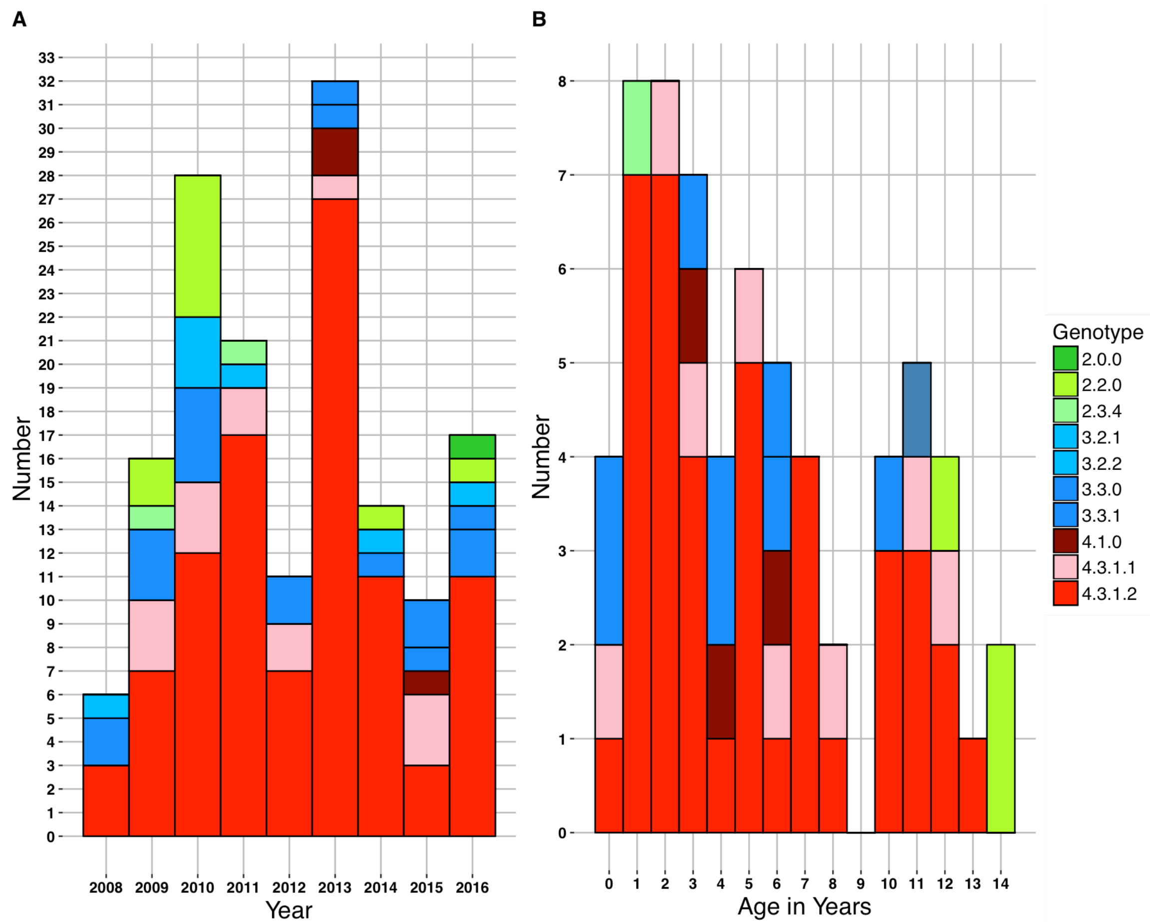


Figure 4.3: Nepal paediatric *S.* Typhi genotypes by year (A) and patient age (B). This figure illustrates the dominance of the H58 strains (4.3.1 genotype) across time and age groups. Further stratification of this genotype revealed that the lineage II strains were greater in proportion

4.3.3. Regional and Global context

To place the novel paediatric isolates in context, a whole genome phylogeny including other *S.* Typhi isolates previously sequenced from adults in Nepal, and a global collection of *S.* Typhi isolates was constructed (**Figure 4.4**; an interactive version of the phylogeny and associated geographical data are also available for exploration online at https://microreact.org/project/SJmU6dhlz). The novel paediatric isolates clustered together with the adult isolates from Nepal, with no evidence of specific genotypes circulating in children more so than adults. In comparison to global isolates, Nepali isolates clustered with those from other regions in the Indian subcontinent, suggesting on-going regional transmission (**Fig 4.4**); indeed 14% of the novel Nepali paediatric isolates and 15% of the previously sequenced Nepali isolates were closest to an isolate from outside Nepal (majority from neighbouring India, Bangladesh or Pakistan), indicating frequent pathogen transfer within the region.

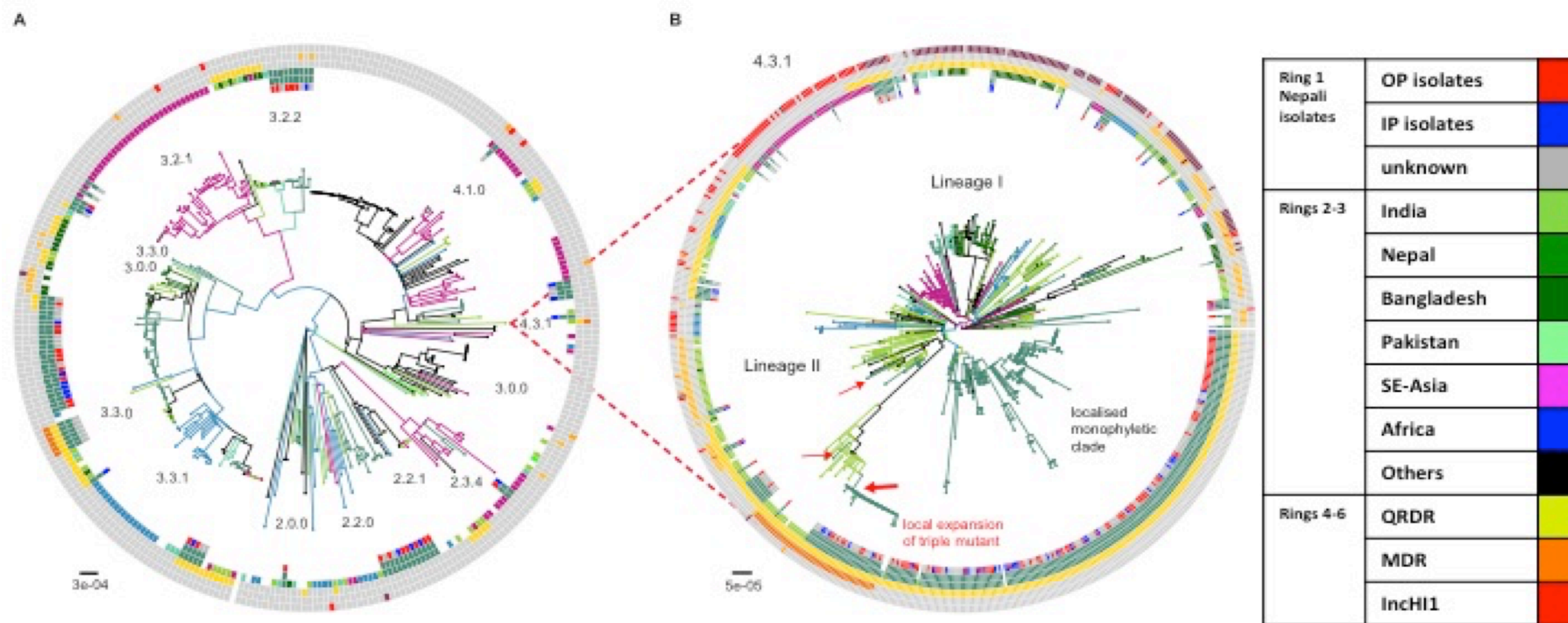


Figure 4.4: Global context of *S.* Typhi in Nepal, shown separately for (A) non-H58 (4.3.1) genotypes and (B) H58 isolates. Rings indicate isolate/patient features according to the inset legend; note the inner-most ring highlights Nepal isolates only. Branch colours indicate country/region of origin according to the ring 2-3 legend; branch lengths are indicative of the estimated number of substitutions rate per variable site; trees are outgroup rooted using *S.* Paratyphi A strain AKU_12601. Genotypes are labeled in panel A, lineages of 4.3.1 are labeled in panel B. The trees show that Nepal isolates are intermingled with isolates from other countries, particularly from the South Asian region, indicative of regional circulation of genotypes. The 4.3.1 tree (B) shows several introductions of 4.3.1 into Nepal from other countries, with one major monophyletic clade that is unique to Nepal, resulting from local clonal expansion; red arrows highlight three separate introductions of QRDR triple-mutant ciprofloxacin-resistant strains, one of which has undergone local transmission and clonal expansion in Nepal.

4.3.4. Evolutionary analysis of H58 (4.3.1 genotype) strains in Nepal

A dated phylogeny of all available *S*. Typhi 4.3.1 from Nepal was constructed, using BEAST2 (**Figure 4.5,** interactive version available at https://microreact.org/project/rJnfyOGxG). This analysis yielded a local substitution rate of 0.8 SNPs per genome per year (95% highest posterior density (HPD), 0.5 – 1.1) or 1.7×10^{-7} genome-wide substitutions per site per year (95% HPD, $1.1 \times 10^{-7} – 2.4 \times 10^{-7}$). The data showed strong temporal structure which were consistent with previous estimates for global *S*. Typhi 4.3.1[83]. The most recent common ancestor (mrca) for all *S*. Typhi 4.3.1 in Nepal existed circa 1993, similar to the mrca estimated globally for *S*. Typhi 4.3.1, which is predicted to have emerged in neighbouring India[83]. Both of the described sublineages of *S*. Typhi 4.3.1 (I and II) were present amongst the Nepali isolates, however lineage II was far more common (67% vs. 10% of paediatric isolates from this study; 68% vs 10% of isolates from other studies). The majority of isolates formed a localised monophyletic clade that was not detected in other countries in the global collection, indicative of local clonal expansion in Nepal (**Figure 4.4**). The relative proportion of local *S*. Typhi infections caused by lineage II increased after 2010 (40% pre-2010 vs 74% from 2011 onwards, $p=1 \times 10^{-7}$), suggesting clonal replacement of the MDR-associated Lineage I with the expansion of the quinolone resistance-associated lineage II over time.

Chapter 7 provides a detailed analysis of the phenotypic characteristics and molecular determinants of AMR in these Nepali strains. For the purpose of describing and differentiating these two lineages of the 4.3.1 genotype (H58) a brief mention of some molecular characteristics are revealed here.

Among these two lineages different AMR patterns were observed (**Figure 4.5**): lineage I was associated with acquired AMR genes (59% of lineage I vs 0 lineage II, $p<1 \times 10^{-15}$), while

lineage II was associated with QRDR mutations (99% of lineage II vs 50% of lineage I, $p<1x10^{-15}$).

Most of the ciprofloxacin resistant triple mutant isolates harboured *gyrA* S83F, *gyrA* D87N, and *parC* S80I and formed a monophyletic subclade of lineage II, together with those previously linked to gatifloxacin failure during the treatment trial conducted in 2013-2014[276]. The mrca of this triple-mutant subclade was dated to 2008 (95% HPD, 1998–2011; see **Figure 4.5**), and comparison with the global tree confirmed it most likely originated in India[276] and was introduced to Nepal at least twice. A distinct ciprofloxacin resistant triple mutant strain (harbouring *gyrA* S83F, *gyrA* D87G, and *parC* E84G) that was isolated from a five-year old girl in 2011 was also noted. This too belonged to *S*. Typhi 4.3.1 lineage II, but shared no particularly close relatives in the Nepali or global collections (**Figure 4.5**).

All isolates with acquired AMR genes belonged to lineage I, and were consistent with at least three distinct acquisition events (**Figure 4.5**): one cluster of isolates (from a previous study conducted by Thanh *et al* 2016) carried the IncHI1 MDR plasmid and had an estimated mrca of 2004 (95% HPD, 1996-2007); two related clusters with the composite transposon inserted in the chromosome after STY3618 shared a mrca in 2001 (95% HPD, 1995-2009); and one cluster had Tn*6029* inserted in the chromosome, with mean mrca 2003 (95% HPD, 1997-2010).

Figure 4.5: Dated phylogeny showing relationships between all *S.* Typhi 4.3.1 (H58) isolates from Nepal that have been sequenced to date. Tree shown is the maximum clade credibility tree inferred using BEAST2; branch lengths are in units of time, according to the scale on the x-axis which indicates calendar years. Tip colours indicate outpatient (red) vs. inpatient (blue) isolates; other source information or features are given in the heatmap, coloured according to the inset legend. The green shaded clade forms a monophyletic Nepal-only subclade in the global phylogenetic tree, indicating results from localised clonal expansion within Nepal; other isolates result from a mix of transfer into Nepal from other countries, and smaller local clonal expansions. The estimated points of acquisitions of AMR genes are indicated on the tree in red, and the most recent common ancestor of the dominant ciprofloxacin-resistant triple-mutant is shown in green.

4.4. Discussion

These data show there is a substantial occurrence of enteric fever amongst children attending Patan hospital (**Table 4.1**). Since this hospital serves as a major referral hospital in the Kathmandu valley it is likely to be representative of the trends among Nepali children seeking hospital care in general. However, since this study is hospital based it may not be representative of trends in the community. This is indeed an issue with most epidemiological enteric fever data as shown in a recent systematic review which reported that 96% were from urban settings, over 95% were hospital based and a majority of these studies did not report data in an age-stratified manner[9]. Nevertheless, the molecular epidemiology data of this chapter provides great insight into understanding the disease trends and an on-going community surveillance study in Nepal is under way to provide age-stratified disease estimations in the Nepali community.

Genomic analysis revealed substantial diversity within the local pathogen population (**Figure 4.2 and Figures 4.3 A&B),** with evidence of transfer of *S*. Typhi between Nepal and neighbouring countries in South Asia (**Figure 4.4**), and intermingling of isolates from adults and children consistent with transmission across age groups (**Figure 4.4 and 4.5**).

4.4.1. H58 strains dominate the molecular structure of the circulating population

Data from 2005-2007 suggested that younger children were more susceptible to a wider range of genotypes. This finding was hypothesised to be due to a naïve immune response by young children who have not been exposed to a wide range of circulating genotypes[128]. A decade later this hypothesis does not hold which could be attributed to the overwhelming dominance of the H58 lineage II population as seen in **Figure 4.2**, which shows the *S*. Typhi 4.3.1 genotype is dominant regardless of the age of the host (and see also a direct comparison between the two studies in **Figure 4.2**). However, as evidenced by recent mathematical modelling data it is probable that the findings in 2005-2007 were indeed true as the *S*. Typhi

4.3.1 population was still gaining its foothold among the circulating pathogen population. These mathematical models identified the introduction of *S*. Typhi 4.3.1, as well as an increase in migration of immunologically naïve 15-25 year olds from outside the Kathmandu Valley, as key drivers of the local enteric fever problem[366] which occurred around the early 2000's. The isolates from this chapter were obtained after *S*. Typhi 4.3.1 was established following the displacement of other genotypes. The further dominance of lineage II strains over lineage I strains of H58 are important findings of this study given the differing capabilities in carrying AMR determinants which is a direct reflection of antimicrobial pressure. Studying the population structure over time also allows for comparisons to be made before and after the introduction of the Vi-TT conjugate vaccine in Nepal as an additional way of assessing vaccine impact.

4.4.2. Disease in the youngest age groups.

The findings of this chapter supplement our understanding of enteric fever in an endemic setting. The occurrence of disease in the <5 years population is in agreement with the other multi-centre data from South Asia[367], underscoring the importance of understanding the disease transmission dynamics and preventive strategies in the most vulnerable populations. The magnitude of disease occurrence in this age group is still an underestimation for several reasons; clinical suspicion of enteric fever in this age group is generally low as evidenced in these data and this trend has also been reported in other endemic regions[368]. The lack of clinical suspicion in the younger age-groups, which in this study is significantly lower that in the older age-groups, leads to a lack of diagnostic testing, which is in itself, fraught with impediments to reliable results. Blood culture, which is the feasible gold standard diagnostic performs poorly in this population owing to the difficulty in obtaining the required amount of blood and due to pre-treatment with antimicrobials prior to obtaining a blood sample[9,367]. Despite the unique challenges associated with diagnosing enteric fever in this population and

the supposed lack of exposure, reports from various endemic regions in addition to the findings of this study continue to reiterate the enormous burden of enteric fever in pre-school children.

4.4.3. Preventive strategies

Children have usually been the focus of vaccination strategies for most diseases. However, in the case of typhoid, children have not been adequately covered historically given the pitfalls of the Ty21a and Vi polysaccharide vaccine. The pre-qualification and SAGE recommendation of the novel Vi-TT conjugate vaccine now makes it possible to protect the youngest age-groups. The Vi conjugate vaccines offer the real possibility of controlling enteric fever but eradication will only become a possibility when the immunization strategy is supplemented by the provision of clean water and improved sanitation. The intermingling of isolates from various studies in the BEAST tree signifies that long-cycle transmission, through contaminated water supplies is probably the dominant mode of transmission in this setting which calls for environmental interventions and that vaccinating only children might not effectively deal with the disease as a whole.

4.4.4. Intra- and inter-region transmission and implications for prevention

From sections **4.3.3** and **4.3.4** it is obvious that enteric fever transmission occurs between various regions of the Indian sub-continent. This finding calls for a coordinated strategy to effectively deal with disease. In the 1970's a successful typhoid vaccine programme nearly eliminated the disease from Thailand. However, introductions from neighbouring South-East Asian countries ensured the continued transmission of enteric fever into Thailand and when AMR began to develop the disease became endemic once again[369]. This could potentially occur in Nepal despite a successful Vi-TT conjugate vaccine coverage if the import of strains from neighbouring countries are not checked. One way of ensuring this is to have a co-ordinated mass vaccination programme across the Indian subcontinent vaccinating children in

addition to young adults. This might however be economically and logistically difficult to implement.

4.4.5. Limitations

The methods and results of this chapter has limitations as all isolates examined were from a single hospital based passive surveillance programme and thus may not be representative of the disease trends in the wider community. For inpatient isolates only stored samples were available (67 out of 102 for *S.* Typhi) for retrospective sequencing analysis, preventing the use of a more formal random sampling technique; however this is unlikely to introduce sampling bias with respect to genotype, as genotypes were not known at the time of storage and there is no reason to suspect particular genotypes would be more likely to have been stored. Consistent with this expectation, the population structure observed here is comparable with that observed in previous studies suggesting that this opportunistic sampling is reasonably representative of the population structure of paediatric typhoid in Nepal from 2008-2016. Outpatient isolates were randomly sampled and expected to be representative, but these were unable to be stratified by age, gender and admission information as outpatient data was limited and did not include any patient level information.

4.5. Conclusion

These data highlight the occurrence of *S.* Typhi infection in children in Nepal while demonstrating the importance of laboratory and molecular surveillance in endemic regions. Those under the age of 5 years contributed most to the occurrence of enteric fever among inpatients who represent the severe spectrum of disease. The substantial contribution of those less than 2 years emphasize the urgent need for the Vi –TT conjugate vaccine in regions such as Nepal where antimicrobial therapy is currently the main modality against enteric fever.

"All the darkness in the world cannot extinguish the light of a single candle."
-St. Francis of Assisi

Chapter 5

The molecular structure of *S.* Paratyphi A strains isolated from Nepali children over a 9 year period

5.1. Introduction

Like *S*. Typhi, *S*. Paratyphi A is also highly clonal and conserved and whole genome sequencing is required to satisfactorily discriminate between lineages among locally circulating populations.

5.1.1. The epidemiology of *S.* Paratyphi A in Nepal

Karkey *et al*, in their 2013 publication reported that exposures related to poor water and socioeconomic status were more important in the risk of acquiring *S.* Typhi infection, whereas in the case of *S.* Paratyphi A infection food consumption habits and migration play more of a role in Nepal[370]. In 2006 it was reported that there was a greater prevalence of *S.* Paratyphi A with higher rates of MDR and ESBL production when compared with *S.* Typhi in Nepal[371].

Zhou *et al* reported that most strains of *S.* Paratyphi A in Nepal belong to lineage A and show no significant shifts in genetic composition when compared with its most recent ancestor[372]. The mechanisms of AMR are similar to that of *S.*Typhi and present similar problems with regard to sustainable treatment regimens[373]. Further information on the molecular epidemiology of *S.* Paratyphi A from Nepal is generally limited particularly regarding paediatric disease.

5.1.2. The global epidemiology of *S.* Paratyphi A

S. Paratyphi A, B and C account for a significant proportion of enteric fever in Asia, with *S.* Paratyphi A being more dominant than the other serovars. While it is rare in the continent of Africa in general, within the continent of Asia, endemic disease is mainly found in the Indian sub-continent, China and South-East Asia[374].

The most recent common ancestor of Paratyphi A existed approximately 450 years ago and recent increases in frequencies of bacterial diseases are due to environmental changes rather than the novel evolution of this serovar[372].

Zhou *et al*, identified seven modern lineages among 149 genomes on the basis of 4,584 SNPs in the core genome of *S*. Paratyphi A[372]. Over the last 450 years the effective population size of *S*. Paratyphi A has undergone a series of changes in response to various selection pressures culminating in recent expansion. Most genetic changes are transient, continuously being

removed by purifying selection, such that the genome of Paratyphi A has not changed extensively over centuries[372].

5.1.3. Rationale for the study

This study was conducted with the aim of identifying the molecular structure of circulating *S.* Paratyphi strains isolated from Nepali children between 2008 and 2016. In addition, these strains were also placed in a regional and worldwide context to gauge genetic relatedness and delineate patterns of intra- and inter-region transmission.

5.2. Methods

The recruitment of patients, collection and processing of samples as well as the DNA extraction, whole genome sequencing and phylogenetic analysis were similar to that elaborated in **sections 2.3** and **4.2** in **chapters 2** and **4** respectively

5.2.1. Mapping of sequences, production of a WGS alignment and SNP analysis

The SNPs obtained from the bioinformatic pipeline were used to assign isolates to previously defined lineages according to a classification scheme developed by Zhou *et al*[372]. This process resulted in a final set of 365 SNPs identified in an alignment length of 4,581,797 bp for the 66 novel Nepali *S*. Paratyphi A isolates. SNP alleles from CT18[84] (accession AL515582) were also included as an outgroup to root the tree.

5.2.2. Regional and global context

To characterize and analyse the genomes of the 66 *S.* Paratyphi A strains, a similar bioinformatic process was adopted using *S.* Paratyphi A AKU_12601[295] (accession no: FM200053) as the reference genome to create an alignment with another 176 isolates from previous studies[375–377], for global context resulting in an alignment of 5,277 SNPs in a total of 242 *S.* Paratyphi isolates, with alleles from *S.* Typhi CT18[84] (accession no: AL515582) included as an outgroup to root the tree.

5.3. Results

5.3.1. Phylogenetic structure and temporal distribution of paediatric isolates from Nepal

The genomes of *S*. Paratyphi A isolated from inpatient surveillance (n=16) and a random selection (every 5th isolate) from outpatients (n=50) were sequenced and subjected to SNP genotyping and phylogenomic analysis as described in section **2.3**. The Nepali *S.* Paratyphi A population was far less diverse than that of the *S*. Typhi strains analysed in **chapter 4**. The resulting phylogeny (**Figure 5.1**) revealed the presence of 3 distinct lineages; A (N=3, 4.5%), A1 (N=41, 62.1%), A2 (N=21, 31.9%), C (N=1, 1.5%). There was clustering of isolates between isolates obtained from children treated as inpatients and outpatients indicating that there was no difference in strains causing mild disease (outpatient disease) vs severe disease (inpatient disease).

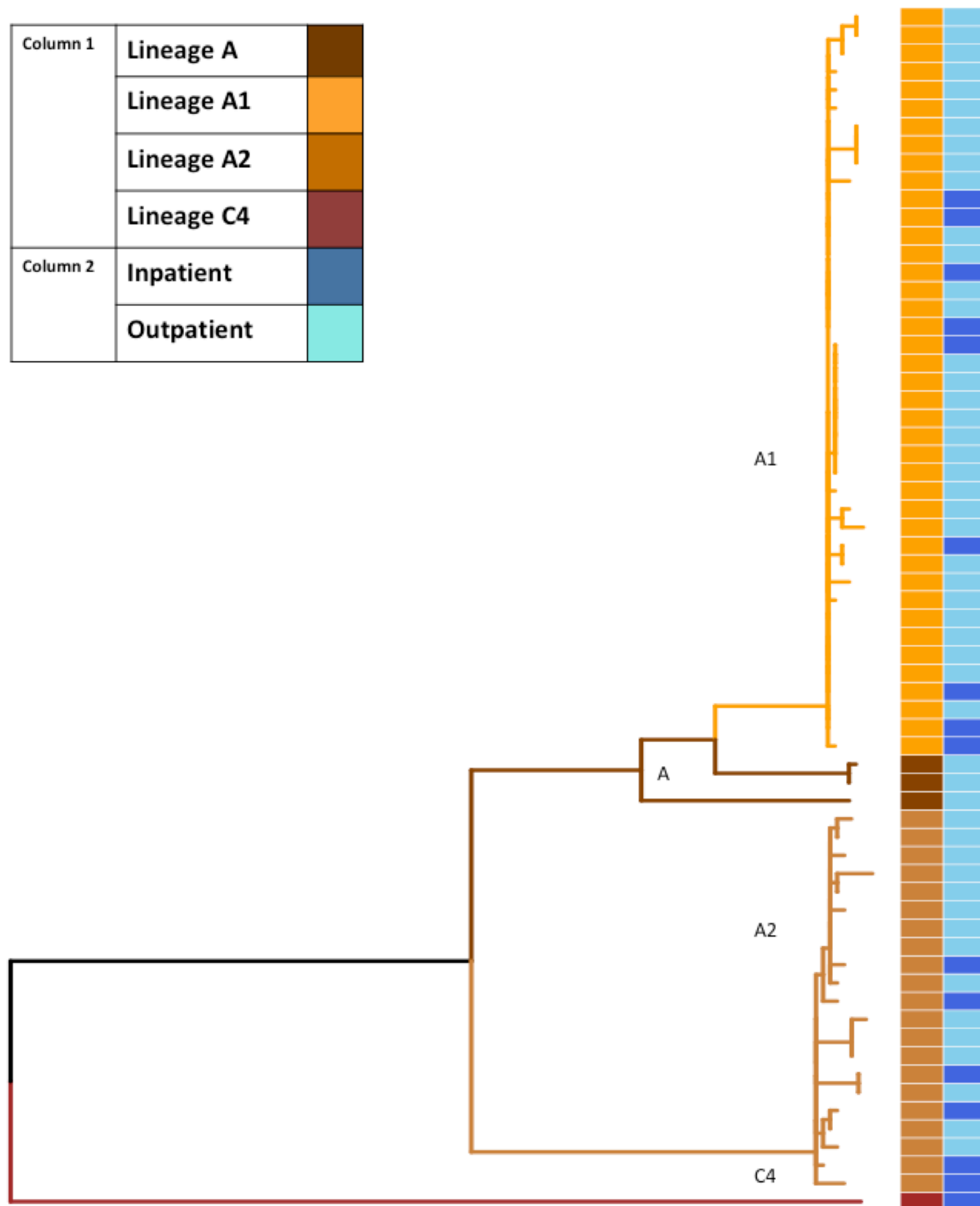


Figure 5.1: The molecular structure of *S.* Paratyphi A strains isolated from children in Nepal between 2008-2016.
This maximum likelihood phylogenetic tree represents the characterisation of isolates between 2008-2016. Branch colours indicate the genotype which are labeled according

There was no significant association between genotype and treatment status (outpatient vs. inpatient), period of isolation or age of patients (**Figures 5.2 and 5.3**). There was also a relative decline in disease in both inpatients and outpatients after 2012. The relatively high occurrence of disease in the youngest age groups is important, as there is uncertainty about the disease burden in the youngest age groups of children.

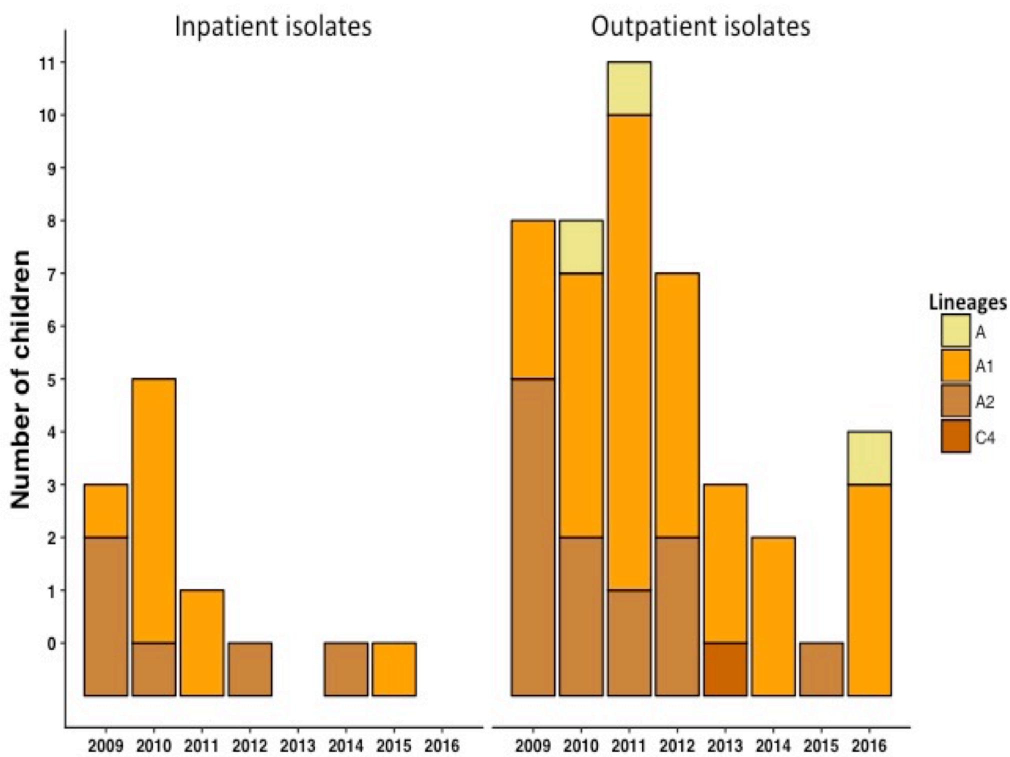


Figure 5.2: **Lineage stratified distribution of *S.* Paratyphi A strains.**
These were isolated from inpatients and outpatients in this study. The main lineages in this study were lineage A1 and A2 and are coloured according to the inset legend.

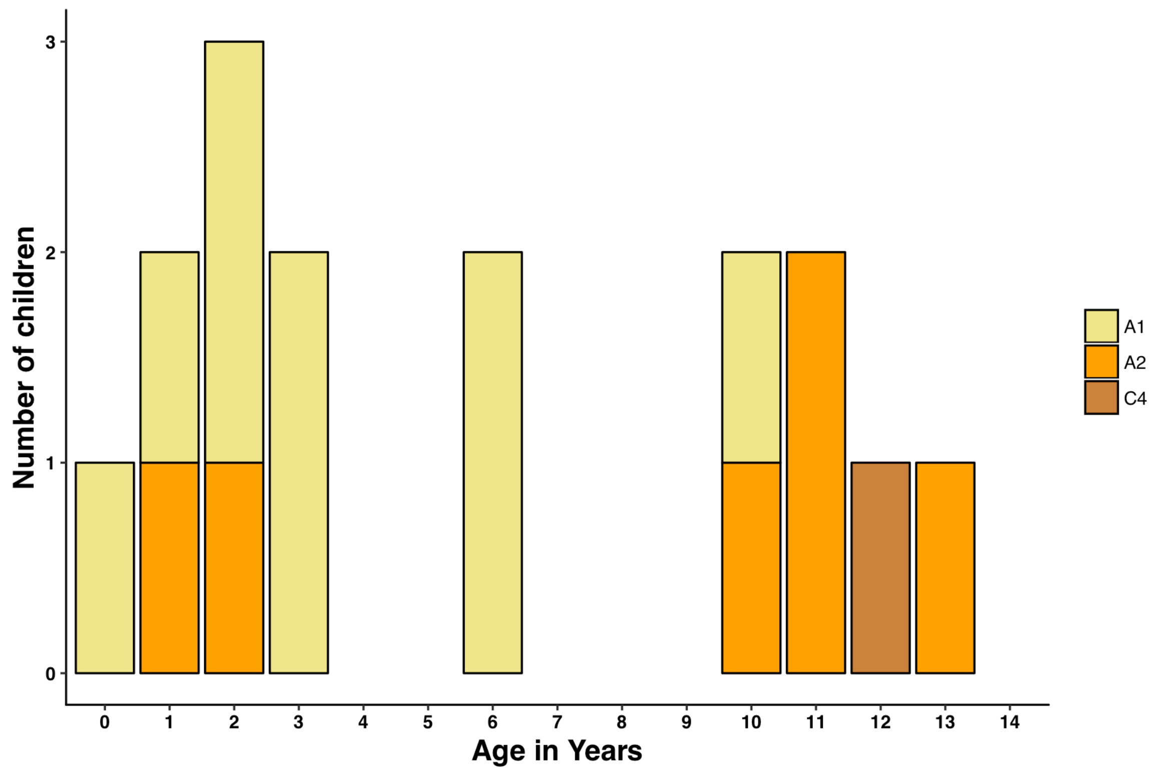


Figure 5.3: Age stratified distribution of *S.* Paratyphi A strains isolated from inpatients.
The main lineages in this study were lineage A1 and A2 and are coloured according to the inset legend.

5.3.2. Regional and Global context

The global *S.* Paratyphi A population was also less diverse than that of the *S.* Typhi global population analysed in **chapter 4**. Akin to *S.* Typhi, the global context of *S.* Paratyphi A also revealed close clustering with isolates from other regions in the Indian subcontinent and to a lesser extent with China. The lineages endemic to South-East Asia formed their own cluster with some evidence of introductions probably *via* travellers to Nepal. There was a small cluster of African isolates belonging to lineage C but these are most likely associated with travel to endemic Asian locations.

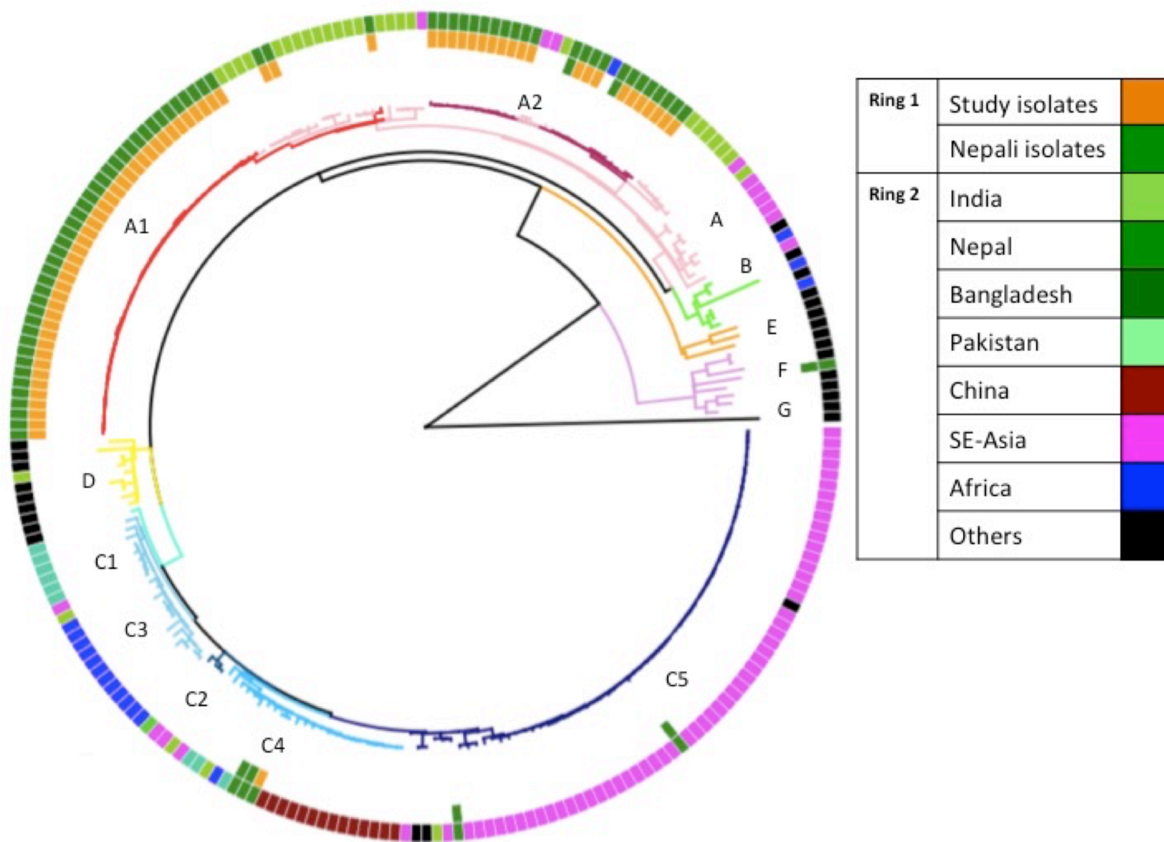


Figure 5.4. Global context of *S.* Paratyphi A genotypes in Nepal. Rings indicate isolate/patient features according to the inset legend; note the inner-most ring highlights Nepali isolates only. Branch colours indicate the lineage of the isolate; branch lengths are indicative of the estimated number of substitutions rate per variable site.

