Antimicrobial Resistance and Gallbladder Carriage of *Salmonella* Typhi and *Salmonella* Paratyphi A in Kathmandu, Nepal

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A thesis submitted for the degree of Doctor of Philosophy

June 2013

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Abstract

Enteric fever remains the most common febrile illness in urban Nepal. Some individuals may have recurrent infection and some may even progress to become long term chronic carriers. The aim of this thesis was to investigate the rate and factors leading to relapse with typhoid fever in patients who were enrolled in clinical treatment trials for acute enteric fever. The results show that relapses in enteric fever is a common complication and is more likely to be associated with the treatment antimicrobial, cefixime. Gallbladder carriage of invasive *Salmonella* is considered fundamental in sustaining enteric fever transmission as humans are the only known natural host. This thesis, therefore, also aimed to investigate the prevalence, characteristics, immunological responses, and mechanism of carriage of invasive *Salmonella* in the gallbladder by examining bile and tissue obtained from individuals who underwent cholecystectomy in Kathmandu. Data presented here demonstrate that *S.* Paratyphi A is almost as prevalent as *S*. Typhi in the gallbladder and that carriage may not be driven by antimicrobial resistance. Gallbladders that contained *Salmonella* were more likely to show evidence of acute inflammation with extensive neutrophil infiltrate. Chronic carriers were found to have dramatically elevated levels of IgG to O:2 and Vi antigens with high bactericidal activity yet low pro-inflammatory cytokine levels suggesting that *Salmonella* are stimulating a constant immunological response, in the form of antibody. *S*. Typhi may be controlling the inflammatory process through the expression of the Vi capsule in the gallbladder. Genome sequencing of *S.* Typhi isolated from chronic carriers were different from those *S.* Typhi causing acute disease. These data question the current dogmas surrounding the carriage of *S*. Typhi in gallbladder and predict a pivotal role of Vi capsule and gallstones in maintaining carriage. Therefore, prospectively identifying these individuals is paramount for rapid local and regional elimination. Furthermore, combining cytokine profiles and antibody levels may be a method of prospectively detecting carriers in the general population.

Acknowledgements

This research project would not have been possible without the help and support from many people. First and foremost, I would like to express my enduring gratitude to my supervisor Dr Stephen Baker for providing me invaluable guidance, advice and support. His willingness to motivate me contributed tremendously to complete this project.

I would like to express my heartfelt thanks to everyone at the Oxford University Clinical Research Unit, Vietnam for providing me help and support. Words are inadequate in offering my thanks particularly to Tran Vu Thieu Nga and Corinne Thompson for helping with Lab work and data analysis. I am also grateful to Prof. Jeremy Farrar and Dr Christiane Dolecek for kind support.

I am grateful to my colleagues at Oxford University Clinical Research Unit, Nepal, particularly Abhilasha Karkey, Bijaya Karanjit, Amit Arjyal, Samir Koirala, and CMAs for their constant help and support. Also, I owe my thanks to Dr Buddha Basnyat for the support and the encouragement throughout my study period.

Finally yet importantly, I would like to express my heartfelt thanks to my husband Jawahar, children Jessica and Jason. They have been my support system with their patience and encouraging me all the way. I would also like to thank my dad who provided me with unwavering moral and emotional support .His care and concern was a big motivating force to complete this work.

Declaration

The majority of work presented in this thesis is my own and was conducted under the supervision of my supervisor Dr Stephen Baker at the Oxford University Clinical Research Unit in Vietnam. Some parts of the work were not my own and they were carried out in collaboration with other persons. The phylogenetic analysis of the *S.* Typhi population was performed in the lab of Prof. Gordon Dougan at Wellcome Trust Sanger Institute. Electron microscopy of *S.* Typhi was performed with the help of Mr David Goulding at the Wellcome Trust Sanger Institute in Cambridge, UK. Similarly, Gallstones recovered from cholecystectomy patients were studied in collaboration with Professor John Gunn at the University of Ohio, USA. Information from this study has not been submitted for any degree or other qualification elsewhere.

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Abbreviations

ATCC	American Type Culture Collection
CDC	Center for disease control
CFU	Colony forming units
CLSI	Clinical and Laboratory Standards Institute
CMA	Community medical assistant
DCA	Desoxycholate citrate agar
ELISA	Enzyme linked immunosorbent assay
ESBL	Extended spectrum beta lactamases
H and E	Hematoxylin and eosin
HIV	Human immune deficiency virus
Ig	Immunoglobulin
IL	Interleukin
LPS	Lipopolysaccharide
LSMC	Lalitpur sub-metropolitan city
MA	MacConkey agar
MH	Mueller-Hinton
MIC	Minimum inhibitory concentration
MDR	Multi drug resistant
NA	Nalidixic acid
NHRC	Nepal health research council

NTS	Non-typhoidal *Salmonella*
OXTREC	Oxford tropical research ethics committee
PBST	Phosphate-buffered saline containing 0.05% Tween 20
PCR	Polymerase chain reaction
RBS	Baby Rabbit Complement
S. Paratyphi	*Salmonella* Paratyphi
S. Typhi	*Salmonella* Typhi
S. Typhimurium	*Salmonella* Typhimurium
SEL	Selenite broth
SNP	Single nucleotide polymorphism
SPI	*Salmonella* pathogenecity island
Spp.	Species
TNF	Tumour necrosis factor
WHO	World Health Organisation
XLD	Xylose Lysine Desoxycholate Agar

1 Introduction

1.1 The genus *Salmonella*

Salmonella are a genus of Gram-negative, facultative anaerobic, rod- shaped, motile bacteria belonging to the family Enterobacteriaceae. The Enterobacteriaceae are named as such as they generally inhabit the intestinal tract of warm and cold-blooded animals. This bacterial family consist of a large number of organisms that share a number of similar phenotypic and biochemical properties. The *Salmonellae* are estimated to have diverged genetically from *Escherichia coli*, also a member of the Enterobacteriaceae, approximately 100-150 million years ago[1]. The two genuses still share common survival and transmission mechanisms. The genus *Salmonella* is named after an American veterinary pathologist Daniel Elmer Salmon, who discovered the genus in 1885 [2].

1.1.1 Nomenclature and classification

The genus *Salmonella* is divided into two species: *Salmonella enterica* and *Salmonella bongori* [3]. *Salmonella enterica* consists of six subspecies, differentiated by their biochemical and antigenic characteristics and their genome phylogeny [4, 5]. Subspecies IIIa and IIIb were historically considered a separate genus, and called Arizonae. *Salmonella bongori* was originally designated as a *S. enterica* subspecies, it is now recognized as a separate species of *Salmonella* [6]. The two *Salmonella* species are

genetically diverse and have adapted over time to colonize many different niches and infect different hosts, including both cold- and warm-blooded animals, and are capable of free survival for extended periods in the environment. Infection with *Salmonella* can cause a range of infections in a range of hosts, that can be asymptomatic, that can be local, e.g. gastroenteritis, or systemic, e.g. typhoid fever [1]. *Salmonella* subspecies organisms are usually only isolated from humans and warm-blooded animals. Serotypes in subspecies I include the pathogens *Salmonella* Typhi (*S*. Typhi) and *Salmonella* Paratyphi A (*S*. Paratyphi A) which cause typhoid (enteric) fever (n.b. the term enteric fever is associated with fever caused by invasive *Salmonella,* typhoid or typhoid fever is enteric fever caused specifically with *Salmonella* Typhi*,* paratyphoid fever is enteric fever caused specifically with *Salmonella* Paratyphi pathovars, these terms will be used where appropriate throughout this thesis). Strains from the remaining subspecies of *S. enterica* and species of *S. bongori* are typically associated with cold-blooded animals and the environment.

The World Health Organization (WHO) Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France [5] has established an international nomenclature system for *Salmonella*. Serotypes (serovars) belonging to *S. enterica* subsp*. enterica* (subspecies I) are designated with a name that is related to the geographical place where the serotype was first isolated. However, confusion in *Salmonella* nomenclature exists because serotypes of *Salmonella* were historically considered different species. To emphasise the fact that they are not separate species, the serotype name is not italicised and the first letter of the serotype is capitalised. In

the first citation of a serotype in a document, the genus name is given followed by the word “serotype” or the abbreviation “ser.” and the serotype name (for example, *Salmonella* serotype or ser. Typhimurium). Table 1.1 summarizes the nomenclature that is currently in use at the US Centers for Disease Control (CDC). Serotypes belonging to the other subspecies are designated by their antigenic formulae following the subspecies name (for example, *S. enterica* subsp. *salamae* ser.50: z: e, n, x or *Salmonella* serotype II 50: z: e, n, x).

Table 1.1: *Salmonella* nomenclature in use at CDC, 2000[5]

Taxonomic position	**Nomenclature**
Genus (italics)	*Salmonella*
Species (italics)	*enterica*, which includes six subspecies I *enterica*, II *salamae*, IIIa *arizonae* IIIb *diarizonae*, IV *houtanae* and VI *indica*
Serotype (capitalised, not italicised)	The first time a serotype is mentioned in the text; the name should be preceded by the word "serotype" or "ser".
	Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori*
	Members of subspecies II, IV and VI and *S. bongori* retain their names if named before 1966

1.1.2 Serotypes of *Salmonella*: The Kauffman-White classification system

For clinical classification of the *Salmonella* genus, the Kauffmann-White serological classification is followed [7]. *Salmonella* serotyping is a subtyping method that has proven to be invaluable for differentiating isolates, particularly for public health purposes such as surveillance and outbreak investigations. First, the 'O' antigen type is determined based on polysaccharides associated with lipopolysaccharide. Then, the flagellar proteins determine the 'H' antigen. H antigens are further divided into phase 1 and phase 2. Currently, 2,610 serotypes of *S. enterica* belonging to six subspecies have been identified [8].

1.1.2.1 Somatic/ polysaccharide antigen ('O' antigen)

The *Salmonella* O-antigen is a polysaccharide antigen that comprises the outermost component of the bacterial lipopolysaccharide (LPS). Figure 1.1 shows a schematic diagram of LPS in Gram negative bacteria. LPS is a polymer of O-antigen subunits; each O-antigen subunit is typically composed of four to six sugars depending on the O-antigen. The outermost surface component of the core polysaccharide is an O polysaccharide consisting of terminal O repeat units. In some *Salmonella* serovars, for example Paratyphi A, this structure is exposed, whereas in others, the O polysaccharide is not exposed, such as *S.* Typhi, whose capsular Vi ("virulence") polysaccharide (a homopolymer of *N*-acetylgalacturonic acid) masks the O polysaccharide [9]. Variation

in the O-antigen results from variation in the sugar components of the O subunit, from variation in the nature of the covalent bond between the sugars of the subunit and finally, from variation in the nature of the linkage between the O subunits that form the O antigen polymer [10]. The terminal O polysaccharide of *Salmonella* varies in structure depending on the sugars and related linkages comprising the core unit. The various serogroups of *Salmonella* are defined, in part, by variation in O-antigen structure. *S.* Paratyphi A falls into serogroup A, *S.* Paratyphi B in serogroup B and *S.* Typhi in serogroup D. Repeating units the O-antigen of *Salmonella* serogroups A, B and D share a common trisaccharide backbone that consists of repeats of mannose, rhamnose and galactose [11]. O-antigens are designated by numbers and are divided into O serogroups or O groups; additionally, O groups are designated by the primary O factor(s) that are associated with the group. Many of the common O groups were originally designated by letter and are still commonly referred to by letter (e.g., serotype Typhimurium belongs to Group O: 4 or Group B, serotype Enteritidis belongs to group O: 9 or Group D1; serotype Paratyphi A belongs to Group O: 2 or Group A).

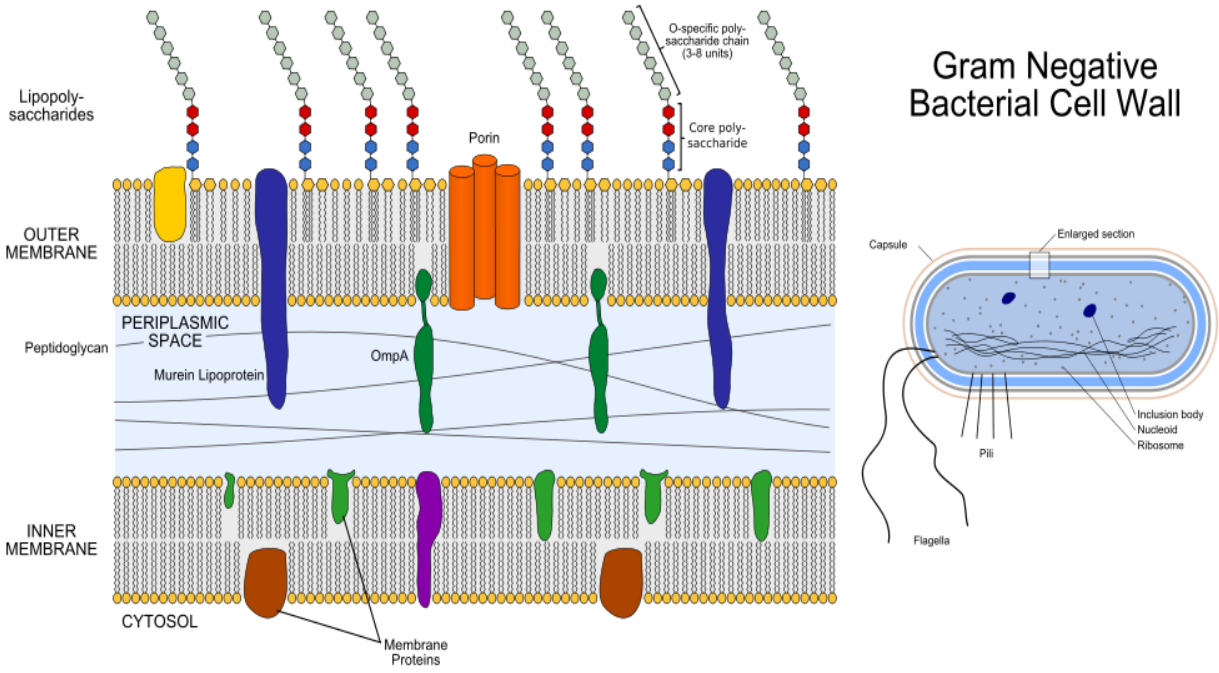


Figure 1.1: Schematic diagram of LPS of Gram negative bacteria

Image reproduced from Wikipedia (http://en.wikipedia.org/wiki/File:Gram negative _cell_wall.svg)

1.1.2.2 Flagellar antigen (H antigen)

The H antigen is the filamentous portion of the bacterial flagella; it is made up of repeating protein subunits called flagellin. The C' and N' termini of flagellin are conserved and give flagella its characteristic filamentous structure. The antigenically variable portion of flagellin is the central region, which is surface-exposed. *Salmonella* is unique among enteric bacteria in that most *Salmonella* can express two different flagellin antigens: phase 1 and phase 2 in a mutually exclusive manner. "Monophasic" isolates are those that express only one flagellin type. Most subspecies in serotypes IIIa and IV are monophasic or they develop through the inactivation of a flagellin gene. *S.* Typhi and *S*. Paratyphi A express only phase 1 flagellar antigens, H:a and H:d, respectively, while *S.* Paratyphi B expresses both phase 1 flagella H:b and phase 2 flagella H:1,2. [10] .

1.1.2.3 Vi polysaccharide capsule (Vi antigen)

The Vi antigen, first identified and described by Felix and Pitt in1934, is found on the surface of *S.* Typhi and *S.* Paratyphi C, as well as some strains of *S.* Dublin and the unrelated organism, *Citrobacter freundii* [12-14]. The polysaccharide capsule is called the "virulence" (Vi) factor because it was historically considered important for the survival and pathogenicity of the bacteria. Purified Vi antigen from *S*. Typhi is a linear homopolymer of alpha (1-4)-D-GalpANAc variably O acetylated at C-3 position [12] (Figure 1.2). Partial O deacetylation is reported to slightly increase the natural

immunogenicity while complete O deacetylation completely eliminates the immunogenicity of Vi [15].

Vi antigen expression is controlled by two separate chromosomal loci, *viaA* and *viaB.* The *viaA* locus is commonly found in many enteric bacteria. In contrast, the *viaB* locus is specific to Vi-expressing strains of *Salmonella* and *Citrobacter.* Genes required for the biosynthesis and the export of the Vi capsular antigen are encoded by the *viaB* locus, which, in *S*. Typhi, is located on a 134-kb *Salmonella* Pathogenicity Island (SPI)-7 [16]. On the basis of functional and bioinformatic analysis, it has been shown that SPI-7 has a mosaic structure and has evolved as a consequence of several independent insertion events [17]. The *viaB* region of *S.* Typhi consists of 10 genes: 5 coding genes for the synthesis of the polysaccharide (*tviA, tviB, tviC, tviD and tviE*) and 5 coding genes for the polysaccharide transportation proteins (*vexA, vexB, vexC, vexD, vexE*) [16, 18]. In addition to the genes in *viaB* locus, 3 other genes are thought to regulate the production of Vi polysaccharide: *rcsB* and *rcsC*, together comprising the *viaA* [13, 19] and the two-component regulator *ompR-envZ* [17, 20].The Vi capsular polysaccharide is expressed during infection and believed to play an important role in the protection of *S.* Typhi from multiple host defence systems through several mechanisms. Firstly, encapsulated strains are more virulent than non-encapsulated strains [21]. Secondly, the expression of Vi capsular polysaccharide is associated with resistance to the action of the anti-O antibody to phagocytosis and complement mediated killing, both of which are mediated by anti-Vi antibody [22]. Additionally, Vi has been demonstrated to enhance survival of *S.* Typhi in cultured macrophages *in vitro* [23]. Furthermore, encapsulated *S.* Typhi

suppresses the production of Tumour Necrosis Factor (TNF) -α in macrophage cell lines in contrast to non-encapsulated strains, which induce high levels of TNF-α secretion [24]. Finally, the capsule of *S.* Typhi is associated with the prevention of the expression of pro-inflammatory, neutrophil chemoattractant Interleukin (IL)-8 in human intestinal mucosa. This is in contrast to mutant strains without capsular expression or strains of *S.* Typhimurium [25].

Recent *in vitro* studies have shown that under conditions of high osmolarity, such as those encountered in the intestinal lumen, capsule expression may be switched off [20]. These data suggest that the Vi antigen may not be expressed in the intestinal lumen, thereby enhancing the ability of *S*. Typhi to invade the intestinal epithelium. Studies have also suggested that *S*. Typhi isolates from blood of enteric fever patients invariably express the Vi antigen [22]. However, SPI-7 may be genetically unstable and can be lost upon laboratory subculture [26, 27]. Furthermore, the amount of Vi antigen produced by *S.* Typhi can decrease following multiple subcultures on agar medium [28]. Therefore, phenotypically Vi negative *S*. Typhi identified through Vi typing agglutination-based approaches should be interpreted with caution as Vi expression is particularly sensitive to the osmolarity of the selected growth media [20]. It is possible that Vi agglutination-negative *S*. Typhi reported from clinical microbiology laboratories may be Vi positive but demonstrate a down regulation of Vi or loss of *viaB* on culturing [29]. Interestingly, an increase in the isolation of Vi agglutination-negative *S*. Typhi has recently been reported from enteric fever endemic countries like India and Malaysia [30, 31]. Reports of Vi negative isolates of *S.* Typhi date back to the 1960s [32]. It has

been established previously that Vi is not essential for the development of enteric fever and Vi negative mutants are still able to cause a enteric fever-like illness in human volunteers [33, 34]. Work from Pakistan has demonstrated the absence of genes encoding the Vi polysaccharide in stored isolates as well as in isolates from patients which suggests that Vi-negative *S*. Typhi strains are not just artefacts of storage but can exist naturally [29, 35]. Vi polysaccharide has been exploited in diagnostics and vaccination strategies, as it is a general characteristic of *S.* Typhi [36-38].

1.2 Morphological and growth conditions of the genus *Salmonella*

The genus *Salmonella* consists of aerobic and facultatively anaerobic Gram-negative rods. Most serotypes in this genus are motile with peritrichous flagella, yet some serotypes are fimbriated or encapsulated. *Salmonella* grow on simple laboratory media such as nutrient agar, yet differential and selective media are generally used in the laboratories in order to isolate the *Salmonella* from faeces and other materials commonly contaminated with other bacteria. The most common selective culture media used for the growth and detection of *Salmonella* are MacConkey agar (MA), Xylose lysine deoxycholate agar (XLD agar), Xylose Lysine Tergitol-4 (XLT) and Deoxycholate Citrate Agar (DCA). The most commonly used enrichment broths for the maximal recovery of *Salmonella* from faecal specimens are tetrathionate broth, tetrathionate broth with brilliant green and Selenite broth (SEL) [39].

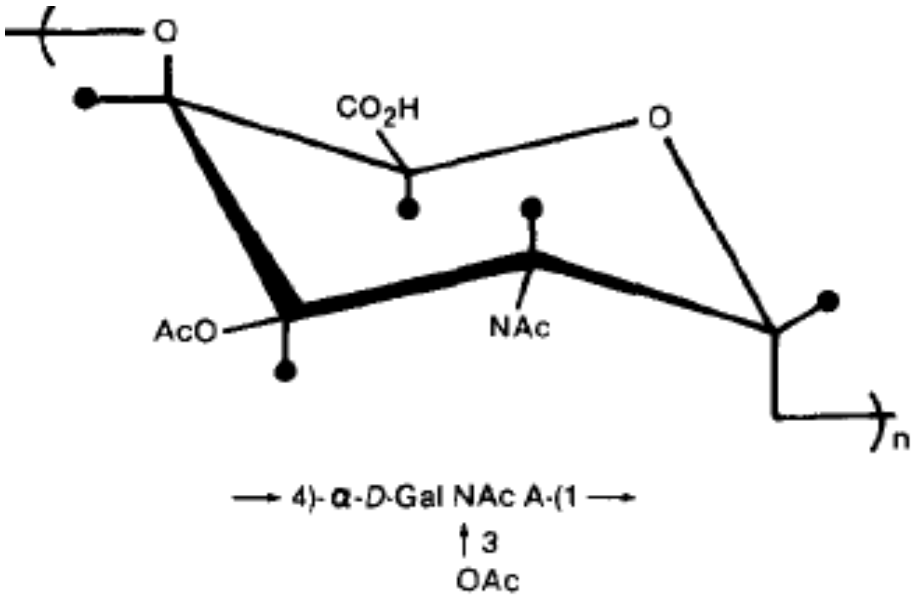


Figure 1.2: Structure of the repeating unit of the Vi capsular polysaccharide of S. Typhi[9]

1.2.1 Identification of *Salmonella* spp.

1.2.1.1 Biochemical Identification

Suspected colonies of *Salmonella* spp can be identified and confirmed biochemically with traditional media in tubes or commercial biochemical systems. Most *Salmonella* strains produce a reaction with alkaline slant/acid with gas and H_2S production (K/AG+). Such a reaction is indicative of glucose fermentation. On these media, *S.* Typhi is characteristically K/AG+ but does not produce gas; additionally, only a small amount of H_2S is visible at the site of the stab and in the stab line. Lysine iron agar is also a useful screening medium because most *Salmonella* isolates, even those that ferment lactose, decarboxylate lysine and produce H_2S [39].