5.4. Discussion

S. Paratyphi A accounts for a substantial proportion of disease among enteric fever affected children attending Patan hospital, Nepal. Molecular analysis suggests that *S.* Paratyphi A is less diverse when compared with the branch lengths of *S*. Typhi. As with *S*. Typhi there is on-going inter-region transmission between Nepal and neighbouring countries in South Asia.

5.4.1. Demographic features and temporal trends.

As with typhoid fever, paratyphoid illness also affects the youngest age groups and even though numbers are small it is plausible that this trend is representative of the trends in other regions of Nepal where the youngest age groups are substantially affected. Interestingly, this study revealed an apparent decrease in both inpatient and outpatient *S.* Paratyphi A infections from 2012 onwards while in the previous chapter there was a sharp spike in *S*. Typhi illness in 2013. There was no apparent reason in the form of a preventive intervention for this decline and it does raise the question of competitive niches among circulating pathogenic strains of typhoidal *Salmonella.* It is not clear why *S*. Paratyphi A infection is not as common as *S*.Typhi in endemic regions and how these two serovars interact with each other in the environment and within the human host. The widespread deployment of the Vi-TT conjugate vaccine, which is thought to be only protective against *S.* Typhi, will shed some light in this area. If indeed the two serovars compete, there will be a spike in cases of *S.* Paratyphi A infection following deployment of the Vi-TT conjugate vaccine and if there is a further reduction in *S.* Paratyphi A it may point towards cross-protection although this is unlikely given that *S.* Paratyphi A does not possess the Vi capsule

5.4.2. The dominance of lineage A in the population structure

The diversity in phylogenetic structure is far less when compared with the phylogenetic tree of *S.* Typhi as evidenced by the length of the branches in the phylogenetic tree. The dominance of lineage A1 and lineage A2 is prominent and potentially reflects endemicity of

disease as previously described by Zhou *et al*[372]. The intermingling of strains isolated from inpatients and outpatients suggests that the infecting strain has no bearing on the severity of disease. The branch lengths in **Figure 5.1** indicative of the estimated number of substitutions rate per variable site are short which suggests that the genomes of *S.* Paratyphi A have not been subject to substitutive changes which could be reflective of the lack of selection pressures although it is difficult to make the argument that the selection pressures differ between *S.* Typhi and *S*. Paratyphi A when the mechanisms of AMR are similar between these serovars[373]. The lack of information regarding circulating lineages of *S.* Paratyphi A in Nepal prior to this study limits interpretation of the changing molecular structure over time and with changing antimicrobial pressures. Nevertheless, these data serve as an important baseline measure of the molecular structure and the monitoring of this structure over time and in response to various direct and indirect selection pressures will aid in estimating the impact of interventions.

5.4.3. Regional and global contextualisation

On a global level, the lineage A1 and A2 strains cluster closely with others from the Indian subcontinent. This suggests that *S.* Paratyphi A transmission occurs mainly between countries of South Asia. It is interesting to note that though *S.* Paratyphi A is endemic in China and the Chinese lineages are not closely related to the Nepali lineages even though these two countries share a border. The inaccessibility between Nepal and China when compared with Nepal and India may be an important reason. It will be important to track the molecular structure of isolates in Nepal given the numerous development projects taking place to improve accessibility between Nepal and China. The single C4 lineage of *S.* Paratyphi A from this study is likely to be an introduction from China. Lineage C5 is endemic to South-East Asia[372] and the isolates belonging to this lineage cluster together in global tree. On a global level it would appear that *S*. Paratyphi A is mainly an Asian disease and there is no

apparent reason why this disease has not been established in Africa. Similarly, it is interesting to note the high burden of *S*. Paratyphi A in China in the absence of a comparable *S.* Typhi burden. Although the use of typhoid vaccines in China maybe part of the reason for this it still does not explain this finding in its entirety.

Although the dynamics of inter-regional transmission are similar in the case of both *S.* Typhi and *S.* Paratyphi A, other gastrointestinal disease such as rotavirus differ. In the case of rotavirus, the molecular structure of circulating strains in Nepal and India differ widely with the G2P genotype being the predominant population in India as opposed to the G12P genotype in Nepal[378,379]. With regard to other Gram-negative bacilli such as *E. Coli* and *Klebsiella species* the lack of high resolution molecular epidemiological data makes analysis regarding transmission dynamics difficult.

5.4.3. Preventive strategies

The preventive strategies against *S.* Typhi and *S*. Paratyphi A are similar in terms of clean water, adequate sanitation and appropriate hygiene (WASH) but the major difference is the lack of *S*. Paratyphi A vaccine. There is no evidence to suggest that the Vi-TT conjugate vaccine is cross protective against *S*. Paratyphi A infection and it has been suggested that the control of typhoid via vaccination might cause an increase in *S*. Paratyphi A. The Vi antigen used in the typhoid vaccine is not present in the *S*. Paratyphi A bacterium and a suitable antigen for *S.* Paratyphi A is still under investigation.

Even with an efficacious vaccine it is unlikely that vaccination alone will eradicate *S*. Paratyphi A given that this is only a short to intermediate term strategy. The real hope is that clean water, adequate sanitation and appropriate hygiene (WASH) are employed in endemic areas, as this will also have a wider impact on other gastrointestinal illnesses. In the case of *S.* Paratyphi A where the risk acquiring the disease more strongly correlated with contaminated

food consumption, the importance of safe food handling and suitable cooking techniques should be also emphasised in addition to WASH[370].

5.4.4. Limitations

Similar to the previous chapter, the methods and results of this chapter has limitations as all isolates examined were from a single hospital based passive surveillance programme and thus may not be representative of the disease trends in the wider community. For inpatient isolates only stored samples were available (17 out of 27 for S. Paratyphi A) for retrospective sequencing analysis, preventing the use of a more formal random sampling technique; however this is unlikely to introduce sampling bias with respect to observed lineages, as lineages were not known at the time of storage and there is no reason to suspect particular lineages would be more likely to have been stored. Consistent with this expectation, the population structure observed here is comparable with that observed in previous local and regional studies suggesting that this opportunistic sampling is reasonably representative of the population structure of *S.* Paratyphi A between 2008-2016. Outpatient isolates were randomly sampled and expected to be representative, but these were unable to be stratified by age, gender and admission information as outpatient data was limited and did not include any patient level information.

5.5. Conclusion

These data highlight the burden of *S*. Paratyphi A infection in children in Nepal while demonstrating the importance of laboratory and molecular surveillance in endemic regions. Those under the age of 5 years contributed substantially to the burden of enteric fever among inpatients who represent the severe spectrum of disease. The lack of a *S*. Paratyphi A vaccine is concerning given its endemic nature in Nepal and potential of causing outbreaks.

"Science brings men nearer to God"
Louis Pasteur

Chapter 6

The laboratory and molecular surveillance of typhoidal *Salmonellae* isolated from patients attending a tertiary care hospital in South India over a 13-month period

6.1. Introduction

The Indian subcontinent is endemic for enteric fever, a febrile illness caused by *Salmonella enterica* serovars Typhi (*S.* Typhi) and Paratyphi A (*S.* Paratyphi A), and on a global scale causes an estimated 26 million cases of febrile illness annually, disproportionally affecting children and young adults[9,380].

6.1.1. Enteric fever in Bengaluru

There have been a number of reports indicating a sharp increase in typhoid cases in the city of Bengaluru leading to concerns of enhanced pathogen virulence or water source contamination[381–385]. A recent study reporting an outbreak of typhoid from this setting, provided some insight into the genetic relatedness of *S.* Typhi strains[386], but further characteristics regarding the molecular structure of circulating strains in this region still need to be determined. Data regarding the age-stratified burden of disease, particularly among the paediatric population are also scarce. This information is vital in planning prevention strategies, particularly the prudent deployment of the Vi-TT conjugate vaccine.

6.1.2. The molecular characterisation of enteric fever in India

The surveillance of enteric fever in India is very limited and there is a paucity of data regarding the molecular structure of circulating strains, which is key in planning sustainable treatment regimens and prevention strategies. A publication in 2016 revealed the dominance of the 4.3.1 genotype of *S.* Typhi in India but this publication reported strains from a global perspective and the Indian strains were not wholly representative of the molecular epidemiology of the disease in the country as the strains were obtained from a limited number of regions[83]. The *S.* Typhi molecular structure in the neighbouring country of Nepal is well characterized and is dominated by the 4.3.1 genotype (H58 population) and it is likely to be similar in India. However, further resolution in the molecular characterisation of strains may be necessary to inform policy. Data regarding *S.* Paratyphi A are even scarcer and very limited information regarding the molecular structure is available.

6.1.3. Rationale for this study

This study was conducted with the aim of identifying the molecular structure of circulating enteric fever strains in Bengaluru, India including the correlation with demographic and laboratory features. In addition, it was aimed to contextualize these strains globally and to

identify possible preventive strategies including the prudent use of the recently pre-qualified Vi- TT conjugate vaccine candidate.

6.2. Methods

6.2.1. Ethics statement

Ethical approval was obtained from the Oxford Tropical Research Ethics Committee (OxTREC 586-16) as well as local institutional approval from the IEC at St. John's Research Institute (140/216) and Health Ministry's Screening Committee, India.

6.2.2. Study Setting

St. John's medical college hospital is a tertiary care hospital catering to approximately 3,500 patients on an outpatient basis daily and has a capacity of 2000 inpatient beds. It is situated in the eastern part of Bengaluru, a city that is densely populated holding a population of over 12 million residents. This hospital is also situated at the confluence of three south Indian states and receives referrals from neighbouring regions in the states of Tamil Nadu and Andhra Pradesh in addition to the referrals from the home state of Karnataka.

Figure 6.1: Location of St. John's Medical College Hospital
This tertiary care facility is located in the city of Bengaluru situated in the confluence of the populous South Indian states of Karnataka, Tamil Nadu and Andhra Pradesh.

6.2.3. Surveillance of culture confirmed enteric fever

The hospital's microbiology laboratory is a 24-hour facility that processes blood for culture and antimicrobial sensitivity on a routine and emergency basis. For the purpose of this study between June 2016 and June 2017, every microbiological specimen that was positive for a typhoidal *Salmonella* organism was identified and stored. The stored isolates were then linked to patient records for relevant demographic and laboratory details that are electronically saved by the hospital.

6.2.4. Genome sequencing and SNP analysis

In June 2017, at the end of the study all the stored isolates were subcultured and bacterial DNA was extracted on-site as described in **section 3.2 of chapter 2**.

The bioinformatic pipeline described in **section 3.3 of chapter 2** was used to identify SNPs in these isolates which in addition to phylogenetic analysis were used to assign isolates to previously defined lineages according to an extended *S.* Typhi genotyping framework[97] (code available at https://github.com/katholt/genotyphi). A final set of 1184 SNPs identified in an alignment length of 4,809,037 bp for the 101 novel Indian *S*. Typhi isolates were used for phylogenetic and SNP analysis. SNP alleles from *S*. Paratyphi A strain AKU_12601[295] (accession FM200053) were also included as an outgroup to root the tree. To provide regional context, a global *S*. Typhi 4.3.1 genotype genome collection[97] (n=1,237) similar to the collection used in **chapter 4**; were subjected to SNP calling and genotyping, resulting in an alignment of 2544 SNPs. An additional analysis of 233 H58 (genotype 4.3.1) from India was carried out in the same manner, resulting in an alignment of 802 SNPs.

To characterize and analyse the genomes of the 14 *S.* Paratyphi A strains, a similar bioinformatic process was adopted using *S.* Paratyphi A AKU_12601[295] (accession no: FM200053) as the reference genome to create an alignment with another selected 242 isolates from previous studies[375–377], for global context resulting in an alignment of 4498 SNPs in a

total of 256 *S.* Paratyphi isolates, with alleles from *S.* Typhi CT18[84] (accession no: AL515582) included as an outgroup to root the tree.

6.3. Results

6.3.1. Enteric fever surveillance over study period.

Between June 2016 and June 2017 there were 19,641 blood cultures performed on blood of patients with a suspected bacterial infection. One hundred and ten (3.4%) of these were positive for enteric fever pathogens across inpatients and outpatients (**Table 6.1**).

Table 6.1: Surveillance of enteric fever

Total blood cultures performed	19,641
Total number of significant cultures	3454 (5.7%)
Total number of *E. coli*	334 (9.7%)
Total number of *Klebsiella* (all species)	153 (4.4%)
Other Gram-negative organisms	180 (5.2%)
Total number of *Salmonellae*	132 (3.8%)
Total number of typhoidal *Salmonellae** *S.* Typhi *S.* Paratyphi A	117 (3.4%) 103 (2.3%) 14 (0.4%)

Organism	Total	Male (%)	Adults (%)	Children (%)
Typhi	103	66 (64.1%)	66 (64.1%)	37 (35.9%)
Paratyphi A	14	8 (61.5%)	11 (76.9%)	3 (23.1%)

* 110 isolates were cultured from blood. Six isolates were cultured from other specimens (3 stool, 2 urine and 1 from joint aspiration)

The temporal distribution, serovar distribution as well as the distribution of isolates cultured from those treated as outpatients vs inpatients are illustrated in **Figure 6.2.** There was no seasonal trend in disease occurrence indicating perennial transmission. Adults made up 65.5% of cases and 62.9% were male (**Figure 6.2**).

The majority of patients were treated on an inpatient basis (68.9%) and there was no difference in likelihood of admission to hospital between those infected with *S.* Typhi vs those infected with *S.* Paratyphi A (OR = 2.96, p = 0.15) or between children and adults (OR

= 0.6, p = 0.25). The duration of admission was available for 63 out of the 79 individuals treated as in-patients (42 adults and 21 children). The median duration of admission among adults was 4.5 days (IQR – 3,6) and when compared with that of children (2 days IQR – 1,4) differed significantly (Wilcoxon rank sum test, p = 0.002). Fever was the common presentation occurring in 97% of cases.

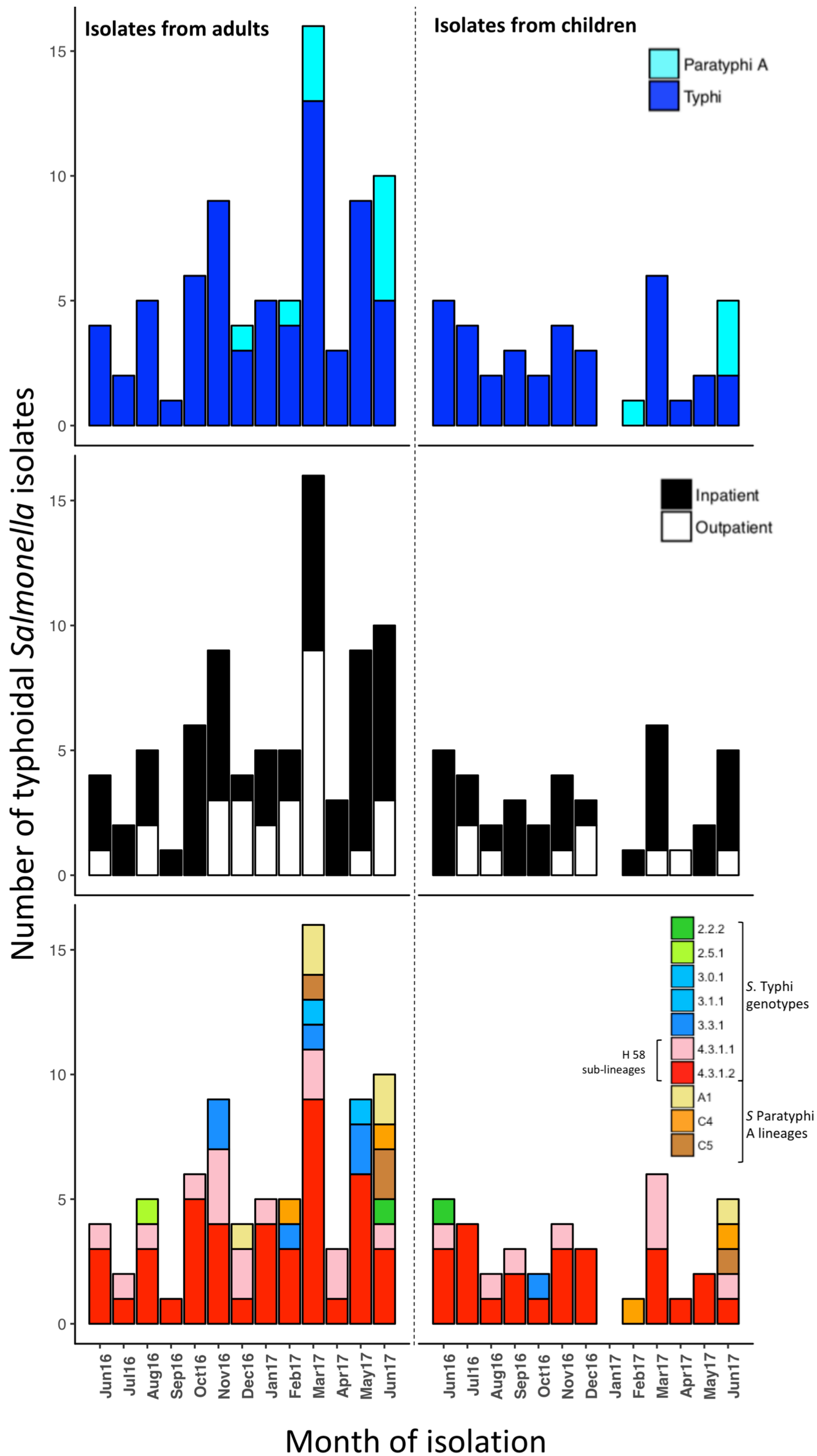
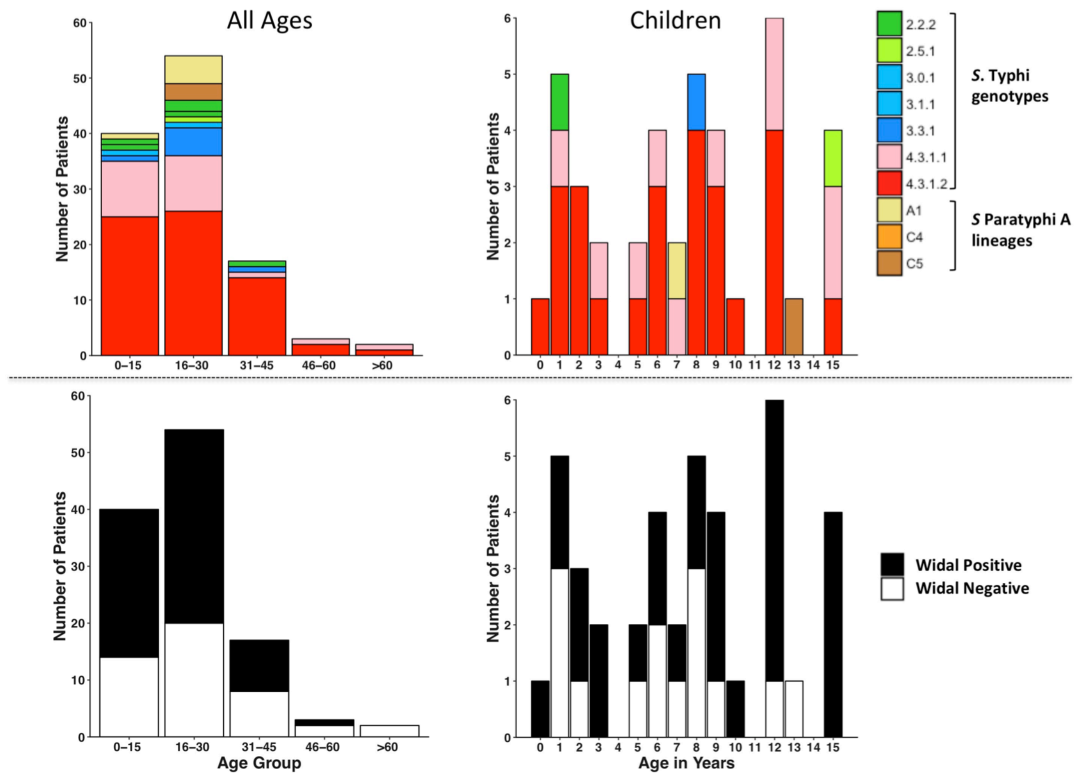


Figure 6.2: **Distribution of typhoidal Salmonella serovars**
Representation of typhoidal serovars, treatment status and genotype distribution as per the inset legends between adults and children over the study period.



Age stratified characteristics of blood-culture positive enteric fever patients					
	Age groups (in years)				
	0-15#	**16-30**	**31-45**	**46-60**	**> 60**
Number (n = 113*)	40	53	15	3	2
Median age in years (IQR)	8 (3-12)	24 (20-27)	36 (33-40)	54 (52-55)	65 (64-66)
Male (%)	27 (67.5)	32 (60)	13 (86.6)	1(33.3)	0 (0)
Inpatient (%)	30 (75)	36 (69.2)	10 (66.6)	1(33.3)	2 (100)
Paediatric enteric fever	**# Paediatric age groups (in years)**				
	n = 40	**0-2**	**3-5**	**6-10**	**11-15**
		9 (22.5%)	4 (10%)	16 (40%)	11 (27.5%)

*There were 117 isolates stored of which four patients had 2 blood culture positive results on two separate occasions. Three of these had 2 positive results on the same admission and these 3 (duplicate) isolates have been excluded from this figure and table.

Figure 6.3:Age stratified distribution of typhoidal *Salmonella* genotypes and Widal positivity with summary measures in the inset table.

6.3.2. Atypical features

A 26-year-old male patient presented with a clinical picture of septic arthritis (CT and MRI illustrated in **Figure 6.4**) and bacteriologic culture of joint fluid aspirate grew *S.* Typhi (genotype 4.3.1.2/H58 lineage II). This patient had no history of a haematologic or immunologic abnormality and his peripheral smear at the time of diagnosis was normocytic and normochromic. Another 5-year old child presented with fever and acute dyspnoea with clinical examination and preliminary laboratory findings suggestive of haemolysis. The child had a haemoglobin (Hb) level of 4 g/dl in addition to a positive blood culture for *S.* Typhi (genotype 4.3.1.2/H58 lineage II). Further haematological work up of this patient revealed a diagnosis of hereditary spherocytosis based on a positive osmotic fragility test with a haemolytic episode precipitated by infection with *S.* Typhi. Among the 103 *S.* Typhi isolates, there were 4 instances where *S*. Typhi was isolated from the same patient on two occasions. In 3 of these cases the organism (*S*. Typhi genotype 4.3.1.2/H58 lineage II) was isolated within the same admission over 48 hours apart highlighting a decreased clearance time. In the last case a confirmed diagnosis was made 2 months apart with two different *S.* Typhi genotypes namely H58 lineage II (4.3.1.2) as well as a 2.2.1 genotype 2 months apart.

6.3.3. Laboratory findings

The numbers of adults and children who had haemoglobin (Hb), total white blood cell (WBC) count and platelet counts on presentation are shown in **Figure 6.5.**

The haematological trends between adults and children are illustrated in **Figure 6.5**. Children had significantly lower (Mann-Whitney U Test, p =0.002) haemoglobin (Hb) values on presentation in comparison with adults. A peripheral smear was done at the same time point as Hb estimation in 31 children and 24 adults and only 8 (3 children and 5 adults) patients had a microcytic hypochromic (MCHC) blood picture suggesting that the majority of anaemia was in fact due to enteric fever as opposed to a nutritional cause which is the most

common cause of anaemia in this region. Abnormal leucocyte counts (leucopoenia or leucocytosis) were seen in a minority of patients on presentation. The WBC count did not differ significantly between adults and children on admission but more surprisingly remained within the normal range until resolution of disease in majority of cases (**Figure 6.5)**. Two adults and two children had abnormally high WBC counts suggestive of a leukaemoid reaction. The two adults had repeated WBC counts during the course of the disease that decreased on initiation of antimicrobial therapy. Thrombocytopenia was seen in 24.7% of patients and it was more common in adults but this difference was not significant.

Fifty-five patients had liver-function test at presentation. Raised liver enzymes were observed in 30 and 5 cases of adults and children respectively. There was a significant difference between adults and children (Mann-Whitney U Test, p =0.001).

Widal test titres at a single time point was done in all patients and was positive (above a titre of 100 for the O and H antigens) in 68% of patients. Among children 26 out of 37 infected with *S.* Typhi (70%) had a positive Widal test vs 44 out of 66 (66%) adults but this difference between adults and children was not significant. There was also no correlation between Widal positivity and infecting genotype of *S.* Typhi.

6.3.4. Molecular structure of the pathogen population

6.3.4.1. The novel Indian isolates

From the 117 collective isolates cultured from patients during the study period, two *S.* Typhi isolates failed quality control tests and were not included in the pathogen genomic analysis. The genomes of 101 *S.* Typhi and 14 *S.* Paratyphi A isolated from children and adults representing both inpatient and outpatient isolates were sequenced and subjected to SNP genotyping and phylogenomic analysis as described in **section 2.3 in chapter 2.**

The resulting *S.* Typhi phylogeny (**Figure 6.6**) revealed the presence of 6 distinct genotypes, each corresponding to a different subclade including 2.2.2 (N=2, 2%) 2.5.1 (N=1, 1%), 3.0.1

(N=1, 1%), 3.1.1 (N=1, %), 3.3.1 (N=7, %), and 4.3.1(N=89, 88.1%) The 4.3.1 genotype was further stratified into lineage I (4.3.1.1, N =22, 21.8 %) and lineage II (4.3.1.2, N= 67, 66.3%). There was no association between genotype and treatment status (outpatient vs. inpatient), month of isolation, (**Figure 6.2**) or patient age (**Figure 6.3**).

In comparison with the *S.* Typhi phylogeny the *S.* Paratyphi A was less diverse comprising of three lineages namely lineage A (N=6, 42.9%), lineage C4 (N=4, 28.6 %) and lineage C5 (N=4, 28.6 %).

6.3.4.2. The Indian context

To place these isolates in regional context, a whole genome phylogeny was constructed comprising other H58 (4.3.1 genotype) *S.* Typhi isolates previously sequenced from a global collection. The Indian H58 *S.*Typhi tree comprised 233 collective isolates and resulted in an alignment length of 802 SNPs **(Figure 6.7).** There was some evidence of clustering among the strains of this chapter although the paucity in the number of publically available *S.* Typhi genomes from the country across time and region are possibly the main factors for this observation. It is evident that a subclade of H58 Lineage II strains is currently expanding possibly due to the antimicrobial pressure exerted by fluoroquinolones. The intermingling of strains isolated from adults and children suggests that transmission occurs between these groups.

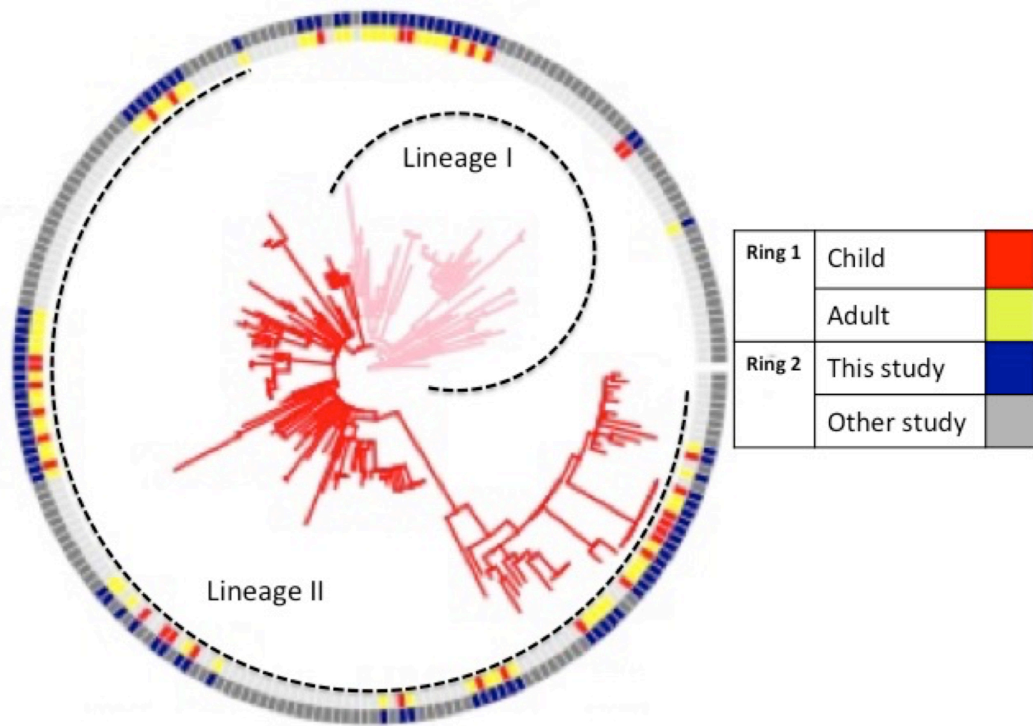


Figure 6.7: Indian contextualization of H58 strains.
This tree is made up of 233 Indian isolates and the rings are coloured according to the inset legend. The clear dominance of lineage II strains with an expanding clade is brought out in the figure. Branch lengths are indicative of the estimated number of substitutions rate per variable site; the tree was outgroup rooted using *S.* Paratyphi A strain AKU_12601

6.3.4.3. The global context

For global contextualization a whole genome phylogeny was assembled including other H58 (4.3.1 genotype) *S.* Typhi isolates previously sequenced from a global collection. This *S.*Typhi tree comprised 1237 collective isolates and resulted in an alignment length of 2544 SNPs **(Figure 6.8).** The strains isolated from countries of the Indian sub-continent clustered closely together (bars in different shades of green in **Figure 6.8**) suggestive of inter-region transmission. A similar cluster was also observed between strains isolated from South-East Asian countries (bars in magenta). In addition to forming a separate cluster depicted in blue, the African strains (East and Southern African countries) were also found between the South-Asian and South-East Asian clusters suggestive of transmission from the endemic Asian areas to East Africa. Lineage II strains dominated in South Asia where as Lineage I strains dominated in South-East Asia and Africa reflecting the varying degrees of antimicrobial pressure.

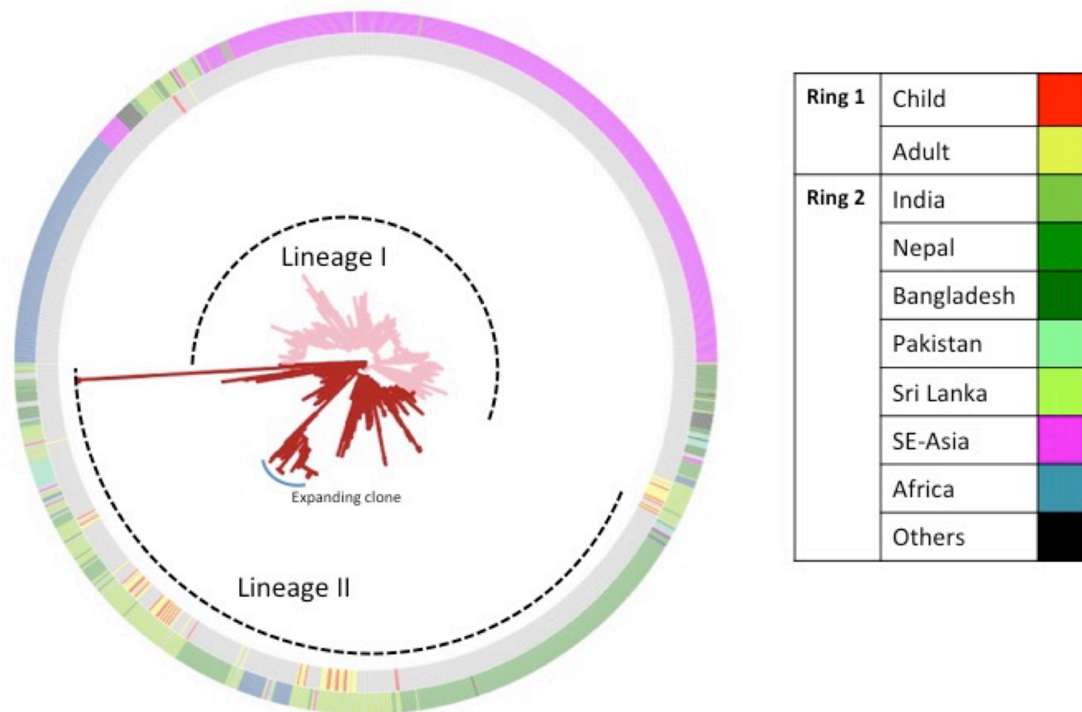


Figure 6.8: Global contextualization of H58 strains.
This tree is made up of 1237 globally representative isolates and the rings are coloured according to the inset legend. The clear dominance of Lineage II strains with an expanding clade in South Asian in contrast to Lineage I strains in South-East Asia and Africa is brought out in the figure. Branch lengths are indicative of the estimated number of substitutions rate per variable site; the tree was outgroup rooted using *S.* Paratyphi A strain AKU_12601

The *S.* Paratyphi A strains were only analysed on a global level as numbers were too few to make meaningful observations on a national level. There were 256 isolates that made up the global collection and belonged to the A1, C4 and C5 lineages and clustered closely with other Indian isolates (**Figure 6.9**). The A1 strains clustered closely with other Indian isolates while the C5 isolates were closely related to the South-East Asian isolates and the C5 isolates clustered with the Chinese isolates

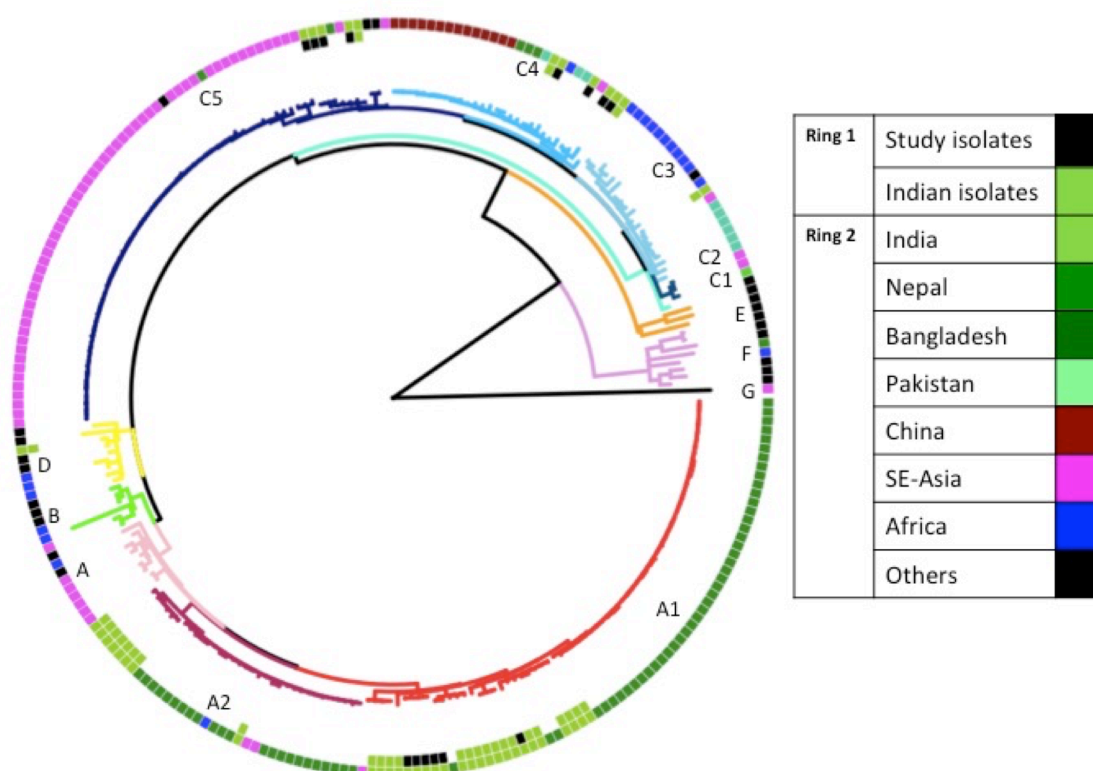


Figure 6.9: **Global contextualization of *S.* Paratyphi A strains.**
This tree is made up of 256 global isolates and the rings are coloured according to the inset legend. The isolates from this chapter belonged to the A1, C4 and C5 lineages and clustered closely with other Indian isolates (Ring 1). On a global level the C5 isolates were closely related to the South-East Asian isolates and the C5 isolates clustered with the Chinese isolates. Branch lengths are indicative of the estimated number of substitutions rate per variable site; the tree was outgroup rooted using *S.* Typhi CT18

6.4. Discussion

These data highlight the occurrence of enteric fever in this setting where 3.4% of significant isolates were typhoidal *Salmonella.* These data also underscore the importance of genotyping and classification of isolates to understand the changes in population structure and possible preventive strategies.

6.4.1. Demographics and temporal features

The occurrence of disease in children and young adults is similar to other endemic regions[135,289,308]. With availability of the Vi –TT conjugate vaccine it is now possible to protect the youngest age groups of children who also account for a substantial proportion of disease. The significantly higher number of patients who were treated as inpatients is a possible reflection of disease severity although this is also likely to be due to bias owing to the more rigorous testing in those patients who have clinical manifestations requiring inpatient care. It is however interesting to note that adults had significantly longer duration of hospital admission when compared with children possibly indicating a more rapid clearance of bacteraemia in children. The perennial nature of disease transmission indicates that vaccination should occur throughout the year in order to have a greater impact. It also suggests that the endemic nature of disease occurs through contamination of water supplies as opposed to a single point-source outbreak.

6.4.2. Haematologic features

From the human challenge model it is known that there is a general decrease in haematological parameters[387]. It was surprising to note the significant difference in Hb on admission between children and adults. Although iron-deficiency is a major problem in this setting only a minority of patients had a microcytic hypochromic blood picture suggesting the anaemia seen in these patients was likely to be due to the disease. The relatively small proportion of patients with abnormal WBC counts was also surprising when compared with

those of the human challenge model[136]. These findings could be due a number of reasons including the discrepancy in sampling times and disease progression and repeated sub-clinical enteric fever infections[187]. There were two adults and two children with high WBC counts suggestive of a leukaemoid reaction which is known to occur in both *Shigella* and typhoidal *Salmonella* infections is thought to be due to the abnormal response to lipopolysaccharide (LPS) present in the cell wall of these organisms and is more commonly seen in children[367].

6.4.3. Widal test and diagnostics

Blood culture is the feasible gold standard test for enteric fever on routine basis. However, as discussed in **chapter 1** it has a variable sensitivity (between 60-90%), which is even lower in the paediatric age group where drawing the required amount of blood during routine phlebotomy is a challenge. Other reasons for the lack of sensitivity include prior antimicrobial treatment (which may be over-the-counter), inappropriate microbial culture protocols and a discrepancy between bacteraemia and phlebotomy. Although the <5 years age group in this cohort of patients made up a substantial proportion of the study population, it is likely to be a gross under-estimate for the aforementioned reasons. The Widal test at a single time point is in an endemic region is considered to be generally uninformative. This is mainly due to the number of falsely high anti-O and anti-H titres that may be due to sub-clinical or past enteric fever infections. However, in the youngest age group (< 2 years) with low exposure, low-probability of past infections and who are unvaccinated it would seem plausible that a single positive Widal test might be useful. However, a cut-off titre needs to be established and with the deployment of the Vi-TT conjugate vaccine possibly with other vaccines of the infant immunization schedule a single positive Widal test would still be of little use.

6.4.4. Molecular structure

The genotyping frame work developed by Wong and colleagues has greatly enhanced the understanding of typhoid population structures and now allows for a more discriminatory level of phylogenetic analysis[97]. In this chapter the dominance of the 4.3.1 genotype is obvious and consistent with other endemic regions of South Asia, South-East Asia, East and Southern Africa. However further characterisation of the 4.3.1 population revealed a greater dominance of 4.3.1.2 strains (Lineage II strains) compared with the 4.3.1.1 strains (Lineage I strains). From **chapter 4** it is known that the 4.3.1.2 strains are more commonly associated with fluoroquinolone resistance and 4.3.1.1 strains are commonly associated with multi-drug resistance (MDR) defined as resistance to ampicillin, cotrimoxazole and chloramphenicol. This finding of the dominance of 4.3.1.2 strains is a reflection of the antimicrobial pressure exerted by fluoroquinolones. A detailed analysis of antimicrobial resistance in *S.* Typhi is undertaken in **chapters 7 and 8** and the molecular mechanisms of resistance are discussed there.

An interesting finding is the intermingling of isolates in the maximum likelihood tree between children and adults, which indicates transmission between these groups as opposed to separate lineages circulating amongst children. It is thus imperative to have a broader vaccination schedule incorporating the vaccination of young adults in order to effectively control the disease and this is also evidenced by mathematical modeling[388]. Nevertheless, the vaccination of children under the age 15 as prioritized by Gavi will have a significant impact on burden of disease.

India is not a Gavi eligible country but the results of this study can supplement data and guide policy making decisions in countries like Nepal which is Gavi eligible and is likely to have a similar population structure (**chapter 4**) of typhoidal strains as seen in **figures 6.7** and **6.8** where there is close clustering of strains isolated from Nepal and India as well as other

countries of the Indian subcontinent. There is also evidence of an expanding clone of Lineage II strains, which were responsible for a substantial proportion of disease in this study. This indicates inter-region transmission and suggests that typhoid prevention strategies require a coordinated approach between these countries. The higher proportion of lineage I strains in other endemic areas of South-East Asia and Africa indicate inter-region transmission and antimicrobial pressure specific to those regions.

Similar findings exist with regard to the *S.* Paratyphi A proportion where strains belonging to the A1 lineage cluster with those of other countries of South Asia while lineages C4 and C5 cluster more closely with those of China and South-East Asia. The lack of *S.* Paratyphi A vaccine limits prevention options and also incites antimicrobial use for the treatment of *S.* Paratyphi A infection further increasing antimicrobial selection pressure.

6.4.5. Atypical features

Salmonella bone and joint infections are more common with non-typhoidal *Salmonella* and most commonly seen in association with sickle cell disease[367]. The young adult with septic arthritis was haematologically normal and had no prior right hip pathology. The absence of these makes this case extremely rare. The isolate cultured from the joint aspirate was a 4.3.1.2 genotype strain and was highly fluoroquinolone resistant. There was no evidence to suggest that this strain was more virulent and a basic immunologic as well haematologic work-up of the patient did not reveal any abnormality. The patient responded well to conservative therapy and his symptoms resolved after 14 days of antimicrobial therapy with cephalosporins.

The incidence of hereditary spherocytosis, like other hereditary conditions in South-India is relatively high owing to the large number of consanguineous marriages. However, acute haemolysis owing to infection with *S.* Typhi has never been documented before. The 5-year old patient was diagnosed with hereditary spherocytosis only after this infection. The exact

mechanism causing haemolysis in enteric fever is not known but could possibly be due to the increased oxidative stress on the red blood cells during an infection. The organism isolated from this patient was a 4.3.1.2 strain and although this strain was highly fluoroquinolone resistant, there was no evidence to suggest that this strain was more virulent. This patient received antimicrobial treatment with cephalosporins and appropriate blood transfusions to treat the anaemia. He was discharged from hospital after 3 days and was doing well when seen as an outpatient 2 weeks following discharge.

It is still not known how many enteric infections are needed to confer life-long protection. Based on the demographic distribution of this study and other similar observations from other endemic areas it appears that infections during childhood and early adulthood confer protection in the later years. But the number of infections and duration between infectious episodes required for this protection are unknown. A 28-year old male was treated on an outpatient basis in April 2017. The infecting agent was a 4.3.1.2 genotype strain. Two months later in June 2017 he was diagnosed with enteric fever again and the infecting agent was 2.2.2 this time. On both occasions the patient was treated with cephalosporins and made a complete recovery. It is unclear whether this difference in genotype caused the 2nd infectious episode or whether this would have happened regardless of the infecting genotype.

From personal communication with Dr. Malick Gibbani who is the lead doctor for a study investigating re-exposure to typhoidal *Salmonella* in the human challenge model, it is seen that there is a 44% attack rate among participants who were re-challenged with *S.* Typhi and 25% attack rate among participants who were re-challenged with *S.* Paratyphi A. These participants were challenged once with the respective agents in the past and the duration between the two challenges ranged from 1-4 years. It is thus likely that a single, immunologically stimulating exposure does not confer protection whether the two exposures are 2 months or 4 years apart.

6.4.6. Implications for preventive strategies

Although the Vi-TT conjugate vaccine is available in India, the dosing schedule and immunization strategy have not been delineated. The continually evolving strains of *S.* Typhi are evidence for the organism's ability to adapt to various selection pressures and underscore the need of eliminating the pathogen from endemic areas. In the short and intermediate term vaccination offers the best chance. Based on the atypical finding of the 28-year old male with two separate infections it is likely that a single dose of the vaccine might not suffice. It is also suggestive that multiple doses will confer protection as the number of cases of enteric fever in patients over 30-years of age in this chapter markedly declined. The large number of children affected is also stark and this population should be the priority of vaccine programmes. However, it is not known how much herd immunity the vaccine confers and what magnitude of protective benefits unvaccinated children will indirectly receive from vaccinating young adults. Mathematical modelling data suggests that vaccination of a wider age range beyond childhood has a greater health impact, but logistically and financially wider population immunisation would be challenging to implement[388]. Finally, a vaccination schedule for travellers to endemic settings is difficult to gauge. Based on observations from the human challenge model, a single dose of Vi-TT vaccination offers a 52% protection from a single exposure, although the conditions in the field are different and the infecting dose in the human challenge model is probably higher. It is not known whether a multiple doses of the vaccine will increase this protective effect.

6.5. Conclusion

The molecular structure of both *S.* Typhi and *S.* Paratyphi A populations suggest that these strains are under a high selection pressure exerted by fluoroquinolones and vaccination should be used as an immediate intervention in reducing the morbidity associated with disease along with the provision of clean water and sanitation. The atypical clinical features

suggest that a high index of suspicion should be maintained in any patient presenting with fever in an endemic setting. These data also highlight the importance of molecular characterisation of strains in studying the dynamics of enteric fever. The changing molecular structure of the pathogen population after the programmatic implementation of the Vi-TT vaccine may serve as an additional metric for assessing vaccine impact.

"You aspire to great things? Begin with little ones"
- Augustine

Chapter 7

A systematic review of antimicrobial resistance in *Salmonella enterica* serovar Typhi, the etiological agent of typhoid

7.1. Introduction

Annually, over 26 million people are culture positive for *S*. Typhi/ Paratyphi[399], and a significant proportion are resistant to multiple antimicrobials[9]. South and South-East Asia, continue to be critical foci for enteric fever, dominated by the "multi-drug resistant" (MDR)-associated H58 haplotype of *S*. Typhi in many regions. Fluoroquinolone resistance is widely prevalent across Asia, in part because of the widespread use of this class of antibiotics.

7.1.1. Multidrug resistant typhoid

MDR typhoid emerged as a public health problem in the early 1990's. In response, therapeutic guidelines for typhoid have changed as antimicrobial resistance (AMR) has evolved. Traditionally, the term MDR *S.* Typhi is used to describe combined resistance to chloramphenicol, co-trimoxazole (trimethoprim-sulfamethoxazole) and ampicillin. These antibiotics are frequently termed first-line antimicrobials in the literature as these were amongst the first to be recommended for typhoid treatment by the WHO[400].

7.1.2. Regional differences between Asia and Africa

MDR *S.* Typhi is now on the decline in regions across South and South-East Asia. This is most likely because first-line antimicrobials are no longer in common use, in view of the previous widespread resistance[189,276]. Empiric antimicrobial treatment for suspected typhoid fever in this region is now predominantly with third-generation cephalosporins including ceftriaxone (parenteral) and cefixime (oral) or azithromycin, since fluoroquinolone resistance is widespread.

In contrast to the situation across Asia, MDR typhoid appears to be increasing in most parts of Africa. Several regions have reported typhoid outbreaks in the last decade and these have been associated with MDR phenotypes. H58 *S.* Typhi disease is moving through areas of East and Southern Africa, while, non-H58 haplotypes are implicated in the Western and Northern regions, illustrating the heterogeneous nature of the disease within the continent[82,83].

7.1.3. Rationale for the study

The historical trend of antibiotic sensitivity and resistance in *S.* Typhi has not been systematically reviewed and reported. Understanding this trend could provide clues for sustaining treatment regimens in endemic areas as well as modelling the potential impact of typhoid vaccines in reducing AMR. This review attempts to summarise such trends.

7.2. Methods

7.2.1. Aims and objectives

The objective of this chapter is to systematically review the temporal trends of antimicrobial resistance (AMR) in the typhoid endemic regions of Asia and Africa.

The aims of this review were two-fold:

1) To systematically delineate the historical trend of expressed phenotypic resistance to first-line antimicrobials, nalidixic acid, ciprofloxacin and cephalosporins.

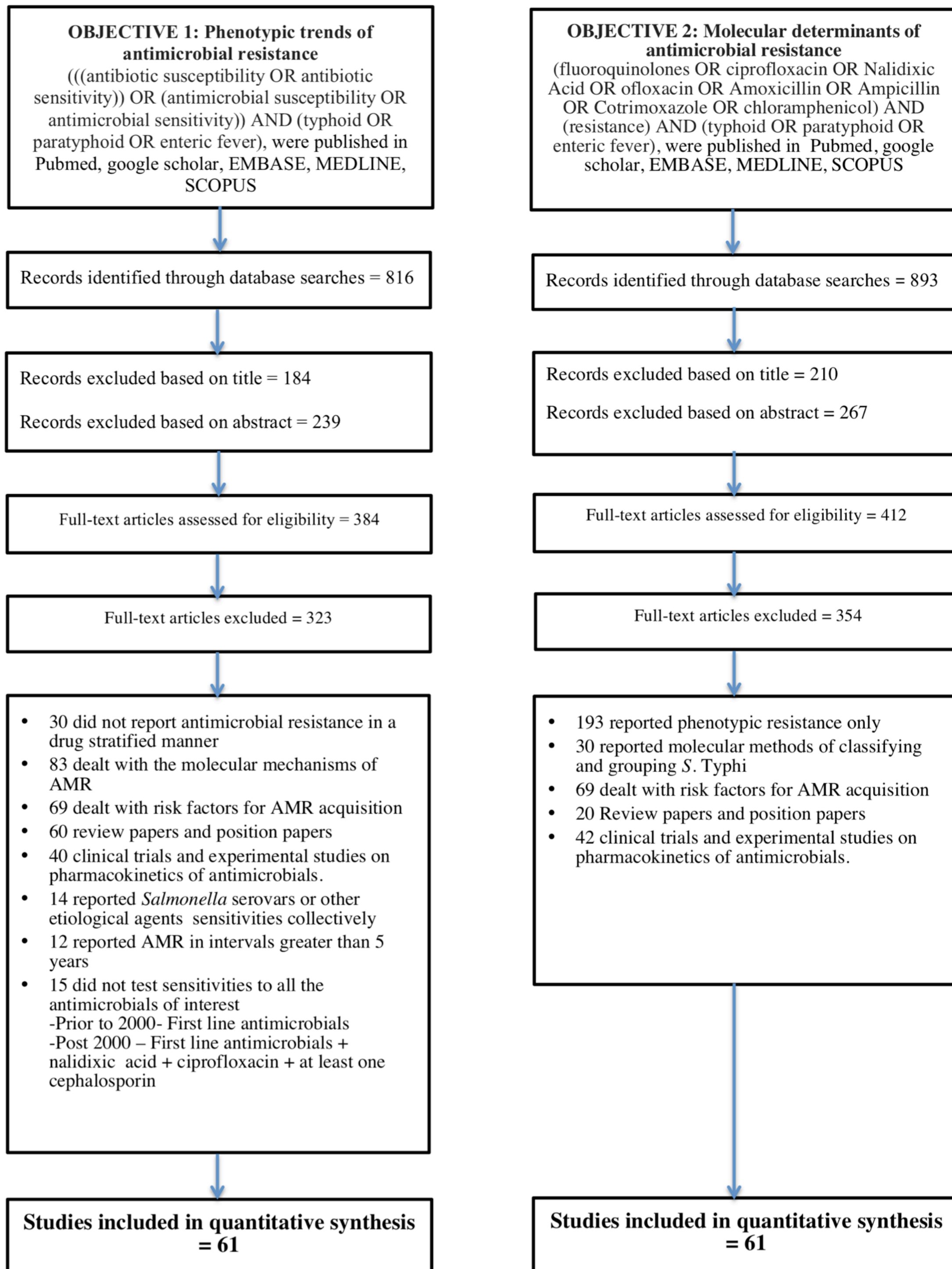
2) To describe the molecular mechanisms of AMR in typhoid.

7.2.2. Search strategy

The search strategies for both objectives are described in **Figure 7.1**.

Exclusion criteria such as time of publication, study design and language were not applied in the search builder in order to ensure complete data collection.

Figure 7.1: Search strategy and characteristics of included studies in the systematic reviews



7.2.3. Phenotypic trends in antimicrobial resistance

An isolate was considered resistant to an antimicrobial if it was reported as "resistant", "intermediately susceptible", "intermediately resistant" or "non-susceptible" based on minimum inhibitory concentration (MIC) values or diameters of zones of inhibition *via* disc diffusion using customary interpretive criteria such as the Clinical & Laboratory Standards Institute (CLSI) or the European Committee on Antimicrobial Susceptibility Testing (EUCAST) standards. For consistency, studies prior to the year 2000, that reported sensitivities of at least the first-line antimicrobials were included while studies conducted after the year 2000, which did not report antimicrobial sensitivities of either chloramphenicol, co-trimoxazole, ampicillin/amoxicillin, nalidixic acid, ciprofloxacin or at least one cephalosporin were excluded. Studies that reported antibiograms collectively and had not stratified these into intervals shorter than 5 years were also excluded.

Isolates identified from reports were then stratified based on year of isolation, geographic location and resistance phenotypes. Stratified isolates that were resistant to each antimicrobial were then expressed as a proportion of all the isolates reported. The trends of antimicrobial resistance were then expressed in 5-year intervals as represented in **Table 8.1**. This process was then repeated on isolates collected from Asia and Africa separately.

Table 7.1: Proportion of antimicrobial non-susceptibility stratified in 5 year intervals

Year	Total no. of isolates	Proportion of resistant isolates					
		CH	**AMP**	**TMX**	**NAL**	**CIP**	**CEPH**
Pre-1991	507	0.31	0.16	0.16	NA	NA	NA
1991-1995	2727	0.53	0.53	0.53	NA	NA	NA
1996-2000	2431	0.44	0.46	0.46	0.22	0.02	0.02
2001-2005	4725	0.29	0.33	0.30	0.45	0.20	0.01
2006-2010	2120	0.18	0.30	0.17	0.48	0.31	0.01
2011-2015	1756	0.13	0.20	0.18	0.80	0.71	0.04

Table 1 represents the proportions of antimicrobial non-susceptibility stratified in 5- year intervals. These data were pooled from 72 published reports from 1973 to 2017 Abbreviations; CH-Chloramphenicol, AMP-Ampicillin, TMX-Cotrimoxazole, NA-Nalidixic Acid, CIP-Ciprofloxacin, CEPH-Cephalosporins

7.2.4. Molecular determinants of antimicrobial resistance

Studies that reported molecular mechanisms of AMR in isolates either collectively or individually were included. These were only stratified based on country of isolation and type of mechanism reported as methods used to study these mechanisms were heterogeneous over the years and techniques employed have also changed thus making temporal comparisons difficult.

7.3. Results

7.3.1. Phenotypic trends of antimicrobial resistance

Sixty-three studies **(Table 7.2)** satisfied the inclusion criteria from which 164 year-wise summaries of antimicrobial resistant *S*. Typhi isolates were obtained. For instance Rahman et al[401] reported the isolates of their study in a year-stratified manner for 13 years, therefore providing 13 serial year-wise summaries. Of these 164 year-wise summaries, 37 were undertaken prior to the year 2000 and more than 80% were retrospective in study design. The year-wise summaries obtained from each report were then pooled into the following temporal intervals; pre-1991, 1991-1995, 1996-2000, 2001-2005, 2006-2010 and 2011-2015 and expressed as a proportion of resistant isolates for each antimicrobial **(Table 7.1)**.

Of the 14,297 isolates obtained from the various reports, 63.2% were isolated from South Asia, 12.8% were from South-East Asia, 15% were from the continent of Africa mostly represented by countries in the East and South-West regions. Isolates that were cultured from travellers returning from endemic regions made up the remainder of the isolates included in this analysis. The number of isolates within each time interval rose steadily until 2001-2005, a period that accounted for the most isolates (4,725 isolates), the subsequent time intervals saw a decline in published data. Nalidixic acid, ciprofloxacin and cephalosporin trends were only analysed from the late 1990's as these drugs were not routinely tested as part of antimicrobial sensitivity studies prior to this period, although preliminary reports of

ciprofloxacin resistance surfaced as early as 1992[78]. **Figure 7.2A** summarises the global AMR trends, which indicate a decline in MDR and a high level of fluoroquinolone resistance. The temporal distribution of isolates obtained from Asia and Africa, when analysed independently, revealed disparate trends as shown in **Figures 7.2B and 7.2C** respectively. The proportion of MDR *S.* Typhi in Asia saw declining trends, accounting for less than 20% of isolates obtained between 2011 and 2015, whereas resistance to fluoroquinolones continued to increase during this period (from 20% in 2001-2005 to 65% in 2011-2015), prompting the use of third-generation cephalosporins in the treatment of enteric fever. Third-generation cephalosporin resistance rose from 1.5% in the 2006-2010 to 4% in the 2011-2015-time interval. Azithromycin is now often used for the treatment of enteric fever, but the numbers of reports on the susceptibility were too few to be presented in this study.

In Africa the scenario is very different, where MDR typhoid is still common, with over 90% resistance in some regions. Interestingly, fluoroquinolone and third-generation cephalosporin resistance are still low (< 1%).

Table 7.2: Publications included in the phenotypic analysis of AMR

No.	Year of Study	Year of Publication	Author	Country/Region of study	PMID	Number of isolates	Study Design
1	2014	2017	Ali A	Pakistan	28303985	155	Retrospective
2	2012	2017	Harichandran D	India	28352198	79	Retrospective
3	2016	2016	Osbourne LG	Travel associated	26243802	1	Case Report
4	2016	2013	Sharvani R	India	27437211	167	Retrospective
5	2013-2014	2016	Misra R	India	25979527	50	Retrospective
6	2014	2015	Khanam F	Bangladesh	25849611	72	Retrospective
7	2013	2015	Mahende C	Tanzania	26138060	17	Prospective
8	2015	2015	Narain U	India	26388636	220	Prospective
9	2002-2013	2015	Nüesch-Inderbinen M	Travel associated	25963025	192	Retrospective
10	2012	2014	Jessica Maltha	Burkina Faso	24551225	12	Prospective
11	2012	2014	Srirangaraj S	India	24817913	16	Retrospective
12	2008	2014	Chiou CS	Bangladesh	25136011	38	Retrospective
13	2012	2014	Chand HJ	Nepal	25390062	56	Prospective
14	2010	2014	Isendahl J	Guinea-Bissau	25526763	3	Prospective
15	2014	2014	Dahiya S	India	28303820	380	Retrospective
16	2010	2013	Choudhary A	India	23703350	322	Retrospective
17	2012	2013	Sultan BA	Pakistan	23905456	1	Case Report
18	2013	2013	Vlieghe E	Cambodia	24094060	31	Retrospective
19	2012	2013	Venkatesh BM	India	24441263	251	Retrospective
20	2008-2010	2013	Gupta V	India	24043999	257	Retrospective
21	2010-2012	2013	Jain S	India	24240035	266	Retrospective
22	2012	2012	Lutterloh E	Malawi-Mozambique border	22357702	42	Retrospective
23	2012	2012	Olut AI	Turkey	22399179	1	Case Report
24	2010	2012	Acharya D	Nepal	22627312	114	Retrospective
25	2008	2011	Kumar Y	India	21444993	128	Retrospective
26	2011	2011	Adhikary	India	22234135	2	Case Report
27	2001-2002 & 2009	2011	Gross U	Ghana	22000360	74	Retrospective
28	2007	2010	Nagshetty K	India	20212336	95	Retrospective
29	2000-2006	2010	Verma S	India	20061765	159	Retrospective
30	2004-2006	2010	Mengo DM		20601792	100	Retrospective
31	2004	2009	Muyembe-Tamfum JJ	DRC	19174300	11	Retrospective
32	2008	2009	Kumar Y	India	19762961	50	Retrospective
33		2009	Yanagi D	Indonesia	19631095	17	Retrospective
34	1999-	2009	Lynch MF	Travel	19706859	2016	Retrospective

	2006			Associated			
35	2005	2008	Mirza SH	Pakistan	18452661	32	Retrospective
36	2008	2008	Prajapati B	Nepal	19558061	195	Retrospective
37	2007	2008	Al-Sanouri TM	Jordan	19741292	48	Retrospective
38	1992	2007	Rodrigues C	India	1307533	74	Retrospective
39	2004	2007	Joshi S	India	16950486	25	Retrospective
40	2007	2007	Parry CM	Vietnam	17145784	187	RCT
41	2003	2007	Bhatta DR	Nepal	17576218	16	Retrospective
42	2004-2005	2007	Akinyemi KO	Nigeria	18330069	89	Prospective
43	2005	2007	Tamang MD	Nepal	17629465	93	Retrospective
44	2002	2007	Capoor MR	India	17873998	178	Retrospective
45	2003	2007	Banerjee A	India	27408039	60	Retrospective
46		2007	Khanal B	Nepal	17615907	132	Retrospective
47	1993-2005	2007	Chau TT	Vietnam, Indonesia, Laos, Pakistan, Nepal, China, India, Bangladesh	17908946	1774	Retrospective
48	1997-2004	2007	Akinyemi KO	Nigeria	18087113	274	Retrospective
49	2004-2005	2006	Manchanda V	India	16687859	56	Retrospective
50	2006	2006	Ray P	India	16926465	70	Cross-Sectional
51	1999-2004	2006	Mohanty S	India	16476168	629	Retrospective
52	1989-2002	2006	Rahman	Bangladesh	16490150	3927	Retrospective
53	2001-2004	2006	Lakshmi V	India	16505555	60	Retrospective
54	2000-2001	2005	Brooks WA	Bangladesh	15752457	49	Prospective
55	2003-2004	2005	Dutta S	India	15793167	379	Retrospective
56	2004	2005	Senthilkumar B	India	15928436	6	Retrospective
57	2002	2004	Madhulika U	India	15347861	157	Cross Sectional
58	2002	2004	Mamun KZ	Bangladesh	16240978	30	Retrospective
59	1997-2001	2002	Gautam V	India	12585971	436	Retrospective
60	2001-2003	2005	Kadhiravan T	India	15904505	50	Retrospective
61	1977	1977	Butler T	Vietnam	324398	87	Retrospective
62	1973	1973	Lawrence RM	Travel Associated	4572522	1	Case Report
63	1973	1973	Overturf G	Travel Associated	4763412	28	Retrospective

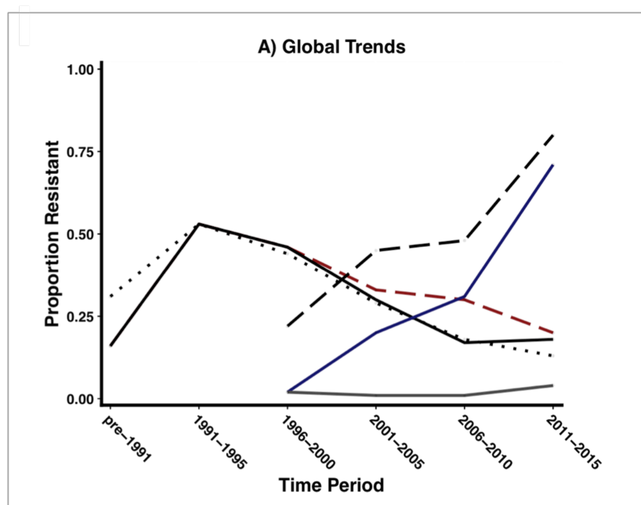
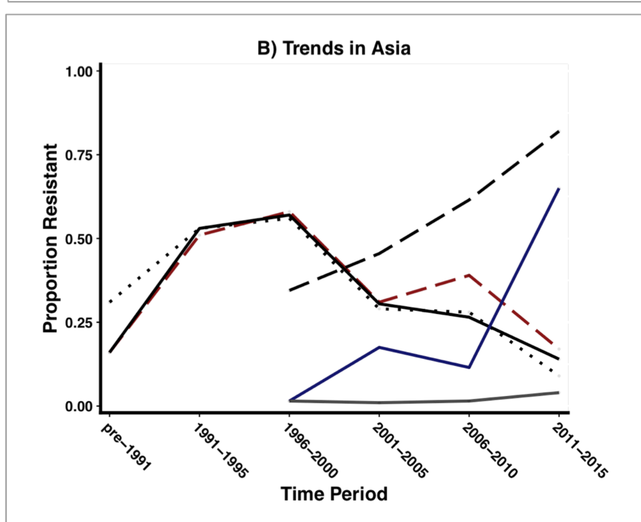


Figure 7.2A is Graphical representation of the proportion of S. Typhi isolates obtained from reports that were resistant to antimicrobials (indicated by coloured lines). Isolates represented in this graph were consolidated from published reports between 1973 and 2017 from endemic and epidemic sources, assembled systematically



In comparison to Figure 7.2A, Figure 8.2B represents the AMR trends obtained from Asian reports. Note the similarity in the trend between 8.2a and 8.2b; it is evident that non-susceptibility to first-line antimicrobials (chloramphenicol, co-trimoxazole and ampicillin) has decreased over time

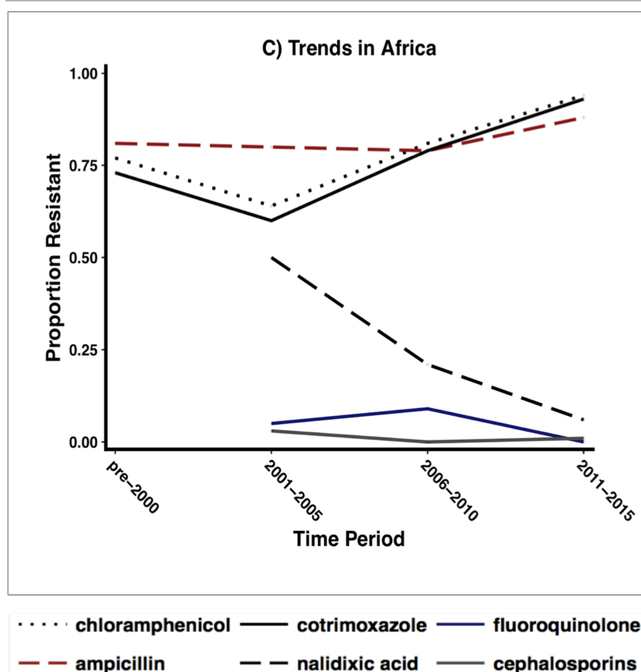


Figure 7.2C represents the AMR trends from African reports. MDR Typhoid is widely prevalent while fluoroquinolone resistance is low.

Figure 8.2:Antimicrobial non-susceptible trends of *S.* Typhi over time A) Global trends, B) Trends in Asia C) Trends in Africa

7.3.2. Molecular determinants of antimicrobial resistance

To meet the second objective of this review 4,226 isolates spanning 61 studies (**Table 7.3**) were included for the analysis of molecular mechanisms. Most studies (66%) incorporated the polymerase chain reaction (PCR) method to study the molecular determinants of anti-microbial resistance. However, four studies[82,83,276,402] reported whole genome sequence analysis of 2,118 isolates and between them provided valuable insights into the development of resistance in *S.* Typhi at a molecular level. In keeping with the phenotypic trends of AMR, the molecular findings of isolates between Africa and Asia were contrasting.

Table 7.3: Publications included in the molecular analysis of AMR

No	Year of Publication	Author	Country/Region of study	PMID	Number of isolates
1	2018	Klemm E	Pakistan	29463654	80
2	2017	Ramachandran A	India	29207706	2
3	2017	Das S	India	27916384	165
4	2016	Gopal M	India	27630841	131
5	2016	Misra	India	27618918	100
6	2016	Ragupathi D	India	27530999	1
7	2016	Elumalai S	India	27166067	1
8	2016	Al-Emran HM	Africa (TSAP)	26933020	11
9	2016	Thanh D	Nepal	26974227	78
10	2016	Wong V	Nigeria	27657909	128
11	2015	García-Fernández A	Italy	26121266	17
12	2015	Nüesch-Inderbinen M	Switzerland	25963025	83
13	2015	Akinyemi KO	Nigeria	25999745	11
14	2015	Ceyssens PJ	Belgium	25385108	62
15	2015	Wong V	Global	25961941	1832
16	2014	Chiou CS	Bangladesh, Indonesia, Taiwan, Vietnam	25136011	38
17	2014	Dutta S	India	25098613	18
18	2014	González-López JJ	Guatemala	25340972	1
19	2014	Dahiya S	India	25027085	18
20	2014	Saleh FO	Egypt	24820472	4
21	2014	Geetha VK	India	24399384	36
22	2013	Lee CJ	Taiwan	23465712	5
23	2013	Jain S	India	24240035	266
24	2012	Lunguya O	Congo	23166855	31
25	2012	Vlieghe ER	Cambodia	23272255	59
26	2012	Emary K	Cambodia	23122884	102
27	2012	Thamizhmani R	India	22885270	6
28	2012	Tatavarthy A	USA	22649021	16
29	2012	Acharya D	Nepal	22627312	11
30	2012	Ahmed D	Bangladesh	22442289	1
31	2012	Koirala KD	Nepal	22371897	1
32	2012	Kumarasamy K	India	22146877	
33	2011	Accou-Demartin M	France	21749778	11
34	2011	Hassing RJ	Netherlands	21227657	11
35	2010	Mohanty S	India	20828458	1
36	2010	Gaborieau V	France	20724089	1
37	2010	Nath G	India	20188522	90
38	2010	Morita M	Japan	20585124	1
39	2010	Wu W	China	20113512	25
40	2010	Dimitrov T	Kuwait	19889623	26
41	2009	Pfeifer Y	Germany	19788837	1
42	2009	Yanagi D	Indonesia	19631095	17
43	2009	Yoon HJ	Korea	19259362	1
44	2009	Dimitrov T	Kuwait	18971360	2
45	2009	Capoor MR	India	18687156	14
46	2008	Dashti AA	Kuwait	18606582	25
47	2008	Rotimi VO	Kuwait	18566147	2
48	2008	Dutta S	India	18280709	2
49	2008	Al-Sanouri	Jordan	19741292	45
50	2007	Chau TT	India	17908946	23
	2007	Chau TT	Pakistan	17908946	34
	2007	Chau TT	Vietnam	17908946	118
51	2007	Capoor MR	India	17873998	12

52	2007	Tamang MD	Nepal	17629465	93
53	2006	Gaind R	India	17071955	8
54	2006	Shirakawa T	Nepal	16466897	30
55	2004	Lee K	South Korea	15504831	11
57	2004	Renuka K	India	15256030	52
58	2002	Mills-Robertson F	Ghana	12399042	21
59	2000	Shanahan PM	Pakistan	10722124	147
	2000	Shanahan PM	Bangladesh	10722124	30
	2000	Shanahan PM	Kuwait	10722124	8
	2000	Shanahan PM	Malaysia	10722124	6
	2000	Shanahan PM	India	10722124	2
	1998	Shanahan PM	India	9620383	20
60	1997	Wain J	Vietnam	9431387	20
61	1996	Panigrahi D	Kuwait	8765450	91

7.3.2.1. Molecular determinants of AMR in Asia

7.3.2.1.1. Genetic determinants of fluoroquinolone resistance

Genetic signatures associated with fluoroquinolone resistance were very distinct amongst isolates studied in Asia. Single nucleotide polymorphisms (SNPs) in *gyrA*, *gyrB*, *parC* and *parE*, which include the quinolone resistance determining region (QRDR) in the *S.* Typhi genome, as well as fluoroquinolone resistance conferring plasmids containing *qnrB2*, *qnrB4* and *qnrS1* genes were reported. From these data it is apparent that fluoroquinolone resistance in *S.* Typhi is frequently linked to mutations with *gyrA* ($p = 0.001$). A frequent position for SNPs in *gyrA* is codon 83 with the S83F being most common occurring in 1189 isolates. S80I was the most common SNP in the *parC* gene, detected in 260 isolates, together with a concordant SNP in S83F. The S83Y mutation was detected in 209 isolates, while 57 isolates harboured the mutation *gyrA* D87N, further underpinning the importance of *gyrA*–associated SNPs, likely in response to antimicrobial selection pressure. Isolates harbouring combinations of three SNPs in *gyrA*, at codons 83 and 87 as well as mutations at codon 80 in *parC* are associated with a high level of ciprofloxacin resistance and designated as 'triple mutants'. These triple mutations were mostly commonly identified in *S*. Typhi isolates from South Asia[82,276], often in distinct sub-groups within the main H58 clonal population[276]. SNPs in *parE* and *gyrB* were also observed but to a much lower extent (3 and 7 isolates respectively). The *qnrB2*, *qnrB4* and *qnrS1* resistance determinants have been found in *S.* Typhi but they are still rare, being identified in 21 *S*. Typhi isolates from Asia. These are usually encoded on plasmids. We can anticipate that such isolates may become more common in the future.