The Identification of *Salmonella* can also be performed by API 20E (bioMérieux, France), which is a standardised system of bacterial identification for Enterobacteriaceae and other non-fastidious Gram-negative rods, utilising 21 biochemical tests in a miniaturised format.

1.2.1.2 Serotyping

Salmonella spp. are further serotyped according to their O, Vi, and H antigens. Slide agglutination for antisera against the heat stable O antigen groups A to E are normally performed first, as 95% of *Salmonella* isolates belong to one of these O groups. If no agglutination occurs in antisera for these O groups, the isolate is then subsequently

tested in pools containing the remaining *Salmonella* O antisera (O11 through O67)[39]. The Vi, a heat-labile capsular polysaccharide, is identified by slide agglutination method and is useful for the identification of *S*. Typhi. The Vi capsular polysaccharide can sometimes mask the O antigens, blocking their reactivity with the O grouping antiserum. In such cases, the bacterial suspension is heated in boiling water for 15 minutes to remove the capsule and subsequently tested again using the same O grouping antisera. H antigens are determined by tube agglutination tests using broth cultures. Isolates are initially tested with H typing antisera, which identify individual or multiple antigens. They are then tested against H single-factor antisera to identify individual antigens. Most *Salmonella* serotypes are either monophasic or biphasic. Biphasic serotypes express antigens from only one phase at any time; however, both phases may be detected in the whole culture [39].

1.3 Pathogenicity

A number of different *Salmonella* species are capable of causing enteric fever and gastroenteritis in humans and other vertebrates. Enteric fever is caused by *S.* Typhi and *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C. Gastroenteritis, however, is generally caused by other *Salmonella*, such as *S.* Enteritidis and *S.* Typhimurium; these infections are referred to as non-typhoidal *Salmonella* (NTS) infections. One of the important distinctions between typhoidal and NTS infections is the ability of non-typhoidal serotypes to colonise both human and non-human hosts where as *S.* Typhi and *S.*

Paratyphi serovars are strictly human pathogens. Gastroenteritis caused by NTS is a localised infection in the intestine and mesenteric lymph nodes in immunocompetent patients and is characterised by rapid onset of symptoms after a short incubation period (12 -72h). The short clinical course of gastroenteritis (< 10 days) suggests that the onset of an adaptive immune response results in clearance of the infection. NTS are usually unable to overcome innate defence mechanisms that limit bacterial dissemination from the intestinal mucosa to systemic sites of infection. However, NTS bacteraemia may occur in patients with impaired immunity [40] as has been shown in patients with human immune deficiency virus (HIV) in sub- Saharan Africa where septicemia with *Salmonella* species were more frequently caused by *S.* Enteritidis and *S.* Typhimurium than by typhoidal *Salmonella.* In Africa, invasive NTS are endemic and are associated with elevated morbidity and mortality in children under three years of age and in adults with HIV infections [41, 42]. However, in Asia NTS are not commonly cultured from patients with bacteraemia, except in individuals with severe immunosuppression [43, 44]. Recent data from Vietnam suggests there may be a transition from typhoidal S*almonella* to NTS related to economic development and increasing prevalence of HIV [45].

Humans are the only natural host and reservoir of infection for *S.* Typhi and *S*. Paratyphi A. Consequently, studying the organism has proven challenging. Understanding of enteric fever pathogenesis is based largely on the murine model in which *S*. Typhimurium causes a systemic infection similar to enteric fever and through the use of human volunteers. For example, the experimental challenge carried out by

Hornick *et al.* in 1970 using the Quailes strain isolated from a carrier revealed that the infectious dose in volunteers varies between 10^3–10^9 organisms (Table 1.2)[21].

Many commensal bacteria persist in the lumen of the intestine without significantly interacting with the epithelia or deeper tissues. However, *Salmonella* are regarded as invasive bacteria as they encode multiple systems for interacting with and penetrating the mucosal epithelia [1]. To establish this intracellular niche, the type three-secretion system (T3SS) encoded on SPI-1 mediates invasion of the intestinal epithelium. The T3SS encoded on SPI-2 is required for the survival within macrophages [46]. These T3SS encode needle-like complexes that inject bacterial proteins, known as effector proteins, directly into the host cells. Such proteins can hijack host cell functions, including those associated with the immune system [1].

Upon translocation to systemic sites, Fields *et al*. demonstrated that bacterial survival within phagocytes is essential for virulence [47]. Bacteria are released from sequestered intracellular habitat like the liver and spleen into the bloodstream during infection. The re-entry of bacteria into the bloodstream (secondary bacteraemia) marks the onset of the clinical illness. The incubation period until symptoms is usually 7 to 14 days. In the bacteraemic phase, the organism is widely disseminated. The most common sites of secondary infection are the spleen, liver, bone marrow, gallbladder and Peyer's patches of the terminal ileum [48], inducing systematic and local humoral and cellular immune responses [48]. Both *S*. Typhi and *S*. Paratyphi A are immuno-modulatory pathogens that avoid detection by the immune host defences [49]. The innate immune

system can distinguish between self and microbial intruders by recognising molecular patterns through pattern recognition receptors (PRRs) including the membrane localized Toll-like receptors (TLRs) and the cytosolic Nod-like receptors (NLRs). It has been hypothesised that *S*. Typhi can evade PRR-mediated host responses. Supporting this hypothesis, pyrogenic cytokines, similar to TNF-α and IL-1β, are found to be elevated in sera of enteric fever patients compared to healthy individuals [50, 51]. Secondly, patients with chronic granulomatous disease (CGD) often develop bacteraemia due to *Salmonella* and other Gram-negative enteric bacteria. CGD comprises a group of disorders associated with mutations in genes encoding subunits of the NADPH oxidase. These defects render phagocytes incapable of killing ingested microorganisms via oxygen-dependent pathways [52]. Additionally, achlorhydria (gastric acid production deficiency) due to ageing, previous history of gastrectomy, treatment with H_2 receptor antagonists, proton-pump inhibitors, large amounts of antacids or *Helicobacter pylori* infection increase susceptibility to enteric fever [53].

Table 1.2: Experimental challenge model of enteric fever; relationship between inoculation ingested, clinical attack rate and incubation period[21]

Dose of pathogenic *S.* Typhi*	**Clinical attack rate**	**Incubation period (Median)**	**Incubation period (Range)**
10^9 CFU	95%	5 days	3-32 days
10^7 CFU	50%	7.5 days	4-56 days
10^5 CFU	28%	9 days	6-33days
10^3 CFU	0%	-	-

*In 45mL of milk

1.4 Epidemiology of Enteric fever

Enteric fever was an important cause of illness and death in the overcrowded and unsanitary urban conditions of the United States and Europe in the 19th century [48]. Then, enteric fever was controlled by dramatic changes in sanitation and the provision of clean water; however, the disease is still endemic in regions of the developing world where sanitary conditions remain poor. Reliable data from which to estimate the burden of disease in these areas are difficult to obtain, since many hospitals lack facilities for blood culture and up to 90% of patients with enteric fever are treated as outpatients [48]. The best global estimates suggest that enteric fever causes 27 million infections every year and 200,000 deaths annually [54]. However, despite the limitations of currently available epidemiologic data, a number of recent trends in enteric disease epidemiology have emerged in Africa, Asia, and Latin America.

The burden of disease due to enteric fever is the least well characterised in sub-Saharan Africa. Hospital based studies indicate that NTS, especially *S*. Enteritidis and *S*. Typhimurium, are a more common cause of bloodstream infections than typhoidal *Salmonella*; as much as 50% of isolated NTS in many parts of Africa are responsible for bacteraemia [55, 56]. Although invasive NTS bacteraemia was first documented in Africa over 20 years ago [57], the magnitude of the problem has only recently been recognised in this region, and is associated with predisposing infections like HIV and malaria [58]. Additionally, outbreaks of enteric fever have also been reported in Africa,

some of which reported multidrug-resistant *S*. Typhi strains and high rates of complications [59].

In Latin America, enteric fever incidence has declined in parallel with both economic transition and improved water and sanitation measures introduced through efforts to control cholera during the last pandemic [60]. For example, a major enteric fever outbreak was reported in Chile in 1977 and lasted until 1986. However, the incidence of enteric fever declined drastically after the government implemented sanitary and education policies to forestall the dissemination of cholera in the country after the outbreak of cholera in 1991 [61].

In Asia, several large, population-based studies have confirmed the high incidence of enteric fever in the region, particularly among children and adolescents [62]. Approximately 80% of the world's enteric fever cases occur in Asia [63]. One of the important concerns arising from recent studies was an increasing incidence of *S*. Paratyphi A infection in South Asia during the past decade [64, 65], which accounts for up to 50% of bloodstream isolates among patients with enteric fever in locations such as Southeast China [64, 66].

Enteric fever remains the most common clinical and blood culture confirmed diagnosis among patients with febrile illness in urban Nepal, where the disease is endemic [67, 68]. The burden of this infection in Nepal is huge. At Patan Hospital in Kathmandu alone, there are over 2,500 suspected cases each year. A retrospective study conducted from 1993 to 2003 reported that 74.5% (9,124 /12,252) of febrile patients admitted to Patan Hospital that were blood-culture positive for a bacterium were positive for *S*.

Typhi or *S*. Paratyphi A [69]. Also a recent study reported that *Salmonella* infections are the leading cause of bloodstream infection among the paediatric population [70]. Additionally, the proportion of enteric fever cases due to *S*. Paratyphi A at Patan Hospital increased from 21.7% during 1993-1997 to 33.8% during 1998-2003 [71]. Contrary to previous suggestions, recent research demonstrated that enteric fever due to *S.* Paratyphi A cannot reliably be distinguished clinically from that due to infection with *S.* Typhi [70-72].

The incidence of enteric fever in endemic areas in Nepal is the highest in children aged 5-15 years old [73, 74]. Rates of disease are typically considered to be low in the first few years of life, peaking in school-aged children and young adults and then falling again during middle age. Studies from Kathmandu have shown that the burden of disease is greatest in school-aged children and young adults, possibly because of a large transient workforce travelling from locations outside the city where the exposure to the infecting organisms may be less common [75]. Older adults are presumably relatively resistant to infection due to constant exposure to the bacteria and subsequent boosting of immunity.

Transmission of enteric fever occurs by ingestion of food or water contaminated by faecal or urinary carriers excreting the organisms [76, 77]. Distinct routes of transmission of enteric fever due to *S.* Typhi and *S.* Paratyphi A have been suggested. Risks for infection with *S.* Paratyphi A are thought to be more likely to occur outside the household due to flooding and consumption of street food from local vendors. Infection

with *S.* Typhi is thought to occur within the household through contact with convalescent carriers or acutely infected individuals due to poor hand-washing hygiene and sharing of food [77]. Other established risk factors for infection include: consuming ice cream, flavoured iced drinks or food from street vendors in addition to raw fruit and vegetables grown in fields fertilised with sewage [53]. Although dogma suggests that chronic carrier is likely to play a major role in transmission, questions remain regarding the precise role of chronic carriers in disease transmission in these endemic settings. Further studies in Kathmandu have noted that indirect transmission, potentially via contaminated water, are likely to play a major role in disease transmission whereas direct human to human transmission plays only a negligible role [78]. Recently developed geospatial mapping analysis methods have also revealed the association of enteric fever cases with low elevation and water spout proximity [78].

The incidence of enteric fever is known to follow seasonal patterns. It is believed that the rate of *S*. Typhi infection in temperate regions peaks during the warmest and wettest time of the year [79]. Some countries have reported a peak in dry, hot months of the year when water sources become stagnant and shallow, allowing the bacteria to concentrate. [76]. Cases of enteric fever were found to be highest in the dry season in Pakistan and Vietnam [76, 80]. However, enteric fever can also peak in the rainy season, as has been reported in Nepal and Bangladesh [68, 81, 82]. This is often attributed to a breakdown in the systems that keep sewage and drinking water separate.

1.5 Clinical manifestations of enteric fever

The clinical manifestations and severity of enteric fever vary widely. Following an incubation period of 7 to 14 days, fever and malaise mark the onset of bacteraemia. Flu-like symptoms with chills (although rigors are rare) and a dull frontal headache are common. The fever, initially low grade, rises progressively, and by the second week is often high and sustained (39–40^{o}C). Other symptoms include anorexia, poorly localised abdominal discomfort, a dry cough and myalgia in addition to a tender abdomen, associated with hepatomegaly and/or splenomegaly [83, 84]. Abdominal pain is usually diffuse and poorly localised, but occasionally sufficiently intense in the right iliac fossa to suggest appendicitis.

Nausea and vomiting are infrequent in uncomplicated enteric fever but are seen with abdominal distension in severe cases. Constipation is generally more common in adults, but in young children and adults with HIV infection, diarrhoea predominates [85-87]. Additionally, a relative bradycardia is considered to be common in enteric fever, although this has not been a consistent feature in all geographic areas. Finally, small blanching erythematous maculopapular lesions known as rose spots are reported in 5 to 30% of cases and are present typically on the abdomen and chest. These lesions are easily missed in dark-skinned patients [48]. There may be a history of intermittent confusion, and many patients have a characteristic apathetic affect. Convulsions may occur in children under five years of age. The haemoglobin level, white cell count and platelet count are usually normal or reduced. The levels of liver enzymes are usually two to three times the upper limit of normal [48]. With appropriate antimicrobial

therapy, the fever reduces within three to four days. Untreated, the fever persists for two weeks or more, defervescence occurs slowly over the following 2–3 weeks and complications can ensue. Convalescence may last for 3–4 months.

1.6 Complications of enteric fever

Although many complications resulting from infection with enteric fever have been described, life- threatening complications include gastrointestinal bleeding, intestinal perforation, enteric fever encephalopathy, shock, myocarditis and hepatitis. Gastrointestinal bleeding occurs in up to 10% of patients and is due to erosion of a necrotic Peyer's patch through the wall of an enteric vessel. Usually the bleeding is minimal and resolves without the need for blood transfusion. However, in 1–2% of cases, bleeding is significant and can be rapidly fatal if a large vessel is involved. Intestinal (usually ileal) perforation is the most serious complication occurring in 1–3% of hospitalised patients [88, 89]. Perforation may present with acute abdominal pain or more covertly with simple worsening of abdominal pain, rising pulse and falling blood pressure in an already ill patient. Finally, enteric fever encephalopathy is associated with a high mortality and is often accompanied by shock [90-92]. Patients can be severely agitated, delirious, or obtounded, but complete stupor or coma is infrequent.

Enteric fever in pregnancy may result in miscarriage, although antimicrobial treatment has made this outcome less common [93]. Vertical intra-uterine transmission from a enteric fever-infected mother may lead to neonatal enteric fever, a rare but severe and life-threatening complication [94]. Other well-known complications of enteric fever are

relapse, reinfection and chronic carriage of the bacteria in the gallbladder. Relapse can occur in 5–10% of patients, and typically occurs 2 to 3 weeks after defervescence. The relapse illness is usually, but not always, milder than the original attack and the relapse *S.* Typhi isolate can have the same susceptibility pattern as in the original episode. Reinfection may also occur which can be distinguished from relapse by molecular typing [95]. Chronic carriers are those patients who continue to shed the bacteria in the faeces for more than one year. However, about 25% of chronic carriers have no history of enteric fever.

1.7 Clinical diagnosis of enteric fever

The clinical diagnosis of enteric fever is difficult because of non-specific symptoms that are present in other febrile illnesses such as dengue, leptospirosis, rickettsial infections and malaria [48, 96]. A study from Nepal confirmed that enteric fever and pneumonia were the most common diagnoses among febrile patients followed by murine typhus, scrub typhus, and leptospirosis. However, no clinical predictors were identified to reliably distinguish between these infections [67]. For patients in countries where enteric fever is not endemic, a travel history is crucial [48]. Signs such as relative bradychardia or leucopoenia may be useful in diagnosis but have low specificity [97]. Complicating this, the classic mode of presentation of enteric fever, namely a slow, progressive rise in fever and toxicity, are rarely present due to antimicrobial treatment available over the counter in settings such as Nepal [35].

1.8 Laboratory diagnosis of enteric fever

1.8.1 Microbiological culture

For the diagnosis of enteric fever, growth of *S*. Typhi or *S*. Paratyphi is required. Potential specimens for culture include blood, bone marrow, stool, urine, duodenal fluid or tissue from rose spots. The gold standard test for diagnosis of enteric fever remains the culture of bone marrow. The sensitivity of this test exceeds 90% and the specificity is 100% regardless of duration of the disease prior to sampling [98, 99]. The increased sensitivity of bone marrow culture is due to a much higher (10x) concentration of viable organisms in bone marrow than in the blood [100]. However, obtaining a bone marrow sample requires an invasive procedure and sophisticated technical expertise and equipment; therefore, blood culture is often used for diagnosis even though the sensitivity of the diagnostic is only approximately 40% -60% [98, 101, 102]. Factors that contribute to the lack of sensitivity of blood culture include the time duration of illness (bacteraemia declines over time), small blood volumes used for blood culture, type of media, and blood broth ratio.

The sensitivity of blood culture, even when a large volume of blood (15mL) is cultured from an adult, is still only 40-70%. Approximately 50% of patients with enteric fever whose blood cultures are positive have less than 1 colony forming unit (CFU)/ml of blood, and only 10% have greater than 50 CFU/ml [103]. Blood culture is more sensitive in the first week of the illness, yet patients do not typically present to hospital until the end of the first week of symptoms. Widespread availability of antimicrobials in

the community also renders blood culture less useful due to antimicrobial activity prior to admission [48]. Fluoroquinolones usually sterilize the blood within 2 days in patients with enteric fever [103]. Reports of the evaluation of different blood culture media suggest that Ox bile broth is superior to rich nutrient media for the isolation of *S.* Typhi from blood [104]. The advantage of Ox bile broth is attributed to inhibition of the antibacterial activity of fresh blood due to lysis of blood cells rather than direct enhancement of growth by bile acids [105]. Other media types commonly used for growing *Salmonella* are tryptone soya broth or brain–heart infusion broth and automated blood culture systems, such as BACTEC (Becton Dickinson, UK) and BactAlert (bioMérieux, France) are also used. Sodium polyanethol sulfonate (SPS) or Liquoid is widely employed as an additive to blood culture broths an anticoagulant and a surface-active agent which is known to neutralise the bactericidal activity of fresh human serum and inhibit phagocytosis [106]. Adequate dilution of blood in broth and the length of the incubation period in the microbiology laboratory are additional important factors for the growth of *Salmonella*. Rapid detection of *S*. Typhi has been reported in blood by culture of the mononuclear cell platelet layer [107]. Other culturing methods include culturing of buffy coat, stool, or from rose spots. However these have not gained widespread use.

Stool culture is an even less reliable diagnostic than blood culture, with <30% sensitivity. From a study in Vietnam, single-admission stool cultures were positive for *S.* Typhi in 14.3% from acute enteric fever patients and was associated with significantly higher bacterial counts in the blood [103]. It has been shown that less than

10% of stool culture samples are positive from blood culture confirmed acute enteric fever patients prior to antimicrobial therapy [108, 109] The sensitivity of stool culture depends on the amount of faeces cultured duration of illness in the patient, as sensitivity increases with length of illness [48]. Enrichment media containing selenite are used to isolate *S.* Typhi from stool because of very large numbers of competing bacteria, especially *Escherichia coli* [110].

A further method of culture involves a string capsule device that, once swallowed by the patient, enters the duodenum and is coated with bile, which is then subsequently cultured. This string device method has proved to be as sensitive as bone marrow culture, however, it is not routinely used as diagnostic specimen due to its invasiveness [111]. *S.* Typhi can be grown from rose spots [98] but rose spots are often difficult to see and may only be present in 4% of cases [103]. *S.* Typhi and *S.* Paratyphi can also be grown from urine but may be associated with urinary tract infection rather than enteric fever [112]. Cultures have also been made from the buffy coat of blood and streptokinase-treated blood clots [101, 107] but these methods are not used routinely. Diagnostic sensitivity is improved by performing cultures on multiple specimens per patient [113].

1.8.2 Antibody detection

The Widal test, developed by F. Widal in 1896, detects the presence of *S.* Typhi specific H and O antigen agglutinating antibodies in the serum of an infected patient. Antibodies

against the O and H antigens typically appear during the end of the first week of disease and peak at the end of the third week, although there is much variability [114]. Although the test has been used for more than 100 years, the role of the Widal test is controversial as the sensitivity, specificity, and predictive values vary considerably among geographic areas [48]. The controversies surrounding the Widal test are linked to the quality of the antigens used and the interpretations of the result, particularly in an endemic regions [115]. *S*. Typhi shares these antigens with other *Salmonella* serotypes in addition to cross-reactive epitopes of other Enterobateriaceae [48].

Ideally, the Widal test requires both acute and convalescent-phase serum samples taken approximately 10 days apart; a positive result is confirmed by a fourfold increase in antibody titre between these two samples [96]. Yet this fourfold increase is not always observed even in blood culture confirmed cases [115, 116] and it has been shown that some patients with enteric fever may not demonstrate any detectable rise in antibody titre [48]. In regions endemic for enteric fever, a single testing of serum with the Widal agglutinin method cannot provide a reliable diagnosis due to repeated exposure to small inocula of *S*. Typhi or other *Salmonella* spp that contain type 9 or 12 antigens, previous typhoid immunisation or other infectious agents such as malaria [115, 116]. Knowledge of background levels of H and O antibodies in the local population may aid interpretation of the Widal test [117, 118]. Unfortunately, in many enteric fever endemic areas, the Widal agglutination test is still used as the primary diagnostic due to limited or non-existent bacterial culture facilities.

Enzyme linked immunosorbent assays (ELISAs) have been used to more precisely define the normal antibody response to enteric fever and its relevance for diagnosis. The antigens used in ELISA-based studies have generally been LPS, H, Vi and outer membrane protein antigens that are found to be more sensitive than the Widal test [119-121].

1.8.3 Rapid diagnostic tests

Several attempts have been made to package serological tests into simple, point-of-care rapid enteric fever diagnostics; such a tool would be invaluable to clinicians in poorly resourced endemic areas. Most of these kits have been developed to detect immunoglobulin (Ig) M antibodies, the presence of which suggests current or recent infection. A major drawback of such rapid diagnostic methods is the lack of an isolated organism and corresponding antimicrobial susceptibility result. In addition, these rapid tests lack sensitivity when compared to culture. Like the Widal test, interpretation is complicated by the background antibody levels in the general population and the cross-reactive nature of selected antigens [96]. Sensitivity and specificity of some of the commonly used rapid tests are shown in Table 1.3.