7.3.2.1.2. Genetic determinants of multiple drug resistance (MDR)

The relatively recent decline in MDR *S*. Typhi in Asia has been accompanied by a decrease in the proportion of isolates carrying IncHI1 plasmids, which often harbour the resistance genes responsible for MDR typhoid. Such resistance genes are clustered on composite transposons and include *catA, sul1, sul2, dfrA,* bla_{TEM-1}, *strA*, *strB, tetA, tetB, tetC and tetD.* These MDR-associated genes can also be found integrated on the chromosome of H58 *S*. Typhi in isolates from countries including India and Bangladesh[83]. Other plasmids identified in *S*. Typhi included R27-like, B7-like and those falling into IncH and IncN, but these are currently relatively uncommon.

7.3.2.1.3. Genetic determinants of cephalosporin resistance

Extended spectrum β lactamase (ESBL) producing *S*. Typhi isolates, which confer resistance to third-generation cephalosporins have been reported in India and Pakistan[247,248]. The Indian isolates carried IncX3 and IncA plasmids which encoded bla_{SHV-12} and bla_{CMY-2} determinants[247], as well as bla_{TEM-1B} and bla_{DHA-1} probably on an IncN plasmid[249]. Other CTX-M producing isolates have been reported from Southern India[403], Japan[404] as well as a traveller returning from and Iraq[405]. A recent publication reported $bla_{CTX-M15}$ producing *S*. Typhi isolates from Pakistan that were cephalosporin resistant in addition to MDR and fluoroquinolone resistant and have been labelled as XDR (extensively drug resistant). All the XDR isolates had a composite transposon as described above and an additional IncY plasmid containing $bla_{CTX-M15}$ and *qnrS* genes[402].

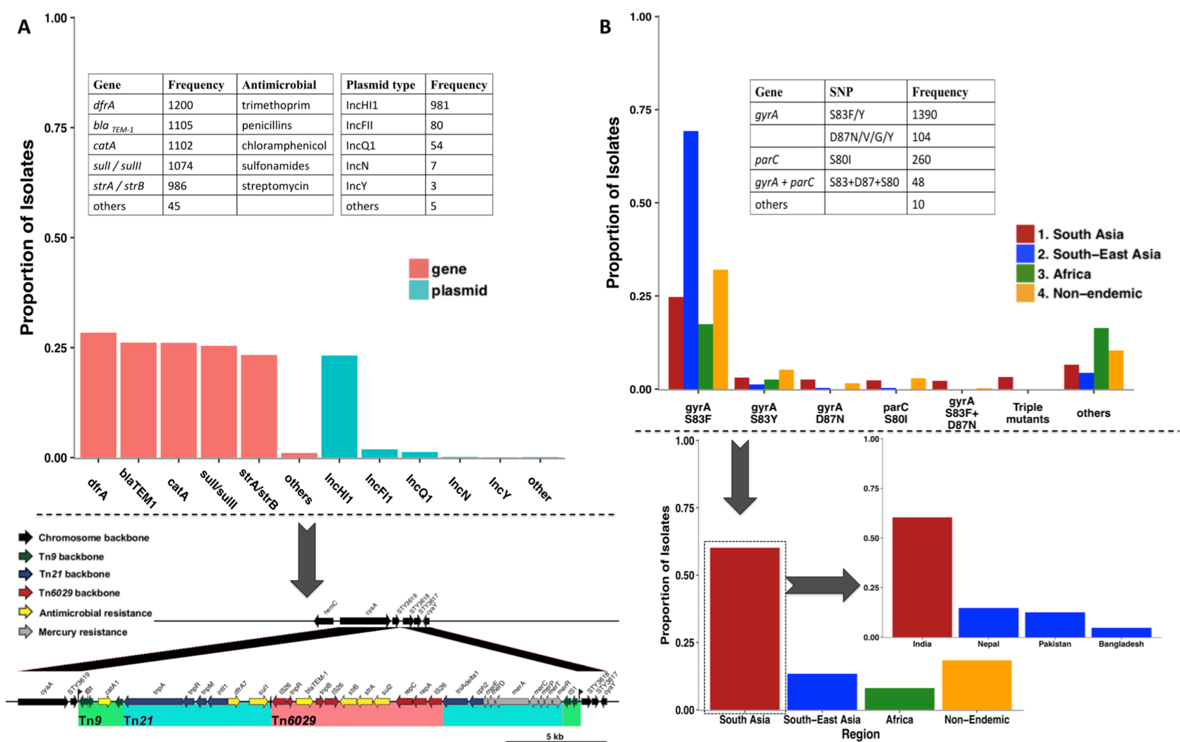
7.3.2.1.4. Genetic determinants of azithromycin resistance

Mediated *via msrD* and *msrA* genes an Indonesian and an Algerian isolate[83] displayed resistance to azithromycin.

7.3.2.2. Molecular determinants of AMR in Africa

The scenario in Africa was very different with MDR being widely prevalent, conferred in part by determinants encoded on IncHI1 plasmids. The H58 clade of *S*. Typhi is associated with much of the typhoid occurring in the last decade in East and Southern Africa, although other haplotypes do occur. The situation is somewhat different in Western Africa, where H58 is still uncommon and AMR typhoid is spread via non-H58 clades[82] with both IncH1 and IncY plasmids being present in the circulating population. Again, as elsewhere in Africa, genetic signatures of fluoroquinolone resistance were present in only a few of the analysed isolates. SNPs in *gyrA*, *gyrB*, *parC* and *parE* were detected in 36 isolates with the S83F SNP

in *gyrA* being the most common. Plasmids encoding the *qnrB2*, *qnrB4* and *qnrS1* determinants have also been reported. Other plasmid-types identified in Africa are illustrated in **Figure 7.3.** More recently, case reports of *S*. Typhi encoding ESBL ($bla_{CTX\text{-}M15}$) has been reported in the Democratic Republic of Congo[406] and Nigeria[407]. There were also reports of azithromycin resistance mediated *via* the *ereA* in an isolate from Algeria.[83]



Gene	Frequency	Antimicrobial
dfrA	1200	trimethoprim
bla TEM-1	1105	penicillins
catA	1102	chloramphenicol
sulI / sulII	1074	sulfonamides
strA / strB	986	streptomycin
others	45	

Plasmid type	Frequency
IncHI1	981
IncFII	80
IncQ1	54
IncN	7
IncY	3
others	5

Gene	SNP	Frequency
gyrA	S83F/Y	1390
	D87N/V/G/Y	104
parC	S80I	260
gyrA + parC	S83+D87+S80	48
others		10

Figure 7.3: Summary of the molecular determinants of *S.* Typhi resistance

- Fluoroquinolone resistance occurs through mutations DNA gyrase enzyme of the bacteria which is encoded by gyrA, gyrB, parC and parE genes (Quinolone resistance determining region; QRDR).
- Number refers to the number of isolates harbouring the respective determinant of antimicrobial resistance as identified through the review.
- Amino acid abbreviations
S – Serine, F- Phenylalanine, Y – Tyrosine, D – Aspargine, N – Aspartic acid, I - Isoleucine

Figure 7.4: The spatial distribution of isolates from endemic areas analysed systematically in this review

7.4. Discussion.

The paucity of reliable point of care diagnostics for typhoid fever compels clinicians in the field to initiate presumptive antimicrobial therapy, often based on clinical judgment. In endemic settings, typhoid features high on the list of causes of undifferentiated febrile illness, and antimicrobial therapy is routinely started empirically with antimicrobials that are thought to be appropriate for local clades of *S.* Typhi. The data presented in this systematic review suggests that such antimicrobial use for the treatment of undifferentiated febrile illness is likely influencing the patterns of AMR in *S.* Typhi.

7.4.1. AMR to first-line antimicrobials

First-line antimicrobials (chloramphenicol, co-trimoxazole and ampicillin) were recommended for treatment of typhoid fever between 1948 and the early 1990s.[78] Unfortunately, the widespread use of these drugs facilitated the emergence of resistance to chloramphenicol and subsequently to ampicillin and co-trimoxazole, leading to MDR typhoid[78]. MDR typhoid became established in parts of Asia in the 1990's and the phenotype was mainly conferred through the acquisition of horizontally acquired plasmids[78] harbouring transposons and integrons encoding resistance-determining genes. The most commonly implicated plasmids found in *S*. Typhi at this time were of the IncHI1 type[78–80]. Bayesian analysis suggests that this plasmid was first acquired by H58 and some other haplotypes of *S.* Typhi in Asia around the early 1990s[83]. With the establishment of widespread MDR typhoid, the use of chloramphenicol, ampicillin and co-trimoxazole became obsolete in this region. However, this analysis indicates that the subsequent circulation of these plasmids within *S.* Typhi in Asia markedly decreased over time, highlighting the adaptability of *S.* Typhi to changing antibiotic pressure. [276,408]

The trend observed in Africa is very different and may partially reflect the more recent introduction of *S*. Typhi isolates into the continent[83]. Transposon-mediated MDR typhoid

associated with composite transposons either on plasmids or in the chromosome is increasingly reported, driven by both H58 and non-H58 clades[82]. Although IncHI1 plasmids are still the most commonly identified, other incompatibility (defined as the inability of two related plasmids to be stably transmitted together[81]) group plasmids such as IncY, IncN and IncFIIK (pKPN3) have also been identified in *S*. Typhi in Africa[82].

7.4.2. AMR to fluoroquinolones

Following the emergence of MDR typhoid, fluoroquinolones were adopted as the treatment of choice for typhoid by the late 1990's. The fluoroquinolone class of antimicrobials were highly effective, could be orally administered and had minimal side effects, although potential adverse effects on the growing epiphysis of long bones was viewed with suspicion and initially restricted in children[78]. Nevertheless, ciprofloxacin and ofloxacin became favoured alternatives to the former first-line antimicrobials and consequently fluoroquinolone resistance began to develop. The antimicrobial pressure associated with fluoroquinolone usage likely facilitated the acquisition of alternative modes of antimicrobial evasion by *S*. Typhi. The spread of fluoroquinolone resistance was accelerated by the emergence of the H58 clade, which dominated circulating *S*. Typhi populations by the late 1990s, with an apparent increased fitness advantage and enhanced transmission success[83,366]. Unlike resistance to first-line antimicrobials, resistance to fluoroquinolones was not initially mediated *via* plasmids but rather by the accumulation of non-synonymous SNPs in the genome inducing conformational changes in DNA gyrase and topoisomerase IV, the main sites of fluoroquinolone action. The genes in which SNPs occur include *gyrA*, *parC*, *parE* and *gyrB*, with *gyrA* SNPs correlating strongly with treatment failure[276]. Unfortunately, the standard method of gauging antimicrobial sensitivity, i.e. disc diffusion, suggested that *S*. Typhi was still relatively sensitive to ciprofloxacin despite ongoing treatment failure and relapse. It then came to light that nalidixic acid break points on disc diffusion correlated more

accurately with ciprofloxacin sensitivity, prompting a revision in the CLSI recommended break points. In our analysis (**Figure 7.2A and 7.2B**) the trend lines of changing nalidixic acid resistance and ciprofloxacin resistance over time seem to converge, which in reality may be due to revisions in the CLSI guidelines. Fluoroquinolone-resistant *S*. Typhi isolates are widespread in Asia with over 60% of isolates in this review demonstrating non-susceptibility. In Africa, 90% of isolates are still susceptible with some reports of *gyrA* SNPs recently emerging[409].

7.4.3. Treatment of typhoid with cephalosporins and macrolides

More recently, third-generation cephalosporins and azithromycin have become the preferred treatment choices for typhoid in the face of MDR and fluoroquinolone resistance, owing to the broad spectrum of activity and the option of oral or intravenous administration. Nevertheless, widespread third-generation cephalosporin resistant typhoid is now on the horizon in South Asia with reports of treatment failure from India[247,249] and Pakistan[248,250]. In South Asia, cephalosporins such as ceftriaxone and cefixime are currently the mainstay of treatment for enteric fever, and are often started empirically, likely driving resistance in typhoid and other Gram-negative bacteria.

Confirmed typhoid and paratyphoid infections make up only a minority of the total proportion of all Gram-negative infections in endemic regions[410,411]. However, empirical antimicrobial treatment with cephalosporins for presumptive enteric fever confers an antimicrobial pressure, which encompasses all Gram-negative bacterial populations. It is thus plausible that the impact of empiric therapy for typhoid is of far greater importance in driving AMR than just as described in this study in *S*. Typhi. The mechanisms of resistance adopted by *S*. Typhi are similar to those among other Gram-negative bacteria[77] and the most contemporary concern stems from the emergence of extended spectrum β lactamases (ESBLs) produced by various Gram-negative species, which has originated as a result of the

widespread cephalosporin use.[297] Preventive approaches warrant a collective approach in tackling Gram-negative resistance as the molecular determinants of resistance are transferrable between Gram-negative organisms and thus reducing the use of cephalosporins for typhoid is likely to have an indirect effect on the other Gram-negative organisms[77].

A 2014 publication suggested that cephalosporins were the most commonly used antimicrobial in India and China, followed by broad-spectrum penicillins, fluoroquinolones and macrolides[396]. This trend might still hold true in 2017 which highlights the mounting antimicrobial pressure exerted by the use of cephalosporins culminating in the production of ESBLs by Gram-negatives, including *S.* Typhi.[247–249] These issues underscore the importance of controlling the spread of typhoid through the deployment of vaccines and prudent antimicrobial use in the short-term.

7.4.4. Potential strategies in sustaining treatment regimens

Single drug therapy (monotherapy) has been common practice in the treatment of typhoid, and monotherapy with former first-line antimicrobials may be a reasonable option in Asia. A single report from Nepal suggests that monotherapy with co-trimoxazole results in complete remission of typhoid fever caused by H58 which was fluoroquinolone-resistant but not MDR[412]. However, a more astute approach in Asia might involve combination therapy with a first-line antimicrobial and perhaps azithromycin. This approach for the treatment of enteric fever in Asia could potentially facilitate the conservation of cephalosporins. The decrease in MDR highlighted in this review following the reduction in use of first-line antibiotics (amoxicillin, chloramphenicol and co-trimoxazole) shows that cycling of these antibiotics for control of typhoid might be an option, where close monitoring of susceptibility is feasible. However, uncoordinated use of these agents would likely lead to a rapid re-emergence of MDR and it is difficult to see how such a programme could be undertaken globally. Immunization could theoretically reduce the number of circulating MDR, fluoroquinolone-

and cephalosporin-resistant strains and, furthermore, decrease the incidence of undifferentiated febrile illness thereby reducing the need for empirical antimicrobial therapy.

7.4.5. Limitations

This study has limitations in that the interpretive criteria employed by majority of studies was the CLSI guidelines which was improved periodically particularly with regard to ciprofloxacin breakpoints in 2012. It is hard to ascertain how quickly individual laboratories made the transition after each revision. Finally, it is also unlikely that true trends of Asian and African isolates are not represented in its entirety, which is mainly due to the lack of published data. Regions from West and Central Africa as well as regions from South-East Asia were under-represented. It was also difficult to account for methodological variations in studying molecular determinants of AMR over time with the rapid evolution of molecular techniques.

7.5. Conclusion

S. Typhi rapidly acquires resistance to the antimicrobials that are being used in the community, but can also lose resistance once these drugs are withdrawn. From these observations, it seems likely that antimicrobial resistance will emerge in areas endemic for typhoid, leading to treatment failure, changes in antimicrobial policy and further resistance developing in *S.* Typhi isolates and other Gram negative bacteria. Therefore, deployment of typhoid conjugate vaccines to control the disease may be the best defence against AMR in *S.* Typhi.

"Messieurs, c'est les microbes qui auront le dernier mot"
-Louis Pasteur

Chapter 8

Phenotypic patterns and molecular determinants of antimicrobial resistance (AMR) in typhoidal *Salmonellae* in Nepal and South India.

8.1. Introduction

The Millennium Developmental Goals (MDGs) set in 1990 greatly augmented access to health-care in many parts of South Asia. The accessibility of essential drugs including antimicrobials has greatly improved as evidenced by a recent study suggesting that the 39% increase in global antimicrobial consumption is mainly driven by low- and middle-income countries (LMICs) particularly in South Asia[389]. This has been possible due to a coalition between public and private sectors but continue to be uncoordinated and inadequately overseen. The legislation of antimicrobial usage as well as antimicrobial stewardship in the

Indian subcontinent has not kept pace with access, which has led to rapidly increasing and poorly regulated availability of antimicrobials. Such widespread unregulated use of antimicrobials has played a prominent role in the development of AMR leading to drugs becoming clinically obsolete, poor treatment outcomes and large economic losses.

8.1.1. The AMR pandemic

This situation is a cause for global concern as entailed in macroeconomist Jim O'Neill's report on the effects AMR suggesting that at least 700,000 annual deaths globally are attributed to AMR and a large proportion of these are likely to be in South Asia[390]. With escalating rates of AMR, we are positioned to lose notable advancements in medical and surgical sciences we have made over the last century which includes but is not limited to combating highly virulent infectious diseases; chemotherapy for malignancies, where antimicrobials are critical in preventing infections in these patients over the course of immunomodulatory treatment; and surgical procedures like organ transplants and caesarean sections, which are currently relatively routine and low risk, thanks to our ability to effectively prevent or treat infections[390]. The drivers of AMR differ between high-income countries (HICs) and LMICs, and the regulations governing the use and prescription of antimicrobials are better legislated in HICs as is antimicrobial stewardship. For instance HICs decreased their antimicrobial consumption by 4% between 2000 and 2015 but the overall global antimicrobial consumption ascended by 75% in the same time period and was mainly due to the use in LMICs[391]. This metric suggests that accessibility to antimicrobials increased in LMICs but it is also likely that this magnitude of antimicrobial use in LMICs is not justifiable which prompted the WHO to report in 2016 that significant gaps in surveillance, standards for methodology, data sharing and coordination continue to persist between LMICs and HICs[392]. This disparity thus calls for a more integrated and collaborative approach in

dealing with AMR particularly in South Asia, which is the hub for the development and spread of AMR.

8.1.2. AMR among Gram-Negatives in India and Nepal

In India alone 60,000 neonates die annually from AMR related causes and Gram-negative organisms resistant to multiple antimicrobials continue to account for the most notable burden of AMR[390]. To underscore this point; in India, between 2008 to 2013, resistance to third-generation cephalosporins and fluoroquinolones in *E. coli* isolates increased from 70% to 83%, and from 78% to 85% respectively[393,394]. More worryingly, with regard to carbapenem resistance 10% *E. coli* isolates were resistant in 2008, and increased to 13% in 2013 while a single centre in Delhi, India reported an increase from 2% in 2002 to 52% in 2009[394] among *K. pneumoniae* isolates.

The situation is likely to be similar in Nepal although there is a dearth of longitudinal data regarding antimicrobial trends over a significant period of time. One single centre study of *K. pneumonia* isolates revealed that plasmids conferring resistance to multiple antimicrobials such as beta-lactams, fluoroquinolones, macrolides, sulphonamides, tetracyclines and chloramphenicol were as high as 31% and 22% of isolates were meropenem resistant[395]. Ninety-eight % of isolates were resistant to at least 2 antimicrobials namely cephalosporins and ciprofloxacin.

8.1.3. Antimicrobial consumption in India and Nepal

Two independent published reports entail the antimicrobial consumption trends in India[389,396]. The first report assessed antimicrobial consumption between 2000 and 2010 and suggested that period global antibiotic consumption increased by 36% based on national pharmaceutical sales[396]. Most notably in 2010, India and China were the world's first and second largest consumers of antibiotics respectively[396]. In India, the three classes of antimicrobials that were most consumed were beta-lactams, macrolides, and fluoroquinolones. The second report

published in 2018 gauged antimicrobial consumption between 2000 and 2015 in defined daily doses (DDDs) as well in DDDs/1000 inhabitants/day and suggested that antimicrobial consumption has increased by 103% between 2000 and 2015 in India with this rise being attributed to the increasing use of cephalosporins in the face rising AMR for other antimicrobials including narrow spectrum beta-lactams and fluoroquinolones[389].

There are no published data on patterns of antimicrobial use in Nepal but these trends are likely to be similar to that of India. Nevertheless, due to the paucity of published data, two senior paediatricians who have been working at Patan Hospital, Kathmandu for the last two decades were interviewed to obtain specific information on antimicrobial usage in Kathmandu, Nepal (**Table 7.1**). The two senior paediatricians were interviewed using a set of pre-designed questions. The two paediatricians were interviewed separately and at two distinct time-points by two separate interviewers. The data obtained from these interviews showed that fluoroquinolones were the mainstay of treatment for children with enteric fever since the early 2000s but has shifted to cephalosporins and azithromycin more recently. Antimicrobials used for the treatment of other community- acquired infectious conditions were gauged through these interviews. It is interesting to note that first-line antimicrobials for enteric fever namely chloramphenicol, co-trimoxazole (sulfamethoxazole-trimethoprim) and ampicillin were never used for enteric fever over the last 2 decades.

Table 8.1: Paediatric antimicrobial usage patterns at Patan Hospital, Kathmandu, Nepal.

Chloramphenicol	
2005-2015	Occasionally used for children admitted with pneumonia and in complicated *Rickettsial* infections. Rarely used and/or available in the community.
Ampicillin and Amoxicillin	
2005-2015	Ampicillin used for children admitted with pneumonia who have had no prior antibiotic treatment.
2005-2015	Amoxicillin commonly used in the community for pneumonia over the last two decades (often with clavulanic acid)
Co-trimoxazole	
2005-2015	Used for UTI prophylaxis and PCP pneumonia.
2005-2014	Widely used in the community for pneumonia until 2014
Fluoroquinolones	
2005-2015	Used for urinary tract infections and dysentery. Also used as second line treatment for **enteric fever** following azithromycin.
2005-2011	Ofloxacin used for outpatient managed **enteric fever.**
Cephalosporins	
2005-2015	Ceftriaxone used as first-line injectable for enteric fever second line inpatient treatment of pneumonia or if the patient has had prior antibiotics in the community.
2010-2015	Cefixime also used for UTIs, **enteric fever**, dysentery, pneumonia
Macrolides	
2013-2015	Azithromycin used for OPD **enteric fever** (empiric therapy).
2005-2015	Azithromycin occasionally used in the community for pneumonia (consistent for last two decades)
Tetracyclines	
2005-2015	Occasionally used in older children for cholera and scrub typhus

8.1.4. Rationale for the study

Therefore, this study was conducted with the aim of determining the antimicrobial resistance patterns of the enteric fever isolates in Nepal and South India, including the examination of how these patterns change over time in Nepal. In addition, it was aimed to identify the molecular determinants of AMR, for each of the main antimicrobial classes being used in South India and Nepal.

8.2. Methods

8.2.1. Study design and objectives

The primary objective of this chapter was to determine the patterns of antimicrobial susceptibility across the enteric fever bacterial population amongst Nepali children between 2008 and 2016 as well as amongst children and adults attending a tertiary care hospital in Bengaluru, India between June 2016 and June 2017. The secondary objective was to determine the molecular determinants of phenotypic AMR for each tested antimicrobial. All of the isolates described in **chapters 4, 5, and 6** which underwent whole genome sequencing, were processed, as described in **section 2.2**, for antimicrobial sensitivities using the disc diffusion method. Antimicrobials tested were chloramphenicol, co-trimoxazole (trimethoprim-sulfamethoxazole), ampicillin, nalidixic acid, ciprofloxacin, ceftriaxone and azithromycin.

8.2.2 Detection of the molecular mechanisms of AMR

S. Typhi and Paratyphi are known to contain AMR genes which are usually acquired horizontally by the means of plasmids as well by single nucleotide polymorphisms (SNPs) in the genome which alters the binding site of known antimicrobials particularly fluoroquinolones. The methods used to identify AMR genes and SNPs are described in **section 2.3**.

8.3 Results

There were 198 *S.* Typhi isolates and 66 *S.* Paratyphi A isolates from the Nepali setting and 115 collective typhoidal *Salmonella* isolates from India

The isolates in this chapter represented

- Outpatient (131 *S*. Typhi isolates + 49 *S.* Paratyphi A) and inpatient (67 *S*. Typhi isolates + 17 *S*. Paratyphi A) isolates from children in Nepal over a 9-year period

- Outpatient and inpatient isolates from children and adults from South India over a 13-month period (101 *S.* Typhi and 14 *S.* Paratyphi A).

8.3.1. Nepali isolates

A graphical representation of the AMR patterns are shown in **Figure 8.1**. There were no differences in the frequency of MDR or fluoroquinolone resistance between the paediatric inpatients and outpatients (OR for MDR = 0.97, 95% CI 0.23 – 4.00; and OR for fluoroquinolone resistance = 1.23, 95% CI 0.62 – 2.44).

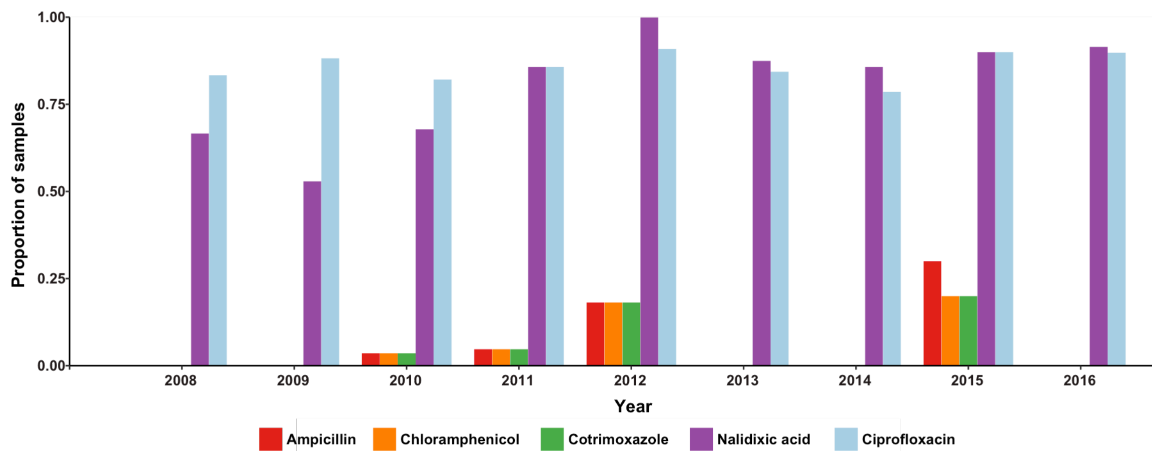


Figure 8.1: **Antimicrobial resistance trends amongst paediatric *S.* Typhi from Nepal (**Reproduced from Britto *et al* 2018). Bars are coloured according to the inset legend, and indicate proportion of isolates per year that displayed resistance to each drug. All isolates were also tested for susceptibility to ceftriaxone, cefixime and azithromycin.

8.3.1.1. Susceptibility of *S.* Typhi isolates to first-line antimicrobials

Amongst the paediatric Nepali isolates analysed, most *S*. Typhi isolates 192 (96%) were susceptible to traditional first-line antibiotics co-trimoxazole, ampicillin and chloramphenicol. Five (3%) isolates were resistant to ampicillin, chloramphenicol and co-trimoxazole (MDR) and 1 (0.5%) isolate was resistant to ampicillin but sensitive to chloramphenicol and cotrimoxazole.

These MDR isolates belonged to *S*. Typhi genotype 4.3.1 and harboured the acquired AMR genes *catA*, *dfrA7, sul1*, *sul2*, *strA, strB* and bla_{TEM-1}, conferring resistance to chloramphenicol, co-trimoxazole, streptomycin and ampicillin respectively. An additional genotype 4.3.1 isolate carried a subset of four of these genes (*sul2*, *strA, strAB* and bla_{TEM-1}) and displayed resistance to ampicillin but was sensitive to co-trimoxazole and chloramphenicol (consistent with the lack of *dfr* and *cat* genes). The full suite of seven acquired AMR genes are common amongst *S*. Typhi globally and are typically located within a composite transposon, comprising Tn*6029* (*sul2*, *strA, strAB* and bla_{TEM-1}) and Tn*21* (*dfrA7, sul1*) inserted within Tn*9* (*catA*)*,* which is most often carried on IncHI1 plasmids[80]. Here, all MDR isolates carried this typical composite transposon, inserted in the chromosome between genes STY3618 and STY3619 and were associated with an 8 bp target site duplication (GGTTTAGA), consistent with integration mediated by the flanking IS*1* transposases of Tn*9* (see **Figure 8.2**). The additional ampicillin resistant isolate (RE2370 in **Figure 8.2.**) carried only transposon Tn*6029*, which was inserted directly into the chromosomal pseudogene *slrP* and associated with an 8 bp target site duplication (TAGCTGAT), consistent with integration mediated by the flanking IS*26* transposases of Tn*6029*.

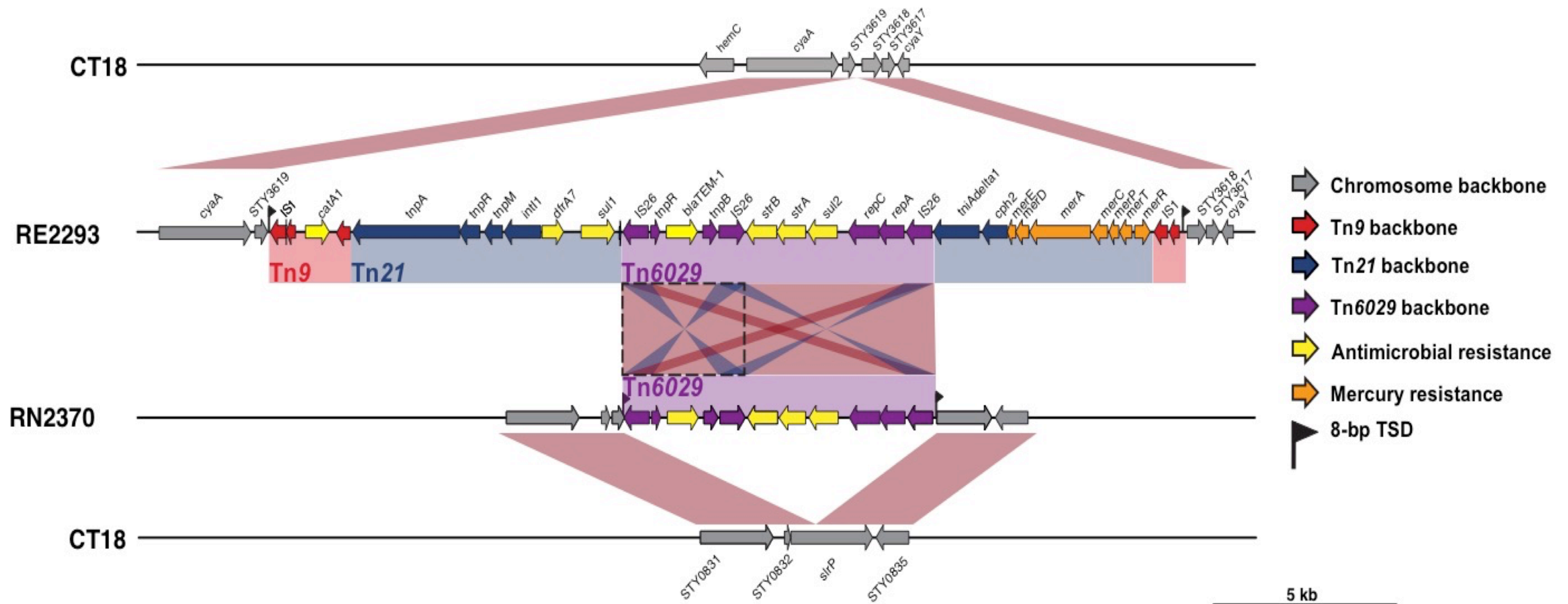


Figure 8.2: Structure and insertion sites of antimicrobial resistance transposons observed in *S.* Typhi from Nepal (Figure reproduced from Britto *et al*, 2018**)**
Arrows represent genes, coloured by functional group according to the inset legend. The boundaries of transposons (Tn*9*, Tn*21* and Tn*2069*) are additionally shaded and labelled. TSD = target site duplication, which are formed by the flanking transposases upon integration into the genome. Red shaded regions indicate homology between the different sequences shown; the reference genome CT18 is included to indicate the sites of transposon integration within the chromosome.

8.3.1.2. Susceptibility of *S.* Typhi isolates to fluoroquinolones

One hundred and seventy one (86%) of *S*. Typhi isolates were resistant to the fluoroquinolone ciprofloxacin (assessed by disk diffusion; **Figure 7.1**). *S*. Typhi isolates displaying fluoroquinolone resistance harboured known QRDR SNPs (**Table 7.2**); the majority of *S*. Typhi isolates had SNPs at codon 83 of *gyrA* where 143 isolates had S83F mutations and 6 isolates had S83Y mutations. The SNP in codon 87 of *gyrA* occurred in combination with other *gyrA* and *parC* SNPs. Sixteen *S*. Typhi isolates (all genotype 4.3.1) were QRDR 'triple mutants', which have SNPs in codons 83 and 87 of gyrA as well as a *parC* SNP at codon 80 or 84 (**Table 7.2**).

Table 8.2: Genetic determinants of AMR detected in the Nepali paediatric isolates

	***S.* Typhi**	***S.* Paratyphi A**
Total isolates	**198**	**66**
QRDR (%)	**164 (82.8%)**	**65 (98%)**
gyrA S83F	143 (72%)	65 (98%)
gyrA S83F only	15 (7.6%)	0
gyrA S83F, *gyrA* D87N	16 (8.1%)	0
gyrA S83F, *gyrA* D87N, *parC* S80I	15 (7.6%)	0
gyrA S83F, *gyrA* D87N, *parC* E84G	1 (0.5%)	0
gyrA S83F, *parC* E84G	1 (0.5%)	0
gyrA S83F, *parE* A364V	5 (2.5%)	0
gyrA S83Y only	6 (3%)	0
parE A364V only	15 (7.6%)	0
Acquired AMR genes	**7 (3.5%)**	**0**
blaTEM-1, *strAB, sul2*	1 (0.5%)	0
catA1, dfrA7, sul1, blaTEM-1, strAB, sul2	6 (3%)	0
+ *gyrA* S83F	4 (2%)	0
+ *gyrA* S83Y	2 (1%)	0

Amino acid abbreviations: S – Serine, F- Phenylalanine, Y – Tyrosine, D – Aspargine, N – Aspartic acid, I – Isoleucine, E - Glutamic Acid , G - Glycine

8.3.1.3. Susceptibility to cephalosporins and azithromycin

All S. Typhi isolates in this chapter were susceptible to cefotaxime, ceftriaxone and azithromycin.