1.8.4 Antigen detection

Polyclonal and monoclonal antibodies have been used to detect *S*. Typhi antigens in body fluids. Polyclonal antibodies recognise multiple epitopes whereas monoclonal antibodies detect only one epitope on the antigen. Antibodies used in these methods have been directed against the same targets used in serological testing namely Vi, O9 and Hd. An ELISA detecting Vi antigen has been shown to be the most sensitive [36, 122]. However, a study from Chile demonstrated that an ELISA assay gave false positive results in 64.7% of 34 culture-proven *S*. Paratyphi A or B patients and 47.1% of 21 patients with other nontyphoidal febrile illnesses [38]. Among patients in whom *S.* Typhi was isolated from blood culture, the ELISA had a sensitivity of 65% when a single urine specimen was examined and 95% when serially collected urine specimens were examined [123]. This data indicates that the intermittent nature of antigen excretion in urine during an infection makes this diagnostic method unreliable [96].

1.8.5 Nucleic acid amplification

Nucleic acid amplification is a technique that rapidly amplifies small numbers of bacilli present in clinical samples for detection. It is generally considered to be an improvement over blood culture due to the faster turn-around-time compared to culture and the ability to detect viable, non-viable and nonculturable bacteria after antimicrobial treatment. Conventional polymerase chain reaction (PCR) detects amplification products using an agarose gel, while real-time PCR amplification is

detected by release of a fluorescent signal. Real-time PCR induces less variability in the assay and the interpretation [96]. Targets for *S.* Typhi PCR-based assays have included the Hd flagella gene *fliC-d* [124], the Ha flagella gene (*fliC-a)*, the Vi capsular gene *viaB* [125], the tyvelose epimerase gene (*tyv*; previously *rfbE*), the paratose synthase gene (*prt*; previously *rfbS*), *groEL* [126] and the 16S rRNA gene [127]. However, PCR performed directly on blood samples may be an unsuitable methodology due to the low number of organisms in the blood sample, which are invariably below the detection limit of the PCR assay [128].

Table 1.3: Laboratory diagnosis of enteric fever by different techniques [35]

Diagnostic test	Sensitivity range (%)	Specificity range (%)
Microbiological tests		
Blood culture	40-80	Not available
Bone marrow culture	55-67	30
Urine culture	0-58	Not available
Stool culture	30	Not available
Molecular diagnostics		
Polymerase chain reaction	100	100
Nested polymerase chain reaction	100	100
Serological diagnosis		
Widal test (tube dilution & slide agglutination)	47-77	50-92
Typhidot	66-88	75-91
Typhidot M	73-95	68-95
Tubex	65-88	63-89
Others		
Urine antigen detection	65-95	Not available

1.9 Treatment of enteric fever

In endemic areas, up to 90% of enteric fever cases are treated in outpatient settings [48, 72]. Treatment of enteric fever involves preventing the onset of severe, complicated or fatal disease. Rapid diagnosis and appropriate antimicrobial treatment are required in addition to adequate rest, hydration and antipyretic therapy. An appropriate antimicrobial regimen should be safe, induce minimum side effects and should resolve the fever and other symptoms within 5-10 days [129]. The antimicrobial regimen must also be designed to prevent relapse and eradicate faecal carriage to reduce potential for onward transmission.

Traditionally, first-line antimicrobials for treatment of enteric fever included chloramphenicol, ampicillin and cotrimoxazole. However, due to the emergence of multidrug resistant strains, fluoroquinolones are now the antimicrobials of choice [130-132]. Fluoroquinolones are recommended as first line therapy for children and adults with uncomplicated, fully sensitive *S*. Typhi and *S*. Paratyphi A as well as those resistant to the traditional antimicrobials. Fluoroquinolones are relatively inexpensive, well tolerated, have excellent tissue penetration and achieve higher active drug levels in the gallbladder than the former first-line drugs [133]. The average fever clearance time is less than four days, and the cure rates exceed 96%. Furthermore, less than 25% of treated patients have persistent faecal carriage or relapse. Results from a meta-analysis of 20 randomised controlled trials suggest that fluoroquinolones are more effective in reducing rates of clinical relapse in adults than chloramphenicol [134]. Ofloxacin given

for 3 or 5 days is effective in the treatment of nalidixic acid (NA) sensitive strains. However, such short treatment duration is not recommended for treating enteric fever caused by strains with reduced susceptibility to fluoroquinolones [129].

In vitro studies have suggested that gatifloxacin, a new generation fluoroquinolone, may be more active than ciprofloxacin and ofloxacin, particularly in isolates with decreased susceptibility to ciprofloxacin [63]. It was shown that gatifloxacin is more effective in reducing syndromic clinical relapse and number of adverse events. Due to shorter treatment duration and lower cost, gatifloxacin should be the preferred treatment of enteric fever in developing countries [109]. Various randomised clinical trials have suggested that azithromycin can also be used effectively for the treatment of uncomplicated enteric fever [135]. Ceftriaxone, a third generation cephalosporin, is also an effective for the treatment of enteric fever [136]. Available antimicrobial treatment options are summarised in Table 1.4.

Table 1.4: Recommended antibiotic treatment of enteric fever [35]

Optimal treatment				**Alternative effective treatment**		
Susceptibility	Drug	Daily dose (mg/kg)	Course (days)	Drug	Daily dose (mg/kg)	Course (days)
Uncomplicated enteric fever						
Fully sensitive	Fluoroquinolone (ofloxacin or ciprofloxacin)	15	5-7*	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				TMP-SMX	1-8	14
Multidrug resistance	Fluoroquinolone or Cefixime	15	5-7	Azithromycin	8-10	7
	Cefixime	15-20	7-14	Cefixime	15-20	7-14
Quinolone resistance**	Azithromycin or	8-10	7	Cefixime	20	7-14
	Ceftriaxone	75	10-14			
Severe enteric fever requiring parenteral treatment						
Fully sensitive	Fluoroquinolone (ofloxacin)	15	10-14	Chloramphenicol	100	14-21
				Ampicillin	100	14
				TMP-SMX	1-8	14
Multidrug resistance	Fluoroquinolone	15	10-14	Ceftriaxone or	60	10-14
				Cefotaxime	80	10-14
Quinolone resistant	Ceftriaxone or	60	10-14	Fluoroquinolone	20	14
	Cefotaxime	80	10-14			

*Three day courses are effective, particularly as in epidemic containment strategy

**Optimum treatment for enteric fever has not been determined. Azithromycin, third generation cephalosporins, or a 10-14 day course of high dose of fluoroquinolne is effective. Combinations of these are now being evaluated.

1.10 Antimicrobial resistance and its mechanisms

Chloramphenicol was introduced for the treatment of enteric fever in 1948 [137]. Resistance was soon reported after only two years but did not become a serious issue until the early 1970s. At that time, outbreaks of chloramphenicol-resistant enteric fever occurred in Mexico, India, Vietnam, Thailand, Korea and Peru [138]. However, in drug sensitive strains, oral chloramphenicol was more effective than parenteral ampicillin or oral co-trimoxazole in reducing fever [139]. After a few years these chloramphenicol-resistant isolates disappeared from Mexico and Peru but persisted at low levels in Asia. However, toward the end of the 1980s and during the 1990s, some *S.* Typhi were reported resistant to all the first-line drugs (chloramphenicol, co-trimoxazole and ampicillin) in China, South and Southeast Asia [48, 138]. These strains are referred to as multi-drug resistant (MDR) strains. Since 1989, such MDR strains have been responsible for numerous outbreaks in countries in the Indian subcontinent, Southeast Asia and Africa [138] (Figure 1.3). MDR *S.* Typhi are still common in many areas, although in some regions fully sensitive strains have re-emerged coinciding with the introduction of fluoroquinolones for the management of enteric fever [65, 69].

Plasmid and chromosomal-mediated resistance mechanisms are most common in *S.* Typhi [48, 138, 140]. Plasmids are extra-chromosomal, self replicating circular pieces of DNA which can carry and transfer multiple resistance genes between bacteria [141]. IncHI1 plasmids were isolated from MDR *S.* Typhi worldwide [142, 143]. IncHI1 plasmids appeared in the 1970s and spread globally. Plasmids of the same incompatibility (Inc) group are related, with similar replication mechanisms and

regulatory circuits for essential plasmid functions; they cannot survive together in the same bacterial cell and so are considered to be incompatible. The H incompatibility complex comprises two Inc groups, IncHI and IncHII, based on their similar H-pilus structure. All IncH plasmids are large molecules with sizes of 150 kb or more and all are temperature sensitive for conjugative transfer. These plasmids have crossed from *S.* Typhi into *S.* Paratyphi A.

NA, a quinolone, is not used for the treatment of enteric fever. However, resistance to NA has been found to correlate with reduced susceptibility to ciprofloxacin [144]. Thus, resistance to NA is a useful marker of isolates with a low level of resistance to fluoroquinolones that is not detected by fluoroquinolone disc testing alone [129, 145, 146]. Since 1997, infection with NA resistant *S*. Typhi with decreased susceptibility to ciprofloxacin has been reported from various parts of the world [144-147]. The first major outbreak of enteric fever due to strains resistant to NA and reduced susceptibility to fluoroquinolones was reported in Tajikistan in 1998 [147]. In a cross-sectional study, 381 *S*. Typhi strains from 8 Asian countries, Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and central Vietnam, were collected from 2002 to 2004. This study found rates of multidrug resistance between 16 to 38% and NA resistance between 5 and 51% [63]. Findings of high and variable levels of resistance have been confirmed in other studies as well [62].

Bacteria most commonly develop resistance to quinolones by non-transmissible, spontaneously occurring point mutations in chromosomal genes *(gyrA*, *gyrB*, *parC,* and *parE).* These point mutations alter the enzymes (DNA gyrase and topoisomerase IV)

that are targets for quinolone drugs. Fluoroquinolone resistance is attributed to chromosomal-mediated mutations in the quinolone resistance-determining region (QRDR) of the topoisomerase gene, *gyr*A, which encodes DNA gyrase [48, 148, 149]. A single mutation in *gyr*A is sufficient to confer resistance to NA and reduced susceptibility to fluoroquinolones [150], whereas a second mutation in the same gene leads to high-level fluoroquinolone resistance [149].

Recent data have raised the concern that NA resistance may no longer be a reliable marker for the detection of decreased susceptibility to fluoroquinolones as there are now reports of NA susceptible isolates with decreased susceptibility to fluoroquinolones [151-154]. This phenotype appears to be mediated by resistance mechanisms outside the gyrA gene [155]. Plasmid mediated quinolone resistance (PMQR) determinants, such as the *qnr* and *aac-6'-Ib-cr* genes are associated with decreased susceptibility to ciprofloxacin but result only modest NA MIC elevations (8-32 ug/mL)[156-160]. However, other mechanisms such as decreased permeability and active efflux of the antimicrobial agent may also be involved [149].

Although ceftriaxone resistance has not been widespread, a gradual rise in the mean MIC has been recorded in India [155]. Sporadic reports have indicated high level resistance to ceftriaxone in *S*. Typhi and *S*. Paratyphi A, although these strains are not common. Extended spectrum beta lactamase (ESBL) production has also been reported from some countries [161, 162]. Resistance to cephlosporins, due to the production of ESBL, is a serious problem worldwide. Patterns of antimicrobial resistance in *Salmonella* are constantly changing. Continual surveillance of resistance levels is critical

for clinicians to keep abreast of treatment options, but is often lacking in resource poor regions of the world with the highest disease burden [163].

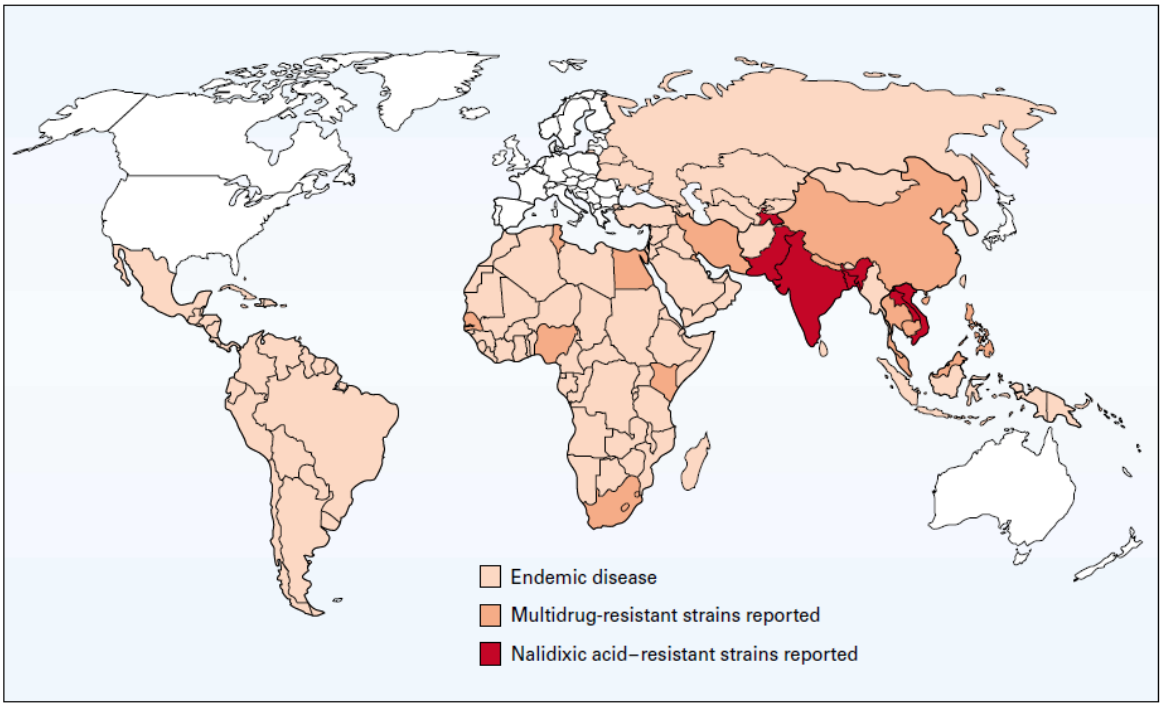


Figure 1.3: Global distribution of antibiotic resistant *S*. Typhi. The figure shows geographical area of multidrug resistance, Nalidixic acid resistance, and areas where no drug resistance has been reported[48]

1.11 Preventing enteric fever

1.11.1 Vaccines

Two safe and licensed vaccines are available for the prevention of enteric fever due to *S.* Typhi. The inactivated whole–cell vaccine, known as Ty21a, is an oral attenuated *S*. Typhi vaccine, and the parenteral polysaccharide vaccine, contains the Vi capsular antigen. These vaccines confer about 70% protection in older children and adults but have not been shown to be effective in young children or infants [164-167]. A conjugate typhoid vaccine, Vi-rEPA, is reported to be safe and immunogenic with more than 90% efficacy in children two to five years old is also about to enter the market [168]. This is a conjugate vaccine that binds Vi to a non-toxic recombinant protein antigenically identical to *Pseudomonas aeruginosa* exotoxin A (Vi-rEPA). Another novel vaccine, Vi-CRM197 (Vi conjugated to a non toxic mutant of diphtheria CRM197), has proved to be immunogenic in animal studies [169]. Routine typhoid vaccination has not been implemented in enteric fever endemic countries due to cost, lack of protection in young children, difficulties integrating the vaccine into Expanded Programmes on Immunization (EPI) schedules and the need for repeated booster doses [170, 171]. Vi capsular polysaccharide-based vaccines, however, are unlikely to provide effective protection against *S.* Paratyphi A since they lack the Vi antigen. Similarly, this vaccine may be ineffective against *S*. Typhi strains not expressing the Vi polysaccharide (Vi negative strains). The emerging importance of *S*. Paratyphi A is of a great concern, particularly in Asia. A combination vaccine that can protect from both serotypes is desired. On going work has focused on developing a bivalent vaccine against both *S.*

Typhi and *S*. Paratyphi A serovars through independent chemical conjugation of the Vi polysaccharide and the O specific polysaccharide of *S.* Paratyphi A to the carrier protein CRM197[172].

1.11.2 Non vaccine measures

Non-vaccine measures for enteric fever prevention encompass both community and individual measures. Community measures include promotion of hand washing, sanitary disposal of human faeces, provision of safe drinking water, sanitary food preparation, pasteurisation of milk and dairy products, implementation of quality control procedures in the food industry and shellfish sanitation. Individual measures for enteric fever prevention include education of patients, and carriers in proper personal hygiene habits in addition to the provision of enteric fever vaccine to persons at high risk due to occupation or travel [171].

1.12 Chronic carriage of *S*. Typhi and *S*. Paratyphi A

1.13 Chronic/persistent bacterial infections

Some pathogenic bacteria are capable of establishing persistent infections in mammalian hosts even in the presence of active inflammatory response, specific antimicrobial mechanisms and a robust adaptive immune response [173-175]. Some persistent bacterial pathogens like *Streptococcus pneumoniae*, *Neisseria meningitidis* and *Haemophilus influenzae* are carried asymptomatically in the nasopharynx in most people as commensal flora, although they still have the ability to cause life-

threatening disease in seemingly immunocompetent individuals [173]. Pathogens like *Helicobacter pylori, S.* Typhi and *Mycobacterium tuberculosis*, after causing an initial disease state, may not be completely cleared from the host and can persist in a privileged niche for long periods of time. Long-term survival of these pathogens in mammalian hosts depends on their ability to avoid elimination by innate and adaptive immune responses. In contrast to the normal host microflora, inhabitation by a persistent pathogen, whether symptomatic or not, represents a burden for the carrier. For example, latent tuberculosis is associated with a risk of reactivation into active disease, whereas carriers of *H. pylori* or *S.* Typhi are at risk of developing malignancies in the gastric and hepatobiliary tracts, respectively [176, 177]. This is further complicated by the fact that many chronic bacterial infections are intrinsically difficult to eradicate through antimicrobial therapy. *S*. Typhi evades host immunity by expressing factors that reduce the host inflammatory response, enabling systemic invasion and colonization of the gallbladder [178].

1.13.1 Epidemiology of chronic *Salmonella* carriers

During an acute enteric fever infection, invasive *Salmonella* cross the intestinal epithelial barrier, invade and survive within macrophages, eventually reaching the bone marrow, liver, pancreas and spleen [179]. The *Salmonella* may enter the biliary tract either via ascending route or descending route. In the ascending route, the bacteria can enter the biliary system via the sphincter of Oddi, which when broken by surgical intervention or by certain pathology, fails to maintain it's function as a mechanical barrier. In the descending or hematogenous route, bacteria enter the

hepatobiliary system from portal circulation. Under normal conditions, Kupffer cells prevent toxic metabolites and bacteria from entering the hepatobiliary system from the portal circulation. Furthermore, the continuous flushing action of bile and the bacteriostatic effect of bile salts normally keep the biliary tract sterile. Secretory immunoglobin A, the predominant immunoglobulin in the bile and the mucus membrane, probably acts by its anti-adherent function to prevent microbial colonisation. Despite these mechanisms, it is likely that organisms in the bile might be derived from blood [180].

It is gallbladder colonisation and faecal shedding that form a central dogma for the transmission and persistence of enteric fever. During the course of infection, three types of carriers can be found: convalescent, temporary and chronic. Convalescent carriers shed the bacilli in faeces for between three weeks to three months post infection. Temporary carriers shed the bacilli for over three months but less than one year and chronic carriers shed the bacilli for more than one year [48, 133]. The relative importance of short-term, convalescent faecal carriers versus long-term, chronic carriers in the dynamics of transmission in endemic areas is not known as limited data exist [129]. Chronic carriers are thought to intermittently shed the bacteria for a prolonged, ill defined period of time in the local environment and thus they may spread the disease in the community and maintain a reservoir of infection [181]. Because of the host restricted nature and the internal localisation of the organism, it is a technically difficult area to investigate.

The majority of chronic carriers are asymptomatic [182]. Up to 5% of acute enteric fever patients develop chronic gallbladder infection and up to 25% of these carriers

may not recall a history of enteric fever [48]. The risk of becoming a chronic carrier following an acute infection increases with age; the risk is also greater for women than for men, and is particularly elevated among persons with cholelithiasis [183, 184]. People with urinary tract abnormalities such as schistosomiasis infection may also become chronic carriers of *Salmonella* [48]. Furthermore, chronic carriers are at increased risk of cancer of the gallbladder and biliary system [177, 185, 186]. It has been hypothesised that bacterial toxins and secondary metabolites produced by persistent bacterial infection might induce carcinogenesis [187, 188].

Epidemiological studies conducted in endemic regions have indicated that there is a strong link between the development of chronic carrier state and the presence of gallstones. Approximately 90% of chronic carriers are also thought to have gallstones [189]. The carrier state is thought to be related to bacterial biofilm formation on the surface of gallstones [181]. A biofilm is defined as a population of one or more organisms attached to each other and a surface by means of a bacterium initiated matrix. These bacteria encase themselves in a hydrated matrix of polysaccharides and protein, forming a protective, slimy layer [190]. Biofilm formation is important due to its inherent resistance to antibiotic chemotherapy. The type of gallstones typically recovered from chronic carriers suggests that composition may play a role in biofilm formation; cholesterol stones recovered from chronic carriers in Mexico were found to have biofilms whereas calcium biluribinate stones were not.

1.13.2 Role of chronic carriers in enteric fever transmission

One of the most infamous *S.* Typhi carriers from history is Mary Mallon, later known as Typhoid Mary. She was the first identified healthy carrier of an infectious disease in United States, as she was symptom free and did not appear to have experienced a bout of acute enteric fever. She worked as a cook in New York and was thought to have infected 54 people through her professional activities, with four deaths from nine different local epidemics. However, Mary Mallon refused to stop working as a cook, ultimately leading to her arrest and involuntary lifelong quarantine. This confinement practice continued even after Mary's death. In 2008, it emerged that 43 female typhoid carriers were quarantined in the Long Grove Asylum in Epsom (Surrey, UK) between 1907 and 1992. Some were held for more than 40 years until the asylum was closed in 1992 [191], well beyond the widespread use of antimicrobials and the increased medical knowledge of the enteric fever carrier state.

Recent studies have shown that transmission of the disease in an endemic setting may be driven less by chronic carriers and more by recently infected acute or convalescing cases who transmit the disease in the household [78, 192]. Similar results were found in a case control study conducted in enteric fever endemic Chile, where Black *et al*. found that chronic carriers within the household accounted for only a small fraction of acute enteric fever cases [193] where high prevalence of chronic carriers of *S.* Typhi was reported at that time (694 per 10,000) [183]. Although the precise role of chronic carriers in disease transmission remains unclear, these asymptomatic carriers may act as a reservoir of diverse *S*. Typhi

strains. Additionally, these individuals are unaffected by currently available typhoid vaccines, which poses a challenge to a control programme involving immunisation [193, 194].

1.13.3 Identification of chronic *Salmonella* carriers

There is no gold standard test for detecting chronic carriers. However, isolating the bacteria by culturing stool samples remain a superior method for the detection of chronic carriers than serological and PCR methods.