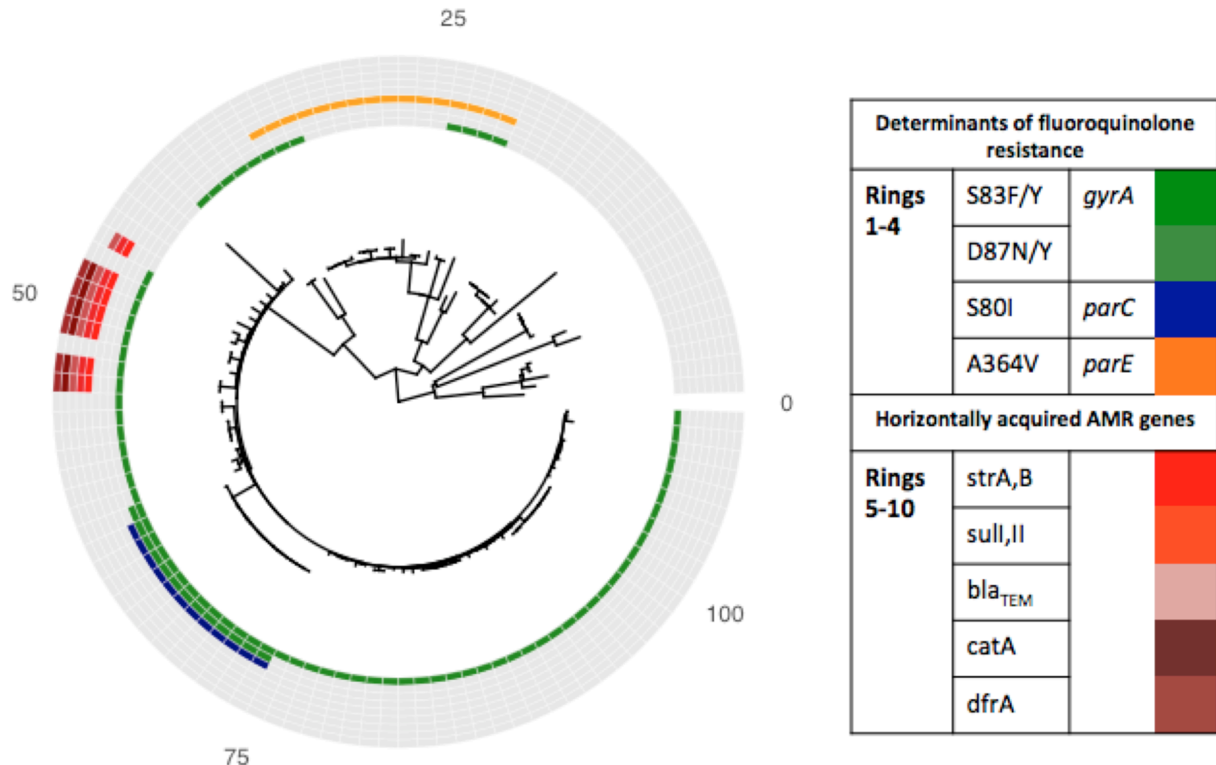


Figure 8.3: Maximum likelihood tree with a graphical representation of QRDR SNPs and acquired AMR genes in *S.* Typhi isolates from the Nepali site.
The first 4 rings depict the QRDR SNPs with the triple mutants clustering together distinct from those strains with other QRDR signatures. The strains with AMR genes cluster together suggesting strong clonality

8.3.1.4. Susceptibility of *S.* Paratyphi A isolates to first-line antimicrobials, cephalosporins and azithromycin

All *S.* Paratyphi A were susceptible to co-trimoxazole, ampicillin, chloramphenicol, cephalosporins and azithromycin on disc diffusion. No acquired AMR genes were detected amongst the *S*. Paratyphi A isolates (**Table 7.2**).

8.3.1.5. Susceptibility of *S.* Paratyphi A to fluoroquinolones

In contrast to other antimicrobials, fluoroquinolone resistance was high and was observed in 65 *S.* Paratyphi A (98%) isolates.

All *S.* Paratyphi A (besides the single lineage C4 isolate) carried the *gyrA* S83F mutation responsible for nalidixic acid resistance and fluoroquinolone resistance (**Table 7.2**)

8.3.3. Indian isolates

8.3.3.1. Susceptibility to first-line antimicrobials, cephalosporins and azithromycin

All 115 isolates (101 *S.* Typhi and 14 *S.* Paratyphi A) were susceptible to ampicillin, co-trimoxazole, chloramphenicol, cefotaxime, ceftriaxone and azithromycin when assessed *via* disc diffusion. There were no known horizontally acquired AMR conferring genes or plasmids identified among these strains.

8.3.3.2. Susceptibility to fluoroquinolones

Ninety-five (94%) *S.* Typhi isolates displayed resistance to ciprofloxacin and ofloxacin. Six *S.*Typhi isolates were phenotypically susceptible to ciprofloxacin of which 5 of these displayed resistance to nalidixic acid. A single *S.* Paratyphi A isolate was susceptible to ciprofloxacin but resistant to nalidixic acid. All 13 other *S.* Paratyphi A isolates were resistant to both nalidixic acid and ciprofloxacin.

All but two isolates (genotype 2.2.1) contained at least one QRDR SNP in their genomes. Six of the seven isolates (*S*. Typhi and *S*. Paratyphi) which were phenotypically sensitive to ciprofloxacin harboured QRDR SNPs highlighting the intrinsic fallacies of disc diffusion in assessing antimicrobial susceptibility. The QRDR mutations are summarized in **Table 8.3** and depicted in **Figure 8.4.** Among those isolates with mutations in the *gyrA* gene in codon 83, 46 had S83F mutations and 50 had S83Y mutations, while in codon 87 a single *S.* Typhi isolate had a D87Y SNP and 22 had the more common D87N SNP. With regard to *parC* mutations in codon 80, 12 isolates from adults and 9 isolates from children harboured this mutation together with simultaneous mutations in gyrA at codons 83 and 87 (triple mutants). Interestingly 7 *S.* Typhi isolates harboured mutations in A364V *parE* and these isolates belonged to genotype 3.3.1 and were distinct from the dominant H58 (genotype 4.3.1) population. There was one isolate that contained a rare *gyrB* S464F SNP. All Paratyphi A isolates had a *gyrA* SNP in codon 83 with S83F being more common.

Table 8.3: Genetic determinants of fluoroquinolone resistance in the Indian isolates

	S. Typhi (n = 101)		*S*. Paratyphi A (n = 14)	
Source	**Adults**	**Children**	**Adults**	**Children**
Total isolates	**68**	**33**	**10**	**4**
QRDR (%)	**67 (99%)**	**32 (97%)**	**10 (100%)**	**4 (100%)**
gyrA S83F	29 (43%)	17 (52%)	7 (70%)	3 (75%)
gyrA S83F only	5 (7%)	4 (12%)	7 (70%)	3 (75%)
gyrA D87N only	1 (1%)	0	0	0
gyrA S83F, *gyrA* D87N, *parC* S80I	12 (18%)	9 (27%)	0	0
gyrA S83F, *parC* E84G	6 (9%)	3 (9%)	0	0
gyrA S83F, *parE* A364V	4 (6%)	1 (3%)	0	0
gyrA S83Y	35 (51%)	15 (45%)	3 (30%)	1 (25%)
gyrA S83Y only	34 (50%)	15 (45%)	3 (30%)	1 (25%)
gyrA S83Y, *parE* A364V	1 (1%)	0	0	0
gyrA D87N, *parE* A364V	1 (1%)	0	0	0
gyrB S464F only	1 (1%)	0	0	0

Amino acid abbreviations: S – Serine, F- Phenylalanine, Y – Tyrosine, D – Aspargine, N – Aspartic acid, I – Isoleucine, E - Glutamic Acid , G - Glycine

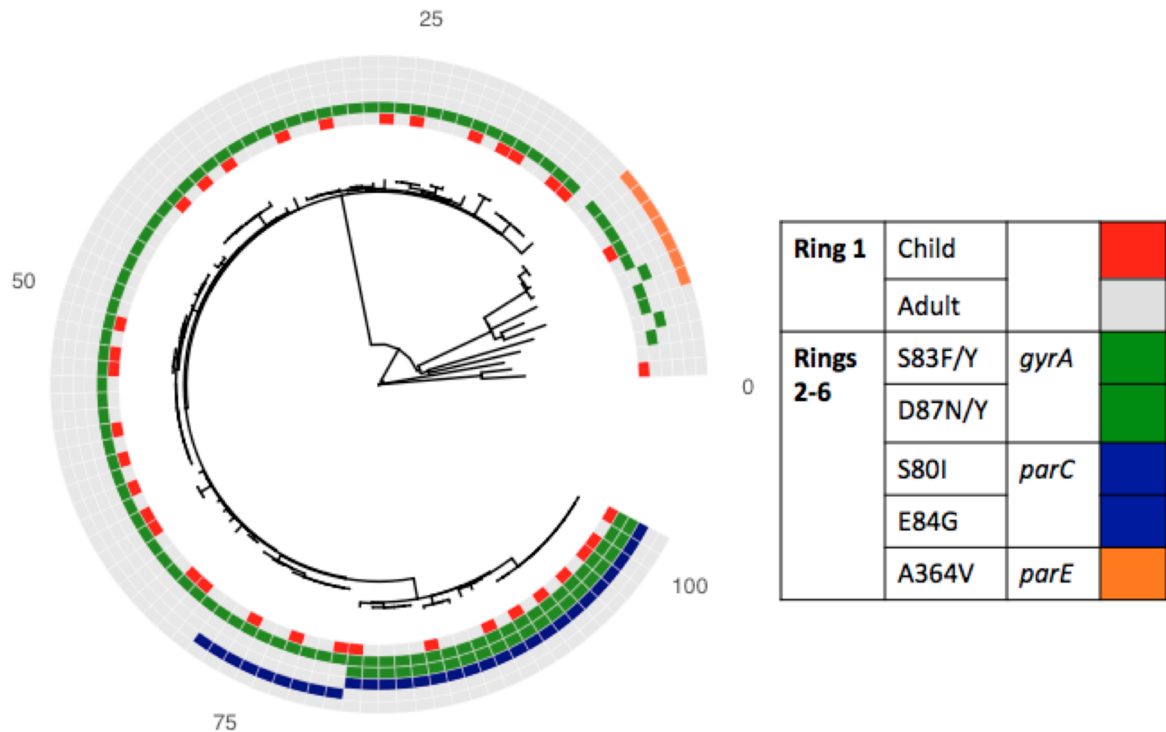


Figure 8.4: Maximum likelihood tree with a graphical representation of QRDR SNPs in *S.* Typhi isolates from the Indian site.
The first ring depicts isolates from children and adults are intermingled suggesting a strong AMR relatedness of strains isolated from these two groups. Rings 2-6 depict the QRDR SNPs with the triple mutants clustering together distinct from those strains with other QRDR signatures.

8.4. Discussion

These data underscore the relevance of understanding AMR from both phenotypic and genotypic aspects culminating in improved patient care by understanding the changing antimicrobial susceptibility trends and the genetic basis for the same. The majority of enteric fever isolates in this study were resistant to fluoroquinolones with MDR strains still circulating at low levels.

8.4.1. Fluoroquinolones have little role to play in the treatment of enteric fever

The high frequency of fluoroquinolone resistance is attributable to uncontrolled use of this class of antimicrobials, which since the turn of the century have been used to treat a range of infections common in the tropics in addition to enteric fever. Fluoroquinolone resistance observed in Nepal[276] and India is most commonly associated with mutations in *gyrA* and *parC*[83]. These data, taken together with findings in **chapter 4** of this thesis suggest that the problem of fluoroquinolone resistant enteric fever in Nepali children is mainly driven by two locally established pathogen variants, namely *S*. Typhi 4.3.1 (H58) Lineage II harbouring the *gyrA*-S83F mutation (accounting for 50% of all enteric fever, 57% of resistant cases, and 66% of all *S*. Typhi) and *S*. Paratyphi A clade A harbouring the *gyrA*-S83F mutation (accounting for 25% of enteric fever, 28% of non-susceptible cases, and 98% of all *S*. Paratyphi A). The universal fluroquinolone resistance demonstrated by the *S*. Paratyphi A population is of great concern particularly since a vaccine against paratyphoid fever is still in development.

Notably, the fluoroquinolone resistant triple mutant *S*. Typhi strain that was first detected in local adults in 2013 and halted the gatifloxacin treatment trial was still causing disease in Nepali children in 2015-2016 based on the results in this chapter. This clonal population is also present among the Indian sites and seems better established possibly indicating the higher antimicrobial pressure in the Indian context. The lack of fully resistant *S*. Paratyphi A is also notable. It has been shown that the *gyrA*-S83F mutation is not associated with a fitness cost in *S*. Typhi and can be maintained in the absence of direct selection from fluoroquinolones.

8.4.2. MDR isolates and current implications

Acquired resistance to other antimicrobials was rare among isolates from Nepal and not identified in any of the Indian isolates. The MDR isolates in the paediatric Nepali population were only associated with strains carrying chromosomally integrated AMR genes as illustrated in **Figure 8.2.** This has not been reported previously in the local *S.* Typhi population, where MDR *S.* Typhi have typically been associated with plasmids[397]. Although similar findings have also been reported from *S.* Typhi strains in other neighbouring countries of India and Bangladesh[83], this is the first description in strains from Nepal. Notably, in addition to the integration of the typical *S*. Typhi MDR composite transposon mediated by IS*1* transposase genes of Tn*9*, we identified for the first time direct integration of Tn*6029* into the *S*. Typhi chromosome (**Figure 8.2**), mediated by IS*26* and conferring ampicillin resistance in the absence of resistance to chloramphenicol or co-trimoxazole.

Given the re-emergence of antimicrobial sensitivity to chloramphenicol and co-trimoxazole as evidenced in this study, it may be logical to shift to these first-line drugs for treating enteric fever; indeed there has already been a case report demonstrating efficacy of co-trimoxazole treatment in the treatment of fluoroquinolone resistant H58 *S*. Typhi in this setting[398]. There is a possibility that typhoidal *Salmonella* strains will acquire resistance to these antimicrobials if they are re-introduced and cycling of antimicrobials is seldom sufficient to effectively prevent MDR in the long-term. However this short-term strategy might be commissioned until the typhoid conjugate vaccines are deployed, in order to conserve cephalosporins and macrolides for the treatment of other tropical infections which require higher-end antimicrobial.

8.4.3. Currently used antimicrobials and emerging resistance

Cephalosporins and azithromycin are currently the first-line treatment for enteric fever in Nepal and India as is the case in majority of Asian settings. No phenotypic or genotypic

cephalosporin resistance was detected in these isolates, however it is anticipated that this will emerge via the acquisition of plasmid-encoded extended-spectrum beta-lactamase genes, as has recently been observed among *S.* Typhi isolates from other parts of India and neighbouring Pakistan[247–250].

Azithromycin is a popular choice for a number of reasons. This drug was sold exclusively by the inventing pharmaceutical company (Pliva) and Pfizer between the early 1990's and 2005, which meant its use was restricted by costs and patent. During this time period it was mainly marketed in North America and the European continent both of which were non-endemic for enteric fever and translated to a very minimal antimicrobial pressure. When the patent lapsed in 2005 this drug began to gain favour due to its convenient dosing schedule and oral route of administration. It was subsequently marketed in South and South-East Asia and soon became a popular option for empirical antimicrobial therapy for community-acquired infections in the background of cheap costs and rising antimicrobial resistance to fluoroquinolones. For enteric fever in particular, this drug has the added advantage of achieving high intra-cellular concentrations and good bioavailability. However, the rampant and largely unrestricted use of this drug for a wide array of tropical infections in addition to enteric fever has created an antimicrobial pressure and reports of azithromycin resistance have already been reported and molecular determinants for the same have been identified in *S.* Typhi isolates[83].

8.4.4. Strengths and limitations

The strengths of this study are the inclusion of isolates from a broad range of time in Nepal and the consecutive sampling strategy used for adults and children in India. Methodologically, assessment of resistance of all of the isolates in Nepal was conducted in the same laboratory using the same guideline. Thus preventing any bias in reporting that would be present if assessment had been done progressively over the decade across which the isolates were collected.

Weaknesses of the study include; the resistance gene database used, and the approach to identifying molecular mechanisms of resistance. The database used is not completely exhaustive and it is conceivable that other resistance genes are present but were not detected because they were not in the database. However, in general there was a good relationship between the detection of a resistance gene and resistance. The approach used in this study, using SRST2, appears to have worked well for the identification of resistance genes.

8.5. Conclusion

These data highlight the significance of laboratory and molecular surveillance of AMR in enteric fever in endemic regions. AMR continues to complicate management protocols and calls for prudent strategies aimed at conserving the currently effective drugs while buying time for vaccine deployment. The Vi conjugate vaccines offer the real possibility of controlling enteric fever and the AMR associated with it but eradication will only become a possibility when the immunization strategy is supplemented by the provision of clean water and improved sanitation.

"There is some good in this world, and it's worth fighting for."
-J.R.R. Tolkien

Chapter 9

Discussion summary and future work

There are significant barriers to the on-going prevention of enteric fever in endemic regions around the world. This thesis provides insight into some of the key issues that have contributed to these impediments. The evidence of disease occurrence in the under 5 years age group as reported in this thesis, now strongly suggests that this age group should also be the focus of vaccine interventions.

The progress in bioinformatic and sequencing technologies in the recent past has provided the opportunity to characterize the molecular structure of circulating strains of enteric fever. These molecular analyses provide an initial picture of the circulating population prior to widespread deployment of the Vi-TT conjugate vaccine. In addition, these data also provide understanding into the various selection pressures at play and also demonstrate the genetic adaptability of typhoidal *Salmonella* to these selection pressures.

9.1. Age characterisation of disease occurrence

The age-characterised trends of disease occurrence in this thesis are in general agreement with larger surveillance studies in Africa and Asia. Data from a multicentre, population-based, prospective surveillance study across 13 sites in 20 sub-Saharan African (TSAP) countries between March 2010 and Jan 2014 revealed that incidence of typhoid fever in those under 15 years was typically higher than those above 15 years. Among children under 15 years those under age the of 4 years had a higher incidence of disease than the other age groups of children at study sites in Burkina Faso, Ghana, Tanzania and Kenya[413]. The next phase of this surveillance project aims to estimate and characterise outcomes of severe typhoid in Africa and is identified as "Severe Typhoid in Africa Program (SETA)"[414].

The Surveillance for Enteric Fever in Asia Project (SEAP) involves a phase 1 retrospective review of existing data and an assessment of health facilities with the potential to participate in a phase II prospective study with the aim of generating data to inform vaccine policy in Nepal, Bangladesh and Pakistan[415,416]. The phase I data revealed a high burden of enteric fever among the bacterial vaccine preventable infections in children. Sites in Bangladesh (between 2013-2014 and 2016-2017) and Pakistan (between 2012-2014 and 2016-2017) reported that children under the age of 5 years accounted for the highest proportion of cases. However, the Indian and Nepali sites reported that the young adults (15-30 years) accounted for the highest number of cases[380] although those under the age of 15 years also accounted for a substantial proportion of disease[380].

Two other surveillance studies on typhoid are also under way to inform the field in the area of age-stratified burden. The Strategic Typhoid alliance across Africa and Asia (STRATAA) study takes a systematic approach to measuring the age-stratified burden of clinical and subclinical disease caused by typhoidal *Salmonellae* infections at three high-incidence urban sites in Malawi, Nepal and Bangladesh[417]. A multi-site, active fever surveillance of paediatric

cohorts is also under way in India with the main aim of identifying the paediatric burden of typhoid fever in order to inform vaccine strategy in India[418].

With the recent pre-qualification of the Vi-TT conjugate vaccine and its prioritisation by Gavi[419], we now have the opportunity to protect the youngest age group of children from typhoid fever particularly those under the age of 2 years. Although the incidence of enteric fever in the under 5 years age group is still largely unknown owing to various reasons alluded to in this thesis, it is clear that this age group suffers from a substantially high proportion of disease among affected children. It is therefore imperative that these children also be recognized as a target age group for vaccination as past typhoid vaccination programmes focused mainly on school-aged children[420]. Given the substantial disease occurrence across the youngest and school-aged population it is thus necessary that a broad vaccine strategy be employed. In addition, it is also important for appropriate diagnostics to be developed which can circumvent the shortcomings of currently available modalities, which consequently lead to an under-estimation of disease occurrence. In Nepal and India, two highly endemic areas for enteric fever, data from this thesis suggests that all age groups of children are mainly infected with 4.3.1 genotype (H58) strains particularly those of lineage II. This signifies that the pathogen population is dominated by this clade indirectly reflecting the currently prevailing antimicrobial pressure in the Indian sub-continent.

9.2 Molecular structure of the circulating population and implications

The molecular structure of strains reported in this thesis suggests that a similar antimicrobial pressure is present across both study sites. More importantly, this structure also provides insight into transmission dynamics suggesting that inter-region transmission occurs between areas of Nepal, India and the broader Indian sub-continent. A similar situation is seen in isolates from South-East Asia and Africa obtained from publically available datasets. It is therefore vital for health organizations from these countries to work together in combating

typhoid across respective regions. The close monitoring of the pathogen structure could be an additional means of surveying the currently prevailing selective pressures and might be used to predict future outbreaks particularly with regard to AMR. It may also serve as a method of assessing vaccine impact and understanding transmission dynamics. In Nepal where there was a 9-year period of strains available, it was interesting to note that the 4.3.1 genotype was constant regardless of age of the host, severity of infection (indicated by treatment status i.e. outpatient vs inpatient) or year of sample collection.

The Bayesian evolutionary analysis performed on the *S.* Typhi strains from Nepal clearly suggests that antimicrobial pressure is the main selective pressure determining the pathogen structure. The currently prevalent fluoroquinolone pressure is probably the most important reason behind the expansion of the 4.3.1 (H58) lineage II strains particularly the localised monophyletic clade as well as the expanding triple mutant population. Similarly the paucity of antimicrobial pressures that were exerted by first-line antimicrobials has resulted in a contraction of lineage I 4.3.1 genotype strains. These observations suggest that ciprofloxacin and other fluoroquinolones have little role to play in the treatment of typhoid but more importantly, provides insight into the rapidly adaptive genomic potential of *S.* Typhi circulating populations to changing antimicrobial pressures.

The differences in the molecular epidemiology of strains also shed light on the dynamics of population evolution and may be important in designing vaccine interventions. It is unknown whether the currently available Vi-TT conjugate vaccine is protective against all genotypes of enteric fever and surveillance post vaccine deployment will thus be necessary. The Typhoid Vaccine Acceleration Consortium (TyVAC) is currently conducting a large population based vaccine trial studying the effects of the Vi-TT conjugate vaccine across sites in Nepal, Malawi as well as Bangladesh and molecular surveillance in these areas post vaccine deployment is being carried out[388]. Additionally Gavi has also prioritised the Vi-TT

conjugate vaccine and co-finances the vaccine for use in routine immunisation programmes in Gavi eligible countries[419]. Molecular surveillance in these regions could be carried out to answer the questions regarding vaccine effectiveness against all genotypes of typhoid fever. Finally, the evolutionary analysis of typhoid bacterial populations can be carried out to assess the change in rates in evolution due to vaccine pressure. These analysis are mainly employed for viral pathogens such as Influenza virus and Human Immunodeficiency virus but have known to be used to assess bacterial population changes in due to vaccine pressure in the case of *B. pertussis*[298,360,363,421]

9.3. The pattern of antimicrobial resistance amongst typhoidal *Salmonella* in South Asia

The molecular characterisation of strains has also shed light on AMR determinants of *S.* Typhi and *S.* Paratyphi A strains. From an overarching perspective, the lineage specific nature of AMR conferring capabilities of the 4.3.1 genotype as seen in this thesis suggest that inferences can be made regarding the selective pressures determining the expansion of lineages and in turn antimicrobial use in the community. In both sites of data collection it was obvious that fluoroquinolones exert a high selection pressure which has resulted in the selection and clonal expansion of fluoroquinolone resistant strains and in particular the triple mutant strains. The correlation between molecular determinants of fluoroquinolone resistance i.e. mutations in QRDRs and phenotypic nalidixic acid/pefloxacin resistance is important as phenotypic resistance of ciprofloxacin correlates poorly with molecular determinants. This might be one of the reasons why the ciprofloxacin treatment failures in the past were reported despite the isolates being apparently susceptible on disc diffusion.

On a more focused note, there were a minority of strains from Nepal that were MDR and an important finding of this thesis was the novel mechanism of AMR where two strains were resistant to only ampicillin with AMR genes within the bacterial chromosome, in addition to classical fluoroquinolone resistance. This is again a testament of the antimicrobial pressures

at play but also provides insight for considering the use of chloramphenicol and cotrimoxazole in current treatment regimens. The uniform display of resistance to fluoroquinolones as well as molecular basis for the same seen amongst all the *S.* Paratyphi A from both sites is a significant finding as a *S.* Paratyphi A vaccine is still in development which leaves antimicrobial therapy as the only short and intermediate strategy to combat *S.* Paratyphi A.

9.4. The global pattern of antimicrobial resistance amongst *S.* Typhi

The data from the Nepali and Indian sites in this thesis reflect trends in South Asia as seen in the global context phylogenetic trees as well as the systematic review in chapter 8. The AMR trends in South-East Asia are similar to that in South Asia while those in Africa are very different. Within the continent of Africa, AMR is mediated by 4.3.1 genotype population in the East and South while non-4.3.1 genotype populations mediate MDR in the West. The MDR strains in Africa contain horizontally acquired plasmids that confer resistance similar to strains that were isolated from patients in South and South-East Asia in the 1990's. Data from African surveillance suggests that majority (91%) of isolates are still sensitive to fluoroquinolones but over 50 % of cumulative isolates across 9 countries are MDR[413].

In Asian settings the high degree of fluoroquinolone resistance is mainly is conferred by QRDR mutations but the recent XDR strains from the Sindh outbreak contained fluoroquinolone resistance plasmids. Although none of the study isolates in this thesis were cephalosporin resistant, the XDR *S.* Typhi outbreak in Sindh, Pakistan is an indicator of the currently prevailing antibiotic pressure exerted by cephalosporins and widespread cephalosporin resistance in *S.* Typhi isolates is probably on the horizon.

9.5. Limitations

In addition to the specific limitations discussed in each chapter, there are some additional limitations to the overall study designs and approach used.

9.5.1. Limitations to the systematic reviews and meta-analysis

The most obvious limitation stems from the fact that the inclusion of studies as well as the risk of bias assessment was carried out by a single reviewer. However, as the data from these chapters have been submitted for publication, all the included studies were reviewed by senior co-authors. The methodology of these chapters was also approved during the peer-reviewed process for publication. Both reviews were also limited due to the unavailability of full text articles from older publications and the inability to adequately translate manuscripts published in certain vernacular languages.

9.5.3 Enteric fever surveillance and sample collection

The invasive bacterial disease database at Patan hospital in Nepal has had different inclusion and exclusion criteria at different stages of its existence which was dependent on funding requirements. The sample collection also varied depending on various stages and has thus not been uniform throughout the years. Finally, the unfortunate natural disasters and political turmoil in the country has resulted in periodic interruptions to data collection and storage of isolates. In both the Nepali and Indian sites the retrospective nature of data collection is subject to shortcomings.

9.5.4. Molecular analysis

Short read sequencing was used which limits certain interpretation of genetic arrangement and virulence regions even when a good quality assembler like unicycler is used. These limitations could have been overcome with longer read technology such as Nanopore or PacBio.

The lack of molecular data limited comparative analytical interpretations in India and greatly reduced the possibility of understanding temporal changes in pathogen population structure.

9.5.3. Limitations to the antimicrobial susceptibility assays

Disc diffusion assays were the only modality employed which are known to be suboptimal particularly with regard to fluoroquinolone susceptibility. The zones of inhibitions were also manually assessed which is subject to human error.

9.6. Further work

9.6.1. Evolutionary analysis of H58 strains from India

The evolutionary analysis of 4.3.1 genotype strains (H58) isolated from patients living in India is a priority in order to obtain a comparison in the rates of evolution between the Nepal and Indian pathogen populations. It will also be helpful in acquiring a baseline to assess vaccine impact given the expected roll out of the Vi-TT conjugate vaccine in India. The groundwork for these analyses is already under way and I should have preliminary results by the end of the summer.

9.6.2. Evolutionary analysis of *S.* Paratyphi A

The evolutionary analysis of the *S.* Paratyphi A isolated from patients in South Asia, China and South-East Asia will provide for comparisons between the evolutionary events and rates of *S.* Typhi and *S.* Paratyphi A. It will most likely also shed light on possible prevention strategies and sustainable treatment regimens. The groundwork for these analyses is also under way and I should have preliminary results by the end of the summer.

9.6.3. Systematic review of the global antimicrobial resistance trends of *S.* Paratyphi A

The phenotypic trends and genotypic determinants of the global trends of *S.* Typhi reported in this thesis revealed very important findings and were part of the WHO SAGE October 2017 meeting document. The group concluded that Vi-TT conjugate vaccine deployment, as a means to decrease AMR in typhoid fever was an important strategy. It is now necessary to analyse the trends of global AMR in *S.* Paratyphi A in comparison with *S.* Typhi and to think

about ways to limit the development of AMR in the absence of a licensed vaccine for *S.* Paratyphi A.

9.6.4. Assessing vaccine impact by monitoring the population structure of *S.* Typhi in Nepal

The evolutionary analysis of 4.3.1 genotype strains from Nepal reported in this thesis has served as a baseline reflection for the selection pressures in Nepal from the 1990's to the current period. The roll out of the Vi-TT conjugate vaccine programmatically in Nepal will create another selection pressure and it will be interesting to analyse the change in population structure as well as evolutionary rates among the 4.3.1 genotype population in Nepal.

9.7. Summary

The youngest age group of children, who in the past were not the focus of immunisation owing to vaccine inadequacies, suffer greatly from enteric fever. The systematic review of age stratification of disease occurrence in endemic regions suggested that all age groups of children are affected and molecular analysis in two South Asian sites showed that there was no difference in the infecting genotypes between age groups.

The dissimilarities between the antimicrobial resistance carrying capabilities of lineage I and lineage II strains of the 4.3.1 genotype as well as novel AMR gene arrangements and finally the temporal trends of AMR in *S.* Typhi shed light on the adaptive and evolutionary potentials of *S* Typhi.

For the first time in history an opportunity has arisen to effectively vaccinate the youngest age group (0-4 years) from typhoid through the Vi-TT conjugate vaccine. The older age groups also suffer greatly from this disease calling for a broad based vaccine strategy. The implications for treatment of enteric fever are however more relevant in the immediate term which suggest that in endemic regions in Asia, fluoroquinolones have little role to play in

treatment protocols while fluoroquinolones are still relevant in the African setting. In Asia, reverting back to former first-line antimicrobials might be an option.

References

1 Crump JA, Luby SP, Mintz ED. The global burden of typhoid fever. *Bull World Health Organ* 2004; **82**: 346–53.

2 CDC. Lesson 1 Understanding the Epidemiologic Triangle through Infectious Disease. .

3 WHO | Typhoid. *WHO* 2015.

4 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.

5 Bäumler AJ, Tsolis RM, Ficht TA, Adams LG. Evolution of host adaptation in Salmonella enterica. *Infect Immun* 1998; **66**: 4579–87.

6 Anastasiou E, Mitchell PD. Palaeopathology and genes: Investigating the genetics of infectious diseases in excavated human skeletal remains and mummies from past populations. *Gene* 2013; **528**: 33–40.

7 Kidgell C, Reichard U, Wain J, *et al.* Salmonella typhi, the causative agent of typhoid fever, is approximately 50,000 years old. *Infect Genet Evol* 2002; **2**: 39–45.

8 Buckle GC, Walker CLF, Black RE. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity and mortality for 2010. *J Glob Health* 2012; **2**: 10401.

9 Azmatullah A, Qamar FN et al. Systematic review of the global epidemiology, clinical and laboratory profile of enteric fever. *J Glob Health* 2015; **5**.

10 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**: 711–25.

11 Darton TC, Jones C, Blohmke CJ, *et al.* Using a Human Challenge Model of Infection to Measure Vaccine Efficacy: A Randomised, Controlled Trial Comparing the Typhoid Vaccines M01ZH09 with Placebo and Ty21a. *PLoS Negl Trop Dis* 2016; **10**: 1–27.

12 Waddington CS, Darton TC, Jones C, *et al.* An outpatient, ambulant-design, controlled human infection model using escalating doses of salmonella typhi challenge delivered in sodium bicarbonate solution. *Clin Infect Dis* 2014; **58**: 1230–40.

13 Parry CM, Hien TT, Dougan G, *et al.* Typhoid fever. *N Engl J Med* 2002; **347**: 1770–82.

14 Mortimer PP. Department of medical history Mr N the milker , and Dr

Koch ' s concept of the healthy carr i e r. *Lancet* 1999; **353**: 1354–6.
15 Marineli F, Tsoucalas G, Karamanou M, Androutsos G. Mary Mallon (1869-1938) and the history of typhoid fever. *Ann Gastroenterol* 2013; **26**: 132–4.
16 Moorhead R. William Budd and typhoid fever. *J R Soc Med* 2002; **95**: 561–4.
17 Wright AE, Semple D. Remarks on Vaccination Against Typhoid Fever. *Br Med J* 1897; : 256–9.
18 Date KA, Bentsi-Enchill A, Marks F, Fox K. Typhoid fever vaccination strategies. *Vaccine* 2015; **33**: C55–61.
19 Sharp PM. Determinants of DNA sequence divergence between Escherichia coli and Salmonella typhimurium: Codon usage, map position, and concerted evolution. *J Mol Evol* 1991; **33**: 23–33.
20 McClelland M, Florea L, Sanderson K, *et al.* Comparison of the Escherichia coli K-12 genome with sampled genomes of a Klebsiella pneumoniae and three salmonella enterica serovars, Typhimurium, Typhi and Paratyphi. *Nucleic Acids Res* 2000; **28**: 4974–86.
21 Mills DM, Bajaj V, Lee CA. A 40 kb chromosomal fragment encoding Salmonella typhimurium invasion genes is absent from the corresponding region of the Escherichia coli K-12 chromosome. *Mol Microbiol* 1995; **15**: 749–59.
22 Brenner FW, Villar RG, Angulo FJ, Tauxe R, Swaminathan B. *Salmonella* Nomenclature. *J Clin Microbiol* 2000; **38**: 2465–7.
23 Health Protection Agency. Kauffmann-White Scheme - 2007. 2007.
24 Francois-Xavier Weill - Salmonella (WHO-CC) • Research - Institut Pasteur. https://research.pasteur.fr/en/whocc/salmonella/ (accessed Dec 20, 2017).
25 Beveridge TJ. Structures of Gram-Negative Cell Walls and Their Derived Membrane Vesicles. *J Bacteriol* 1999; **181**: 4725–33.
26 Figueira R, Holden DW. Functions of the Salmonella pathogenicity island 2 (SPI-2) type III secretion system effectors. *Microbiology* 2012; **158**: 1147–61.
27 Choen GN. Microbial Biochemistry, 2nd edn. Springer Netherlands, 2011.
28 Chart H, Cheasty T, De Pinna E, *et al.* Serodiagnosis of Salmonella enterica serovar Typhi and S. enterica serovars Paratyphi A, B and C human infections. *J Med Microbiol* 2007; **56**: 1161–6.
29 Samuel G, Reeves P. Biosynthesis of O-antigens: Genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydr Res* 2003; **338**: 2503–19.
30 Reeves P. Evolution of< i> Salmonella</i> O antigen variation by interspecific gene transfer on a large scale. *Trends Genet* 1993; **9**: 17–

22.
31 Gibson DL, White AP, Snyder SD, *et al.* Salmonella produces an O-antigen capsule regulated by AgfD and important for environmental persistence. *J Bacteriol* 2006; **188**: 7722–30.
32 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**: 5341–9.
33 Koebnik R, Locher KP, Van Gelder P. Structure and function of bacterial outer membrane proteins: barrels in a nutshell. *Mol Microbiol* 2000; **37**: 239–53.
34 Hamid N, Jain SK. Characterization of an outer membrane protein of Salmonella enterica serovar Typhimurium that confers protection against typhoid. *Clin Vaccine Immunol* 2008; **15**: 1461–71.
35 Chooneea D, Karlsson R, Encheva V, Arnold C, Appleton H, Shah H. Elucidation of the outer membrane proteome of *Salmonella enterica* serovar *Typhimurium* utilising a lipid-based protein immobilization technique. *BMC Microbiol* 2010; **10**: 44.
36 Reidl J. Outer Membrane Vesicle Biosynthesis in *Salmonella* : Is There More to Gram-Negative Bacteria? *MBio* 2016; **7**: e01282-16.
37 Brown L, Wolf JM, Prados-Rosales R, Casadevall A. Through the wall: extracellular vesicles in Gram-positive bacteria, mycobacteria and fungi. *Nat Rev Microbiol* 2015; **13**: 620–30.
38 Mortimer CKB, Gharbia SE, Logan JMJ, Peters TM, Arnold C. Flagellin gene sequence evolution in Salmonella. *Infect Genet Evol* 2007; **7**: 411–5.
39 DeRosier DJ. Spinning tails. *Curr Opin Struct Biol* 1995; **5**: 187–93.
40 DeRosier DJ. The turn of the screw: The bacterial flagellar motor. *Cell* 1998; **93**: 17–20.
41 Chilcott GS, Hughes KT. Coupling of Flagellar Gene Expression to Flagellar Assembly in Salmonella enterica Serovar Typhimurium and Escherichia coli. *Microbiol Mol Biol Rev* 2000; **64**: 694–708.
42 Simon M, Zieg J, Silverman M, Mandel G, Doolittle R. Phase variation: evolution of a controlling element. *Science (80-)* 1980; **209**.
43 Schreiber F, Kay S, Frankel G, *et al.* The Hd, Hj, and Hz66 flagella variants of Salmonella enterica serovar Typhi modify host responses and cellular interactions. *Sci Rep* 2015; **5**: 7947.
44 Smith NH, Beltran P, Selander RK. Recombination of Salmonella Phase 1 Flagellin Genes Generates New Serovars. *J Bacteriol* 1990; **172**: 2209–16.
45 Salazar-gonzalez RM, Mcsorley SJ. Salmonella flagellin , a microbial target of the innate and adaptive immune system. *Trends Microbiol* 2004; **101**: 117–22.

46 Huang Y, Smith BS, Chen LX, Baxter RHG, Deisenhofer J. Insights into pilus assembly and secretion from the structure and functional characterization of usher PapC. *Proc Natl Acad Sci U S A* 2009; **106**: 7403–7.
47 Humphries AD, Townsend SM, Kingsley R a, Nicholson TL, Tsolis M, Ba AJ. Role of fimbriae as antigens and intestinal colonization factors of Salmonella serovars. 2001; **201**: 121–5.
48 Jacob-Dubuisson F, Striker R, Hultgren SJ. Chaperone-assisted self-assembly of pili independent of cellular energy. *J Biol Chem* 1994; **269**: 12447–55.
49 Dodson KW, Jacob-Dubuisson F, Striker RT, Hultgren SJ. Outer-membrane PapC molecular usher discriminately recognizes periplasmic chaperone-pilus subunit complexes. *Proc Natl Acad Sci* 1993; **90**: 3670–4.
50 Townsend SM, Kramer NE, Edwards R, *et al.* Salmonella enterica Serovar Typhi Possesses a Unique Repertoire of Fimbrial Gene Sequences Salmonella enterica Serovar Typhi Possesses a Unique Repertoire of Fimbrial Gene Sequences. 2001; **69**: 2894–901.
51 Sokurenko E V, Chesnokova V, Dykhuizen DE, *et al.* Pathogenic adaptation of Escherichia coli by natural variation of the FimH adhesin. *Proc Natl Acad Sci U S A* 1998; **95**: 8922–6.
52 Pratap CB, Kumar G, Patel SK, *et al.* Targeting of putative fimbrial gene for detection of S. typhi in typhoid fever and chronic typhoid carriers by nested PCR. *J Infect Dev Ctries* 2013; **7**: 520–7.
53 EFSA Panel on Biological Hazards. Scientific Opinion on monitoring and assessment of the public health risk. *Eur Food Saf Auth J* 2010; **8**: 1–48.
54 Wain J, House D, Zafar A, *et al.* Vi antigen expression in Salmonella enterica serovar typhi clinical isolates from Pakistan. *J Clin Microbiol* 2005; **43**: 1158–65.
55 Johnson EM, Krauskopf B, Baron LS. Genetic Mapping of Vi and Somatic Antigenic Determinants in Salmonella. *J Bacteriol* 1965; **90**.
56 Tran QT, Gomez G, Khare S, *et al.* The Salmonella enterica Serotype Typhi Vi Capsular Antigen Is Expressed after the Bacterium Enters the Ileal Mucosa . *Infect Immun* 2010; **78**: 527–35.
57 Virlogeux I, Waxin H, Ecobichon C, Popoff MY. Role of the viaB locus in synthesis , transport and expression of Salmonella typhi Vi antigen. *Microbiology* 2017; : 3039–47.
58 Pickard D, Li J, Roberts M, *et al.* Characterization of Defined ompR Mutants of Salmonella typhi : ompR Is Involved in the Regulation of Vi Polysaccharide Expression. 1994; **62**: 3984–93.
59 Mitra M, Shah N, Ghosh A, *et al.* Efficacy and Safety of Vi-Tetanus

Toxoid conjugated Typhoid vaccine (PedaTyph) in Indian Children: School Based Cluster Randomized Study. *Hum Vaccin Immunother* 2016; : 0.
60 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. *Clin Infect Dis* 2015; **61**: 393–402.
61 Hacker J, Blum-Oehler G, Muhldorfer I, Tschape H. Pathogenicity islands of virulent bacteria: structure, function and impact on microbial evolution. *Mol Microbiol* 1997; **23**: 1089–97.
62 Mueller CA, Broz P, Cornelis GR. The type III secretion system tip complex and translocon. *Mol Microbiol* 2008; **68**: 1085–95.
63 Que F, Wu S, Huang R. Salmonella pathogenicity Island 1(SPI-1) at work. *Curr Microbiol* 2013; **66**: 582–7.
64 Ellermeier JR, Slauch JM. Adaptation to the host environment: regulation of the SPI1 type III secretion system in Salmonella enterica serovar Typhimurium. *Curr Opin Microbiol* 2007; **10**: 24–9.
65 Ellermeier CD, Ellermeier JR, Slauch JM. HilD, HilC and RtsA constitute a feed forward loop that controls expression of the SPI1 type three secretion system regulator hilA in Salmonella enterica serovar Typhimurium. *Mol Microbiol* 2005; **57**: 691–705.
66 Blanc-Potard AB, Solomon F, Kayser J, Groisman EA. The SPI-3 pathogenicity island of Salmonella enterica. *J Bacteriol* 1999; **181**: 998–1004.
67 Amavisit P, Lightfoot D, Browning GF, Markham PF. Variation between pathogenic serovars within Salmonella pathogenicity islands. *J Bacteriol* 2003; **185**: 3624–35.
68 Parkhill J, Dougan G, James KD, *et al.* Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *Nature* 2001; **413**: 848–52.
69 Saroj SD, Shashidhar R, Karani M, Bandekar JR. Distribution of Salmonella pathogenicity island (SPI)-8 and SPI-10 among different serotypes of Salmonella. *J Med Microbiol* 2008; **57**: 424–7.
70 Seth-Smith HM. SPI-7: Salmonella's Vi-encoding Pathogenicity Island. *J Infect Dev Countriesfile///Users/carl/Downloads/citations.nbib* 2008; **2**: 267–71.
71 Vernikos GS, Parkhill J. Interpolated variable order motifs for identification of horizontally acquired DNA: revisiting the Salmonella pathogenicity islands. *Bioinformatics* 2006; **22**: 2196–203.
72 Galán JE. Typhoid toxin provides a window into typhoid fever and the biology of Salmonella Typhi. *Proc Natl Acad Sci United States Am* 2016.

DOI:10.1073/pnas.1606335113.
73 Fowler CC, Chang S, Gao X, Geiger T, Stack G, Galan JE. Emerging insights into the biology of typhoid toxin. *Curr Opin Microbiol* 2017; : 70–7.
74 Spano SUJE, Galan JE. Delivery of a Salmonella Typhi Exotoxin from a Host Intracellular Compartment. *Cell Host Microbe* 2008; **3**: 30–8.
75 Song J, Gao X, Galán JE. Structure and function of the Salmonella Typhi chimaeric A(2)B(5) typhoid toxin. *Nature* 2013; **499**: 350–4.
76 Redfield RJ. Do bacteria have sex? *Nat Rev Genet* 2001; **2**: 634–9.
77 Bennett PM. Plasmid encoded antibiotic resistance: acquisition and transfer of antibiotic resistance genes in bacteria. *Br J Pharmacol* 2008; **153 Suppl**: S347–57.
78 Ugboko H, De N. Mechanisms of Antibiotic resistance in Salmonella typhi. *IntJCurrMicrobiolAppSci* 2014; **3**: 461–76.
79 Wain J, Diem Nga LT, Kidgell C, *et al.* Molecular analysis of incHI1 antimicrobial resistance plasmids from Salmonella serovar Typhi strains associated with typhoid fever. *Antimicrob Agents Chemother* 2003; **47**: 2732–9.
80 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**. DOI:10.1371/journal.pntd.0001245.
81 Carattoli A, Zankari E, García-Fernández A, *et al.* In Silico Detection and Typing of Plasmids using PlasmidFinder and Plasmid Multilocus Sequence Typing. DOI:10.1128/AAC.02412-14.
82 Wong VK, Holt KE, Okoro C, *et al.* Molecular Surveillance Identifies Multiple Transmissions of Typhoid in West Africa. *PLoS Negl Trop Dis* 2016; : 1–22.
83 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**.
84 Parkhill J, Dougan G, James KD, *et al.* Complete genome sequence of a multiple drug resistant Salmonella enterica serovar Typhi CT18. *Nature* 2001; **413**: 848–52.
85 Porwollik S, Wong RM-Y, McClelland M. Evolutionary genomics of *Salmonella*: Gene acquisitions revealed by microarray analysis. *Proc Natl Acad Sci* 2002; **99**: 8956–61.
86 Dorrell N, Hinchliffe SJ, Wren BW. Comparative phylogenomics of pathogenic bacteria by microarray analysis. *Curr Opin Microbiol* 2005; **8**: 620–6.
87 McClelland M, Sanderson KE, Clifton SW, *et al.* Comparison of genome degradation in Paratyphi A and Typhi, human-restricted serovars of

Salmonella enterica that cause typhoid. *Nat Genet* 2004; **36**: 1268–74.
88 Octavia S, Lan R. Multiple-locus variable-number tandem-repeat analysis of Salmonella enterica serovar Typhi. *J Clin Microbiol* 2009; **47**: 2369–76.
89 Ramisse V, Houssu P, Hernandez E, *et al.* Variable number of tandem repeats in Salmonella enterica subsp. enterica for typing purposes. *J Clin* 2004; **42**: 5722–30.
90 Wang H, Diao B, Cui Z, Yan M, Kan B. Genotyping of Salmonella Typhi using 8-loci multi locus VNTR analysis. *Gut Pathog* 2016; **8**: 14.
91 Achtman M. Evolution, population structure, and phylogeography of genetically monomorphic bacterial pathogens. *Annu Rev Microbiol* 2008; **62**: 53–70.
92 Roumagnac P, Weill F, Dolecek C, *et al.* Evolutionary History of Salmonella Typhi. *Science (80-)* 2006; **314**: 1301–4.
93 Altwegg M, Hickman-Brenner FW, Farmer JJ. Ribosomal RNA Gene Restriction Patterns Provide Increased Sensitivity for Typing Salmonella Typhi Strains. *J Infect Dis* 1989; **160**: 145–9.
94 Boyd EF, Wang FS, Whittam TS, Selander RK. Molecular genetic relationships of the salmonellae. *Appl Environ Microbiol* 1996; **62**: 804–8.
95 Boyd EF, Li J, Ochman H, Selander RK. Comparative genetics of the inv-spa invasion gene complex of Salmonella enterica. *J Bacteriol* 1997; **179**: 1985–91.
96 Christensen H, Nordentoft S, Olsen JE. Phylogenetic relationships of Salmonella based on rRNA sequences. *Int J Syst Bacteriol* 1998; **48 Pt 2**: 605–10.
97 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; : 1–11.
98 Achtman M, Wagner M. Microbial diversity and the genetic nature of microbial species. *Nat Rev Microbiol* 2008; **6**: 431–40.
99 Navarro F, Llovet T,et al . Molecular typing of Salmonella enterica serovar typhi. *J Clin Microbiol* 1996; **34**: 2831–4.
100 Nair S, Poh CL, Lim YS, Tay L, Goh KT. Genome fingerprinting of Salmonella typhi by pulsed-field gel electrophoresis for subtyping common phage types. *Epidemiol Infect* 1994; **113**: 391–402.
101 Chandel DS, Chaudhry R, Dey AB, Malhotra P. Molecular typing reveals a unique clone of Salmonella enterica serotype Typhi among Indian strains [2]. *J Clin Microbiol* 2006; **44**: 2673–5.
102 Sanger F, Air GM, Barrell BG, *et al.* Nucleotide sequence of bacteriophage phi X174 DNA. *Nature* 1977; **265**: 687–95.
103 Goodwin S, McPherson JD, McCombie WR. Coming of age: ten years of

next-generation sequencing technologies. *Nat Rev Genet* 2016; **17**: 333–51.

104 Bentley DR, Gormley NA, Baybayan PA, *et al.* Accurate whole human genome sequencing using reversible terminator chemistry. *Nature* 2008; **456**: 53–9.

105 Stankiewicz P, Lupski JR. Structural variation in the human genome and its role in disease. *Annu Rev Med* 2010; **61**: 437–55.

106 Mirkin SM. Expandable DNA repeats and human disease. *Nature* 2007; **447**: 932–40.

107 McCarroll SA, Altshuler DM. Copy-number variation and association studies of human disease. *Nat Genet* 2007; **39**: S37-42.

108 Bhan MK, Bahl R, Sazawal S, *et al.* Association between Helicobacter pylori infection and increased risk of typhoid fever. *J Infect Dis* 2002; **186**: 1857–60.

109 Vollaard AM, Verspaget HW, Ali S, *et al.* Helicobacter pylori infection and typhoid fever in Jakarta, Indonesia. *Epidemiol Infect* 2006; **134**: 163–70.

110 Waddington CS, Darton TC, Jones C, *et al.* An outpatient, ambulant-design, controlled human infection model using escalating doses of Salmonella Typhi challenge delivered in sodium bicarbonate solution. *Clin Infect Dis* 2014; **58**: 1230–40.

111 Hornick R.B, Greisman S.E, Woodward T.E, DuPont H.L, Dawkins A.T SM. Typhoid fever: pathogenesis and immunologic control (first of two parts). *N Engl J Med* 1970; **283**: 686–91.

112 Dunstan SJ, Hue NT, Han B, *et al.* Variation at HLA-DRB1 is associated with resistance to enteric fever. *Nat Genet* 2014; : 1–6.

113 Pier GB, Grout M, Zaidi T, *et al.* Salmonella typhi uses CFTR to enter intestinal epithelial cells. *Nature* 1998; **393**: 79–82.

114 van de Vosse E, Ali S, de Visser AW, *et al.* Susceptibility to typhoid fever is associated with a polymorphism in the cystic fibrosis transmembrane conductance regulator (CFTR). *Hum Genet* 2005; **118**: 138–40.

115 Pulickal AS, Gautam S, Clutterbuck EA, *et al.* Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin Vaccine Immunol* 2009; **16**: 1413–9.

116 Marmion D, Naylor G, Stewart I. Second attacks of typhoid fever. *J Hyg (Lond)* 1953; **51**: 260–7.

117 Hornick RB, Greisman SE, Woodward TE, DuPont HL, Dawkins AT, Snyder MJ. Typhoid fever: pathogenesis and immunologic control. *N Engl J Med* 1970; **283**: 686–91.

118 Dupont HL, Hornick RB, Snyder MJ, Dawkins AT, Heiner GG, Woodward TE. Studies of immunity in typhoid fever. Protection

induced by killed oral antigens or by primary infection. *Bull World Health Organ* 1971; **44**: 667–72.
119 Saul A, Smith T, Maire N. Stochastic Simulation of Endemic Salmonella enterica Serovar Typhi: The Importance of Long Lasting Immunity and the Carrier State. *PLoS One* 2013; **8**. DOI:10.1371/journal.pone.0074097.
120 Ferreccio C, Levine M, Manterola A, *et al.* Benign bacteremia caused by Salmonella typhi and paratyphi in children younger than 2 years. *J Pediatr* 1984; **104**: 899–901.
121 Sen SK, Mahakur AC. Enteric fever--a comparative study of adult and paediatric cases. *Indian J Pediatr* 1972; **39**: 354–60.
122 Mahle WT, Levine MM. Salmonella typhi infection in children younger than five years of age. *Pediatr Infect Dis J* 1993; **12**: 627–31.
123 Kapoor JP, Mohan M, Talwar V, Daral TS, Bhargava SK. Typhoid fever in young children. *Indian Pediatr* 1985; **22**: 811–3.
124 Khuri-Bulos N. Enteric fevers in children. The importance of age in the varying clinical picture. *Clin Pediatr (Phila)* 1981; **20**: 448–52.
125 Butler T, Islam A, Kabir I, Jones PK. Patterns of morbidity and mortality in typhoid fever dependent on age and gender: review of 552 hospitalized patients with diarrhea. *Rev Infect Dis* 1991; **13**: 85–90.
126 Sinha A, Sazawal S, Kumar R, *et al.* Typhoid fever in children aged less than 5 years. *Lancet* 1999; **354**: 734–7.
127 Brooks WA, Hossain A, Goswami D, *et al.* Bacteremic typhoid fever in children in an urban slum, Bangladesh. *Emerg Infect Dis* 2005; **11**: 326–9.
128 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**: 144.
129 Kumar S, Rizvi M, Berry N. Rising prevalence of enteric fever due to multidrug-resistant Salmonella: An epidemiological study. *J Med Microbiol* 2008; **57**: 1247–50.
130 Feasey N, Archer BN, Heyderman RS, *et al.* Typhoid fever and invasive nontyphoid salmonellosis, Malawi and South Africa. *Emerg Infect Dis* 2010; **16**: 1448–51.
131 Ganesh R, Janakiraman L, Vasanthi T, Sathiyasekeran M. Profile of typhoid fever in children from a tertiary care hospital in Chennai-South India. *Indian J Pediatr* 2010; **77**: 1089–92.
132 Dewan AM, Corner R, Hashizume M, Ongee ET. Typhoid Fever and Its Association with Environmental Factors in the Dhaka Metropolitan Area of Bangladesh: A Spatial and Time-Series Approach. *PLoS Negl Trop Dis* 2013; **7**: e1998.

133 Ochiai RL. a study of typhoid fever in five Asian countries: disease burden and implications for controls. *Bull World Health Organ* 2008; **86**: 260–8.
134 Dougan G, Baker S. Salmonella enterica serovar typhi and the pathogenesis of typhoid fever. Annu. Rev. Microbiol. 2014; **68**: 317–36.
135 Ghosh S, Batabyal P, Rajendran K, Palit A. Typhoid fever in rural communities of West Bengal, India-an age-wise perspective. *Jpn J Infect Dis* 2010; **63**: 219–21.
136 Gibani M, Darton TC. The challenge with typhoid. PLOS Negl Trop Diis. 2016 Aug 17:10
137 Hassan RH, Fouda AE, Kandil SM. Fever of Unknown Origin in Children: A 6 year- Experience in a Tertiary Pediatric Egyptian Hospital. Int. J. Health Sci. (Qassim). 2014; **8**: 13–9.
138 Bhutta ZA. Impact of age and drug resistance on mortality in typhoid fever. *Arch Dis Child* 1996; **75**: 214–7.
139 Who. Background document : The diagnosis, treatment and prevention of typhoid fever. *Commun Dis Surveill Response Vaccines Biol* 2003.
140 Topley JM. Mild typhoid fever. *Arch Dis Child* 1986; **61**: 164–7.
141 Arora_1991_Plant-Science. .
142 Rasaily R, Dutta P, Saha MR, Mitra U, Lahiri M, Pal SC. Multi-drug resistant typhoid fever in hospitalised children. Clinical, bacteriological and epidemiological profiles. *Eur J Epidemiol* 1994; **10**: 41–6.
143 Shwe TN, Nyein MM, Yi W, Mon A. Blood culture isolates from children admitted to Medical Unit III, Yangon Children's Hospital, 1998. *Southeast Asian J Trop Med Public Heal* 2002; **33**: 764–71.
144 Bhan MK, Bahl R, Bhatnagar S. Typhoid and paratyphoid fever. *Lancet* 2015; **366**: 749–62.
145 Ahmad KA, Khan LH, Roshan B, Bhutta Z a. Factors associated with typhoid relapse in the era of multiple drug resistant strains. *J Infect Dev Ctries* 2011; **5**: 727–31.
146 Nasir AA, Abdur-Rahman LO, Adeniran JO. Predictor of mortality in children with typhoid intestinal perforation in a Tertiary Hospital in Nigeria. *Pediatr Surg Int* 2011; **27**: 1317–21.
147 Onen A, Dokucu AI, Cigdem MK, Ozturk H, Otcu S, Yucesan S. Factors effecting morbidity in typhoid intestinal perforation in children. *Pediatr Surg Int* 2002; **18**: 696–700.
148 Alexander L, Allen S, Bindoff NL. Summary for Policymakers. *Eff Br mindfulness Interv acute pain Exp An Exam Individ Differ* 2013; **1**: 1–36.

149 Malik AS. Complications of bacteriologically confirmed typhoid fever in children. *J Trop Pediatr* 2002; **48**: 102–8.
150 Verma M. Brief Reports Enteric Fever Below 2 Years of Age. 1996; **33**: 229–30.
151 Huang DB, DuPont HL. Problem pathogens: extra-intestinal complications of Salmonella enterica serotype Typhi infection. *Lancet Infect Dis* 2005; **5**: 341–8.
152 Khair AM, Elmagrabi D. Febrile Seizures and Febrile Seizure Syndromes: An Updated Overview of Old and Current Knowledge. *Neurol Res Int* 2015; **2015**: 1–7.
153 Hoffman SL, Punjabi NH, Kumala S, *et al.* Reduction of mortality in chloramphenicol-treated severe typhoid fever by high-dose dexamethasone. *N Engl J Med* 1984; **310**: 82–8.
154 Heitmann D, Ziehr H, Müthing J. Large scale purification of gangliosides G(M3)(Neu5Ac) and G(M3)(Neu5Gc) by trimethylaminoethyl-fractogel high-performance liquid chromatography. *J Chromatogr B Biomed Appl* 1998; **710**: 1–8.
155 Uysal H, Karademir A, Kilinc M, Erturk O. Salmonella encephalopathy with seizure and frontal intermittent rhythmic delta activity. *Infection* 2001; **29**: 103–6.
156 Desai JD, Toczek MT, Mitchell WG. Frontal intermittent rhythmic delta activity (FIRDA): is there a clinical significance in children and adolescents? *Eur J Paediatr Neurol* 2012; **16**: 138–41.
157 Balasubramanian S, Shivbalan S, Miranda PKL. Pseudotumour cerebri as an unusual manifestation of typhoid. Ann. Trop. Paediatr. 2003; **23**: 223–4.
158 Bhatt GC, Dewan V, Dewan T, Yadav TP. Pseudotumour cerebri with multiple cranial nerve palsies in enteric fever. Indian J. Pediatr. 2014; **81**: 196–7.
159 Mehndiratta S, Rajeshwari K, Dubey AP. Guillain-Barre syndrome as a complication of typhoid fever in a child. Neurol. India. 2012; **60**: 433–5.
160 May W, Senitiri I. Guillain-Barre syndrome associated with typhoid fever. A case study in the Fiji Islands. *Pac Health Dialog* 2010; **16**: 85–8.
161 Kapoor K, Jain S, Jajoo M, Talukdar B. A rare neurological complication of typhoid fever: Guillain-Barre' syndrome. *J Pediatr Neurosci* 2014; **9**: 148–9.
162 Donoso R. Guillain-Barre syndrome following typhoid fever/letter. *Ann Neurol* 1988; **23**: 627.
163 Datta V, Sahare P, Chaturved P. Guillain-Barre syndrome as a complication of enteric fever. *J Indian Med Assoc* 2004; **102**: 172–3.

164 Chanmugam D, Waniganetti A. Guillain-Barre syndrome associated with typhoid fever. *Br Med J* 1969; **1**: 95–6.
165 Berger JR, Ayyar DR, Kaszovitz B. Guillain-Barre syndrome complicating typhoid fever. Ann. Neurol. 1986; **20**: 649–50.
166 Aldrey JM, Fernandez-Rial A, Lopez-Gonzalez FJ, Doval JC, de la Fuente-Fernandez R. Guillain-Barre syndrome as first manifestation of typhoid fever. *Clin Infect Dis* 1999; **28**: 1171–2.
167 Moodley M, Coovadia HM. Benign intracranial hypertension in typhoid fever. A case report. *S Afr Med J* 1990; **78**: 608–9.
168 Chow CB, Wang PS, Leung NK. Typhoid fever in Hong Kong children. *Aust Paediatr J* 1989; **25**: 147–50.
169 Hanel RA, Araujo JC, Antoniuk A, da Silva Ditzel LF, Flenik Martins LT, Linhares MN. Multiple brain abscesses caused by Salmonella typhi: case report. *Surg Neurol* 2000; **53**: 86–90.
170 Incecik F, Herguner MO, Mert G, Alabaz D, Altunbasak S. Acute cerebellar ataxia associated with enteric fever in a child: a case report. *Turk J Pediatr* 2013; **55**: 441–2.
171 Okunola PO, Ofovwe GE, Abiodun MT, Azunna CP. Superior sagittal sinus thrombosis complicating typhoid Fever in a teenager. *Case Rep Pediatr* 2012; **2012**: 201203.
172 Rajeshwari K, Yadav S, Puri RK, Khanijo CM, Sethi Y. Cerebritis in typhoid fever. *Indian Pediatr* 1995; **32**: 1305–7.
173 Singh RR, Chaudhary SK, Bhatta NK, Khanal B, Shah D. Clinical and etiological profile of acute febrile encephalopathy in eastern Nepal. *Indian J Pediatr* 2009; **76**: 1109–11.
174 Ozen H, Cemeroglu P, Ecevit Z, Secmeer G, Kanra G. Unusual neurologic complications of typhoid fever (aphasia, mononeuritis multiplex, and Guillain-Barre syndrome): a report of two cases. *Turk J Pediatr* 1993; **35**: 141–4.
175 Malik a S, Malik RH. Typhoid fever in Malaysian children. *Med J Malaysia* 2001; **56**: 478–90.
176 Shetty AK, Mital SR, Bahrainwala AH, Khubchandani RP, Kumta NB. Typhoid hepatitis in children. *J Trop Pediatr* 1999; **45**: 287–90.
177 Ali G, Kamili MA, Rashid S, Mansoor A, Lone BA, Allaqaband GQ. Spontaneous splenic rupture in typhoid fever. *Postgrad Med J* 1994; **70**: 513–4.
178 Julia J, Canet JJ, Lacasa XM, Gonzalez G, Garau J. Spontaneous spleen rupture during typhoid fever. *Int J Infect Dis* 2000; **4**: 108–9.
179 Nesbitt A, Mirza NB. Salmonella septicaemias in Kenyan children. *J Trop Pediatr* 1989; **35**: 35–9.
180 Lai X-H, Xu Y, Chen X-M, Ren Y. Macrophage cell death upon intracellular bacterial infection. *Macrophage* 2015; : 1–11.

181 Butler T, Islam MR, Bardhan PK. The leukemoid reaction in shigellosis. *Am J Dis Child* 1984; **138**: 162–5.
182 Azim T, Qadri F, Ahmed S, *et al.* Lipopolysaccharide-specific antibodies in plasma and stools of children with Shigella-associated leukemoid reaction and hemolytic-uremic syndrome. *Clin Diagn Lab Immunol* 1996; **3**: 701–5.
183 Pitzer VE, Bowles CC, Baker S, *et al.* Predicting the Impact of Vaccination on the Transmission Dynamics of Typhoid in South Asia: A Mathematical Modeling Study. DOI:10.1371/journal.pntd.0002642.
184 Dongol S, Thompson CN, Clare S, *et al.* The Microbiological and Clinical Characteristics of Invasive Salmonella in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal. *PLoS One* 2012; **7**: 1–7.
185 Pitzer VE, Bowles CC, Baker S, *et al.* Predicting the Impact of Vaccination on the Transmission Dynamics of Typhoid in South Asia: A Mathematical Modeling Study. *PLoS Negl Trop Dis* 2014; **8**. DOI:10.1371/journal.pntd.0002642.
186 Siddiqui TR, Bibi S, Mustufa MA, Ayaz SM, Khan A. High prevalence of typhoidal Salmonella enterica serovars excreting food handlers in Karachi-Pakistan: a probable factor for regional typhoid endemicity. *J Heal Popul Nutr* 2015; **33**: 27.
187 Pulickal AS, Gautam S, Clutterbuck EA, *et al.* Kinetics of the natural, humoral immune response to Salmonella enterica serovar Typhi in Kathmandu, Nepal. *Clin Vaccine Immunol* 2009; **16**: 1413–9.
188 Corner RJ, Dewan AM, Hashizume M. Modelling typhoid risk in Dhaka Metropolitan Area of Bangladesh: the role of socio-economic and environmental factors. DOI:10.1186/1476-072X-12-13.
189 Thanh DP, Thompson CN, Rabaa MA, *et al.* The Molecular and Spatial Epidemiology of Typhoid Fever in Rural Cambodia. *PLoS Negl Trop Dis* 2016; **10**: 1–16.
190 Akullian 1☯ A, Ng ’eno 2☯ E, Matheson AI, *et al.* Environmental Transmission of Typhoid Fever in an Urban Slum. *Am Centers Dis Control Prev*; **2**. DOI:10.1371/journal.pntd.0004212.
191 Cruz Espinoza LM, Nichols C, Adu-Sarkodie Y, *et al.* Variations of Invasive Salmonella Infections by Population Size in Asante Akim North Municipal, Ghana. *Clin Infect Dis* 2016; **62 Suppl 1**: S17-22.
192 Bousfield R, Thyl M, Samol O, *et al.* A retrospective study of factors which determine a negative blood culture in Cambodian children diagnosed with enteric fever Short Communication A retrospective study of factors which determine a negative blood c. *Paediatr Int Child Health* 2016; **9047**. DOI:10.1080/20469047.2015.1109242.
193 Moore CE, Pan-Ngum W, Wijedoru LPM, *et al.* Evaluation of the

diagnostic accuracy of a typhoid IgM flow assay for the diagnosis of typhoid fever in Cambodian children using a Bayesian latent class model assuming an imperfect gold standard. *Am J Trop Med Hyg* 2014; **90**: 114–20.
194 Naheed A, Ram PK, Brooks WA, *et al.* Burden of typhoid and paratyphoid fever in a densely populated urban community, Dhaka, Bangladesh. *Int J Infect Dis* 2010; **14 Suppl 3**: e93-9.
195 Wain J, To Song D, Phan Van Be B, *et al.* Specimens and culture media for the laboratory diagnosis of typhoid fever. *J Infect Dev Ctries* 2008; **2**: 469–674.
196 Echeverria P, Chaicumpa W, Ruangkunaporn Y, Burr D. Salmonella typhi antigen in urine . Diagnosis of Typhoid Fever by Detection of Salmonella typhi Antigen in Urine. 1992; **30**: 2513–5.
197 Hoffman SL, Punjabi NH, Rockhill RC, Sutomo A, Rivai AR, Pulungsih SP. Duodenal string-capsule culture compared with bone-marrow, blood, and rectal-swab cultures for diagnosing typhoid and paratyphoid fever. *J Infect Dis* 1984; **149**: 157–61.
198 Khanam F, Sheikh A, Sayeed MA, *et al.* Evaluation of a typhoid/paratyphoid diagnostic assay (TPTest) detecting anti-Salmonella IgA in secretions of peripheral blood lymphocytes in patients in Dhaka, Bangladesh. *PLoS Negl Trop Dis* 2013; **7**: e2316.
199 Gupta AK, Rao KM. Simultaneous detection of Salmonella typhi antigen and antibody in serum by counter-immunoelectrophoresis for an early and rapid diagnosis of typhoid fever. *J Immunol Methods* 1979; **30**: 349–53.
200 Sundararaj T, Ilango B, Subramanian S. A study on the usefulness of counter immuno-electrophoresis for the detection of Salmonella typhi antigen in the sera of suspected cases of enteric fever. *Trans R Soc Trop Med Hyg* 1983; **77**: 194–7.
201 Mukherjee C, Malik A, Khan HM. Rapid diagnosis of typhoid fever by co-agglutination in an Indian hospital. *J Med Microbiol* 1993; **39**: 74–7.
202 John TJ, Sivadasan K, Kurien B. Evaluation of passive bacterial agglutination for the diagnosis of typhoid fever. *J Clin Microbiol* 1984; **20**: 751–3.
203 Rao PS, Prasad S V, Arunkumar G, Shivananda PG. Salmonella typhi VI antigen co-agglutination test for the rapid diagnosis of typhoid fever. *Indian J Med Sci* 1999; **53**: 7–9.
204 Liang L, Juarez S, Nga TVT, *et al.* Immune profiling with a Salmonella Typhi antigen microarray identifies new diagnostic biomarkers of human typhoid. *Sci Rep* 2013; **3**: 1043.
205 Zhou L, Pollard AJ. A novel method of selective removal of human DNA improves PCR sensitivity for detection of Salmonella Typhi in blood

samples. *BMC Infect Dis* 2012; **12**. DOI:10.1186/1471-2334-12-164.
206 Gasem MH, Dolmans WM, Isbandrio BB, Wahyono H, Keuter M, Djokomoeljanto R. Culture of Salmonella typhi and Salmonella paratyphi from blood and bone marrow in suspected typhoid fever. *Trop Geogr Med* 1995; **47**: 164–7.
207 Gilman RH, Terminel M, Levine MM, Hernandez-Mendoza P, Hornick RB. Relative efficacy of blood, urine, rectal swab, bone-marrow, and rose-spot cultures for recovery of Salmonella typhi in typhoid fever. *Lancet* 1975; **1**: 1211–3.
208 Dance D, Richens JE, Ho M, Acharya G, Pokhrel B, Tuladhar NR. Blood and bone marrow cultures in enteric fever. J. Clin. Pathol. 1991; **44**: 1038.
209 Wain J, Diep TS, Ho VA, *et al.* Quantitation of bacteria in blood of typhoid fever patients and relationship between counts and clinical features, transmissibility, and antibiotic resistance. *J Clin Microbiol* 1998; **36**: 1683–7.
210 Gasem MH, Keuter M, Dolmans WM V, Ven-jongekrijg J Van Der, Djokomoeljanto R, Meer JWM Van Der. Persistence of Salmonellae in Blood and Bone Marrow: Randomized Controlled Trial Comparing Cipro oxacin and Chloramphenicol Treatments against Enteric Fever. *Microbiology* 2003; **47**: 1727–31.
211 Dutta S, Sur D, Manna B, *et al.* Evaluation of new-generation serologic tests for the diagnosis of typhoid fever: data from a community-based surveillance in Calcutta, India. *Diagn Microbiol Infect Dis* 2006; **56**: 359–65.
212 Dong B, Galindo CM, Shin E, *et al.* Optimizing typhoid fever case definitions by combining serological tests in a large population study in Hechi City, China. *Epidemiol Infect* 2007; **135**: 1014–20.
213 Olsen SJ, Pruckler J, Bibb W, *et al.* Evaluation of rapid diagnostic tests for typhoid fever. *J Clin Microbiol* 2004; **42**: 1885–9.
214 Lim PL, Tam FC, Cheong YM, Jegathesan M. One-step 2-minute test to detect typhoid-specific antibodies based on particle separation in tubes. *J Clin Microbiol* 1998; **36**: 2271–8.
215 Naheed A, Ram PK, Brooks WA, *et al.* Clinical value of Tubex and Typhidot rapid diagnostic tests for typhoid fever in an urban community clinic in Bangladesh. *Diagn Microbiol Infect Dis* 2008; **61**: 381–6.
216 Choo KE, Oppenheimer SJ, Ismail AB, Ong KH. Rapid serodiagnosis of typhoid fever by dot enzyme immunoassay in an endemic area. *Clin Infect Dis* 1994; **19**: 172–6.
217 Jackson AA, Ismail A, Ibrahim TA, Kader ZS, Nawi NM. Retrospective review of dot enzyme immunoassay test for typhoid fever in an

endemic area. *Southeast Asian J Trop Med Public Health* 1995; **26**: 625–30.
218 Choo KE, Davis TM, Ismail A, Tuan Ibrahim TA, Ghazali WN. Rapid and reliable serological diagnosis of enteric fever: comparative sensitivity and specificity of Typhidot and Typhidot-M tests in febrile Malaysian children. *Acta Trop* 1999; **72**: 175–83.
219 Gopalakrishnan V, Sekhar WY, Soo EH, Vinsent RA, Devi S. Typhoid fever in Kuala Lumpur and a comparative evaluation of two commercial diagnostic kits for the detection of antibodies to Salmonella typhi. *Singapore Med J* 2002; **43**: 354–8.
220 Narayanappa D, Sripathi R, Jagdishkumar K, Rajani HS. Comparative study of dot enzyme immunoassay (Typhidot-M) and Widal test in the diagnosis of typhoid fever. *Indian Pediatr* 2010; **47**: 331–3.
221 Kawano RL, Leano SA, Agdamag DMA. Comparison of serological test kits for diagnosis of typhoid fever in the Philippines. *J Clin Microbiol* 2007; **45**: 246–7.
222 Nizami SQ, Bhutta ZA, Siddiqui AA, Lubbad L. Enhanced detection rate of typhoid fever in children in a periurban slum in Karachi, Pakistan using polymerase chain reaction technology. *Scand J Clin Lab Invest* 2006; **66**: 429–36.
223 Baker S, Duy PT, Nga T V, *et al.* Fitness benefits in fluoroquinolone-resistant Salmonella Typhi in the absence of antimicrobial pressure. *Elife* 2013; **2**: e01229.
224 Arjyal A, Basnyat B, Nhan HT, *et al.* Gatifloxacin versus ceftriaxone for uncomplicated enteric fever in Nepal: an open-label, two-centre, randomised controlled trial. *Lancet Infect Dis* 2016; **3099**: 1–11.
225 Ecevit Z. Ofloxacin versus co-trimoxazolz in the treatment o typhoid fever in children. 1996; : 39–42.
226 Adabara NU, Ezugwu BU, Momojimoh A, Madzu A, Hashiimu Z, Damisa D. The prevalence and antibiotic susceptibility pattern of Salmonella typhi among patients attending a military hospital in Minna, Nigeria. *Adv Prev Med* 2012; **2012**: Article ID 875419.
227 Edmiston CE, Suarez EC, Walker AP, *et al.* Penetration of ciprofloxacin and fleroxacin into biliary tract. *Antimicrob Agents Chemother* 1996; **40**: 787–91.
228 Adefurin A, Sammons H, Jacqz-Aigrain E, Choonara I. Ciprofloxacin safety in paediatrics: a systematic review. *Arch Dis Child* 2011; **96**: 874–80.
229 Kubin R. Safety and efficacy of ciprofloxacin in paediatric patients--review. *Infection*; **21**: 413–21.
230 Bethell DB, Hien TT, Phi LT, *et al.* Effects on growth of single short courses of fluoroquinolones. *Arch Dis Child* 1996; **74**: 44–6.