As chronic carriers of *Salmonella* are asymptomatic, monitoring *Salmonella* in the stool is one option for identification of chronic carriers but is hampered by low level or sporadic shedding of the organism. Rectal swab cultures from individuals known to have high anti-Vi antibody were found to be negative [194]. In addition, stool sampling at a routine level is expensive and time consuming [49]. Culture methods using a string capsule device have also been employed for the identification of chronic carriers in the past [195], however, these are invasive methods and not feasible for the detection of chronic carriers in the community.

Studies have shown that typhoid carriers may produce higher levels of Vi antibodies over extended periods of time compared to acutely infected typhoid patients [196]. Antibodies against the Vi antigen generally appear too late in the course of typhoid illness to be helpful for diagnosis [197, 198]. In contrast, 90% of chronic gallbladder carriers of *S*. Typhi manifest high titres of serum Vi antibody [37, 199]. Therefore, the detection of IgG to the Vi antigen has been proposed as a method to detect

chronic carriers [37, 200, 201]. In a study in Chile, anti-Vi antibody titres ≥160 had a 75% sensitivity and between 92% and 97% specificity for detecting chronic typhoid carriers, and a positive predictive value of between 8% and 17% in the general adult population [199]. However, a community based sero-survey conducted in Vietnam detected high prevalence of elevated anti-Vi antibody titres in the population but failed to isolate the bacteria from stool samples [194]. Background levels of elevated anti-Vi antibody titres in typhoid endemic areas appear to be high despite a low prevalence of true chronic carriers. Therefore, the results of antibody detection techniques should be interpreted in the context of the background antibody levels in the local population [184]. Furthermore, Vi antigen is not expressed by *S*. Paratyphi A and reports have demonstrated that carriage of *S*. Paratyphi A in the gallbladder is almost as common as *S*. Typhi in some enteric fever endemic regions [202]. Thus, there is a clear need for the development of techniques that are less invasive and can identify chronic *S.* Paratyphi A in addition to *S.* Typhi carriers.

Detection of IgA may be an important marker of chronic carriage as IgA antibodies are known to be stimulated in the mucosal membrane if bacteria are intermittently shed into the lumen from the gallbladder giving a persistent stimulation to the immune response [203].

1.13.4 Treatment of chronic *Salmonella* carriers

1.13.4.1 Antimicrobial therapy

Various antimicrobials have been used to treat chronic carriers, however such treatment has not proved to be effective in the resolution of chronic colonisation of the gallbladder. Even prolonged, high-dose antimicrobial therapy resolves less than two-thirds of chronic infections, although treatment with ampicillin has been shown to be effective in patients without gallstones [204, 205]. The mechanisms of resistance to antimicrobials in bacterial biofilms are based on three hypotheses. The first is the possibility of slow or incomplete penetration of the antimicrobial into the biofilm. Secondly, the biofilm could represent an altered chemical microenvironment in which the antimicrobial cannot function fully. Finally, it is thought that a sub-population of microorganisms in a biofilm can form a unique and highly protected phenotypic state similar to spore formation [190]. Chloramphenicol and sulfonamides are unable to eradicate the carrier state effectively. Moderately successful results have been obtained with the use of oral ampicillin [206]. Norfloxacin and ciprofloxacin were found to be effective and well-tolerated antimicrobial agents for eradicating the chronic carrier state [207, 208]. However, the emergence of MDR *S*. Typhi during 1980s has led to the failure of fluoroquinolones as a drug of choice for the treatment of enteric fever. A study from Nepal showed no MDR strains among the *Salmonella* isolated from chronic carriers [209] whereas, from India, 19.4% of the *S*. Typhi isolates from chronic carriers were found to be MDR; ceftriaxone resistance was also noted in 17% of the isolates [210].

In the presence of cholelithiasis, antibiotic therapy as well as cholecystectomy may be required [48].

1.13.4.2 Surgical methods

Removal of gallbladder (cholecystectomy) increases the cure rate, but does not guarantee elimination of the carrier state [211]. Biofilm infections usually persist until the colonised surface is surgically removed from the body [190]. Additional foci of infection can persist in the biliary tree, mesenteric lymph nodes or liver [212, 213].

1.14 Hypothesis of the study

Several studies from Nepal have shown that enteric fever is still an endemic disease. Humans are the only known natural host where the organisms colonise the gallbladder. It is gallbladder colonisation and faecal shedding that is thought to form a central dogma for the transmission and persistence of enteric fever. Epidemiological data related to chronic carriers are none existent and in an endemic region like Nepal, acute shedders outnumber chronic carriers. Therefore, I hypothesise that chronic carriers may play a minor role in disease transmission in an endemic setting. However, they remain reservoirs of *Salmonella* of different genetic variation and such carriers will become more important if current transmission mechanisms are disturbed with the introduction of an effective intervention strategy like vaccine and water and sanitation quality improvement.

Furthermore, I predict that antimicrobial resistance is pivotal, with those infected with highly resistant organism being more likely to relapse and more likely to progress to carriage. Therefore, I aimed to study the epidemiological and microscopic characteristics of chronic carriers, and the phenotypic and genotypic characteristics of *Salmonella* isolated from chronic carriers and patients with acute enteric fever.

1.15 Aims and objectives of the study

1. To examine the overall antimicrobial susceptibility pattern of *S*. Typhi and *S*. Paratyphi A isolated from three randomised controlled trials conducted from 2005 to 2011.

2. To examine and compare *Salmonella* strains that caused relapse with non-relapsing strains by genotypic and phenotypic characteristics.

3. To identify the clinical risk factors for the development of relapse in enteric fever.

4. To calculate the prevalence of chronic biliary carriage of *S.* Typhi and *S*. Paratyphi A among patients with gallbladder diseases and define their microbiological and epidemiological characteristics.

5. To investigate the antibody responses against *S.* Typhi and *S.* Paratyphi A in individuals with acute enteric fever, chronic carriers and the general hospital population.

6. To identify the pro-inflammatory cytokines that play key role during acute and chronic infection.

7. To study the serum bactericidal activity of plasma from acute enteric fever patients and chronic carriers.

8. To explain the mechanism of survival and interaction between *Salmonella* and gallbladder tissues by microscopy.

2 Study subjects, materials and methods

2.1 Ethics statement

The bacterial strains used for antimicrobial susceptibility testing and genotyping were collected during three clinical trials conducted for the treatment of uncomplicated enteric fever at Patan Hospital. These trials were gatifloxacin versus cefixime (ISRCTN75784880) [108], gatifloxacin versus chloramphenicol (ISRCTN53258327)[109] and gatifloxacin versus ofloxacin (ISRCTN63006567). These trials were conducted according to the principles expressed in the Declaration of Helsinki and was approved by the institutional ethical review boards of Patan Hospital, The Nepal Health Research Council (NHRC) and The Oxford University Tropical Research Ethics Committee (OXTREC). Similarly, ethical clearance was obtained from these boards for the gallbladder characteristics study (Reference number: 2108).

2.2 Informed consent procedure

All enrolees in both the clinical trials and the gallbladder study were required to provide written informed consent (Appendix 9.1 and 9.2) for the collection and storage of all samples and subsequent data analysis. In the case of those less than 18 years of age, a parent or guardian was asked to provide written informed consent.

2.3 Setting

Nepal is a landlocked country in South Asia positioned between the Tibet Autonomous Region of the People's Republic of China to the north and the Republic of India to the south (Figure 2.1). Divided into 14 zones with 75 districts, the nation has a population of 26.5 million according to 2011 census. It is listed by the United Nations as a Least Developed Country (LDC) on the basis of per capita income, human development indices and economic criteria.

The work for this thesis was conducted at Patan Hospital, a 450-bed government hospital located in the Lalitpur Sub-Metropolitan City (LSMC) in the Kathmandu valley, Nepal. Patan Hospital provides both emergency and elective inpatient services. More than 300,000 people, both inpatient and outpatient, attend this hospital every year for healthcare service and treatment. Enteric fever is a common complaint at Patan Hospital and *S*. Typhi and *S.* Paratyphi A are the most common bacteria cultured from blood of febrile patients in this location. Patan is one of the few locations in LSMC capable of performing a blood culture and diagnosing enteric fever through microbiology. At Patan Hospital, all febrile patients with suspected bacteraemia have a blood culture performed.

Additionally, antimicrobials are available without prescription in the community in a variety of public and private outlets; there are numerous private physician clinics where patients may seek advice and clinical diagnosis for febrile disease. There has been no widespread implementation of a typhoid vaccine in this area, yet a generic typhoid Vi vaccine is available for purchase in some health care settings. However, at the time of this investigation there was limited community uptake of the vaccine.

Figure 2.1: Location of Nepal in South Asia, between China to the North and India to the south. Also shown is Kathmandu, the capital of Nepal

2.4 Study population

This work consisted of three separate study populations: suspected enteric fever patients enrolled in clinical trials, surgical patients undergoing cholecystectomies and a general hospital population derived from a plasma bank.

2.4.1 Suspected enteric fever patients enrolled in clinical trials

Three separate clinical treatment trials were conducted at Patan Hospital from 2005 – 2011. Inclusion and exclusion criteria for each study were the same and all patients were derived from the same broad population. Patients visiting the Outpatient-Department at Patan Hospital were eligible to be enrolled. Patients with a fever lasting for more than 3 days who were clinically diagnosed with enteric fever (undifferentiated fever with no clear focus of infection on preliminary physical exam and appropriate laboratory tests) whose residence was in a pre-designated area of about 20 km^2 in urban Lalitpur (Figure 2.2) and who gave fully informed, written consent were eligible for the study. Exclusion criteria included pregnancy or lactation, age under 2 years or weight less than 10 kg, presence of shock, jaundice, gastrointestinal bleeding or any other signs of severe typhoid fever, previous history of hypersensitivity to either of the trial drugs, or known previous treatment with chloramphenicol, quinolone antibiotic, third generation cephalosporin, or macrolide within one week of hospital admission. Patients who reported a history of treatment with amoxicillin or cotrimoxazole were included as long as they did not show evidence of clinical response.

Each enrolled patient was managed as an outpatient and attended to by trained community medical assistants (CMAs). The CMAs visited each patient's house every 12 hours for 10 or 14 days (depending upon the antimicrobial treatment arm) or until the patient was cured. The physicians re-examined the patients on days 8 and 15, and at 1, 3, and 6 months. All examinations were standardised and entered into case report forms. A short questionnaire was administered detailing recent history of illness in both the patient and members of the patient's household (Appendix 9.4). During the period of follow up in this study (6 months) incidence of febrile episodes in household members were recorded at hospital via a questionnaire. Complete blood counts were done on days 1, 8, and 15 of each patient in the study. On day 1, serum creatinine, bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were also measured [109]. Blood culture was performed as described in Section 2.6.1 in all patients on the day of enrolment (day 1), in the culture positive patients or clinically symptomatic on day 8. Stool cultures were done on day 1 in all patients, and in culture-positive patients after completion of treatment and at the 1 month, 3 month, and 6 month visits (Section 2.6.2).

Figure 2.2: The enteric fever catchment area surrounding Patan Hospital [75].

Patan is part of the metropolitan area of greater Kathmandu and lies to the south of the city of Kathmandu, separated by the Bagmati River. This Google Earth image has been drawn to display our study catchment area surrounding Patan Hospital, labeled as A. The position of residences of enteric fever patients who have been enrolled in clinical studies and are farthest from the hospital in each direction have been highlighted on the map with yellow markers and joined to calculate the catchment area. The point denoted as B marks the location of the residence farthest from the hospital and represents a distance of 6 km; the total catchment area surrounding the hospital is approximately 28.5 km^2.

2.4.2 Surgical patients undergoing cholecystectomies

The surgical department of Patan Hospital performs approximately 400 cholecystectomies annually. For the purposes of this study, consecutive patients admitted to the surgical ward from June 2007 to October 2010 for either open cholecystectomy or laparoscopic surgery for symptomatic cholelithiasis between 8am and 4pm were approached for participation. All patients who gave written informed consent were eligible for the study; there were no exclusion criteria. A questionnaire related to the patient's health and demographics was administered prior to surgery (Appendix 9.3). The subjects were also required to provide a stool sample for culture and blood sample for serology purposes. Figure 2.3 shows surgeons performing the surgery and collecting bile samples and gallbladder tissue during the procedure.

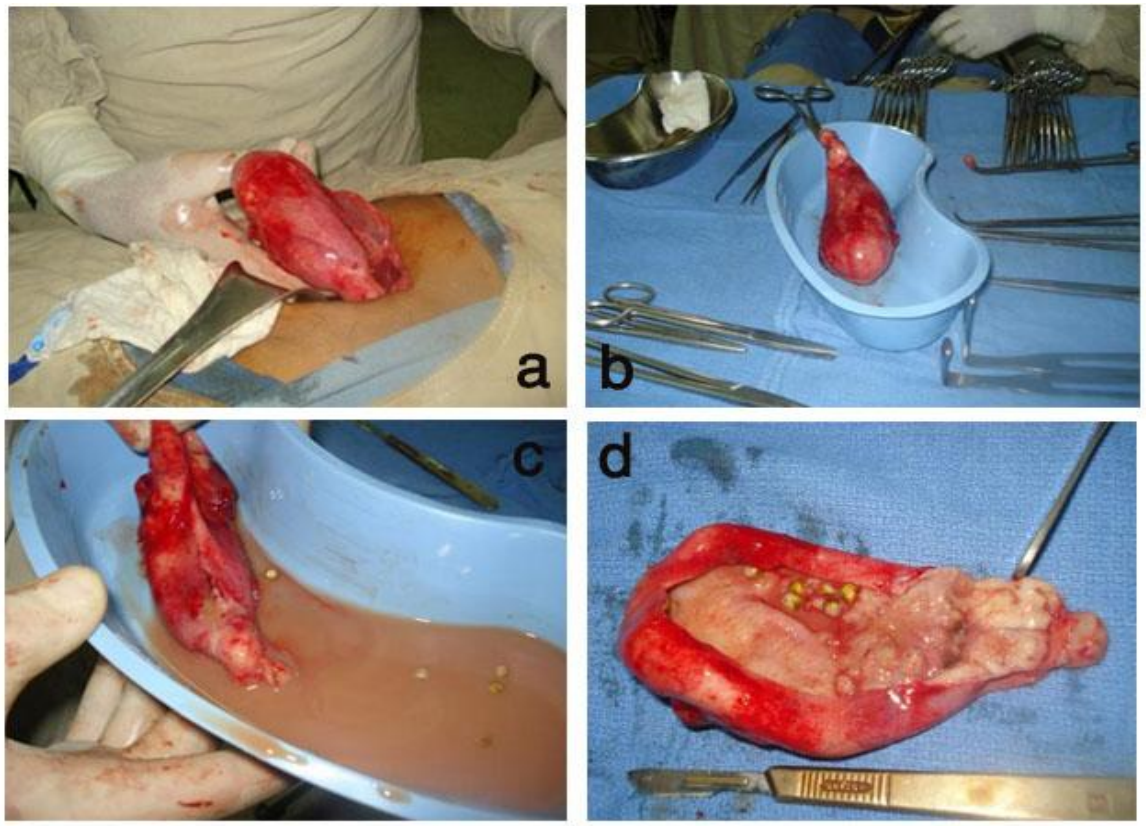


Figure 2.3: Patient undergoing cholecystectomy at Patan Hospital, Kathmandu. **a;** A gallbladder from a patient with acute cholecystitis, **b;** Gallbladder post-removal, **c;** Draining the gallbladder, **d;** Bile and stones are removed and gallbladder fixed in formaldehyde to be sent to the histopathology department. Bile is inoculated onto peptone broth and Selenite F broth and sent for culture to the Microbiology laboratory. Gallstone was stored in PBS and stored at -80^{o}C for electron microscopy examination.

2.4.3 General Hospital population derived from a plasma bank

This group consisted of patients who visited Patan Hospital Emergency Department from January 2010 to September 2010. A total of 795 plasma samples collected from an age group spanning from 2 months to 65 years of age. A total of 620 plasma samples were tested from 2 months to 30 years old age group, each age band consisted of 30 samples. From age 31 to 60 years old, a total of 150 samples were tested each age band comprised of 10 plasma samples. Finally, a total of 25 samples from 61 to 65 years old age were tested each band consisted of 5 samples.These samples were evaluated for the distribution and prevalence of antibodies expressed against *S.* Typhi- Vi antigen and *S*. Paratyphi A- O:2 antigen. A total of 2 ml of blood was collected in EDTA K3 (Golden Vac) containing 10% EDTA. Plasma was separated by centrifuging at 300rpm for 3 minutes and transferred onto a cryovial and stored at -20^{o}C until the tests were performed.

2.5 Methods applicable throughout thesis

2.5.1 Microbiological culture, identification and antimicrobial susceptibility testing

All isolates grown from blood, stool and bile were identified by API 20E, confirmed by serotyping and subjected to antimicrobial susceptibility testing.

2.5.1.1 Identification of isolates by API 20E system

All bacterial isolates were identified using the API 20E (bioMérieux, France) biochemical identification kit that contains 21 biochemical tests. The API 20E strip consists of 20 microtubes containing dehydrated substrates of 2-nitrophenyl-βDgalactopyranoside (ONPG), arginine (ADH), lysine (LDC), ornithine (ODC), trisodium citrate (CIT), sodium thiosulfate (H_2S), urea (URE), tryptophane (TDA), sodium pyruvate (IND), acetoin (VP), gelatin (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose(SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA). The procedure was performed according to the manufacturer's recommendations. Briefly, the test substrates were inoculated with a bacterial suspension in sterile saline that reconstituted the media. After filling the cupules of ADH, LDC, ODC, H_2S and URE, the microtubes were overlaid with mineral oil. The incubation box was prepared by adding 5 ml of sterile water to the honeycombed wells of the tray to create a humid atmosphere, and the API strip was placed within the incubation box and incubated at 36°C ±2°C for 24 hours. During incubation, metabolism of the organism produced colour changes in the various tests spontaneously or in the case of VP, TDA and IND through the addition of reagents**.** Additionally, an oxidase test was performed to detect the ability of the microorganism to produce the enzyme cytochrome c oxidase. Each biochemical reaction was assessed according to the set score system that created a numerical seven digit profile for the organism which was used for identification following manufacturer's instructions (Figure 2.4). The API 20E test strip includes reactions for ONPG (activation of the β- galactosidase), ADH (dehydrolation of the L- arginine by arginine deydrolase), LDC (decarboxylation of the lysine by lysine decarboxylase), ODC (decarboxylation of the L-ornitine by ornithine decarboxylase),

CIT (utilisation of citrate), H_2S (production of hydrogen sulphide), URE (activation of urease), TDA (activation of tryptophan deaminase), IND (production of indole, which is detected by the addition of Kovac's reagent), VP (production of acetoin, which is detected by the Voges Prosakuer test), GEL (activation of gelatinase), GLU (fermentation of glucose), MAN (fermentation of mannose), INO (fermentation of inositol), SOR (fermentation of sorbitol), RHA (fermentation of rhamnose), SAC (fermentation of sucrose), MEL (fermentation of melibiose), AMY (fermentation of amygdalin), ARA (fermentation of arabinose) and OX (production of cytochrome c oxidase).

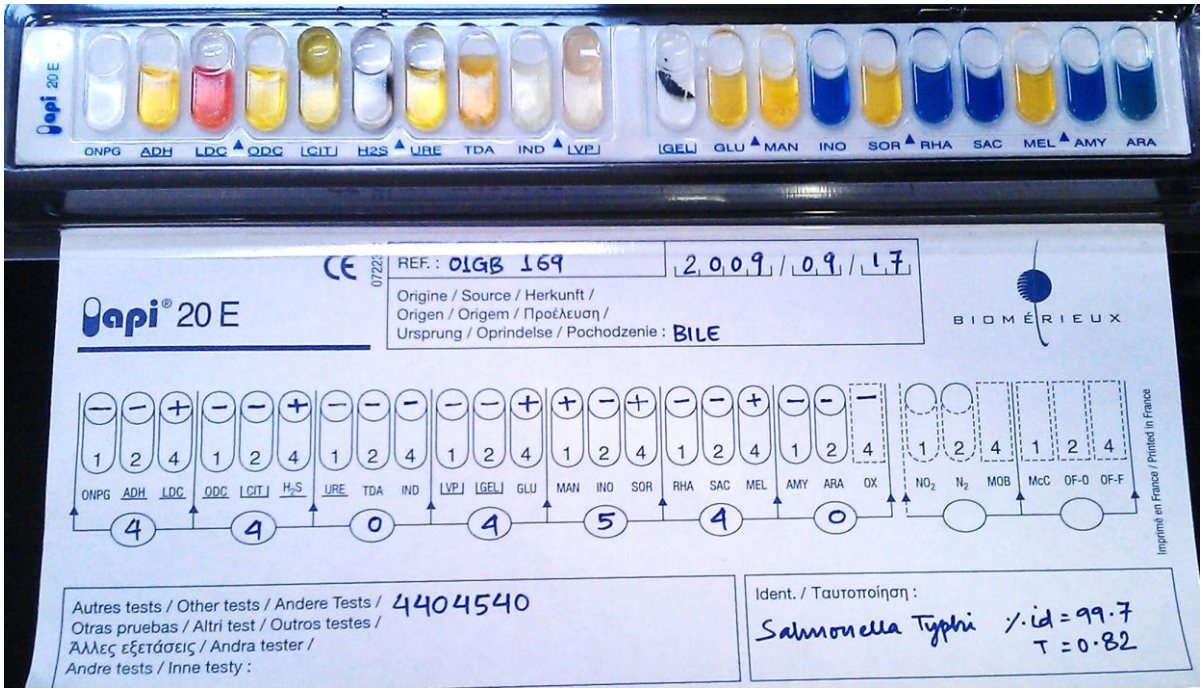


Figure 2.4: Representative biochemical characteristic of *S*. Typhi using API 20E test

The API 20E test strip includes reactions for ONPG (activation of the β-galactosidase), ADH (dehydrolation of the L- arginine by arginine deydrolase), LDC (decarboxylation of the Llysine by lysine decarboxylase), ODC (decarboxylation of the L-ornitine by ornithine decarboxylase), CIT (utilisation of citrate), H_2S (production of hydrogen sulphide), URE (activation of urease), TDA (activation of tryptophan deaminase), IND (production of indole, which is detected by the addition of Kovac's reagent), VP (production of acetoin, which is detected by the Voges Prosakuer test), GEL (activation of gelatinase), GLU (fermentation of glucose), MAN (fermentation of mannose), INO (fermentation of inositol), SOR (fermentation of sorbitol), RHA (fermentation of rhamnose), SAC (fermentation of sucrose), MEL (fermentation of melibiose), AMY (fermentation of amygdalin), ARA (fermentation of arabinose) and OX (production of cytochrome c oxidase).

2.5.1.2 Serotyping of *Salmonella* isolates

Isolates identified as *S*. Typhi and *S*. Paratyphi A by API 20E were confirmed by slide agglutination by specific antisera (Murex, Dartford, UK). For *Salmonella* serovars, *S.* Typhi strains were defined as isolates exhibiting agglutinations with the poly O, O:9 and Vi antisera (Murex, Dartford, UK). *S.* Paratyphi A strains were defined as isolates exhibiting agglutination to poly O, and O:2 antisera.