231 Doherty CP, Saha SK, Cutting WA. Typhoid fever, ciprofloxacin and growth in young children. *Ann Trop Paediatr* 2000; **20**: 297–303.
232 Yee CL, Duffy C, Gerbino PG, Stryker S, Noel GJ. Tendon or joint disorders in children after treatment with fluoroquinolones or azithromycin. *Pediatr Infect Dis J* 2002; **21**: 525–9.
233 Bacci C, Galli L, de Martino M, Chiappini E. Fluoroquinolones in children: Update of the literature. *J Chemother* 2015; **27**: 257–65.
234 Vinh H, Duong NM, Phuong LT, *et al.* Comparative trial of short-course ofloxacin for uncomplicated typhoid fever in Vietnamese children. *Ann Trop Paediatr* 2005; **25**: 17–22.
235 Soe GB, Overturf GD. Treatment of typhoid fever and other systemic salmonelloses with cefotaxime, ceftriaxone, cefoperazone, and other newer cephalosporins. *Rev Infect Dis*; **9**: 719–36.
236 Islam A, Butler T, Nath SK, *et al.* Randomized treatment of patients with typhoid fever by using ceftriaxone or chloramphenicol. *J Infect Dis* 1988; **158**: 742–7.
237 Islam A, Butler T, Kabir I, Alam NH. Treatment of typhoid fever with ceftriaxone for 5 days or chloramphenicol for 14 days: a randomized clinical trial. *Antimicrob Agents Chemother* 1993; **37**: 1572–5.
238 Lasserre R, Sangalang RP, Santiago L. Three-day treatment of typhoid fever with two different doses of ceftriaxone, compared to 14-day therapy with chloramphenicol: a randomized trial. *J Antimicrob Chemother* 1991; **28**: 765–72.
239 Acharya G, Butler T, Ho M, *et al.* Treatment of typhoid fever: randomized trial of a three-day course of ceftriaxone versus a fourteen-day course of chloramphenicol. *Am J Trop Med Hyg* 1995; **52**: 162–5.
240 Tatli MM, Aktas G, Kosecik M, Yilmaz A. Treatment of typhoid fever in children with a flexible-duration of ceftriaxone, compared with 14-day treatment with chloramphenicol. *Int J Antimicrob Agents* 2003; **21**: 350–3.
241 Bhutta ZA, Khan IA, Shadmani M. Failure of short-course ceftriaxone chemotherapy for multidrug-resistant typhoid fever in children: A randomized controlled trial in Pakistan. *Antimicrob Agents Chemother* 2000; **44**: 450–2.
242 Bhutta ZA, Khan IA, Molla AM. Therapy of multidrug-resistant typhoid fever with oral cefixime vs. intravenous ceftriaxone. *Pediatr Infect Dis J* 1994; **13**: 990–4.
243 Pandit A, Arjyal A, Day JN, *et al.* An open randomized comparison of gatifloxacin versus cefixime for the treatment of uncomplicated enteric fever. *PLoS One* 2007; **2**. DOI:http://dx.doi.org/10.1371/journal.pone.0000542.

244 Cao XT, Kneen R, Nguyen TA, Truong DL, White NJ, Parry CM. A comparative study of ofloxacin and cefixime for treatment of typhoid fever in children. The Dong Nai Pediatric Center Typhoid Study Group. *Pediatr Infect Dis J* 1999; **18**: 245–8.
245 Shakur MS, Arzuman SAL, Jesmin H, Hasan M, Mesbahuddin A. Cefpodoxime proxetil compared with cefixime for treatment of typhoid fever in children. *Indian Pediatr* 2007; **44**: 838–41.
246 Jain S, Chugh T Das. Antimicrobial resistance among blood culture isolates of Salmonella enterica in New Delhi. *J Infect Dev Ctries* 2013; **7**: 788–95.
247 Rodrigues C, Kapil A, Sharma A, *et al.* Whole-Genome Shotgun Sequencing of Cephalosporin-Resistant Salmonella enterica Serovar Typhi. *Genome Announc* 2017; **5**: e01639-16.
248 Munir T, Lodhi M, Ansari JK, Andleeb S, Ahmed M. Extended Spectrum Beta Lactamase producing Cephalosporin resistant Salmonella Typhi, reported from Rawalpindi, Pakistan. *J Pak Med Assoc* 2016; **66**: 1035–6.
249 Devanga Ragupathi NK, Muthuirulandi Sethuvel DP, Shankar BA, Munusamy E, Anandan S, Veeraraghavan B. Draft genome sequence of blaTEM-1-mediated cephalosporin-resistant Salmonella enterica serovar Typhi from bloodstream infection. *J Glob Antimicrob Resist* 2016; **7**: 11–2.
250 Qamar FN, Saleem K, Shakoor S, *et al.* Proceedings of 10th International Conference on Typhoid and other Invasive Salmonelloses. Kampala, Uganda, 2017: 40.
251 Panteix G, Guillaumond B, Harf R, *et al.* In-vitro concentration of azithromycin in human phagocytic cells. *J Antimicrob Chemother* 1993; **31 Suppl E**: 1–4.
252 Dolecek C, La TTP, Rang NN, *et al.* A multi-center randomised controlled trial of gatifloxacin versus azithromycin for the treatment of uncomplicated typhoid fever in children and adults in Vietnam. *PLoS One* 2008; **3**. DOI:10.1371/journal.pone.0002188.
253 Parry CM, Ho VA, Phuong le T, *et al.* Randomized controlled comparison of ofloxacin, azithromycin, and an ofloxacin-azithromycin combination for treatment of multidrug-resistant and nalidixic acid-resistant typhoid fever. *Antimicrob Agents Chemother* 2007; **51**: 819–25.
254 Frenck Jr RW, Mansour A, Nakhla I, *et al.* Short-course azithromycin for the treatment of uncomplicated typhoid fever in children and adolescents. *Clin Infect Dis* 2004; **38**: 951–7.
255 Chandey M, Multani AS. A comparative study of efficacy and safety of azithromycin and ofloxacin in uncomplicated typhoid fever: A

randomised, open labelled study. *J Clin Diagnostic Res* 2012; **6**: 1736–9.

256 Chinh NT, Parry CM, Ly NT, *et al.* A randomized controlled comparison of azithromycin and ofloxacin for treatment of multidrug-resistant or nalidixic acid-resistant enteric fever. *Antimicrob Agents Chemother* 2000; **44**: 1855–9.

257 Girgis NI, Butler T, Frenck RW, *et al.* Azithromycin versus ciprofloxacin for treatment of uncomplicated typhoid fever in a randomized trial in Egypt that included patients with multidrug resistance. *Antimicrob Agents Chemother* 1999; **43**: 1441–4.

258 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**: 1134–8.

259 Begum B, Haque MA, Ahmed MS, *et al.* Comparison between azithromycin and cefixime in the treatment of typhoid fever in children. *Mymensingh Med J* 2014; **23**: 441–8.

260 Jain S, Chugh TD. Antimicrobial resistance among blood culture isolates of Salmonella enterica in New Delhi. *J Infect Dev Ctries* 2013; **7**: 788–95.

261 Molloy A, Nair S, Cooke FJ, *et al.* First Report of Salmonella enterica Serotype Paratyphi A Azithromycin Resistance Leading to Treatment Failure. *J Clin Microbiol* 2010; **48**: 4655–7.

262 Hassing RJ, Goessens WHF, van Pelt W, *et al.* Salmonella subtypes with increased MICs for azithromycin in travelers returned to the Netherlands. *Emerg Infect Dis* 2014; **20**: 705–8.

263 Molloy ES, Powell FC, Doran MF, *et al.* An unusual case of Behçet's syndrome: Triggered by typhoid vaccination? *Clin Exp Rheumatol* 2004; **22**: S71–4.

264 Kobayashi T, Hayakawa K, Mawatari M, *et al.* Case report: Failure under azithromycin treatment in a case of bacteremia due to Salmonella enterica Paratyphi A. *BMC Infect Dis* 2014; **14**. DOI:http://dx.doi.org/10.1186/1471-2334-14-404.

265 Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid fever. *N Engl J Med* 2002; **347**: 1770–82.

266 Punjabi NH, Hoffman SL, Edman DC, *et al.* Treatment of severe typhoid fever in children with high dose dexamethasone. Pediatr. Infect. Dis. J. 1988; **7**: 598–600.

267 Chisti MJ, Bardhan PK, Huq S, *et al.* High-dose intravenous dexamethasone in the management of diarrheal patients with enteric fever and encephalopathy. *Southeast Asian J Trop Med Public Health* 2009; **40**: 1065–73.

268 Vaidya A, Supe A, Samsi AB, Ramakantan R. Continuous intra arterial

vasopressin infusion for control of typhoid hemorrhage. *Indian J Gastroenterol* 1990; **9**: 225–6.
269 Lee JH, Kim JJ, Jung JH, *et al.* Colonoscopic manifestations of typhoid fever with lower gastrointestinal bleeding. *Dig Liver Dis* 2004; **36**: 141–6.
270 Shaikhani MAR, Husein HAB, Karbuli TA, Mohamed MA. Colonoscopic findings and management of patients with outbreak typhoid fever presenting with lower gastrointestinal bleeding. *Indian J Gastroenterol* 2013; **32**: 335–40.
271 Lim WY, H'ng MWC, Punamiya S. An uncommon case of haemorrhagic enteric fever treated successfully with super-selective embolisation using polyvinyl alcohol particles and coils. *Singapore Med J* 2013; **54**: e53–5.
272 van Basten JP, Stockenbrügger R. Typhoid perforation. A review of the literature since 1960. *Trop Geogr Med* 1994; **46**: 336–9.
273 Bitar R, Tarpley J. Intestinal perforation in typhoid fever: a historical and state-of-the-art review. *Rev Infect Dis*; **7**: 257–71.
274 Tayyab M, Aurangzeb M, Hussain A, Ahmad N. The prognostic factors in Typhoid ileal perforation. *J Med Sci* 2010; **18**: 79–82.
275 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**: 901–37.
276 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**: 1–13.
277 Geetha VK, Yugendran T, Srinivasan R, Harish BN. Plasmid-mediated quinolone resistance in typhoidal Salmonellae: a preliminary report from South India. *Indian J Med Microbiol* 2014; **32**: 31–4.
278 Parry CM, Vo Anh H, Le Thi P, *et al.* Randomized controlled comparison of ofloxacin, azithromycin, and an ofloxacin-azithromycin combination for treatment of multidrug-resistant and nalidixic acid-resistant typhoid fever. *Antimicrob Agents Chemother* 2007; **51**: 819–25.
279 Girgis NI, Tribble DR, Sultan Y, Farid Z. Short course chemotherapy with cefixime in children with multidrug-resistant Salmonella typhi Septicaemia. *J Trop Pediatr* 1995; **41**: 364–5.
280 Girgis NI, Sultan Y, Hammad O, Farid Z. Comparison of the efficacy, safety and cost of cefixime, ceftriaxone and aztreonam in the treatment of multidrug-resistant Salmonella typhi septicemia in children. *Pediatr Infect Dis J* 1995; **14**: 603–5.
281 Arjyal A, Basnyat B, Koirala S, *et al.* Gatifloxacin versus

chloramphenicol for uncomplicated enteric fever: An open-label, randomised, controlled trial. *Lancet Infect Dis* 2011; **11**: 445–54.
282 Dipika, Sur. Ochiai, Leon. Bhattacharya. Sjith, Clemens. John. New England Journal. *N Engl J Med* 2009; **361**: 865–75.
283 Khan MI, Soofi SB, Ochiai RL, *et al.* Effectiveness of Vi capsular polysaccharide typhoid vaccine among children: A cluster randomized trial in Karachi, Pakistan. *Vaccine* 2012; **30**: 5389–95.
284 Saul A, Smith T, Maire N. Stochastic Simulation of Endemic Salmonella enterica Serovar Typhi: The Importance of Long Lasting Immunity and the Carrier State. *PLoS One* 2013; **8**. DOI:10.1371/journal.pone.0074097.
285 Thiem VD, Lin FY, Canh do G, *et al.* The Vi conjugate typhoid vaccine is safe, elicits protective levels of IgG anti-Vi, and is compatible with routine infant vaccines. *Clin Vaccine Immunol* 2011; **18**: 730–5.
286 Micoli F, Rondini S, Gavini M, *et al.* O:2-CRM197 Conjugates against Salmonella Paratyphi A. *PLoS One* 2012; **7**. DOI:10.1371/journal.pone.0047039.
287 Battersby AJ, Gibbons DL. The gut mucosal immune system in the neonatal period. *Pediatr Allergy Immunol* 2013; **24**: 414–21.
288 Ygberg S, Nilsson A. The developing immune system - From foetus to toddler. *Acta Paediatr Int J Paediatr* 2012; **101**: 120–7.
289 Akinyemi KO, Oshundare YO, Oyeyinka OG, Coker AO. Original Article A retrospective study of community-acquired Salmonella infections in patients attending public hospitals in Lagos , Nigeria. *J Infect Dev Ctries* 2012; **5**: 387–95.
290 France GL, Marmer DJ, Steele RW. Breast-feeding and Salmonella infection. *Am J Dis Child* 1980; **134**: 147–52.
291 Bhutta ZA, Capeding MR, Bavdekar A, *et al.* lmmunogenicity and safety of the Vi-CRM197 conjugate vaccine against typhoid fever in adults, children, and infants in south and southeast Asia: results from two randomised, observer-blind, age de-escalation, phase 2 trials. *Lancet Infect Dis* 2014; **14**: 119–29.
292 Hayden JA, van der Windt DA, Cartwright JL, Côté P, Bombardier C. Assessing Bias in Studies of Prognostic Factors. *Ann Intern Med* 2013; **158**: 280.
293 The Joanna Briggs Institute. Rev. Man. 2014. https://joannabriggs.org/assets/docs/sumari/ReviewersManual-2014.pdf (accessed April 11, 2018).
294 Performance Standards for Antimicrobial Susceptibility Testing An informational supplement for global application developed through the Clinical and Laboratory Standards Institute. http://www.facm.ucl.ac.be/intranet/CLSI/CLSI-2017-M100-S27.pdf

(accessed April 19, 2018).
295 Holt,K.E Thomson,N.R Wain,J., Langridge,G.C Hasan, R Bhutta,Z.A Quail,MA Norbertczak,H. Walker,D Simmonds,M White,B Bason,N Mungall,K Dougan,G Parkhill J. Salmonella enterica subsp. enterica serovar Paratyphi A str. AKU_12601 complete genome, strain AKU_12601. Bioinformatics. 2009; **25**: 2078–9.
296 Langmead BSS. Fast gapped-read alignment with Bowtie 2. *Nat Commun* 2012; **9**: 357–9.
297 Li H, Handsaker B, Wysoker A, *et al.* The Sequence Alignment/Map format and SAMtools. *Bioinformatics* 2009; **25**: 2078–9.
298 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–e15.
299 Stamatakis A. RAxML-VI-HPC: maximum likelihood-based phylogenetic analyses with thousands of taxa and mixed models. *Bioinformatics* 2006; **22**: 2688–90.
300 Argimón S, Abudahab K, Goater RJE, *et al.* Microreact: visualizing and sharing data for genomic epidemiology and phylogeography. *Microb genomics* 2016; **2**: e000093.
301 Yu G, Smith DK, Zhu H, Guan Y, Lam TT-Y. ggtree : an r package for visualization and annotation of phylogenetic trees with their covariates and other associated data. *Methods Ecol Evol* 2017; **8**: 28–36.
302 Inouye M, Dashnow H, Raven L, *et al.* SRST2 : Rapid genomic surveillance for public health and hospital microbiology labs. 2014; : 1–16.
303 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.
304 Zankari E, Hasman H, Cosentino S, *et al.* Identification of acquired antimicrobial resistance genes. DOI:10.1093/jac/dks261.
305 Bankevich A, Nurk S, Antipov D, *et al.* SPAdes: A New Genome Assembly Algorithm and Its Applications to Single-Cell Sequencing. DOI:10.1089/cmb.2012.0021.
306 Wick RR, Judd LM, Gorrie CL, Holt KE. Unicycler: Resolving bacterial genome assemblies from short and long sequencing reads. DOI:10.1371/journal.pcbi.1005595.
307 Wick RR, Schultz MB, Zobel J, Holt KE. Bandage: interactive visualization of de novo genome assemblies. DOI:10.1093/bioinformatics/btv383.
308 Feasey NA, Masesa C, Jassi C, *et al.* Three epidemics of invasive multidrug-resistant salmonella bloodstream infection in Blantyre,

Malawi, 1998-2014. *Clin Infect Dis* 2015; **61**: S363–71.
309 Saha SK, Baqui AH, Hanif M, *et al.* Typhoid fever in Bangladesh: implications for vaccination policy. Pediatr Infect Dis J. 2001; **20**: 521–4.
310 United N. World Population Prospects The 2015 Revison. *Dep Econ Soc Aff* 2015; **53**: 1689–99.
311 The Nordic Cochrane Centre, The Cochrane Collaboration. Review Manager (RevMan) [Computer program]. Version 5.3. Copenhagen: 2014.
312 DerSimonian R, Laird N. Meta-analysis in clinical trials. *Control Clin Trials* 1986; **7**: 177–88.
313 Parry CM, Thompson C, Vinh H, *et al.* Risk factors for the development of severe typhoid fever in Vietnam. *BMC Infect Dis* 2014; **14**. DOI:http://dx.doi.org/10.1186/1471-2334-14-73.
314 Pradhan R, Shrestha U, Gautam SC, *et al.* Bloodstream Infection among Children Presenting to a General Hospital Outpatient Clinic in Urban Nepal. *PLoS One* 2012; **7**. DOI:10.1371/journal.pone.0047531.
315 Saha MR, Palit A, Chatterjee NS, Dutta P, Mitra U, Bhattacharya SK. A prospective study of phage types & biotypes of Salmonella enterica serotype Typhi isolated from hospitalized children in Kolkata, India. *Indian J Med Res* 2003; **117**: 201–4.
316 Shakva, KN. Baral, MR. Shrestha R. A study of Atypical Manifestations of Enteric Fever in Childlren. *Nepal Heal Res Counc* 2008; **6**: 1–4.
317 Verma M, Parashar Y, Singh A, Kamoji R. Current pattern of enteric fever: A prospective clinical and microbiological study. *J Indian Med Assoc* 2007; **105**: 582–91.
318 Walia M, Gaind R, Paul P, Mehta R, Aggarwal P, Kalaivani M. Age-related clinical and microbiological characteristics of enteric fever in India. *Trans R Soc Trop Med Hyg* 2006; **100**: 942–8.
319 Abucejo PE, Capeding MR, Lupisan SP, Arcay J. Blood Culture Confirmed Typhoid Fever in a Provincial Hospital in the Phillipines. *Southeast Asian J Trop Med Public Heal* 2001; **32**: 3–8.
320 Bajracharya D, Khan MI, Pach A, *et al.* 25 Years after Vi typhoid vaccine efficacy study, typhoid affects significant number of population in Nepal. *PLoS One* 2014; **9**: 1–4.
321 Karkey A, Arjyal A, Anders KL, *et al.* The burden and characteristics of enteric fever at a healthcare facility in a densely populated area of Kathmandu. *PLoS One* 2010; **5**: e13988.
322 Kelly DF, Thorson S, Maskey M, *et al.* International Journal of Infectious Diseases The burden of vaccine-preventable invasive bacterial infections and pneumonia in children admitted to hospital in urban Nepal. *Int J Infect Dis* 2011; **15**: e17–23.

323 Malla, T. Malla, KK. Thapalial, A. Shaw C. Enteric Fever: A retrospective 6-year analysis of 82 paediatric cases in a teaching hospital. *Kathmandu Univ Med J* 2007; **5**: 181–7.

324 Breiman RF, Cosmas L, Njuguna H, *et al.* Population-based incidence of typhoid fever in an urban informal settlement and a rural area in Kenya: implications for typhoid vaccine use in Africa. *PLoS One* 2012; **7**: e29119.

325 Hendriksen RS, Leekitcharoenphon P, Lukjancenko O, *et al.* Genomic signature of multidrug-resistant Salmonella enterica serovar typhi isolates related to a massive outbreak in Zambia between 2010 and 2012. *J Clin Microbiol* 2015; **53**: 262–72.

326 Lunguya O, Lejon V, Phoba M-F, *et al.* Salmonella typhi in the democratic republic of the congo: fluoroquinolone decreased susceptibility on the rise. *PLoS Negl Trop Dis* 2012; **6**: e1921.

327 Maltha J, Guiraud I, Kaboré B, *et al.* Frequency of severe malaria and invasive bacterial infections among children admitted to a rural hospital in Burkina Faso. *PLoS One* 2014; **9**: e89103.

328 Marks, Florian. Adu-Sarkodie, Yaw. Hunger, Frank. Sarpong, Nimako. Ekuban, Samuel. May J. Typhoid Fever among Children, Ghana. *Emergung Infect Dis* 2010; **16**: Letter to the editor.

329 Phoba M-F, De Boeck H, Ifeka BB, *et al.* Epidemic increase in Salmonella bloodstream infection in children, Bwamanda, the Democratic Republic of Congo. *Eur J Clin Microbiol Infect Dis* 2014; **33**: 79–87.

330 Mai Ngoc L, Phan Van B, Vo Anh H, *et al.* Persistent efficacy of Vi conjugate vaccine against typhoid fever in young children. *N Engl J Med* 2003; **349**: 1390–1.

331 Kossaczka Z, Lin FY, Ho VA, *et al.* Safety and immunogenicity of Vi conjugate vaccines for typhoid fever in adults, teenagers, and 2- to 4-year-old children in Vietnam. *Infect Immun* 1999; **67**: 5806–10.

332 Khan MI, Pach III A, Khan GM, *et al.* Typhoid vaccine introduction: An evidence-based pilot implementation project in Nepal and Pakistan. *Vaccine* 2015; **33**: C62–7.

333 Owais A, Sultana S, Zaman U, Rizvi A, Zaidi AKM. Incidence of typhoid bacteremia in infants and young children in Southern Coastal Pakistan. *Pediatr Infect Dis J* 2010; **29**: 1035–9.

334 Khan MI, Soofi SB, Ochiai RL, *et al.* Original Article Epidemiology , clinical presentation , and patterns of drug resistance of Salmonella Typhi in Karachi , Pakistan. *J Infect Dev Ctries* 2012; **6**: 704–14.

335 Siddiqui FJ, Haider SR, Bhutta ZA. Risk factors for typhoid fever in children in squatter settlements of Karachi: a nested case-control study. *J Infect Public Health* 2008; **1**: 113–20.

336 Arndt MB, Mosites EM, Tian M, *et al.* Estimating the Burden of Paratyphoid A in Asia and Africa. *PLoS Negl Trop Dis* 2014; **8**. DOI:10.1371/journal.pntd.0002925.
337 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**: e0004616.
338 Shanta D, Dipika S, Byomkesh M, Bhattacharya SK, Deen JL, Clemens JD. Rollback of Salmonella enterica serotype typhi resistance to chloramphenicol and other antimicrobials in Kolkata, India. *Antimicrob Agents Chemother* 2005; **49**: 1662–3.
339 Hendriksen RS, Leekitcharoenphon P, Lukjancenko O, *et al.* Genomic signature of multidrug-resistant salmonella enterica serovar Typhi isolates related to a massive outbreak in Zambia between 2010 and 2012. *J Clin Microbiol* 2015; **53**: 262–72.
340 Lutterloh E, Likaka A, Sejvar J, *et al.* Multidrug-resistant typhoid fever with neurologic findings on the Malawi-Mozambique border. *Clin Infect Dis* 2012; **54**: 1100–6.
341 Karki S, Shakya P, Cheng AC, Dumre SP, Leder K. Trends of etiology and drug resistance in enteric fever in the last two decades in Nepal: a systematic review and meta-analysis. *Clin Infect Dis* 2013; **57**: e167-76.
342 Lakshmi V, Ashok R, Susmita J, Shailaja V V. Changing trends in the antibiograms of Salmonella isolates at a tertiary care hospital in Hyderabad. *Indian J Med Microbiol* 2006; **24**: 45–8.
343 Yashwant K, Anshu S, Mani KR. Re-emergence of susceptibility to conventionally used drugs among strains of Salmonella Typhi in central west India. *J Infect Dev Ctries* 2011; **5**: 227–30.
344 Maskey AP, Day JN, Phung QT, *et al.* Salmonella enterica serovar Paratyphi A and S. enterica serovar Typhi cause indistinguishable clinical syndromes in Kathmandu, Nepal. *Clin Infect Dis* 2006; **42**: 1247–53.
345 Thaver D, Zaidi AKM, Critchley J, Azmatullah A, Madni SA, Bhutta ZA. A comparison of fluoroquinolones versus other antibiotics for treating enteric fever: Meta-analysis. *BMJ* 2009; **338**: 1371.
346 Khan MI, Ochiai RL, Clemens JD. Population impact of Vi capsular polysaccharide vaccine. *Expert Rev Vaccines* 2010; **9**: 485–96.
347 Maskey AP, Basnyat B, Thwaites GE, Campbell JI, Farrar JJ, Zimmerman MD. Emerging trends in enteric fever in Nepal: 9124 cases confirmed by blood culture 1993-2003. *Trans R Soc Trop Med Hyg* 2008; **102**: 91–5.
348 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**.
DOI:http://dx.doi.org/10.1371/journal.pntd.0003748.
349 Al-Emran HM, Eibach D, Krumkamp R, *et al.* A Multicountry Molecular Analysis of Salmonella enterica Serovar Typhi With Reduced Susceptibility to Ciprofloxacin in Sub-Saharan Africa. *Clin Infect Dis* 2016; **62 Suppl 1**: S42-6.
350 Crump JA, Heyderman RS. A perspective on invasive salmonella disease in Africa. *Clin Infect Dis* 2015; **61**: S235–40.
351 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**: S325–31.
352 Nichols C, Cruz Espinoza LM, Von Kalckreuth V, *et al.* Bloodstream infections and frequency of pretreatment associated with age and hospitalization status in Sub-Saharan Africa. *Clin Infect Dis* 2015; **61**: S372–9.
353 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 Suppl 1**: S9–16.
354 Baker S, Holt KE, Clements ACA, *et al.* Combined high-resolution genotyping and geospatial analysis reveals modes of endemic urban typhoid fever transmission. *Open Biol* 2011; **1**: 110008.
355 World Bank. datahelpdesk.worldbank.org. Available:https://datahelpdesk.worldbank.org/knowledgebase/articles/906519-world-bank-country-and-lending-groups. .
356 Available at. UNICEF. https://data.unicef.org/topic/child-survival/under-five-mortality/. .
357 World Bank. Available at. http://www.worldbank.org/en/news/feature/2013/04/01/managing-nepals-urban-transition. .
358 IndexMundi. Nepal Age Structure. IndexMundi.com. 2013.
359 Williams EJ, Thorson S, Maskey M, *et al.* Hospital-based surveillance of invasive pneumococcal disease among young children in urban Nepal. *Clin Infect Dis* 2009; **48**. DOI:10.1086/596488.
360 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.
361 Core Team R. A language and environment for statistical computing. *R Found Stat Comput Vienna, Austria*.
362 Bouckaert R, Heled J, Kühnert D, *et al.* BEAST 2: A Software Platform for Bayesian Evolutionary Analysis. *PLoS Comput Biol* 2014; **10**: e1003537.

363 Duchêne S, Holt KE, Weill F-X, *et al.* Genome-scale rates of evolutionary change in bacteria. DOI:10.1099/mgen.0.000094.
364 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. DOI:10.1093/molbev/msq088.
365 Luo X, Zhao B. A Statistical Tree Annotator and Its Applications. 2011; : 1230–8.
366 Saad NJ, Bowles CC, Grenfell BT, *et al.* The impact of migration and antimicrobial resistance on the transmission dynamics of typhoid fever in Kathmandu, Nepal: A mathematical modelling study. *PLoS Negl Trop Dis* 2017; **11**. DOI:10.1371/journal.pntd.0005547.
367 Britto C, Pollard AJ, Voysey M, Blohmke CJ. An appraisal of the clinical features of pediatric enteric fever: Systematic review and meta-analysis of the age-stratified disease occurrence. *Clin Infect Dis* 2017; **64**. DOI:10.1093/cid/cix229.
368 Britto C, Pollard AJ, Voysey M, Blohmke CJ. An Appraisal of the Clinical Features of Pediatric Enteric Fever: Systematic Review and Meta-analysis of the Age- Stratified Disease Occurrence. *Clin Infect Dis* 2017. DOI:10.1093/cid/cix229.
369 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. DOI:10.1371/journal.pntd.0005274.
370 Karkey A, Thompson CN, Tran Vu Thieu N, *et al.* Differential Epidemiology of Salmonella Typhi and Paratyphi A in Kathmandu, Nepal: A Matched Case Control Investigation in a Highly Endemic Enteric Fever Setting. *PLoS Negl Trop Dis* 2013; **7**: e2391.
371 Pokharel BM, Koirala J, Dahal RK, Mishra SK, Khadga PK, Tuladhar NR. Multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing Salmonella enterica (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: surveillance of resistance and a search for newer alternatives. *Netherlands Int J Infect Dis* 2006; **10**: 434–8.
372 Zhou Z, Mccann A, Weill F-X, *et al.* Transient Darwinian selection in Salmonella enterica serovar Paratyphi A during 450 years of global spread of enteric fever. DOI:10.1073/pnas.1411012111.
373 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**. DOI:10.1371/journal.pntd.0001245.
374 Arndt MB, Mosites EM, Tian M, *et al.* Estimating the Burden of Paratyphoid A in Asia and Africa. *PLoS Negl Trop Dis* 2014; **8**: e2925.
375 Zhou Z, McCann A, Weill F-X, *et al.* Transient Darwinian selection in

Salmonella enterica serovar Paratyphi A during 450 years of global spread of enteric fever. *Proc Natl Acad Sci U S A* 2014; **111**: 12199–204.
376 Yan M, Yang B, Wang Z, *et al.* A Large-Scale Community-Based Outbreak of Paratyphoid Fever Caused by Hospital-Derived Transmission in Southern China. *PLoS Negl Trop Dis* 2015; **9**: e0003859.
377 Kuijpers LMF, Le Hello S, Fawal N, *et al.* Genomic analysis of Salmonella enterica serotype Paratyphi A during an outbreak in Cambodia, 2013-2015. *Microb genomics* 2016; **2**: e000092.
378 Giri S, Priya Hemavathy R, Arumugam R, *et al.* Molecular epidemiology of rotaviruses in the south-east Asian region from 2009 to 2015. *Vaccine* 2018; published online March. DOI:10.1016/j.vaccine.2018.02.092.
379 Rajendran P, Kang G. Molecular epidemiology of rotavirus in children and animals and characterization of an unusual G10P[15] strain associated with bovine diarrhea in south India. *Vaccine* 2014; **32**: A89–94.
380 Garrett D. At a Glance: Surveillance for Enteric Fever in Asia Project (SEAP). .
381 200 cases of typhoid in Bengaluru in one week, BBMP advises boiled water for drinking | The News Minute. https://www.thenewsminute.com/article/200-cases-typhoid-bengaluru-one-week-bbmp-advises-boiled-water-drinking-67614 (accessed Jan 4, 2018).
382 Flood brings home the typhoid bug - Bangalore Mirror. http://bangaloremirror.indiatimes.com/bangalore/others/flood-brings-home-the-typhoid-bug/articleshow/60266183.cms (accessed Jan 4, 2018).
383 Alert! Typhoid is back - Bangalore Mirror. http://bangaloremirror.indiatimes.com/bangalore/others/alert-typhoid-is-back/articleshow/55099538.cms (accessed Jan 4, 2018).
384 Diseases on the rise in city after drain bursts - Bangalore Mirror. http://bangaloremirror.indiatimes.com/bangalore/others/bangalore/others/diseases-on-the-rise-in-city-after-drain-bursts/articleshow/60146942.cms (accessed Jan 4, 2018).
385 Typhoid News – Take on Typhoid. http://www.coalitionagainsttyphoid.org/facts/typhoid-news/ (accessed Jan 4, 2018).
386 Purighalla S, Esakimuthu S, Reddy M, *et al.* Investigation into a community outbreak of Salmonella Typhi in Bengaluru, India. *Indian J Med Res* 2017; **146**: S15–22.

387 Waddington CS, Darton TC, Jones C, *et al.* An outpatient, ambulant-design, controlled human infection model using escalating doses of Salmonella Typhi challenge delivered in sodium bicarbonate solution. *Clin Infect Dis* 2014; **58**: 1230–40.
388 Meiring JE, Gibani M, Basnyat B, *et al.* The Typhoid Vaccine Acceleration Consortium (TyVAC): Vaccine effectiveness study designs: Accelerating the introduction of typhoid conjugate vaccines and reducing the global burden of enteric fever. Report from a meeting held on 26â€“27 October 2016, Oxford, UK. *Vaccine* 2017; **35**: 5081–8.
389 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. DOI:10.1073/pnas.1717295115.
390 Neill JIMO. Tackling drug-resistant infections globally: final report and recommendations. The review on antimicrobial resistance. 2016.
391 Antibiotic use is rapidly increasing in developing countries - Daily chart. https://www.economist.com/blogs/graphicdetail/2018/04/daily-chart (accessed April 5, 2018).
392 WHO | Antimicrobial resistance: global report on surveillance 2014. *WHO* 2016.
393 Kakkar M, Chatterjee P, Chauhan A, et al. Antimicrobial resistance in South East Asia. Global Health Action. 2018; 11(1)
394 Datta S, Wattal C, Goel N, Oberoi JK, Raveendran R, Prasad KJ. A ten year analysis of multi-drug resistant blood stream infections caused by Escherichia coli & Klebsiella pneumoniae in a tertiary care hospital. *Indian J Med Res* 2012; **135**: 907–12.
395 Shrestha S, Adhikari N, Rai BK, Shreepaili A. Antibiotic resistance pattern of bacterial isolates in neonatal care unit. *JNMA J Nepal Med Assoc*; **50**: 277–81.
396 Van Boeckel TP, Gandra S, Ashok A, *et al.* Global antibiotic consumption 2000 to 2010: An analysis of national pharmaceutical sales data. *Lancet Infect Dis* 2014; **14**: 742–50.
397 Tamang MD, Oh JY, Seol SY, *et al.* Emergence of multidrug-resistant Salmonella enterica serovar Typhi associated with a class 1 integron carrying the dfrA7 gene cassette in Nepal. *Int J Antimicrob Agents* 2007; **30**: 330–5.
398 Karki M, Pandit S, Baker S, Basnyat B. Cotrimoxazole treats fluoroquinolone-resistant Salmonella typhi H58 infection. *BMJ Case Rep* 2016; **2016**. DOI:10.1136/bcr-2016-217223.
399 Buckle GC, Fischer Walker CL, Black RE. Typhoid fever and paratyphoid fever: Systematic review to estimate global morbidity

and mortality for 2010. *J Glob Health* 2012; **2**.

400 Parry CM, Hien TT, Dougan G, White NJ, Farrar JJ. Typhoid Fever. *N Engl J Med* 2002; **347**: 1770–82.

401 Rahman M, Siddique AK, Shoma S, *et al.* Emergence of multidrug-resistant Salmonella enterica serotype Typhi with decreased ciprofloxacin susceptibility in Bangladesh. *Epidemiol Infect* 2005; **134**: 433.

402 Klemm EJ, Shakoor S, Page AJ, *et al.* Emergence of an Extensively Drug-ResistantSalmonella entericaSerovar Typhi Clone Harboring a Promiscuous Plasmid Encoding Resistance to Fluoroquinolones and Third-Generation Cephalosporins. *MBio* 2018; **9**: e00105-18.

403 Ramachandran A, Shanthi M, Sekar U. Detection of blaCTX-M Extended Spectrum Betalactamase Producing Salmonella enterica Serotype Typhi in a Tertiary Care Centre. *J Clin Diagnostic Res* 2017; **11**: DC21-DC24.

404 Morita M, Takai N, Terajima J, *et al.* Plasmid-mediated resistance to cephalosporins in Salmonella enterica serovar Typhi. *Antimicrob Agents Chemother* 2010; **54**: 3991–2.

405 Pfeifer Y, Matten J, Rabsch W. Salmonella enterica serovar Typhi with CTX-M beta-lactamase, Germany. Emerg. Infect. Dis. 2009; **15**: 1533–5.

406 Phoba M-F, 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**: 1229–31.

407 Akinyemi KO, Iwalokun BA, Alafe OO, Mudashiru SA, Fakorede C. bla CTX-M-I group extended spectrum beta lactamase-producing Salmonella typhi from hospitalized patients in Lagos, Nigeria. *Infect Drug Resist* 2015; **8**: 99–106.

408 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.

409 Al-Emran HM, Eibach D, Krumkamp R, *et al.* A Multicountry Molecular Analysis of Salmonella enterica Serovar Typhi With Reduced Susceptibility to Ciprofloxacin in Sub-Saharan Africa. *Clin Infect Dis* 2016; **62(S1)**: S42-6.

410 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.

411 Mokdad AH. Estimates of global, regional, and national morbidity, mortality, and aetiologies of diarrhoeal diseases: a systematic analysis for the Global Burden of Disease Study 2015. *Lancet Infect Dis* 2017;

17: 909–48.
412 Karki M, Pandit S, Baker S, Basnyat B. Cotrimoxazole treats fluoroquinolone-resistant Salmonella typhi H58 infection. *BMJ Case Rep* 2016; : bcr-2016-217223.
413 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**: e310–23.
414 Se Eun Park. Severe Typhoid Fever Surveillance in Africa (SETA) Program: An Overview. In: 10th Internaltioal Conference on Typhoid and Other Invasive Salmonelloses. Kampala, 2017.
415 Garrett D. The surveillance for enteric fever in Asia project (SEAP): Estimating the community burden of enteric fever. *Int J Infect Dis* 2016; **45**: 64.
416 Surveillance for Enteric-Fever in Asia Project (SEAP): Phase II. http://chrfbd.org/surveillance-for-enteric-fever-in-asia-project-seap-phase-ii/ (accessed June 10, 2018).
417 Darton, Tc, Meiring JE, Tonks S PA. 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**. DOI:10.1136/bmjopen-2017-016283.
418 John J, Bavdekar A, Rongsen-Chandola T, Dutta S, Collaborators N, 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**. DOI:10.1186/s12889-018-5498-2.
419 Typhoid vaccine - Gavi, the Vaccine Alliance. https://www.gavi.org/support/nvs/typhoid/ (accessed June 10, 2018).
420 Record E. Typhoid vaccines: WHO position paper. *Wkly Epidemiol Rec* 2008; **83**: 49–59.
421 Xu Y, Liu B, Gröndahl-Yli-Hannuksila K, *et al.* Whole-genome sequencing reveals the effect of vaccination on the evolution of Bordetella pertussis. *Nat Publ Gr* 2015. DOI:10.1038/srep12888.

Appendices

Appendix 1: List of isolates and accession numbers

Sample accession number	Lane accession number	Sanger_lane	Organism
ERS1545626	ERR2213822	22027_3#5	S. Typhi
ERS1545622	ERR2213818	22027_3#1	S. Typhi
ERS1545631	ERR2213827	22027_3#10	S. Typhi
ERS1545197	ERR2213854	22027_3#37	S. Typhi
ERS1545223	ERR2213879	22027_3#62	S. Typhi
ERS1545201	ERR2213857	22027_3#40	S. Typhi
ERS1545632	ERR2213828	22027_3#11	S. Typhi
ERS1545633	ERR2213829	22027_3#12	S. Typhi
ERS1545634	ERR2213830	22027_3#13	S. Typhi
ERS1545635	ERR2213831	22027_3#14	S. Typhi
ERS1545636	ERR2213832	22027_3#15	S. Typhi
ERS1545637	ERR2213833	22027_3#16	S. Typhi
ERS1545638	ERR2213834	22027_3#17	S. Typhi
ERS1545639	ERR2213835	22027_3#18	S. Typhi
ERS1545640	ERR2213836	22027_3#19	S. Typhi
ERS1545623	ERR2213819	22027_3#2	S. Typhi
ERS1545641	ERR2213837	22027_3#20	S. Typhi
ERS1545642	ERR2213838	22027_3#21	S. Typhi
ERS1545643	ERR2213839	22027_3#22	S. Typhi
ERS1545644	ERR2213840	22027_3#23	S. Typhi
ERS1545645	ERR2213841	22027_3#24	S. Typhi
ERS1545646	ERR2213842	22027_3#25	S. Typhi
ERS1545647	ERR2213843	22027_3#26	S. Typhi
ERS1545648	ERR2213844	22027_3#27	S. Typhi
ERS1545649	ERR2213845	22027_3#28	S. Typhi
ERS1545650	ERR2213846	22027_3#29	S. Typhi
ERS1545624	ERR2213820	22027_3#3	S. Typhi
ERS1545651	ERR2213847	22027_3#30	S. Typhi
ERS1545652	ERR2213848	22027_3#31	S. Typhi
ERS1545653	ERR2213849	22027_3#32	S. Typhi
ERS1545654	ERR2213850	22027_3#33	S. Typhi
ERS1545655	ERR2213851	22027_3#34	S. Typhi
ERS1545656	ERR2213852	22027_3#35	S. Typhi
ERS1545657	ERR2213853	22027_3#36	S. Typhi
ERS1545200	ERR2213856	22027_3#39	S. Typhi
ERS1545625	ERR2213821	22027_3#4	S. Typhi
ERS1545202	ERR2213858	22027_3#41	S. Typhi
ERS1545203	ERR2213859	22027_3#42	S. Typhi

ERS1545204	ERR2213860	22027_3#43	S. Typhi
ERS1545205	ERR2213861	22027_3#44	S. Typhi
ERS1545206	ERR2213862	22027_3#45	S. Typhi
ERS1545207	ERR2213863	22027_3#46	S. Typhi
ERS1545208	ERR2213864	22027_3#47	S. Typhi
ERS1545209	ERR2213865	22027_3#48	S. Typhi
ERS1545211	ERR2213867	22027_3#50	S. Typhi
ERS1545212	ERR2213868	22027_3#51	S. Typhi
ERS1545213	ERR2213869	22027_3#52	S. Typhi
ERS1545214	ERR2213870	22027_3#53	S. Typhi
ERS1545216	ERR2213872	22027_3#55	S. Typhi
ERS1545217	ERR2213873	22027_3#56	S. Typhi
ERS1545218	ERR2213874	22027_3#57	S. Typhi
ERS1545219	ERR2213875	22027_3#58	S. Typhi
ERS1545220	ERR2213876	22027_3#59	S. Typhi
ERS1545627	ERR2213823	22027_3#6	S. Typhi
ERS1545221	ERR2213877	22027_3#60	S. Typhi
ERS1545222	ERR2213878	22027_3#61	S. Typhi
ERS1545628	ERR2213824	22027_3#7	S. Typhi
ERS1545629	ERR2213825	22027_3#8	S. Typhi
ERS1545630	ERR2213826	22027_3#9	S. Typhi
ERS1545621	ERR1842968	20949_8#40	S. Typhi
ERS1545589	ERR1842964	20949_8#8	S. Typhi
ERS1545568	ERR1837290	20621_8#78	S. Typhi
ERS1545567	ERR1837289	20621_8#77	S. Typhi
ERS1545566	ERR1837288	20621_8#76	S. Typhi
ERS1545565	ERR1837287	20621_8#75	S. Typhi
ERS1545564	ERR1837286	20621_8#74	S. Typhi
ERS1545563	ERR1837285	20621_8#73	S. Typhi
ERS1545562	ERR1837284	20621_8#72	S. Typhi
ERS1545561	ERR1837283	20621_8#71	S. Typhi
ERS1545560	ERR1837282	20621_8#70	S. Typhi
ERS1545558	ERR1837281	20621_8#69	S. Typhi
ERS1545557	ERR1837280	20621_8#68	S. Typhi
ERS1545555	ERR1837279	20621_8#67	S. Typhi
ERS1545554	ERR1837278	20621_8#66	S. Typhi
ERS1545551	ERR1837275	20621_8#63	S. Typhi
ERS1545550	ERR1837274	20621_8#62	S. Typhi
ERS1545549	ERR1837273	20621_8#61	S. Typhi
ERS1545548	ERR1837272	20621_8#60	S. Typhi
ERS1545547	ERR1837271	20621_8#59	S. Typhi
ERS1545546	ERR1837270	20621_8#58	S. Typhi
ERS1545545	ERR1837269	20621_8#57	S. Typhi
ERS1545544	ERR1837268	20621_8#56	S. Typhi
ERS1545543	ERR1837267	20621_8#55	S. Typhi

ERS1545542	ERR1837266	20621_8#54	S. Typhi
ERS1545541	ERR1837265	20621_8#53	S. Typhi
ERS1545540	ERR1837264	20621_8#52	S. Typhi
ERS1545539	ERR1837263	20621_8#51	S. Typhi
ERS1545538	ERR1837262	20621_8#50	S. Typhi
ERS1545537	ERR1837261	20621_8#49	S. Typhi
ERS1545536	ERR1837260	20621_8#48	S. Typhi
ERS1545535	ERR1837259	20621_8#47	S. Typhi
ERS1545534	ERR1837258	20621_8#46	S. Typhi
ERS1545533	ERR1837257	20621_8#45	S. Typhi
ERS1545532	ERR1837256	20621_8#44	S. Typhi
ERS1545531	ERR1837255	20621_8#43	S. Typhi
ERS1545530	ERR1837254	20621_8#42	S. Typhi
ERS1545529	ERR1837253	20621_8#41	S. Typhi
ERS1545528	ERR1837252	20621_8#40	S. Typhi
ERS1545527	ERR1837251	20621_8#39	S. Typhi
ERS1545526	ERR1837250	20621_8#38	S. Typhi
ERS1545525	ERR1837249	20621_8#37	S. Typhi
ERS1545524	ERR1837248	20621_8#36	S. Typhi
ERS1545523	ERR1837247	20621_8#35	S. Typhi
ERS1545522	ERR1837246	20621_8#34	S. Typhi
ERS1545521	ERR1837245	20621_8#33	S. Typhi
ERS1545520	ERR1837244	20621_8#32	S. Typhi
ERS1545519	ERR1837243	20621_8#31	S. Typhi
ERS1545518	ERR1837242	20621_8#30	S. Typhi
ERS1545517	ERR1837241	20621_8#29	S. Typhi
ERS1545516	ERR1837240	20621_8#28	S. Typhi
ERS1545515	ERR1837239	20621_8#27	S. Typhi
ERS1545514	ERR1837238	20621_8#26	S. Typhi
ERS1545513	ERR1837237	20621_8#25	S. Typhi
ERS1545512	ERR1837236	20621_8#24	S. Typhi
ERS1545511	ERR1837235	20621_8#23	S. Typhi
ERS1545509	ERR1837233	20621_8#21	S. Typhi
ERS1545508	ERR1837232	20621_8#20	S. Typhi
ERS1545507	ERR1837231	20621_8#19	S. Typhi
ERS1545506	ERR1837230	20621_8#18	S. Typhi
ERS1545505	ERR1837229	20621_8#17	S. Typhi
ERS1545504	ERR1837228	20621_8#16	S. Typhi
ERS1545503	ERR1837227	20621_8#15	S. Typhi
ERS1545502	ERR1837226	20621_8#14	S. Typhi
ERS1545501	ERR1837225	20621_8#13	S. Typhi
ERS1545500	ERR1837224	20621_8#12	S. Typhi
ERS1545499	ERR1837223	20621_8#11	S. Typhi
ERS1545498	ERR1837222	20621_8#10	S. Typhi
ERS1545497	ERR1837221	20621_8#9	S. Typhi

ERS1545496	ERR1837220	20621_8#8	S. Typhi
ERS1545495	ERR1837219	20621_8#7	S. Typhi
ERS1545494	ERR1837218	20621_8#6	S. Typhi
ERS1545493	ERR1837217	20621_8#5	S. Typhi
ERS1545492	ERR1837216	20621_8#4	S. Typhi
ERS1545491	ERR1837215	20621_8#3	S. Typhi
ERS1545490	ERR1837214	20621_8#2	S. Typhi
ERS1545489	ERR1837213	20621_8#1	S. Typhi
ERS1545488	ERR1837212	20087_8#79	S. Typhi
ERS1545487	ERR1837211	20087_8#78	S. Typhi
ERS1545486	ERR1837210	20087_8#77	S. Typhi
ERS1545481	ERR1837205	20087_8#72	S. Typhi
ERS1545479	ERR1837203	20087_8#70	S. Typhi
ERS1545478	ERR1837202	20087_8#69	S. Typhi
ERS1545477	ERR1837201	20087_8#68	S. Typhi
ERS1545472	ERR1837196	20087_8#63	S. Typhi
ERS1545469	ERR1837193	20087_8#60	S. Typhi
ERS1545468	ERR1837192	20087_8#59	S. Typhi
ERS1545467	ERR1837191	20087_8#58	S. Typhi
ERS1545466	ERR1837190	20087_8#57	S. Typhi
ERS1545465	ERR1837189	20087_8#56	S. Typhi
ERS1545464	ERR1837188	20087_8#55	S. Typhi
ERS1545463	ERR1837187	20087_8#54	S. Typhi
ERS1545460	ERR1837184	20087_8#51	S. Typhi
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ERS1545456	ERR1837180	20087_8#47	S. Typhi
ERS1545455	ERR1837179	20087_8#46	S. Typhi
ERS1545454	ERR1837178	20087_8#45	S. Typhi
ERS1545453	ERR1837177	20087_8#44	S. Typhi
ERS1545452	ERR1837176	20087_8#43	S. Typhi
ERS1545451	ERR1837175	20087_8#42	S. Typhi
ERS1545450	ERR1837174	20087_8#41	S. Typhi
ERS1545449	ERR1837173	20087_8#40	S. Typhi
ERS1545448	ERR1837172	20087_8#39	S. Typhi
ERS1545447	ERR1837171	20087_8#38	S. Typhi
ERS1545445	ERR1837169	20087_8#36	S. Typhi
ERS1545444	ERR1837168	20087_8#35	S. Typhi
ERS1545443	ERR1837167	20087_8#34	S. Typhi
ERS1545442	ERR1837166	20087_8#33	S. Typhi
ERS1545441	ERR1837165	20087_8#32	S. Typhi
ERS1545440	ERR1837164	20087_8#31	S. Typhi
ERS1545439	ERR1837163	20087_8#30	S. Typhi
ERS1545438	ERR1837162	20087_8#29	S. Typhi
ERS1545437	ERR1837161	20087_8#28	S. Typhi

ERS1545436	ERR1837160	20087_8#27	S. Typhi
ERS1545435	ERR1837159	20087_8#26	S. Typhi
ERS1545434	ERR1837158	20087_8#25	S. Typhi
ERS1545433	ERR1837157	20087_8#24	S. Typhi
ERS1545432	ERR1837156	20087_8#23	S. Typhi
ERS1545431	ERR1837155	20087_8#22	S. Typhi
ERS1545430	ERR1837154	20087_8#21	S. Typhi
ERS1545428	ERR1837152	20087_8#19	S. Typhi
ERS1545427	ERR1837151	20087_8#18	S. Typhi
ERS1545426	ERR1837150	20087_8#17	S. Typhi
ERS1545425	ERR1837149	20087_8#16	S. Typhi
ERS1545424	ERR1837148	20087_8#15	S. Typhi
ERS1545423	ERR1837147	20087_8#14	S. Typhi
ERS1545422	ERR1837146	20087_8#13	S. Typhi
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ERS1545416	ERR1837140	20087_8#7	S. Typhi
ERS1545415	ERR1837139	20087_8#6	S. Typhi
ERS1545414	ERR1837138	20087_8#5	S. Typhi
ERS1545413	ERR1837137	20087_8#4	S. Typhi
ERS1545412	ERR1837136	20087_8#3	S. Typhi
ERS1545411	ERR1837135	20087_8#2	S. Typhi
ERS1545410	ERR1837134	20087_8#1	S. Typhi
ERS1545421	ERR1837145	20087_8#12	S. Paratyphi A
ERS1545429	ERR1837153	20087_8#20	S. Paratyphi A
ERS1545457	ERR1837181	20087_8#48	S. Paratyphi A
ERS1545461	ERR1837185	20087_8#52	S. Paratyphi A
ERS1545462	ERR1837186	20087_8#53	S. Paratyphi A
ERS1545470	ERR1837194	20087_8#61	S. Paratyphi A
ERS1545471	ERR1837195	20087_8#62	S. Paratyphi A
ERS1545474	ERR1837198	20087_8#65	S. Paratyphi A
ERS1545475	ERR1837199	20087_8#66	S. Paratyphi A
ERS1545476	ERR1837200	20087_8#67	S. Paratyphi A
ERS1545480	ERR1837204	20087_8#71	S. Paratyphi A
ERS1545483	ERR1837207	20087_8#74	S. Paratyphi A
ERS1545484	ERR1837208	20087_8#75	S. Paratyphi A
ERS1545485	ERR1837209	20087_8#76	S. Paratyphi A
ERS1545510	ERR1837234	20621_8#22	S. Paratyphi A
ERS1545553	ERR1837277	20621_8#65	S. Paratyphi A
ERS1545569	ERR1837291	20621_8#79	S. Paratyphi A
ERS1545570	ERR1837292	20621_8#80	S. Paratyphi A
ERS1545571	ERR1837293	20621_8#81	S. Paratyphi A
ERS1545572	ERR1837294	20621_8#82	S. Paratyphi A

ERS1545573	ERR1837295	20621_8#83	S. Paratyphi A
ERS1545574	ERR1837296	20621_8#84	S. Paratyphi A
ERS1545575	ERR1837297	20621_8#85	S. Paratyphi A
ERS1545576	ERR1837298	20621_8#86	S. Paratyphi A
ERS1545577	ERR1837299	20621_8#87	S. Paratyphi A
ERS1545578	ERR1837300	20621_8#88	S. Paratyphi A
ERS1545579	ERR1837301	20621_8#89	S. Paratyphi A
ERS1545580	ERR1837302	20621_8#90	S. Paratyphi A
ERS1545581	ERR1837303	20621_8#91	S. Paratyphi A
ERS1545582	ERR1837304	20949_8#1	S. Paratyphi A
ERS1545591	ERR1837307	20949_8#10	S. Paratyphi A
ERS1545592	ERR1837308	20949_8#11	S. Paratyphi A
ERS1545593	ERR1837309	20949_8#12	S. Paratyphi A
ERS1545594	ERR1837310	20949_8#13	S. Paratyphi A
ERS1545595	ERR1837311	20949_8#14	S. Paratyphi A
ERS1545596	ERR1837312	20949_8#15	S. Paratyphi A
ERS1545597	ERR1837313	20949_8#16	S. Paratyphi A
ERS1545598	ERR1837314	20949_8#17	S. Paratyphi A
ERS1545600	ERR1837316	20949_8#19	S. Paratyphi A
ERS1545583	ERR1837305	20949_8#2	S. Paratyphi A
ERS1545602	ERR1837318	20949_8#21	S. Paratyphi A
ERS1545603	ERR1837319	20949_8#22	S. Paratyphi A
ERS1545604	ERR1837320	20949_8#23	S. Paratyphi A
ERS1545605	ERR1837321	20949_8#24	S. Paratyphi A
ERS1545606	ERR1837322	20949_8#25	S. Paratyphi A
ERS1545607	ERR1837323	20949_8#26	S. Paratyphi A
ERS1545608	ERR1837324	20949_8#27	S. Paratyphi A
ERS1545609	ERR1837325	20949_8#28	S. Paratyphi A
ERS1545610	ERR1837326	20949_8#29	S. Paratyphi A
ERS1545611	ERR1837327	20949_8#30	S. Paratyphi A
ERS1545614	ERR1837330	20949_8#33	S. Paratyphi A
ERS1545616	ERR1837332	20949_8#35	S. Paratyphi A
ERS1545617	ERR1837333	20949_8#36	S. Paratyphi A
ERS1545618	ERR1837334	20949_8#37	S. Paratyphi A
ERS1545619	ERR1842966	20949_8#38	S. Paratyphi A
ERS1545620	ERR1842967	20949_8#39	S. Paratyphi A
ERS1545585	ERR1842960	20949_8#4	S. Paratyphi A
ERS1545586	ERR1842961	20949_8#5	S. Paratyphi A
ERS1545587	ERR1842962	20949_8#6	S. Paratyphi A
ERS1545588	ERR1842963	20949_8#7	S. Paratyphi A
ERS1545590	ERR1842965	20949_8#9	S. Paratyphi A
ERS1545210	ERR2213866	22027_3#49	S. Paratyphi A
ERS1545248	ERR2213880	22027_3#63	S. Paratyphi A
ERS1545249	ERR2213881	22027_3#64	S. Paratyphi A
ERS1545250	ERR2213882	22027_3#65	S. Paratyphi A

ERS1545251	ERR2213883	22027_3#66	S. Paratyphi A

Appendix 2: Risk of bias assessment for studies included in chapter 3

Included Studies	Risk of bias domains (QUIPS)				
	Study population	**Measurement of outcome(s)**	**Study Attrition**	**Statistical analysis and reporting**	**Risk of Bias**
First author	Domain 1	Domain 2	Domain 3	Domain 4	
Breiman (2012)[324]	Low	Low	Low	Low	**Low**
Feasy (2015)[308]	Low	Low	Low	Low	**Low**
Hendriksen (2014)[325]	Low	Low	Low	Low	**Low**
Lungya (2012)[326]	Low	Low	Low	Low	**Low**
Feasy (2010)[130]	Low	Low	Low	Low	**Low**
Maltha (2014)[327]	Moderate	Low	Low	Low	**Low**
Marks (2010)[328]	Low	Low	Low	Low	**Low**
Phoba (2014)[329]	Low	Moderate	Low	Low	**Low**
Maltha (2014)[327]	Low	Low	Low	Low	**Low**
Marks (2010)[328]	Low	Low	Low	Low	**Low**
Phoba (2014)[329]		Moderate	Low	Low	**Low**
Abucejo (2001)[319]	Low	Low	Low	Low	**Low**
Bajrachatya (2014)[320]	Low	Low	Moderate	Low	**Low**
Gosh (2010)[135]	Low	Low	Low	Moderate	**Moderate**
Holt (2010)[128]	Moderate	Low	Low	Low	**Low**
Karkey (2010)[321]	Low	Low	Low	Low	**Low**
Kelly (2010)[322]	Low	Low	Low	Low	**Low**
Kumar (2008)[129]	Low	Low	Low	Low	**Low**
Malla (2007)[323]	Low	Low	Low	Low	**Low**
Ochiai (2008)[133]	Low	Low	Low	Low	**Low**
Ochiai (2008)[133]	Low	Low	Low	Low	**Low**
Parry (2014)[313]	Low	Low	Low	Low	**Low**
Pradhan (2012)[314]	Low	Low	Low	Low	**Low**
Saha (2001)[309]	Moderate	Low	Moderate	Low	**Moderate**
Saha (2003)[315]	Low	Low	Low	Low	**Low**
Shakva (2008)[316]	Low	Low	Low	Low	**Low**
Verma (2007)[317]	Low	Low	Low	Low	**Low**
Walia (2006)[318]	Low	Low	Low	Low	**Low**

Appendix 3: Risk of bias assessment for studies included in chapter 8

Objective 1: Phenotypic trends of AMR in typhoid fever													
Included Studies			Risk of bias domains (QUIPS)					Risk of bias domains (JBI)					
			Study population	**Measurement of outcome(s)**	**Study Attrition**	**Statistical analysis and reporting**	**Risk of Bias**	**Population description**	**Prospective sampling**	**Microbiologic methods**	**Sample size >200**	**Performance standards used**	**Risk of Bias**
First author	Publication year	PubMed IDentifier	Domain 1	Domain 2	Domain 3	Domain 4		Q1	Q2	Q3	Q4	Q5	
Ali A	2017	28303985	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Harichandran D	2017	28352198	Low	Low	Low	Moderate	**Low**	Yes	No	Yes	Yes	Yes	**No**
Osbourne LG	2016	26243802	Low	Low	NA	Low	**Low**	Yes	NA	Yes	Yes	Yes	**Yes**
Sharvani R	2013	27437211	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Misra R	2016	25979527	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Khanam F	2015	25849611	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Mahende C	2015	26138060	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	Yes	Yes	**No**
Narain U	2015	26388636	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Nüesch-Inderbinen M	2015	25963025	Moderate	Low	Low	Low	**Low**	No	No	Yes	Yes	Yes	**No**
Jessica Maltha	2014	24551225	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	Yes	Yes	**No**
Srirangaraj S	2014	24817913	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Chiou CS	2014	25136011	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Chand HJ	2014	25390062	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	Yes	Yes	**No**
Isendahl J	2014	25526763	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	Yes	Yes	**Yes**
Dahiya S	2014	28303820	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Choudhary A	2013	23703350	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Sultan BA	2013	23905456	Low	Low	NA	Low	**Low**	No	NA	Yes	No	Yes	**Yes**

Vlieghe E	2013	24094060	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Venkatesh BM	2013	24441263	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Gupta V	2013	24043999	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Jain S	2013	24240035	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Lutterloh E	2012	22357702	Moderate	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Olut AI	2012	22399179	Low	Low	NA	Low	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Acharya D	2012	22627312	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Kumar Y	2011	21444993	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Adhikary	2011	22234135	Low	Low	NA	Low	Low	Yes	NA	Yes	No	Yes	**Yes**
Gross U	2011	22000360	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Nagshetty K	2010	20212336	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Verma S	2010	20061765	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Mengo DM	2010	20601792	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Muyembe-Tamfum JJ	2009	19174300	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Kumar Y	2009	19762961	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Yanagi D	2009	19631095	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Lynch MF	2009	19706859	Moderate	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Mirza SH	2008	18452661	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Prajapati B	2008	19558061	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Al-Sanouri TM	2008	19741292	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Rodrigues C	2007	1307533	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Joshi S	2007	16950486	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Parry CM	2007	17145784	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	No	Yes	**No**
Bhatta DR	2007	17576218	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Akinyemi KO	2007	18330069	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	No	Yes	**No**
Tamang MD	2007	17629465	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Capoor MR	2007	17873998	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Banerjee A	2007	27408039	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Khanal B	2007	17615907	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Chau TT	2007	17908946	Low	Low	Low	Low	**Low**	Yes	No	Yes	Low	Yes	**No**
Akinyemi KO	2007	18087113	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Manchanda V	2006	16687859	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Ray P	2006	16926465	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Mohanty S	2006	16476168	Low	Low	Low	Low	**Low**	Yes	No	Yes	Low	Yes	**No**
Rahman	2006	16490150	Low	Low	Low	Low	**Low**	Yes	No	Yes	Low	Yes	**No**

Lakshmi V	2006	16505555	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Brooks WA	2005	15752457	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Dutta S	2005	15793167	Low	Low	Low	Low	**Low**	Yes	No	Yes	Low	Yes	**No**
Senthilkumar B	2005	15928436	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Madhulika U	2004	15347861	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Mamun KZ	2004	16240978	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Gautam V	2002	12585971	Low	Low	Low	Low	**Low**	Yes	No	Yes	Low	Yes	**No**
Kadhiravan T	2005	15904505	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Butler T	1977	324398	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Lawrence RM	1973	4572522	Low	Low	NA	Low	**Low**	No	NA	Yes	No	Yes	**Yes**
Overturf G	1973	4763412	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**

Included Studies			Risk of bias domains (QUIPS) (Hayden et al. 2013)					Risk of bias domains (JBI)					
			Study population	**Measurement of outcome(s)**	**Study Attrition**	**Statistical analysis and reporting**	**Risk of Bias**	**Population description**	**Prospective sampling**	**Bacteriologic culture Methods**	**Sample size over 100**	**Genotyping method**	**Risk of Bias**
First author	Publication year	PubMed IDentifier	Domain 1	Domain 2	Domain 3	Domain 4		Q1	Q2	Q3	Q4	Q5	
Klemm E	2018	29463654	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Ramachandran A	2017	29207706	Low	Low	Moderate	Low	**Low**	Yes	No	Yes	No	No	**No**
Das S	2017	27916384	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	No	**No**
Gopal M	2016	27630841	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	No	**No**
Misra	2016	27618918	Low	Low	Low	NA	**Low**	Yes	No	Yes	Yes	No	**No**
Ragupathi D	2016	27530999	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**No**
Elumalai S	2016	27166067	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	No	**Yes**
Al-Emran HM	2016	26933020	Low	Low	Low	Low	**Low**	Yes	Yes	Yes	No	Yes	**No**
Thanh D	2016	26974227	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	Yes	**No**
Wong V	2016	27657909	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
García-Fernández A	2015	26121266	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**Yes**
Nüesch-Inderbinen M	2015	25963025	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**Yes**
Akinyemi KO	2015	25999745	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**Yes**
Ceyssens PJ	2015	25385108	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**Yes**
Wong V	2015	25961941	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	Yes	**No**
Chiou CS	2014	25136011	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Dutta S	2014	25098613	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
González-López JJ	2014	25340972	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Dahiya S	2014	25027085	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Saleh FO	2014	24820472	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Geetha VK	2014	24399384	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Lee CJ	2013	23465712	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Jain S	2013	24240035	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	No	**No**
Lunguya O	2012	23166855	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Vlieghe ER	2012	23272255	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Emary K	2012	23122884	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	No	**No**
Thamizhmani R	2012	22885270	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Tatavarthy A	2012	22649021	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**

Acharya D	2012	22627312	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Ahmed D	2012	22442289	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Koirala KD	2012	22371897	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Kumarasamy K	2012	22146877	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Accou-Demartin M	2011	21749778	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Hassing RJ	2011	21227657	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Mohanty S	2010	20828458	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Gaborieau V	2010	20724089	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Nath G	2010	20188522	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Morita M	2010	20585124	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Wu W	2010	20113512	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Dimitrov T	2010	19889623	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Pfeifer Y	2009	19788837	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Yanagi D	2009	19631095	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Yoon HJ	2009	19259362	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Dimitrov T	2009	18971360	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Capoor MR	2009	18687156	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Dashti AA	2008	18606582	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Rotimi VO	2008	18566147	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Dutta S	2008	18280709	Low	Low	Moderate	NA	**Low**	Yes	NA	Yes	No	Yes	**Yes**
Al-Sanouri	2008	19741292	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Chau TT	2007	17908946	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Chau TT	2007	17908946	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Chau TT	2007	17908946	Low	Low	Low	Low	**Low**	Yes	No	Yes	Yes	No	**No**
Capoor MR	2007	17873998	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Tamang MD	2007	17629465	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Gaind R	2006	17071955	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shirakawa T	2006	16466897	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Lee K	2004	15504831	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Renuka K	2004	15256030	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Mills-Robertson F	2002	12399042	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shanahan PM	2000	10722124	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shanahan PM	2000	10722124	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shanahan PM	2000	10722124	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shanahan PM	2000	10722124	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Shanahan PM	2000	10722124	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**

Shanahan PM	1998	9620383	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Wain J	1997	9431387	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**
Panigrahi D	1996	8765450	Low	Low	Low	Low	**Low**	Yes	No	Yes	No	No	**No**

Quality In Prognosis Studies tool (QUIPS) Domains : (Hayden et al, 2013)

Domain 1: **Study population**	The study sample sufficiently represents the population of interest to minimise the potential risk of bias to the results.
Domain 2: **Measurement of outcomes**	The outcomes of interest measured adequately and similarly for all isolates in the study to minimise potential risk of bias to the results.
Domain 3: **Study Attrition**	The available study data sufficiently represent the entire study sample
Domain 4: **Exposure measurement**	*Not applicable to this systematic review*
Domain 5: **Statistical data, analysis and reporting**	Reliable data, appropriate statistical analysis for study design, and all outcome measures are reported

Joanna Briggs Institute (JBI) tool (The Joanna Briggs Institute - Reviewer's manual) and modifications from Tadesse *et al, 2018*

Quality item 1:	Was the sampling/target population described?
Quality item 2:	Was sampling prospective?
Quality item 3:	Was microbiological culture and adequate quality control of disc diffusion/MIC methods applied?
Quality item 4:	Was the sample size >200 for Objective 1 and > 100 for phenotype 2
Quality item 5:	Performance standards adequately and appropriately described for objective 1 and whether whole genome sequencing was used for objective 2

The first tool aids in reporting whether each study has a low, moderate or high risk of bias while the second tool reports whether there is a risk of bias or not as a simple binary outcome.

Appendix 4: Antibiotic disc concentrations and respective breakpoints taken from the CSLI breakpoint table.

Antibiotic disc	Disc concentration (μg)	Sensitive if diameter greater than or equal to (mm)	Resistant if less than (mm)
Chloramphenicol	30	21	21
Erythromycin	15	22	19
Tetracycline	30	25	22
Trimethoprim-sulfamethoxazole[1]	1.25-23.75	18	15
Oxacillin (benzylpenicillin breakpoint)	1	20	20
Oxacillin (amoxicillin breakpoint)	1	8	8
Norfloxacin	10	12	12