To perform the agglutination, one drop of the appropriate agglutinating sera was placed on a clean glass slide. One colony of the test strain picked from a nutrient plate was mixed with the agglutinating serum. After thorough mixing, the slide was gently rotated for approximately 15 seconds and observed for agglutination with the naked eye. Positive and negative controls were performed in tandem with an American Type Culture Collection (ATCC) strain and sterile water respectively.

2.5.1.3 Antimicrobial susceptibility testing

2.5.1.3.1Disc diffusion method

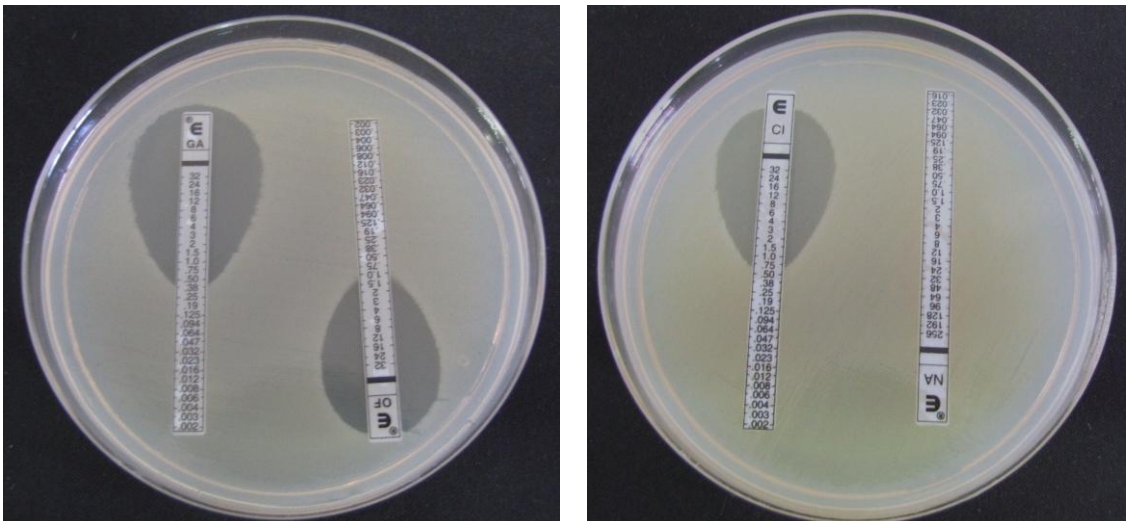
The susceptibility of isolates to antimicrobials was determined using the disc diffusion method on Mueller-Hinton (MH) agar plates, according to the Clinical and Laboratory Standards Institute (CLSI) procedures and interpreted following CLSI guidelines [214]. The antimicrobials tested were amoxicillin, chloramphenicol, co-trimoxazole, NA, ciprofloxacin, ofloxacin, ceftriaxone, gatifloxacin, gentamycin, azithromycin, amikacin, cefixime. At least three to five well-isolated colonies of the

same morphological type were selected from an 18- to 24-hour non-selective agar medium like nutrient agar. The top of a colony was touched with a loop, and transferred into a tube containing 1 ml of sterile distilled water. The suspension was adjusted to match the 0.5 McFarland turbidity standard. A sterile cotton swab was dipped into the adjusted suspension and streaked evenly over the surface of the medium in three directions, the plate was rotated through approximately 60^{o} to ensure even distribution. Before applying discs, the plate was dried until there was no visible surface moisture. Antimicrobial discs were pressed down to ensure complete contact with the agar surface. After incubating for 16 to 18 hours at 37^{o}C, the diameter of the clear zone of inhibition of growth was measured to the nearest whole millimetre, using a ruler [214].

2.5.1.3.2 Minimum inhibitory concentration

The minimum inhibitory concentration (MIC) is the most commonly used parameter to describe the efficacy of an antimicrobial agent against a bacterial strain. MICs were performed for amoxicillin, augmentin, cefixime, ceftriaxone, chloramphenicol, ciprofloxacin, cotrimoxazole, gatifloxacin, nalidixic acid, ofloxacin by E-test (AB Biodisk, Sweden). The results were interpreted using CLSI guidelines 2012. Susceptibility to ciprofloxacin was evaluated using a new breakpoint and for ofloxacin since CLSI 2012 has not adapted a new breakpoint, we evaluated using newly suggested susceptibly breakpoint of 0.25 µg/ml [215]. Similar to the disc diffusion method, an MH plate was swabbed with the bacterial suspension and

allowed to dry before placing E-test strip on the surface. The plates with E-test strips were incubated for 20 hours at 37^{o}C. The MIC was then read from the scale where the ellipse edge intersects the strip and read at the point of inhibition of all growth. When growth occurs along the entire strip and no inhibition is seen, the MIC was reported as greater than the highest value on the reading scale. When the ellipse is so large that the zone edge does not intersects the strip, the MIC was read as less than the lowest reading on the scale [216] (Figure 2.5)



a) **b)**

Figure 2.5: Representative image of MIC E-tests for examining antimicrobial agents

a) *S*. Typhi isolate from blood, demonstrating sensitivity to gatifloxacin (GA) and ofloxacin (OF). The MIC of GA (0.50 µg/ml) and OF (1.0µg/ml) to this organism were read according to manufacturers recommendations.

b) *S.* Typhi isolate from blood, demonstrating sensitivity to ciprofloxacin CI and nalidixic acid (NA). The MIC of CI (0.75 µg/ml) and NA (>256µg/ml) to this organism were read according to the manufacturers recommendations

Table 2.1: Zone diameter and minimal inhibitory concentration (MIC) interpretive standard based on CLSI guidelines 2012

Antimicrobial agents	Disc content (µg)	Zone diameter Nearest Whole (mm)			MIC interpretative Criteria (µg/ml)		
		S	I	R	S	I	R
Ampicillin	10	≥17	14-16	≤13	≤8	16	≥32
Amikacin	30	≥17	15-16	≤14	≤16	32	≥64
Augmentin	10/20	≥18	14-17	≤13	≤8/4	8-16	≥32/16
Ceftriaxone	30	≥23	20-22	≤19	≤1	2	≥4
Cefotaxime	30	≥26	23-25	≤22	≤1	2	≥4
Cefixime	5	≥19	16-18	≤15	≤1	2	≥4
Chloramphenicol	30	≥18	13-17	≤12	≤8	16	≥32
Ciprofloxacin	5	≥31	21-30	≤20	≤0.06	0.12-0.5	≥1
Gentamicin	10	≥17	35	≤13	≤8	54	≥32
Gatifloxacin	5	≥18	15-17	≤14	≤2	4	≥8
Nalidixic Acid	30	≥19	14-18	≤13	≤16	-	≥32
Ofloxacin	5	≥16	13-15	≤12	≤2	4	≥8
Tetracycline	30	≥15	12-14	≤11	≤4	8	≥16
Trimethoprim-sulphamethoxazole	1.25/23.75	≥16	11-15	≤10	≤2/38	-	≥4/76

2.6 Methods specifically related to chapter 3

2.6.1 Blood culture

Blood was collected from individuals who were enrolled in the clinical trials for suspected typhoid fever. A volume of 6 ml and 3 ml of blood was used from those over 12 years of age and those 12 years of age or less, respectively. Blood was inoculated into 30-50 ml of medium containing tryptone soya broth containing 0.05% of sodium polyanethanol sulphonate. The inoculated medium was incubated at 37°C and examined daily for bacterial growth over a 7-day period. If the broth became turbid, indicative of growth, the inoculated broth was sub-cultured onto MA medium to isolate invasive *Salmonella*. Any colonies presumptive of *S*. Typhi and *S*. Paratyphi A were identified as described in Section 2.5.1 using API 20E system tests and serotype-specific antisera (Murex Biotech, Dartford, UK). All *S.* Typhi and *S.* Paratyphi A strains were stored at -20°C in protect beads. Duplicates were dispatched for secondary verification at the microbiology laboratory at Oxford University Clinical Research Unit in Ho Chi Minh City, Vietnam [108, 109].

2.6.2 Stool culture

Stool samples were collected on Cary Blair transport medium from acute enteric fever patients on day 1 prior to the antimicrobial therapy. Samples were inoculated onto 10 ml of SEL-F broth for enrichment and incubated at 37°C. After the overnight incubation, one loopful of broth was subcultured onto MA and XLD agar media. Any colonies presumptive of *S*. Typhi or *S*. Paratyphi A were identified as described in

Section 2.5.1 using standard biochemical tests and serotyping. Stool cultures were performed at the end of the first, third and sixth month of their enrolment.

2.6.3 Molecular examination of *Salmonella* isolates

Further analysis was carried out on *S*. Typhi and *S*. Paratyphi A isolated from blood culture and bile culture.

2.6.3.1 Bacterial DNA extraction

DNA was extracted by using the Wizard ® Genomic DNA purification kit. All *S*. Typhi and *S*. Paratyphi A strains from which DNA was extracted were grown on nutrient agar plates at 37^{o}C. Colonies of bacteria were scraped from approximately half a media plate and suspended in 1ml of nutrient broth. The mixture was thoroughly vortexed to form an even suspension. The suspension was centrifuged for 2 minutes at 15,000 X g (Thermo Fischer Scientific, IEC Micro CL17, and Germany) and the supernatant was discarded. To the cell pellet, 600 µl of nuclei lysis solution was added and mixed by gently pipetting the suspension. The solution was incubated for 5 minutes at 80°C and then allowed to cool at room temperature. Following this, 3µl of RNAse solution was added to the suspension, mixed and incubated at 37°C for 30 minutes, and then allowed to cool to ambient temperature. Subsequently, 200 µl of protein precipitation solution was added and vortexed vigorously. The suspension was then incubated at -20°C for 10 minutes before centrifugation at 15,000 X g in a bench top microfuge for 10 minutes. The supernatant was transferred to a sterile microfuge tube containing 600 µl of isopropanol and gently mixed. The suspension

was then centrifuged for two minutes at 15,000 X g and the supernatant was discarded. To the solution 600µl of room temperature 70% ethanol was added and mixed by gentle pipetting. The suspension was centrifuged for two minutes at 15,000 X g, the supernatant was removed and the DNA pellet was air dried at ambient temperature. The DNA pellet was rehydrated with 100 µl of rehydration solution, and stored at -20°C until required.

2.6.3.2 Single nucleotide polymorphism (SNP) genotyping

DNA was extracted from *S.* Typhi isolates (Appendix 9.9) as described in Section 2.6.3.1. DNA quality and concentration was assessed using the Quant-IT kit (Invitrogen, USA) prior to SNP typing. Extracted DNA was subjected to SNP typing at the Sanger Institute in the laboratory of Prof. Gordon Dougan. SNP typing is a high resolution method of genotyping the *S*. Typhi population. Alleles at 113 predetermined [217, 218] *S*. Typhi chromosomal loci were evaluated using the iPLEX Gold assay (Sequenom Inc, USA). Samples were amplified in multiplexed PCR reactions before allele specific extension. Allelic discrimination was obtained by analysis with a MassARRAY Analyzer Compact mass spectrometer. Assays for all SNPs were designed using the MassARRAY Assay Design software version 3.1 (Sequenom Inc, USA). Genotypes were automatically assigned and manually confirmed using MassArray TyperAnalyzer software version 4.0 (Sequenom Inc, USA). The resulting alleles were used to assign each *S.* Typhi isolate to previously defined haplotypes as described previously [219-221].

2.6.3.3 Data analysis

Data from the three enteric fever treatment trials was gathered and stored in Microsoft Access and Excel. These data were cleaned and combined using STATA v9.2 (TX, USA). Antimicrobial resistance classification into sensitive, intermediate and resistant was based on MIC values.

A relapse case was defined as any person who (1) was culture positive for *S.* Typhi or *S.* Paratyphi A within week one of enrolment and again at any point >14 days post enrolment; (2) was culture positive for *S.* Typhi or *S.* Paratyphi A within one week of enrolment and relapsed with a fever >14 days post enrolment; (3) was culture negative but relapsed with a fever >14 days post enrolment.

Differences in demographic, clinical, household, and antimicrobial characteristics between relapse and non-relapse cases were evaluated using the chi-squared test, Fisher's exact test or the Kruskal-Wallis test for non-parametric, continuous data. Logistic regression was used to generate an odds ratio, 95% confidence interval and p-value to quantify the risk of relapse for each factor. P values ≤ 0.05 were considered statistically significant throughout.

2.7 Methods specifically related to Chapter 4

2.7.1 Routine examination before surgery

2.7.1.1 Gallbladder Morphology

Patients were routinely examined by ultrasonography before surgery to assess the presence of gallstones and to detect inflammation. Assessing the thickness of the gallbladder wall (stratified into three categories, thick: more than 4 mm, normal: 4 mm and thin: less than 4 mm), the presence and the number of gallstones, the presence and characteristics of fluid (pus: empyema, mucoid/clear/watery: mucocele and sludge) and overall morphology (contracted or distended).

2.7.1.2 Laboratory investigations

Complete blood count (CBC) (Hematocrit, total leukocytes with differential count) and biochemical tests (total bilirubin, conjugated bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and amylase) were measured prior to surgical intervention.

A 3 mL venous blood was collected in gel/clot activator tubes (Golden Vac). After the formation of clot, the tubes were centrifused at 3000rpm/min for 3 minutes. The serum thus separated was used for the biochemical tests. All biochemical tests were performed using Vitros 250 Chemistry System.

A 3 ml blood was collected in EDTA K3 (Golden Vac) containing 10% EDTA. The blood was mixed and CBC was performed using Sysmex XS 1000i.

2.7.1.3 Stool culture

A stool sample was obtained from patients prior to surgery. The sample was inoculated onto 10 ml of SEL-F broth for enrichment. Following an overnight incubation, a loopful of broth was subcultured onto MA and XLD agar plates and incubated at 37^{o}C. Any colonies suspected to be *S*. Typhi and *S*. Paratyphi A were identified by the API system and specific serological confirmation and antimicrobial susceptibility tests were performed as mentioned in Section 2.5.1.

2.7.1 Examination of samples after cholecystectomy

2.7.1.1 Bile culture

Bile collected during the surgery was cultured from all patients who underwent a cholecystectomy. Bile was inoculated into equal volumes of SEL-F broth and Peptone broth and incubated at 37^{o}C overnight. Broths were sub-cultured onto MA and XLD agar. After overnight incubation at 37^{o}C the plates were examined for the growth of Gram-negative bacteria and identification and antimicrobial susceptibility testing were performed as described in Section 2.5.1.

2.7.1.2 Molecular examination on stored bile samples

A bile sample was also collected from each patient in a separate cryogenic vial during surgery and was stored at -20^{0}C until the experiments were performed. These stored bile samples were subjected to real time PCR for the detection of *Salmonella* in bile.

2.7.1.2.1 DNA extraction from bile

DNA extraction from bile was performed using QIAamp DNA stool mini kit (QIAGEN system). Stored bile sample was brought to room temperature. A total of 300µl of bile was transferred to a micro centrifuge tube. To this sample, 1.4ml buffer ASL was added and continuously vortexed for 1 minute or more until the bile sample was completely homogenized thoroughly. This suspension was heated for 5 minutes at 70°C. It was then vortexed for 15 seconds and centrifuged at 15,000 X g for 1 minute. Then, 1.2 ml of the supernatant was pipetted into a new 2 ml microcentrifuge tube and the pellet was discarded. One InhinitEX tablet was added to the sample and immediately vortexed continuously until the tablet was completely suspended. The suspension was incubated for 1 minute at room temperature to allow inhibitors to adsorb to the InhibitEX matrix. The sample was centrifuged at 15,000 X g for 3 minutes to pellet inhibitors bound to InhibitEX matrix. All the supernatant was pipetted into a new 1.5ml microcentrifuge tube and the pellet was discarded. The sample was centrifuged at 15,000 X g for 3 minutes. Following this, 200µl of supernatant was transferred into a new 1.5 microcentrifuge tube containing 15µl of proteinase K. It was thoroughly mixed and 200µl of buffer AL was added and vortexed for 15 seconds. The suspension was incubated at 70°C for 10 minutes. To this lysate, 200µl of ethanol (96-100%) was added, and vortexed. The tube was centrifuged briefly to remove drops from inside of the tube lid. The lid of a new QIAamp spin column was labeled and placed in a 2 ml collection tube. The complete lysate was carefully applied to the QIAamp spin column without moistening the rim. The cap was closed and centrifuged at full speed for 1 minute.

The QIAamp spin column was placed in a new 2 ml collection tube, and the tube containing the filtrate was discarded. The QIAamp spin column was carefully opened and 500µl of Buffer AW2 was added. The cap was closed and centrifuged at 15,000 X g for 3 minutes. The collection tube containing the filtrate was discarded. The QIAamp spin column was placed in a new 2 ml collection tube and the old collection tube with the filtrate was discarded. It was centrifuged for 1 minute at 15,000 X g. This step helps to eliminate the chance of possible Buffer AW2 carryover. The QIAamp spin column was transferred to a new, labelled 1.5ml microcentrifuge tube. The QIAamp spin column was carefully opened and 200µl of Buffer AE was pipetted directly onto the QIAamp membrane. The cap was closed and incubated at room temperature for 1 minute, and then it was centrifuged at 15,000 X g for 1 minute to elute DNA. For maximum PCR robustness, 0.1 µg/µl of BSA was added to the eluate. It was then stored at -20^{o}C until required.

2.7.1.2.2 Multiplex Real time PCR on bile samples

Multiplex Real-time PCR was performed on all extracted DNA from bile samples to detect DNA sequences specific for *S.* Typhi and *S*. Paratyphi A. These assays were performed by me in the laboratory of Dr. Stephen Baker at Oxford University Clinical Research Unit in Vietnam in collaboration with Ms. Tran Vu Thieu Nga [128]. These PCRs were established and validated using the following methods.

2.7.1.2.3 Target sequence selection

Sequences unique to *S.* Typhi or *S.* Paratyphi A were identified using a whole-genome comparison of *S.* Typhi strain CT18 (GenBank AL513382) [16] and *S.* Paratyphi A strain AKU12601 (GenBank FM200053) [222], conducted using BLASTn and visualized using the Artemis Comparison Tool (ACT). To confirm whether these sequences were likely to discriminate more generally between members of the *S.* Typhi and *S.* Paratyphi A populations, we searched for sequences in all available *S.* Typhi (finished sequence for strain Ty2 (GenBank AE014613) and 17 additional 454 shotgun-sequenced strains (GenBank CAAV01000001-CAAV01003682)[220] and *S.* Paratyphi A strains (finished sequence for strain ATCC9150 (GenBank CP000026)). Genomic data from the recent *S.* Typhi and *S.* Paratyphi A sequencing projects were mined to find genes that were specific for each serovar [220, 222]. The criteria for selection were: a lack of homology with other genes in other pathogens or human sequences (to ensure no cross-reactivity) and the sequence was required to be conserved in all the re-sequenced and previously sequenced strains.

2.7.1.2.4 Manipulation, bacterial strains and construction of internal control

Strain *E. coli* VU1 was constructed by PCR amplifying the gB gene from Phocid herpes virus using the primers phHV-1 forward and reverse [223]. The gB gene amplicon was cloned into plasmid pCR 2.1-TOPO (Invitrogen). *E. coli* VU1 was to act as an internal control to monitor DNA extraction and amplification efficiency in all PCR reactions using primers phHV-1 forward and reverse and a specific probe

[223]. PCR amplicons for all target sequences were produced by monoplex conventional PCR using the primer sequences outlined below. *E. coli* TOP10 cells (Invitrogen) were transformed with purified plasmid DNA containing target DNA sequence and PCR amplicons were sequenced (Applied Biosystems) to ensure accurate amplification. Purified plasmid DNA was used as template in all subsequent experiments which utilized a standard curve.

2.7.1.2.5 Primers and PCR conditions

Primers and probes for the detection of *S*. Typhi and *S*. Paratyphi A were designed using Primer Express Software (Applied biosystems) and manufactured by Sigma - Proligo (Singapore). Primers and probe sequences were shown in Table 2.2. PCR reactions were performed in 25 µl reaction volumes consisting of 5 mM $MgCl_2$, 2.5 mM each deoxynucleotide triphosphate, 1 U of Hot start *Taq* DNA polymerase (Qiagen) and 5 µl of template DNA. Final reaction concentrations of the three primer and probe sets for internal control, *S.* Typhi and *S.* Paratyphi A were 0.8 µM of each primer and 0.25 µM of each probe. PCR was performed on a Bio-Rad Chromo 4 real-time PCR system and fluorescence was released via the TaqMan 5' to 3' exonuclease activity. All PCRs were cycled under the following conditions; 15 minutes at 95°C and 45 cycles of 15 seconds at 95°C, 15 seconds at 60°C and 45 seconds at 72°C.

Table 2.2: Oligonucleotide primers and probes used in the PCR reaction for the detection of *S.* Typhi and *S*. Paratyphi A DNA sequence

Organism	**Primer and Probe sequence**
Salmonella Typhi	
	ST- Frt 5'CGCGAAGTCAGAGTCGACATAG 3'
	ST-Rrt 5'AAGACCTCAACGCCGATCAC 3'
	ST- Probe 5' FAMCATTTGTTCTGGAGCAGGCTGACGG-TAMRA 3'
Salmonella Paratyphi A	
	Pa-Frt 5'ACGATGATGACTGATTTATCGAAC 3'
	Pa-Rrt 5' TGAAAAGATATCTCTCAGAGCTGG 3'
	Pa-Probe 5' Cy5-CCCATACAATTTCATTCTTATTGAGAATGCGC-BHQ5 3'
Phocid herpes virus	
	PhHV-Frt 5' GGGCGAATCACAGATTGAATC 3'
	PhHV-Frt 5' GCGGTTCCAAACGTACCAA 3'
	phHVProbe- hex 5' Hex-TTTTTATGTGTCCGCCACCATCTGGATC-TAMRA 3'

2.7.1.2.6 Real time PCR quantification

Plasmid DNA with cloned target DNA sequences (*S.* Typhi and *S.* Paratyphi A) were purified and concentrations (µg/ml) were calculated by a NanoDrop spectrophotometer (Thermo-Scientific). Concentrations were converted to copy number using the formula; mol/g x molecules/mol=molecules/g, via a DNA copy number calculator http://www.uri.edu/research/gsc/resources/cndna.html. Plasmid solutions were diluted in 10-fold serial dilutions ranging from 100 to 105 plasmid copies per µl. Serially diluted plasmid DNA was mixed in increasing (*S.* Typhi target) and decreasing (*S.* Paratyphi A target) concentrations and subjected to a real-time PCR amplification. Standard curves for *S.* Typhi and *S.* Paratyphi A copy number were constructed by plotting the C*t* value against the plasmid DNA copy number.

2.7.1.3 Histopathlogical examination of gallbladder

All extracted gallbladder tissue was fixed in 4% formaldehyde and sent to the histopathology department of Patan Hospital for examination to assess and confirm the extent of the inflammation; all histopathology was performed by the skilled technician who was blinded to presence or absence of bacteria within the bile. All sections were examined by light microscopy after staining with heamatoxylin and eosin. Inflammation was identified by tissue morphology and the presence of neutrophils (indicative of acute inflammation) and lymphocytes (chronic inflammation).

Once the tissue was fixed, it was then processed into a form in order to make thin microscopic sections. First, water from the tissues was removed by dehydrating with a series of different concentrations of isopropanol (50%, 70% and 100%) followed by series of different concentrations of methanol (50%, 70% and 100%), letting the samples stand for 1 hour in each solution. This was followed by clearing of dehydrant with a clearing agent, xylene, for 1 hour in 2 series. The tissues were embedded into moulds along with liquid embedding material, paraffin wax, which then hardens when cooled. Sectioning was performed by a steel knife mounted in a microtome and tissues were cut 3 micrometer (μm) thick tissue sections which were mounted on a glass microscope slide. Then the mounted sections were treated with H and E staining.

2.7.1.3.1 Hematoxylin and Eosin (H and E) staining

Hematoxylin and eosin (H and E) is a common stain used almost ever histopathology department, samples for this study were stained in the histopathology department of Patan Hospital. The embedding process was reversed in order to remove the paraffin wax from the tissue and allow water soluble dyes to penetrate the sections. Slides were deparaffinized by passing through two changes of xylene for 5 minutes each. Then the slides were rehydrated by passing the slides through graded alcoholic solutions of 100% ethanol followed by 90% ethanol, 70% ethanol then into tap water, letting the slides stand for 5 minutes in each solution.

As a dye with a basic pH, hematoxylin has an affinity for the nucleic acids of the cell nucleus. Eosin is an acidic dye with an affinity for cytoplasmic components of the cell. The slide carrier was kept into a container filled with hematoxylin for 4 minutes then rinsed with tap water for 5 minutes to remove the hematoxylin. The slide carrier was dipped into a jar containing 0.1% acid alcohol (1 ml HCL in 99 ml propanol) 3 times and then into tap water 3 to 4 times. This is also known as regressive staining as it removes the cytoplasm staining but retains the nucleus staining. The slides were stained in 1% eosin for 2 minutes for cytoplasmic staining. 2-3 drops of mountant was dropped onto the slide and a cover glass was then put onto the slide.

2.7.2 Data Analysis

Data were entered into a database using Excel 2007 (Microsoft). Data cleaning and analysis was performed in Stata/IC version 9.2 (StataCorp, TX, USA). Clinical, socioeconomic, haematological, antimicrobial resistance and gallbladder characteristics were compared between those who were bile culture positive for *Salmonella,* those who were bile culture positive for Gram-negative bacteria except *Salmonella,* and bile culture negative patients. Chi-square and Fisher's exact tests were used to compare proportions between groups and Mann-Whitney U tests were used for continuous non-parametric data. P-values < 0.05 were considered to be statistically significant.

2.8 Methods specifically related to chapter 5

Plasma samples were collected from following group of patients for the detection of antibodies against *S*. Typhi antigen (Vi) and *S*. Parayphi A antigen (O:2) by ELISA, serum bactericidal assay and cytokine detection. Serological assays were performed by me in the laboratory of Dr Stephen Baker at Oxford University Clinical Research Unit in Vietnam in collaboration with Ms Tran Vu Thieu Nga.

2.8.1 Blood collection for serology from enteric fever patients

A total of 2 ml blood was collected and stored as described in Section 2.4.3 from enteric fever patients on day 1, day 8, month 1 and month 3.

2.8.2 Samples from surgical patients

2.8.2.1 Blood collection

A 2 ml blood sample was drawn from patients admitted to the surgical ward who underwent cholecystectomy. Plasma was separated as described in Section 2.4.3 until serological tests were performed.

2.8.2.2 Oral swab collection

Oral swab samples from surgical patients were collected by using Oracol swab (Malvern Medical Developments Ltd., Worcester, UK) on the day of their first follow up (day 7) after surgery. Oracol swab is a cylindrical plastic sponge mounted on a

short plastic stick. The enrolled subject used the Oracol sponge to brush their teeth and gums for a period of 60 seconds. The sponge was then placed into an Oracol tube with 1 ml of phosphate buffered saline, pH 7.2, containing 0.2% Tween 20 and 10% faetal bovine serum (Sigma chemical co. Germany). Saliva was extracted by vortexing and centrifugation at 3000rpm for 10 minutes, before the pad or sponge was removed [224]. The extracted saliva was then stored at -20^{o}C.

2.8.3 General hospital population group

Blood samples were collected from patients visiting Emergency Department of Patan Hospital from January – September 2010 were subjected to antibody detection assay against Vi and O:2 antigens. Separation and stoarage of plasma was doe as mentioned in Section 2.4.3.

2.8.4 Enzyme linked immunosorbent assay (ELISA)

ELISAs were developed to measure immunoglobulin (Ig) G antibodies to the Vi and O2 antigens. The indirect ELISA assays, performed on the plasma samples, were performed by me in the laboratory of Dr Stephen Baker at the Hospital for Tropical Diseases, Ho Chi Minh city, Vietnam. These assays were performed according to the protocol provided by the Novartis Vaccine Institute for Global Health (NVGH) protocol [169].

Briefly, Nunc flat-bottomed 96-well plates were coated with 100 µl of purified Vi antigen at a concentration 1µg/ml and O2 antigen at a concentration 15µg/ml in

carbonate buffer (0.05 M; pH 9.6). The immunoplates were incubated at 4°C overnight. The wells were blocked with 200 µl of 5% skimmed milk powder (Fluka) in phosphate-buffered saline containing 0.05% Tween 20 (PBST) and incubated at room temperature (RT) for 2 hours. The wells were then washed three times with PBST using automatic ELISA washer. Then, 100µl/well of the diluted sera diluted 1:200 in PBST with 0.1% bovine serum albumin (BSA), were incubated for 2 hours at RT. Each sample was run in duplicates. In each ELISA plate, standard was run in duplicates. For the detection of IgG against Vi, a standard curve was generated from human anti Vi standard serum provided by NVGH. The reference serum was diluted 1:25 in dilution buffer (0.1% BSA in PBS with 0.05% Tween 20), serially double diluted up to ten times. Standard for IgG against O:2 was generated by a pool of plasma from *S*. Paratyphi A confirmed patients with high level of IgG. Similarly, Standard for IgM against Vi and O:2 were generated by a pool of plasma from *S*. Typhi and *S*. Paratyphi A confirmed patients who had high level of antibody expressed against Vi and O:2 respectively, which had been briefly screened prior to actual quantification. After washing the plates as mentioned above, secondary antibody was added 100µl/well and incubated at RT for one hour. The secondary antibody used was alkaline phosphatase-conjugated anti human IgG (γ-chain specific, A3187, SIGMA) and anti human IgM (µ- chain specific) diluted in 1:10,000 and 1:2500 in PBST with 0.1% of BSA respectively. Washing process was repeated at the end of the incubation. Alkaline phosphatase substrate, p-nitrophenyl phosphate (Sigma N2770, pNPP) was added 100µl/well. Substrate was freshly prepared 30 minutes before loading it by adding one tablet of pNPP and one tablet of buffer to aluminium foil wrapped tube containing 20 ml of water. The ELISA

plates were incubated at RT in the dark for 1 hour. Absorbance of samples was read against a blank (antigen in buffer) at dual filter 490nm and 405nm on an ELISA reader (Bio-Rad 550- Microplate reader). ELISA units were expressed relative to anti Vi and anti O:2 standard serum curves, with best 4 parameter fit determined by modified Hill Plot. One ELISA unit was defined as the reciprocal of the dilution of the standard serum that gave an absorbance value equal to 1 [169, 172]. The cut off value of the ELISAs was defined as the optical density of blank control wells plus 2 SD.

2.8.5 Serum bactericidal assay (SBA)

Serum bactericidal assay was performed on plasma samples collected from acute enteric fever patients, chronic carriers and bile culture negative patients. The assay was performed as described by Micoli F *et al*. [172].

S. Paratyphi A was grown in Luria Bertani (LB) plates incubating at 37^{o}C. Approximately 5 colonies were inoculated in 50 ml of LB broth, incubated at 37^{o}C with agitation at 150 rpm until optical density of 0.2 is obtained. The bacterial cultures were then diluted 1:15,000 in assay buffer (0.9mM $CaCl_2.2H_2O$, 0.5mM $MgCl_2.6H2O$ in 1X PBS added 10% BSA) to reach the concentration of approximately 1.5-2x10^4 CFU/ml and distributed into sterile polystyrene U bottom 96 well microtiter plates (12.5µl/well). Eight point 3-fold serial dilution of plasma which had been heated at 56^{0}C for 30mins to inactivate endogenous complement, and was prepared in assay buffer starting at 1:40. The final volume of reaction mixture was 50 µl which included plasma, 10% Baby Rabbit Complement (BRC, Pel-Freez

Biologicals 04) and 12.5 µl of diluted bacterial culture. Then, 7 µl of reaction mixture from each well was allowed to flow down in lanes on a nutrient agar plates (11 mm in diameter) at time zero to access initial CFU, and at 1.5 hours and 3 hours of incubation at 37^0C. Another 7 µl of the reaction mixture from each well at each time point was plated on nutrient agar plate as described above. The plates were incubated at 37^oC and total CFU were counted the following day. The assays were perfomed in triplicate and in parallel with inactivated BRC (heated at 56^oC for 30mins). The results were interpreted as the total number of bacteria in three wells of the triplicate. Bactericidal capability was determined as a percent CFU of test sera dilution with active or inactive BRC, compared with CFU of negative control. The percentage of bacteria killed/alive was the amount of bacteria counted from the wells with active BRC over those from the wells with inactive BRC (was calculated at every level of dilution at each time point). The samples were scored from 1 to 8 according to eight levels of dilution (1 is the least and 8 is the greatest dilution) at which ≥50% of the bacteria in the assay using active BRC were killed compared to the control containing inactive BRC.

2.8.6 Cytokine measurements

Cytokine measurements were performed on plasma samples collected from acute enteric fever patients, chronic carriers and bile culture negative patients. Ten cytokines were measured: IL-1, IL-2, IL-4, IL-5, IL-6, IL-10, IL-12, IL-13, TNF-alpha and Interferon (IFN)-gamma. Cytokine levels were measured using a Bio-Plex Prescision Pro (Bio-rad, USA). The assays were run following the recommendations

of the manufacture. Briefly, the filter plate was pre-wet with 200 µl of assay buffer. Beads were diluted according to recommendation from the manufacture then 50 µl of the diluted beads was loaded into each well. The plate was then washed twice with 100 µl of wash buffer using washing station. Standards and samples, which had been prepared following the manufacturers instructions, were assayed into separate wells, in a volume of 50 µl per well. The plate was sealed, agitated at 1,100 rpm for 30 seconds then maintained at 300 rpm for 30 minutes in the dark. After incubation without light, the plate was washed 3 times with 100µl of wash buffer. Twenty-five µl of prepared detection antibody was added to each well. The plate was incubated under the same conditions as described above and each well was had 50 µl of ready Streptavidin-PE added. The plate was sealed, incubated and washed as described above. Beads bound with cytokines (integrating a detection antibody which were captured by streptavidin-PE) were diluted in 150µl of assay buffer per well. The plate was sealed again and shaken at 1,100 rpm for 30 seconds. The seal was removed the plate was read immediately on the Bio-plex system.

2.8.7 Data analysis

Data were entered into a database using Excel 2007 (Microsoft) and analysed using using R software. To investigate if there was an association between anti-Vi IgG, anti-O:2, and age the data were stratified into four age groups (0-10 years, 11-20 years, 21-40 years and 41-65 years) and IgG values were plotted against each other and Spearman's values were calculated. P values were calculated for the various pairwise comparisons comparing median antibody titers (IgG upper and IgM lower)

against antigens Vi and O:2 in plasma throughout an acute infection time series. Similarly, p values were also calculated for the various pairwise comparisons comparing median antibody titers (O:2 upper and Vi lower) in patients with a negative bile culture and positive bile culture for *S.* Typhi and *S.* Paratyphi A.

2.9 Methods specifically related to chapter 6

Immuno-fluorescence staining and electron microscopy were performed on sections of gallbladder from *S*. Typhi chronic carriers and bile culture negative patients.

2.9.1 Immunofluorescence staining

Immunofluorescence staining was performed by indirect staining method. An unlabeled primary antibody (Vi or O:9) was first bound to the cellular antigen. Then, a fluorochrome-conjugated secondary antibody that binds specifically to the primary antibody was added. Phalloidin and DAPI were used as the immunofluorescent stain. Phalloidin is a phallotoxin that binds relatively tightly to F-actin in microfilaments. DAPI is a small water soluble fluorescent molecule with extreme avidity and specificity for DNA, preferentially binding to the A:T rich regions of DNA. After removing the slide from water as mentioned in the methods of H and E staining (Section 2.7.1.3.1), a circle was drawn around the tissue with a wax pen. The slide was washed in PBS (pH 7.4) 2 times for 2 minutes per wash and blocked with blocking buffer (PBS containing 5% fish gelatin, 10% goat serum, 0.05% tween 20, 1% bovine serum albumin, 1% sodium azide) for 30 minutes at room temperature. Excess blocking serum was removed by tapping on a paper

towel and the areas around the tissue were wiped with a paper towel and washed with normal saline. Primary antibody (goat anti-*Salmonella* antibody and rabbit anti-Vi antibody (murex) was diluted in blocking buffer (1:200). 40 µl of diluted primary antibody was applied to the tissue and the slide was incubated at room temperature in a humidity chamber for one hour. At the end of incubation, the slide was washed 3 times in saline for 5 minutes per wash. Blocking buffer, containing secondary antibody, was added and then incubated at room temperature for 1 hour in the dark (wrapped in aluminium foil). The secondary antibodies used were either Alexa Fluor 594 Chicken anti Rabbit IgG (red) or Alexa Fluor 350 Donkey anti Rabbit IgG (green) diluted in blocking buffer at a ratio of 1:200 and 1,400, respectively. The tissue samples were then washed 5 times in PBS and mounted in Prolong gold with Dapi (aqueous mounting media). The mounting media was allowed to set around the edges and was then sealed with nail varnish, before a final coat of nail varnish was applied for examination under epifluorescence microscope (Zeiss Scope A1).

2.9.2 Electron microscopy on gallbladder tissues

Transmission electron microscopy (TEM) was performed on gallbladder tissues obtained from chronic *S*. Typhi carriers by David Goulding at the Wellcome Trust Sanger Institute in Cambridge, UK. A paraffin-embedded human gall bladder, removed by cholecystectomy and previously screened by immunocytochemistry for *S.* Typhi, was selected for TEM analysis. An adjacent 50µm microtome section within the infected area visualised by fluorescence microscopy was mounted, cleared with histosol, rehydrated, gently washed from the glass slide and then processed for TEM.

Primary fixation was in 2% paraformaldehyde with 2% glutaraldehyde and secondary in 1% osmium tetroxide in 0.1M sodium cacodylate buffer followed by mordanting in 1% tannic acid and dehydration through an ethanol series staining with 2% uranyl acetate at the 30% stage. Finally the gall bladder ring was laid flat and embedded in TAAB resin.

50nm ultrathin sections from the region of interest were cut on a Leica EM UC6, contrasted with uranyl acetate and lead citrate and imaged on an 120kV FEI Spirit Biotwin TEM with a Tietz F4.15 CCD camera.

2.9.3 Scanning microscopy on gallstones

Gallstones recovered from *S.* Typhi chronic carriers and bile culture negative patients were rinsed in sterile PBS, fixed in 2% gluteraldehyde, and air dried in an incubator [225]. Examination of biofilm on the gallstone surfaces were performed at the Laboratory of Professor John Gunn, University of Ohio.

3 Characteristics of enteric fever relapse in a highly endemic setting

3.1 Abstract

Relapse is one of the most common complications of typhoid fever, known to occur in approximately 10% of acutely infected typhoid fever patients. Factors that influence a predisposition for relapse are not well understood. However, studies suggest that differences in relapse rates may be partly dependent on the antimicrobial used for therapy of the initial infection. Therefore, I hypothesised that antimicrobial therapy is one of the main factors leading to relapse with typhoid fever, possibly dependent on the mechanism of the antimicrobial group used. To investigate the rate and factors leading to relapse with typhoid fever I examined enteric fever relapse in patients who were enrolled in one of three separate clinical treatment trials for acute enteric fever performed at Patan Hospital in Kathmandu, Nepal. Demographic, socioeconomic and clinical information in addition to antimicrobial susceptibility patterns were compared between relapse and non-relapse cases of enteric fever. I found that the majority of relapse cases were associated with diarrhoea, were more likely to report a household member with an illness during follow up and were eventually diagnosed with enteric fever. The majority of relapse occurred in patients treated with cefixime rather than other antimicrobials (gatifloxacin, ofloxacin and chloramphenicol). Furthermore, in order to differentiate between relapse and reinfection, *Salmonella* isolates from the primary infection were compared with the isolates from secondary infection

(relapse cases) through genotyping and antimicrobial resistance patterns. The antimicrobial resistance patterns of secondary isolates during relapse were comparable to primary isolates. However, *S*. Paratyphi A were more likely to show antimicrobial resistance than *S.* Typhi isolates. Genotyping of paired isolates of *S.* Typhi revealed that majority of the strains causing secondary infection were identical to the primary strains. The results show that relapse in enteric fever is a common complication and is associated with the treatment antimicrobial and its resistance pattern.

3.2 Introduction

The case fatality rate of enteric fever has fallen dramatically from 20% in the pre-antibiotic era to <1% currently due to the use of antimicrobials in clinical management of cases [60]. Chloramphenicol was the first antimicrobial used for the treatment of enteric fever and significantly reduced mortality and frequency of complications. However, the prolonged course of 2 to 3 weeks required to prevent relapse and convalescent faecal carriage poses significant treatment completion issues [48]. An additional challenge for, the treatment of enteric fever is the development of multidrug resistance through decreased susceptibility to ciprofloxacin and the development of resistance to 3rd generation cephalosporins through ESBLs. Appropriate antimicrobial selection for the treatment of typhoid fever depends upon local patterns of antimicrobial resistance and other factors such

as severity of the disease, availability of drug supply, and the cost of the antimicrobial course [226].

Recurrent typhoid fever is one of the most common complications of enteric fever. Recurrence of symptoms may be due to either relapse or reinfection and relapse is most likely to result from recrudescence of bacteria that lie quiescent within host tissues such as the gallbladder and the bone marrow [227]. However, identifying relapse cases is challenging and requires the bacterial isolation of identical primary and secondary strains. If the primary and secondary isolates differ, it is likely that second bout of syndromic disease is reinfection with a new strain [95]. Relapse occurs in an estimated 10% of acutely infected patients, commonly between two to three weeks and within eight weeks after the resolution of the initial fever. Generally, a relapse isolate will have an identical antimicrobial susceptibility pattern to the original isolate [48, 228]. Despite a large relapse burden, factors influencing a predisposition for relapse are not well understood. Evidence suggests that differences in relapse rates are partly dependent on the antimicrobial used for therapy of the initial infection [139]. Additionally, a large study conducted in Pakistan regarding enteric fever relapse in 1,650 children over a period of 15 years revealed an increase in relapse rates starting in the early 1990s concurrent with the emergence of MDR strains. Furthermore, relapse cases are also reported to be related to the acquisition of multiple strains at the time of the initial infection [229].

I sought to investigate the factors associated with enteric fever relapse in an endemic region. Demographic, socioeconomic and clinical information in addition to

antimicrobial susceptibility patterns were compared between relapse and non-relapse cases of enteric fever. Patients for this evaluation were those enrolled in three separate clinical trials performed at Patan Hospital in Kathmandu, Nepal. The primary aim of this work was to explore important drivers of relapse in this population. I hypothesised that relapse may be dependent on the antimicrobial resistance patterns of the invasive *Salmonella* and the treatment arm of the patient (which was determined by the trial and were gatifloxacin, cefixime, chloramphenicol, and ofloxacin). Furthermore, recurrent infection may be relapse (fever due to same strain of *Salmonella*) or reinfection (fever due to different serotype or genotype). To verify this, secondary *Salmonella* strains isolated from relapse cases were genotyped and compared with the primary isolate.

3.3 Results

From June 2005 to August 2011, 762 invasive *Salmonella* were isolated from enteric fever patients enrolled in three clinical trials, gatifloxacin versus cefixime (ISRCTN75784880) [108], gatifloxacin versus chloramphenicol (ISRCTN53258327)[109] and gatifloxacin versus ofloxacin (ISRCTN63006567). From a total of 1,872 suspected enteric fever patients enrolled in one of three different clinical treatment trials, 91 (4.9%) patients met the definition for relapse. Relapse is defined as a second episode of febrile illness after 14 days and within the six months of primary infection. Relapse cases were further divided into culture relapse and fever relapse as shown in Table 3.1. Culture relapse cases were blood

culture positive at primary infection as well as secondary infection. Fever relapse cases had fever on both episodes of infections and may or may not have had a positive-culture on both occasions.

As shown in Table 3.1, of those patients with a defined fever relapse, 96% (87/91) had at least one positive stool or blood culture, 77% (70/91) of which occurred during the first week of enrolment. In the non-relapse cases, 38% (675/1,781) had at least one positive stool or blood culture ($p<0.001$, Fisher's exact test, compared to relapse patients). Overall, 44% (40/91) of the relapse cases were culture relapse (culture positive on entry and >14 days) and 42% (38/91) experienced fever relapse (fever on entry and at >14 days). Culture/no fever relapse was the most common type (42%, 38/91) although this was largely due to a lack of recorded fever status when the secondary culture sample. A sizeable proportion of relapse cases had either one culture positive result with fever relapse (35%, 31/91) or were culture negative at entry yet culture positive at some point >14 days post-enrolment (17%, 15/91) without a fever relapse. A small proportion of relapse cases (4%, 4/91) were entirely culture negative but did experience an episode of fever relapse.

Table 3.1: Description of relapse cases and types of relapse

Characteristic	Relapse n=91	Non Relapse n=1,781
Culture Positive		
Total	87/91 (95.6)	675/1,781 (37.9)
Primary (1st week)	70/91 (76.9)	675/1,781 (37.9)
Secondary (>1st week)	56/91 (61.5)	0/1,781 (0)
Type of relapse		
Culture Relapse	40/91 (44.0)	
Fever Relapse	38/91 (41.8)	
Specific type of relapse		
Culture Relapse/Fever Relapse	2/91 (2.2)	
Culture Relapse/No Fever Relapse	38/91 (41.8)	
No Culture Relapse/Fever Relapse	32/91 (35.2)	
No Culture Relapse/No Fever Relapse	15/91 (16.5)	
Culture Negative/Fever Relapse	4/91 (4.4)	

3.3.1 Baseline characteristics of patients

As shown in Table 3.2, relapse cases were, on average, slightly younger than non-relapse cases with a median age of 14 years compared to 17 years (p=0.031, Mann-Whitney U test). Males predominated in both, although were slightly more common in relapse cases (74%, 67/91) than non-relapse (64%, 1,140/1,779) (p=0.063, χ^2 test).

Relapse cases were much more likely to originate from the DM study (cefixime v. gatifloxacin) (p<0.001, χ^2 test), and specifically associated with a treatment regime of cefixime (p<0.001, χ^2 test) compared to chloramphenicol, gatifloxacin and ofloxacin. Consistent with these findings, almost half of the relapse cases occurred in 2005 (45%, 41/91), which is when the DM study was conducted.

Of the 87 culture positive relapse cases, 61% (53) were positive for *S.* Typhi, 29% (25) for *S.* Paratyphi A and 10% (9) for both serovars. In the non-relapse patients, 68% (458/677) of culture positive cases were diagnosed with *S.* Typhi and 32% (218/677) with *S.* Paratyphi A.

Table 3.2: Baseline characteristics of relapse and non-relapse patients from three clinical trials at Patan Hospital, n (%) or as indicated

Characteristic	Relapse n=91	Non-Relapse n=1781	p^
Median age (IQR)	14 (8-19)	17 (9-23)	0.031*
Male sex	67/91 (73.6)	1140/1779 (64.1)	0.063
Study			
DM	41/91 (45.1)	349/1781 (19.6)	
ED	26/91 (28.6)	827/1781 (46.4)	<0.001*
01TY	24/91 (26.4)	605/1781 (34.0)	
Medication			
Cefixime	22/91 (24.2)	164/1780 (9.2)	
Chloramphenicol	19/91 (20.9)	403/1780 (22.6)	<0.001*
Gatifloxacin	37/91 (40.7)	910/1780 (51.1)	
Ofloxacin	13/91 (14.3)	303/1780 (17.0)	
Year admitted			
2005	41/91 (45.1)	349/1780 (19.6)	
2006	12/91 (13.2)	402/1780 (22.6)	
2007	11/91 (12.1)	286/1780 (16.1)	
2008	6/91 (6.6)	213/1780 (12.0)	<0.001*
2009	8/91 (8.8)	185/1780 (10.4)	
2010	6/91 (6.6)	235/1780 (13.2)	
2011	7/91 (7.7)	110/1780 (6.2)	
Culture Positive			
Total	87/91 (95.6)	675/1781 (37.9)	<0.001*
S. Typhi	53/87 (60.9)	458/677 (67.7)	
S. Paratyphi	25/87 (28.7)	218/677 (32.2)	<0.001*
Mixed	9/87 (10.3)	0/677 (0)	

^From χ^2 test, Fisher's exact test or Mann-Whitney U test, as appropriate; * indicates $p<0.05$

3.3.2 Clinical history and examination characteristics of patients

As shown in Table 3.3, a large proportion of both relapse and non-relapse patients had taken some form of treatment in the two weeks prior to enrolment. Additionally, most patients reported a history of fever and headache and many reported anorexia. Relapse patients were more likely to report a history of diarrhoea (30%, 27/90) than non-relapse patients (18%, 324/1775) (OR: 1.92, 95%CI: 1.20-3.06, p=0.006). Abdominal pain (41%, 768/1863), cough (36%, 664/1864), constipation (12%, 218/1865), nausea (30%, 562/1867) and vomiting (18%, 343/1863) were also reported with little or no difference between relapse and non-relapse patients. Both relapse (15%, 14/91) and non-relapse cases (16%, 290/1779) reported a history of previous enteric fever.

On medical examination on entry, patients who would go on to relapse were less likely to appear sick on general appearance (OR: 0.49, 95%CI: 0.25-0.97, p=0.041) than non-relapse as shown in Table 3.4. Very few patients presented with pallor, icterus, roseolae or enlarged lymph nodes. Patients who would go on to relapse presented with a higher median pulse (116 beats per minute) than those who did not (108 beats per minute) (OR: 1.02, 95%CI: 1.00-1.02, p=0.004). The median respiratory rate of relapse patients was also slightly higher (22 breaths per minute, IQR: 20-28) than non-relapse patients (20 breaths per minute, IQR: 18-6) (OR: 1.02, 95%CI: 0.99-1.04, p=0.068). Presence of abdominal tenderness (5%, 90/1870), splenomegaly (9%, 163/1870) and hepatomegaly (7%, 139/1870) were also low.

Table 3.3: Clinical history characteristics of relapse and non- relapse patients, n (%) or as indicated

Clinical History	Relapse n=91	Non-Relapse n=1781	OR	95%CI	p
Treatment in the last 2 weeks	72/88 (81.8)	1298/1659 (78.2)	1.22	0.71-2.11	0.477
General symptoms					
Duration of illness, days	5 (4-7)	5 (4-7)	0.99	0.92-1.08	0.902
Fever	91/91 (100)	1777/1780 (99.8)	-	-	-
Headache	82/91 (90.1)	1591/1778 (89.5)	1.07	0.53-2.17	0.849
Abdominal pain	40/91 (44.0)	728/1772 (41.1)	1.12	0.74-1.72	0.587
Cough	31/91 (34.1)	633/1773 (35.7)	0.93	0.60-1.45	0.751
Diarrhoea	27/90 (30.0)	324/1775 (18.3)	1.92	1.20-3.06	0.006*
Anorexia	73/91 (80.2)	1315/1777 (74.0)	1.42	0.84-2.41	0.188
Constipation	12/91 (13.2)	206/1774 (11.6)	1.16	0.62-2.16	0.649
Vomit	12/91 (13.2)	331/1772 (18.7)	0.66	0.36-1.23	0.190
Nausea	30/91 (33.0)	532/1776 (29.3)	1.15	0.73-1.80	0.542
Previous infections					
Previous enteric fever	14/91 (15.4)	290/1779 (16.3)	0.93	0.52-1.67	0.817
Previous tuberculosis	0/91 (0)	22/1765 (1.2)	-	-	-
Previous hepatitis	8/91 (8.8)	204/1779 (11.5)	0.74	0.36-1.56	0.434

*indicates $p<0.05$

Table 3.4: Patient characteristics and symptoms recorded during medical examination on entry to the study, n (%) or median (interquartile range) as appropriate

Examination on entry	Relapse n=91	Non-Relapse n=1781	OR	95%CI	p
General appearance					
Sick	11/50 (22.0)	519/1427 (36.4)	0.49	0.25-0.97	0.041*
Fair	39/50 (78.0)	908/1427 (63.7)	1.00	-	-
Symptoms on exam					
Pallor	0/50 (0)	2/1430 (0.1)	-		
Icterus	0/50 (0)	3/1431 (0.2)	-		
Roseolae	0/91 (0)	2/1780 (0.1)	-		
Enlarged lymph nodes	1/91 (1.1)	48/1780 (2.7)	0.4	0.05-2.94	0.368
Temperature (^{0}C)	39 (38.3-39.5)	38.8 (38.2-39.4)	1.17	0.89-1.55	0.264
Pulse (bpm[1])	116 (100-120)	108 (96-120)	1.02	1.00-1.03	0.004*
Respiratory rate (bpm[2])	22 (20-28)	20 (18-26)	1.02	0.99-1.04	0.068
Abdominal tenderness	4/91 (4.4)	86/1779 (4.8)	0.91	0.32-2.52	0.849
Splenomegaly	7/91 (7.7)	156/1779 (8.8)	0.87	0.39-1.91	0.723
Hepatomegaly	4/91 (4.4)	135/1779 (7.6)	0.56	0.20-1.55	0.264

bpm[1]: beats per minute; bpm[2]: breaths per minute; * indicates $p<0.05$

3.3.3 Household characteristics and household member illness

As displayed in Table 3.5, patients who would eventually relapse had a higher median count of individuals living in the same household (20 people) than non-relapse (15) (OR: 1.01, 95%CI: 1.00-1.01, p=0.044) although the median number of people sharing a kitchen was not significantly different (4.5 and 4, respectively). The majority of patients reported gathering water for general use as well as drinking from the municipal supply (56%, 822/1,481) followed by stone spouts (23%, 340/1481) and well water (17%, 254/1,481), although no major differences existed between relapse and non-relapse patients. When questioned about water treatment method, relapse cases were more likely to report filtering (52%, 26/50) than non-relapse cases (37%, 518/1414) although this was not a significant risk factor. Over a third of both relapse cases (24%, 17/50) and non-relapse cases (28%, 537/1414) reported using untreated water on a regular basis. Finally, patients who would go on to relapse reported eating in a common mess (26%, 13/50) than those that did not (15%, 214/1430) (OR: 1.99, 95%CI: 1.04-3.82, p=0.037). Similar proportions of relapse and non-relapse reported consuming food frequently from a street vendor (16%, 8/50 and 17%, 246/1431, respectively), which has shown to be a risk factor for acquiring the infection in this setting previously.

Table 3.5: Household characteristics of relapse and non-relapse cases, median (interquartile range) or n (%) as appropriate

Household characteristics	Relapse n=91	Non-Relapse n=1781	OR	95%CI	p
No people in household	20 (11-30)	15 (8-22)	1.01	1.00-1.01	0.044*
No people sharing kitchen	4.5 (3-6)	4 (3-5)	1.01	0.99-1.02	0.255
Water type					
Tap water use	28/50 (56.0)	794/1431 (55.5)	1.02	0.58-1.80	0.943
Well water use	11/50 (22.0)	243/1431 (17.0)	1.38	0.70-2.73	0.357
Tube well use	3/50 (6.0)	76/1431 (5.3)	1.14	0.35-3.74	0.831
Stone spout use	11/50 (22.0)	329/1431 (23.0)	0.94	0.48-1.87	0.870
Other water use	2/50 (4.0)	117/1431 (8.2)	0.47	0.11-1.95	0.297
Water treatment type					
Filter	26/50 (52.0)	518/1414 (36.6)	1.59	0.85-2.93	0.147
Boil	2/50 (4.0)	169/1414 (12.0)	0.37	0.09-1.63	0.191
Chlorinate	1/50 (2.0)	52/1414 (3.7)	0.61	0.08-4.66	0.631
Combination	3/50 (6.0)	100/1414 (7.1)	0.95	0.27-3.29	0.933
Other	1/50 (2.0)	38/1414 (2.7)	0.83	0.11-6.42	0.859
Untreated	17/50 (34.0)	537/1414 (38.0)	1.00	-	-
Common eating place					
Home	44/50 (88.0)	1299/1431 (90.8)	0.75	0.31-1.78	0.508
Common mess	13/50 (26.0)	214/1430 (15.0)	1.99	1.04-3.82	0.037*
Party	1/50 (2.0)	54/1431 (3.8)	0.52	0.07-3.84	0.522
Street vendor	8/50 (16.0)	246/1431 (17.2)	0.92	0.43-1.98	0.826
Other	4/50 (8.0)	102/1431 (7.1)	1.13	0.40-3.21	0.814

* indicates $p<0.05$

Relapse cases were much more likely to report a household member with an illness during follow up (34%, 31/91) than non-relapse (14%, 251/1781) (OR: 3.20, 95%CI: 2.03-5.05, p<0.001) (Table 3.6). Additionally, household members of relapse cases were much more likely to be admitted to hospital (OR: 5.42, 95%CI: 1.28-23.07, p=0.022), be diagnosed with enteric fever (OR: 36.5, 95%CI: 11.6-115.4, p<0.001) and to subsequently enter an enteric fever clinical treatment trial run at Patan Hospital (OR: 19.6, 95%CI: 8.1-47.6, p<0.001).

Table 3.6: Household member (HHM) illness episodes in relapse and non-relapse, n (%)

HHM Characteristic	Relapse n=91	Non-Relapse n=1781	OR	95%CI	p
HHM unwell	31/91 (34.1)	251/1781 (14.1)	3.20	2.03-5.05	<0.001*
0 people unwell	60/91 (65.9)	1530/1781 (85.9)	1.00	-	-
1 person unwell	20/91 (22.0)	218/1781 (12.2)	2.38	1.41-4.03	0.001*
2 people unwell	7/91 (7.7)	28/1781 (1.6)	6.46	2.72-15.46	<0.001*
3 people unwell	4/91 (4.4)	5/1781 (0.3)	20.76	5.43-79.30	<0.001*
HHM admitted	4/30 (13.3)	4/145 (2.8)	5.42	1.28-23.07	0.022*
HHM diagnosed with EF	26/30 (86.7)	21/139 (15.1)	36.52	11.56-115.4	<0.001*
HHM enter trial	23/31 (74.2)	31/242 (12.8)	19.57	8.05-47.58	<0.001*

HHM: household member; EF: enteric fever; * indicates p<0.05

3.3.4 Antimicrobial resistance profiles

All strains (primary and relapse/reinfection) were subjected to antimicrobial susceptibility testing against 10 antimicrobials (Outlined in Table 3.7) The proportion of primary isolates that were designated as either resistant or of intermediate sensitivity was comparable between the relapse and non-relapse cases, as shown in Table3.7 and Figure 3.1. Resistance or intermediate sensitivity to ciprofloxacin was high in both groups (81%, 58/72 in relapse cases; 80%, 518/651 in non-relapse cases) as was resistance to nalidixic acid (81%, 58/72; 78%, 508/653, respectively). New CLSI guideline [214]was used for the evaluation of ciprofloxacin MIC breakpoints (sensitive ≤ 0.06μg/ml, Intermediate 0.12-0.5 μg/ml, and resistant ≥1μg/ml)

Table 3.7: Proportion of primary isolates from relapse cases resistant or with reduced susceptibility to various antimicrobials

Antimicrobial	Relapse n=72	Non-Relapse n=654	OR	95%CI	p
Any reduced sus.	58/72 (80.6)	524/654 (80.1)	1.03	0.56-1.90	0.930
Amoxicillin	0/20 (0)	3/328 (0.9)	-		
Augmentin	0/14 (0)	3/201 (1.5)	-		
Cefixime	0/37 (0)	1/124 (0.8)	-		
Ceftriaxone	0/51 (0)	4/325 (1.2)	-		
Chloramphenicol	2/51 (3.9)	8/325 (2.5)	1.62	0.33-7.84	0.551
Ciprofloxacin	58/72 (80.6)	518/651 (79.6)	1.06	0.58-1.97	0.844
Cotrimoxazole	0/13 (0)	4/196 (2.0)	-		
Gatifloxacin	0/51 (0)	0/322 (0)	-		
Nalidixic acid	58/72 (80.6)	508/653 (77.8)	1.18	0.64-2.18	0.591
Ofloxacin	2/72 (2.8)	8/654 (1.2)	2.31	0.48-11.08	0.296

Some odds ratios were unable to be calculated due to a lack of resistance to some antimicrobials.

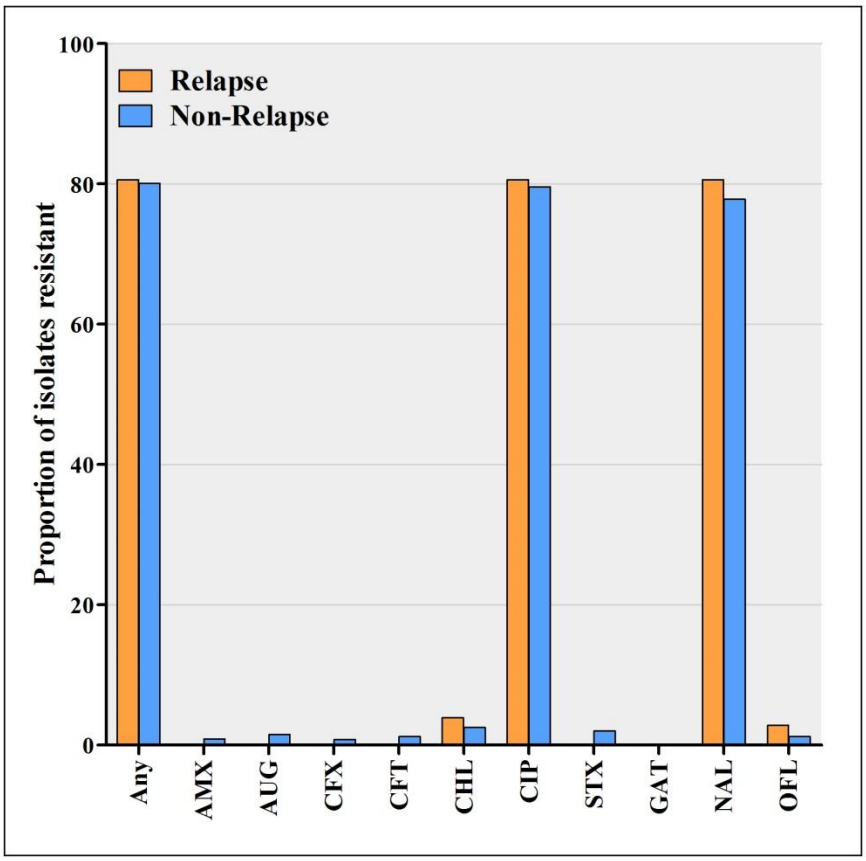


Figure 3.1: Proportion of isolates resistant or with a reduced susceptibility to a variety of antimicrobials from relapse and non-relapse cases. Abbreviations – Any: any antimicrobial; AMX: Amoxicillin; AUG: Augmentin; CFX: Cefixime; CFT: Ceftriaxone; CHL: Chloramphenicol; CIP: Ciprofloxacin; STX: Cotrimoxazole; GAT: Gatifloxacin; NAL: Nalidixic acid; OFL: Ofloxacin

In the case of *S.* Typhi specifically, primary isolates from relapse cases were more often resistant or have intermediately susceptible to nalidixic acid (79%, 41/52) than primary non-relapse isolates (69%, 303/439) (OR: 1.67, 95%CI: 0.83-3.35, p=0.147) as shown in Table 3.8 and Figure 3.2. Similarly, *S.* Typhi primary isolates from relapse cases were additionally more likely to display resistance or intermediate susceptibility to ciprofloxacin (79%, 41/52 and 72%, 314/437, respectively) (OR: 1.46, 95%CI: 0.723-2.93, p=0.287). Conversely, *S.* Paratyphi A primary isolates from relapse cases were less likely to be resistant to nalidixic acid (85%, 17/20) than non-relapse cases (96%, 205/214) (OR: 0.25, 95%CI: 0.06-1.01, p=0.051) and to ciprofloxacin (17/20, 85% and 204/213, 95%, respectively) (OR: 0.28, 95%CI: 0.07-1.11, p=0.069). However, *S.* Paratyphi A primary isolates from relapse cases were more often resistant or intermediately susceptible to chloramphenicol (13%, 2/16) than non-relapse isolates (3.4%, 4/116) (OR: 4.00, 95%CI: 0.67-23.9, p=0.128). The same trend was found for ofloxacin (10%, 2/20 and 3%, 7/214, respectively) (OR: 3.29, 95%CI: 0.64-17.00, p=0.156), although the total sample size for *S.* Paratyphi A was small (n=21).

Overall, *S*. Paratyphi A isolates were more likely to show antimicrobial resistance or intermediate susceptibility (96%, 225/235) than *S.* Typhi isolates (74%, 363/492) (p<0.001, chi square test). The median MIC values against ciprofloxacin and nalidixic acid were higher in the *S*. Typhi relapse isolates than the non-relapse isolates (Table 3.9). Additionally, an increase in the MIC range of ofloxacin was observed in the *S*. Typhi isolates from the relapse cases (median: 0.38, IQR: 0.22-

0.50) than non-relapse cases (median: 0.38, IQR: 0.04-0.50) (p=0.021, Mann-Whitney U test).

Table 3.10 shows median MIC levels to various antimicrobials for *S.* Paratyphi A isolates, higher MICs to chloramphenicol were found in cases that eventually relapsed (median: 8.0, IQR: 5.0-8.0) compared to non-relapse cases (median: 6.0, IQR: 4.0-8.0) (p=0.067, Mann-Whitney U test). The *S.* Paratyphi A strains from relapse cases exhibited a lower range of MIC values against gatifloxacin (median: 0.25, IQR: 0.25-0.25) than non-relapse strains (median: 0.27, IQR: 0.25-0.38) (p=0.060, Mann-Whitney U test). The proportion of isolates in each MIC level is displayed visually in Figures 3.3, 3.4 and 3.5.

Table 3.8: Proportion of primary isolates from relapse and non-relapse cases by *Salmonella* serotype resistant or with reduced susceptibility to various antimicrobials

	Relapse		**Non-Relapse**							
Antimicrobial	*S*. Typhi n=52	*S*. Paratyphi A n=21	*S*. Typhi n=443	*S*. Paratyphi A n=215	**OR[1]**	**95%CI[1]**	**p[1]**	**OR[2]**	**95%CI[2]**	**p[2]**
Any reduced sus.	41/52 (78.8)	17/20 (85.0)	319/440 (72.5)	205/214 (95.8)	1.41	0.70-2.84	0.331	0.25	0.06-1.01	0.051
Amoxicillin	0/17 (0)	0/3 (0)	3/230 (1.3)	0/98 (0)	-			-		
Augmentin	0/9 (0)	0/5 (0)	1/122 (0.8)	2/79 (2.5)	-			-		
Cefixime	0/26 (0)	0/11 (0)	0/87 (0)	1/37 (2.7)	-			-		
Ceftriaxone	0/35 (0)	0/16 (0)	1/209 (0.5)	3/116 (2.6)	-			-		
Chloramphenicol	0/35 (0)	2/16 (12.5)	4/209 (1.9)	4/116 (3.4)	-			4.00	0.67-23.9	0.128
Ciprofloxacin	41/52 (78.8)	17/20 (85.0)	314/437 (71.9)	204/214 (95.3)	1.46	0.73-2.93	0.287	0.28	0.07-1.11	0.069
Cotrimoxazole	0/8 (0)	0/5 (0)	2/117 (1.7)	2/79 (2.5)	-			-		
Gatifloxacin	0/35 (0)	0/1 (0)	0/206 (0)	0/116 (0)	-			-		
Nalidixic acid	41/52 (78.8)	17/20 (85.0)	303/439 (69.0)	205/214 (95.8)	1.67	0.83-3.35	0.147	0.25	0.06-1.01	0.051
Ofloxacin	0/52 (0)	2/20 (10.0)	1/440 (0.2)	7/214 (3.3)				3.29	0.64-17.00	0.156

OR[1], 95%CI[1] and p[1] comparing *S.* Typhi isolates between relapse and non-relapse cases; OR[2], 95%CI[2], p[2] comparing *S.* Paratyphi A isolates between relapse and non-relapse cases

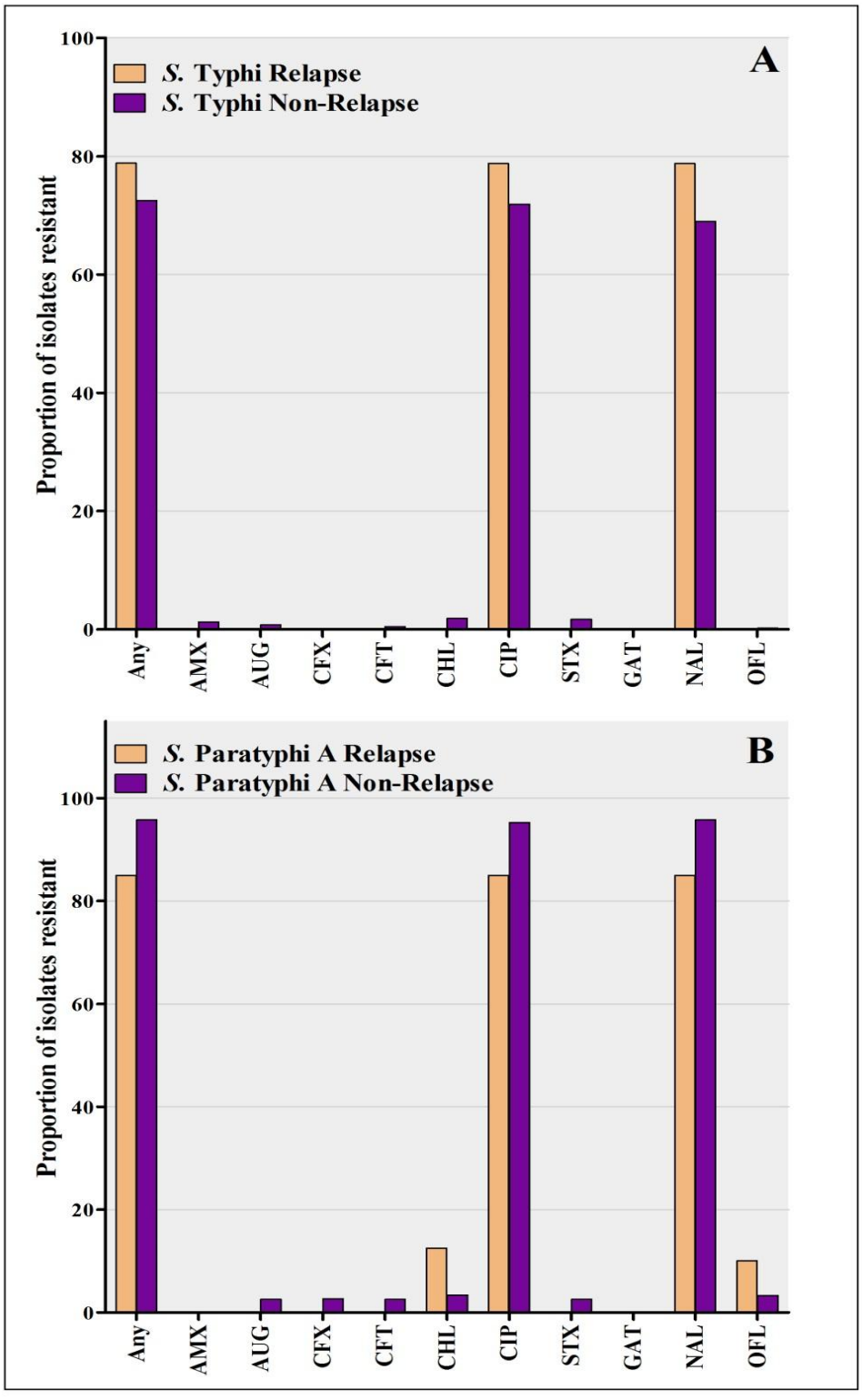


Figure 3.2: Proportion of isolates resistant or with reduced susceptibility to a variety of antimicrobials from relapse and non-relapse cases found to be culture-positive for *S.*Typhi (A) or *S.* Paratyphi A (B).

Abbreviations – Any: any antimicrobial; AMX: Amoxicillin; AUG: Augmentin; CFX: Cefixime; CFT: Ceftriaxone; CHL: Chloramphenicol; CIP: Ciprofloxacin; STX: Cotrimoxazole; GAT: Gatifloxacin; NAL: Nalidixic acid; OFL: Ofloxacin

Table 3.9: Median MIC (µg/ml) and interquartile ranges of *S*. Typhi relapse and non-relapse cases

Antimicrobial	**Relapse**		**Non-Relapse**		**p**
	N	Median (IQR)	N	Median (IQR)	
Amoxicillin	17	0.75 (0.5-0.75)	230	0.5 (0.5-0.75)	0.373
Augmentin	9	0.5 (0.5-0.75)	119	0.75 (0.5-0.75)	0.208
Cefixime	26	0.38 (0.13-0.38)	87	0.38 (0.13-0.38)	0.585
Ceftriaxone	35	0.13 (0.09-0.19)	206	0.13 (0.09-0.19)	0.344
Chloramphenicol	35	6.0 (3.0-8.0)	206	4.0 (3.0-6.0)	0.257
Ciprofloxacin	52	0.25 (0.16-0.38)	437	0.25 (0.02-0.38)	0.039*
Gatifloxacin	35	0.094 (0.09-0.13)	206	0.09 (0.01-0.13)	0.355
Nalidixic acid	52	256 (256-256)	437	256 (2.0-256)	0.035*
Ofloxacin	52	0.38 (0.22-0.50)	437	0.38 (0.04-0.50)	0.021*

* indicates $p<0.05$

Table 3.10: Median MIC (μg/ml) and interquartile ranges of *S*. Paratyphi A relapse and non-relapse cases

Antimicrobial	**Relapse**		**Non-Relapse**		**p**
	N	Median (IQR)	N	Median (IQR)	
Amoxicillin	3	1.5 (1.0-1.5)	98	1.5 (1.0-1.5)	0.734
Augmentin	5	1.0 (1.0-1.0)	79	1.0 (1.0-1.5)	0.349
Cefixime	11	0.25 (0.25-0.38)	37	0.38 (0.25-0.38)	0.333
Ceftriaxone	16	0.19 (0.16-0.22)	116	0.19 (0.19-0.25)	0.145
Chloramphenicol	16	8.0 (5.0-8.0)	116	6.0 (4.0-8.0)	0.067
Ciprofloxacin	20	0.50 (0.38-0.75)	214	0.5 (0.38-0.50)	0.770
Gatifloxacin	16	0.25 (0.25-0.25)	116	0.265 (0.25-0.38)	0.060
Nalidixic acid	20	256 (256-256)	214	256 (256-256)	0.441
Ofloxacin	20	1.5 (0.88-2.0)	214	1.5 (1.0-1.5)	0.699

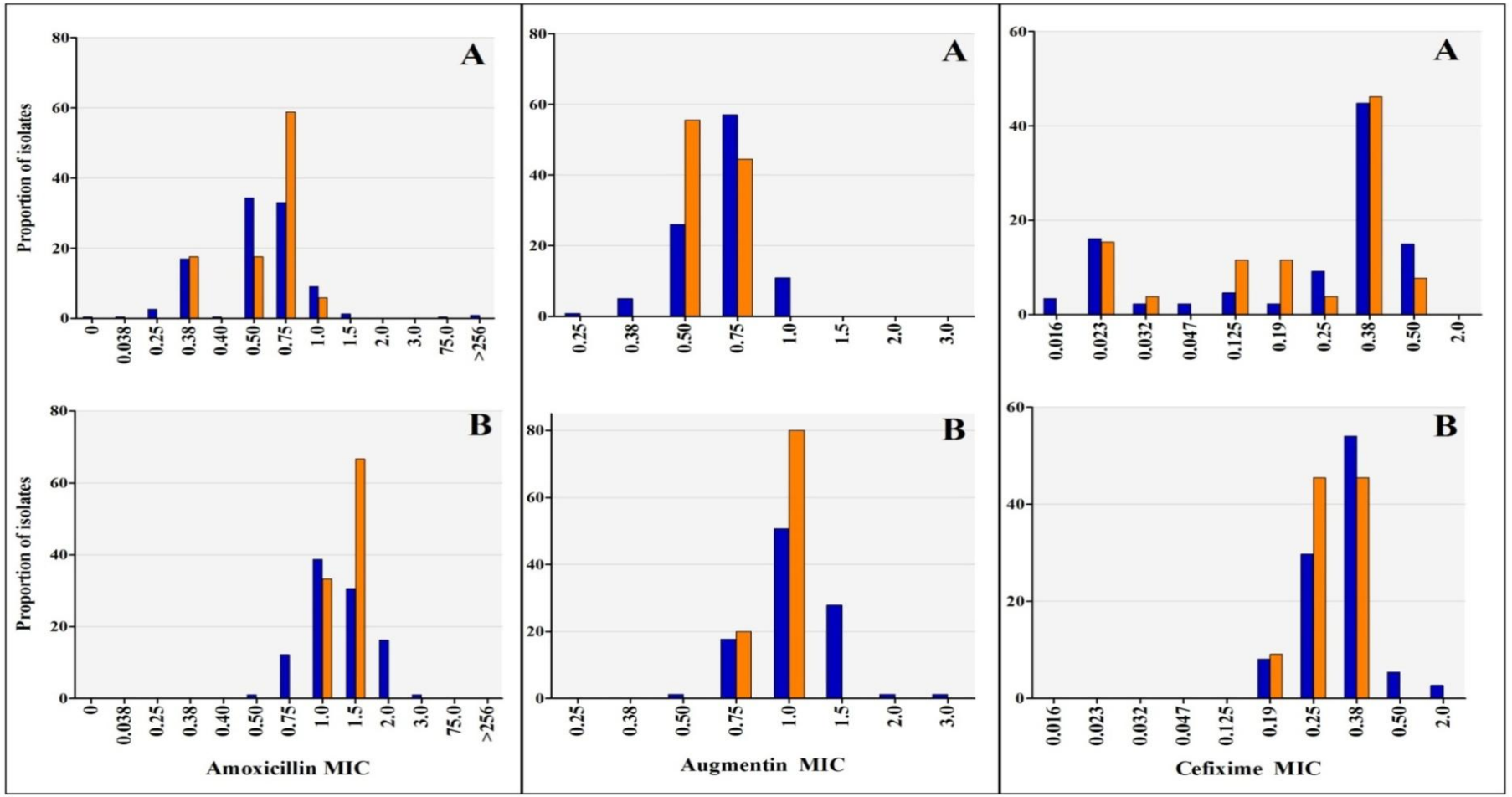


Figure 3.3: Proportion of primary isolates with varying susceptibilities to Amoxicillin, Augmentin and Cefixime from culture-positive (A) *S*. Typhi and (B) *S*. Paratyphi A. Relapse cases are shown in orange, non-relapse cases are shown in blue.

3. Characteristics of enteric fever relapse in a highly endemic setting

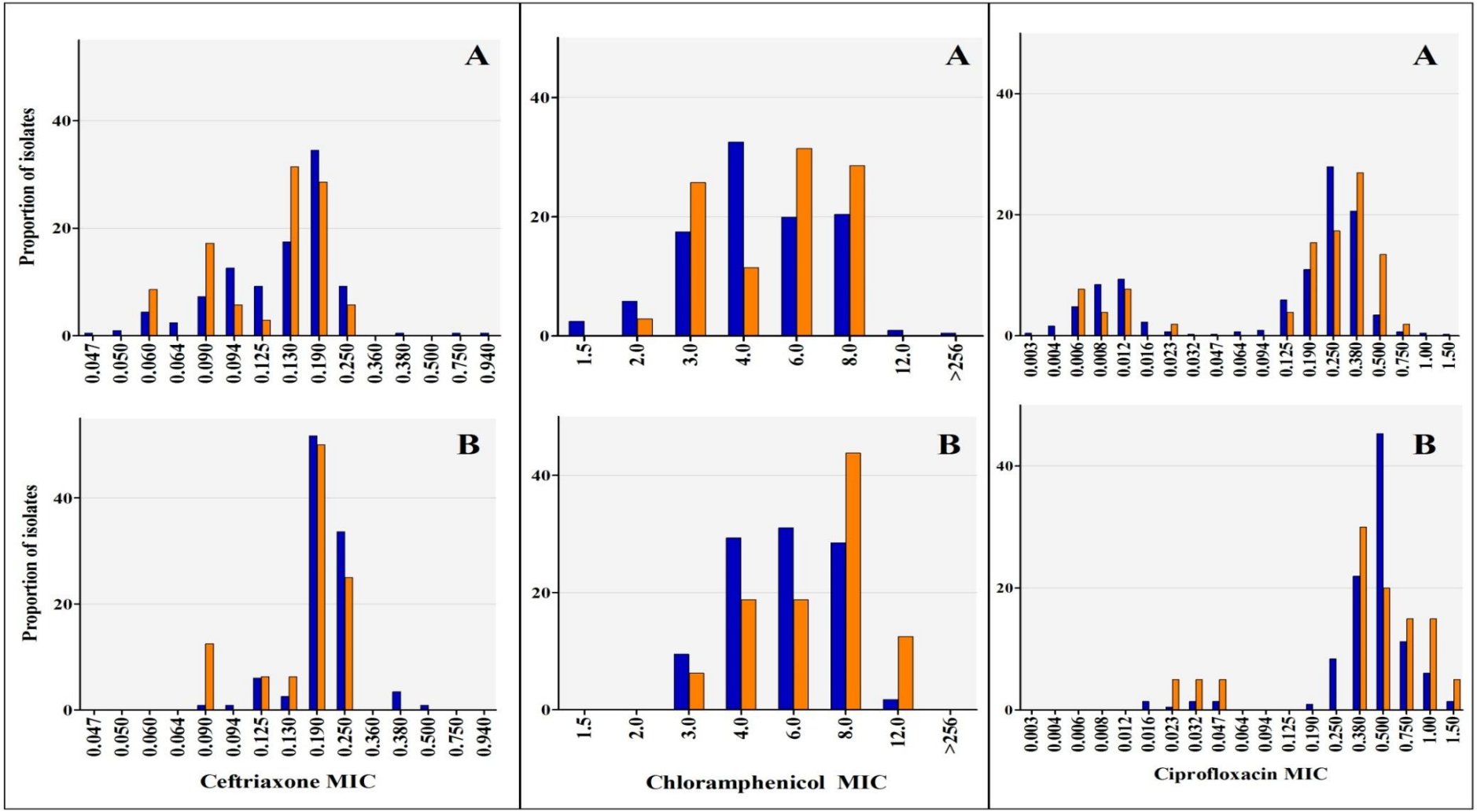


Figure 3.4: Proportion of primary isolates with varying susceptibilities to Ceftriaxone, Chloramphenicol and Ciprofloxacin from culture-positive (A) *S*. Typhi and (B) *S*. Paratyphi A.Relapse cases are shown in orange, non-relapse cases are shown in blue

3. Characteristics of enteric fever relapse in a highly endemic setting

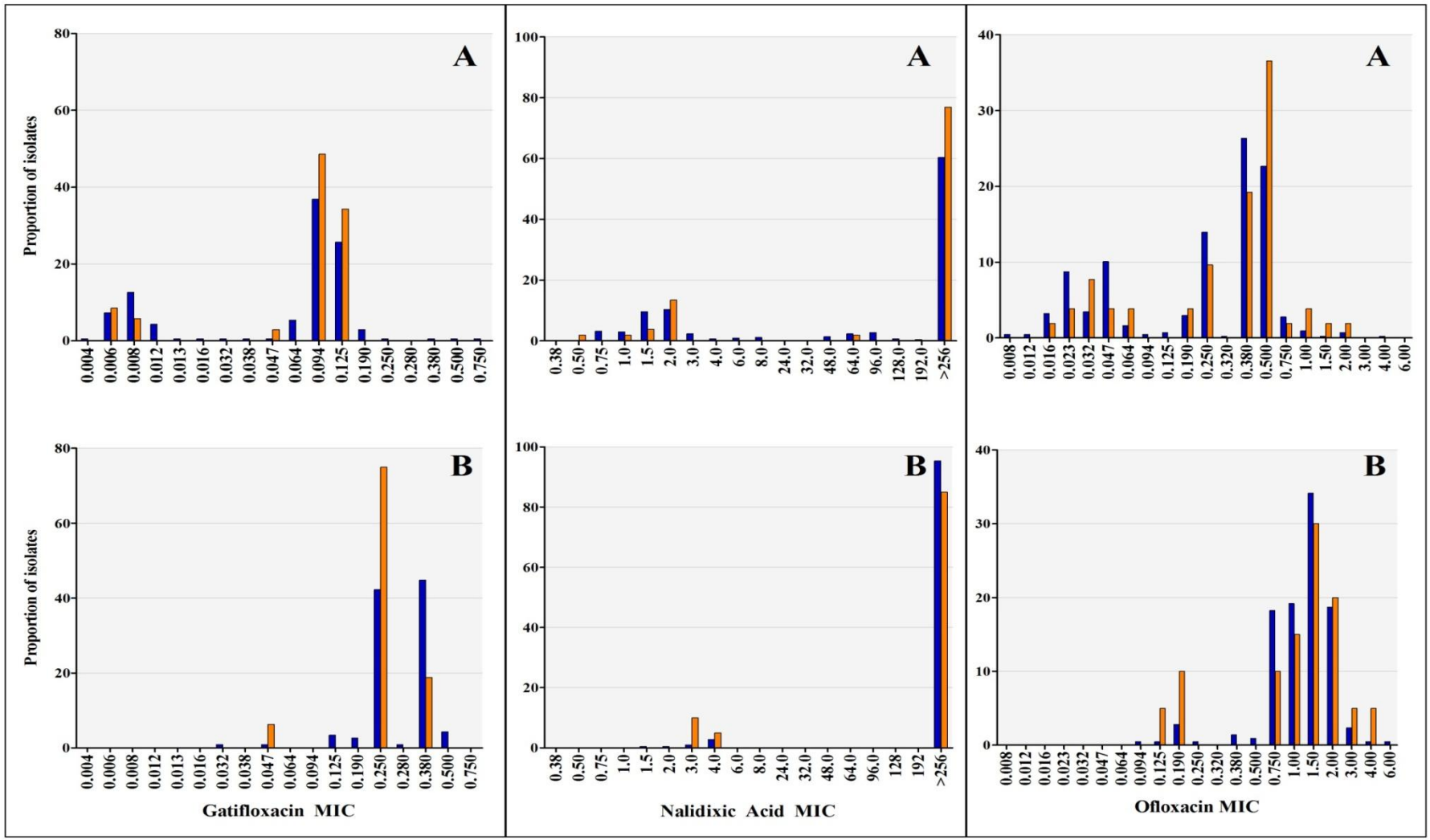


Figure 3.5: Proportion of primary isolates with varying susceptibilities to Gatifloxacin, Nalidixic acid and Ofloxacin from culture-positive (A) *S*. Typhi and (B) *S*. Paratyphi A. Relapse cases are shown in orange, non-relapse cases are shown in blue.

Finally, as shown in Table 3.11 and Figure 3.6, secondary isolates of relapse cases exhibited largely similar antimicrobial resistance patterns to the primary isolates. However, secondary isolates of *S.* Paratyphi A demonstrated resistance to a greater number of antimicrobials compared to primary isolates, as shown in Figure 3.7, this is in fact due to one isolate which was resistant to 6 separate antimicrobials and a low number of secondary *S.* Paratyphi A isolates that were screened for antimicrobial susceptibility (n=23).

Table 3.11: Proportion of primary and secondary isolates from relapse cases resistant or with a reduced susceptibility to various antimicrobials

Antimicrobial	**Primary** n=73	**Secondary** n=46
Any reduced sus.	58/72 (80.6)	38/46 (82.6)
Amoxicillin	0/20 (0)	-
Augmentin	0/14 (0)	1/13 (7.7)
Cefixime	0/37 (0)	0/11 (0)
Ceftriaxone	0/51 (0)	1/24 (4.2)
Chloramphenicol	2/51 (3.9)	1/24 (4.2)
Ciprofloxacin	58/72 (80.6)	38/46 (82.6)
Cotrimoxazole	0/13 (0)	1/13 (7.7)
Gatifloxacin	0/51 (0)	0/24 (0)
Nalidixic acid	58/72 (80.6)	38/46 (82.6)
Ofloxacin	2/72 (2.8)	2/46 (4.3)

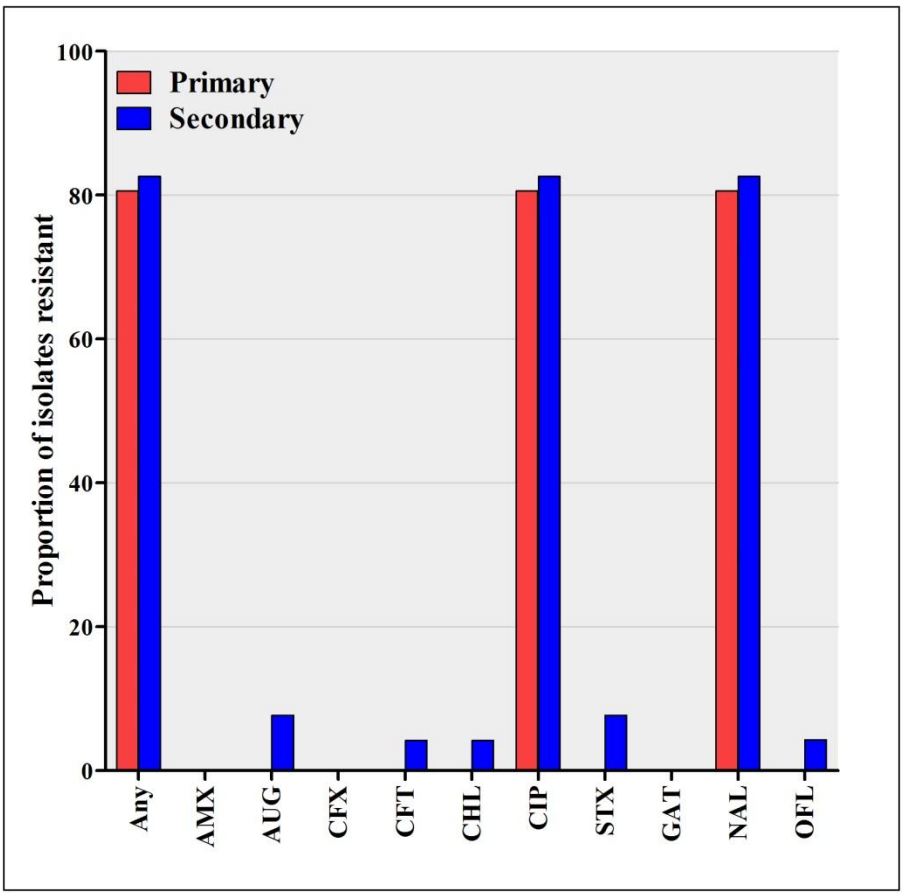


Figure 3.6: Proportion of primary and secondary isolates resistant or with a reduced susceptibility to a variety of antimicrobials from relapse cases only. Abbreviations – Any: any antimicrobial; AMX: Amoxicillin; AUG: Augmentin; CFX: Cefixime; CFT: Ceftriaxone; CHL: Chloramphenicol; CIP: Ciprofloxacin; STX: Cotrimoxazole; GAT: Gatifloxacin; NAL: Nalidixic acid; OFL: Ofloxacin

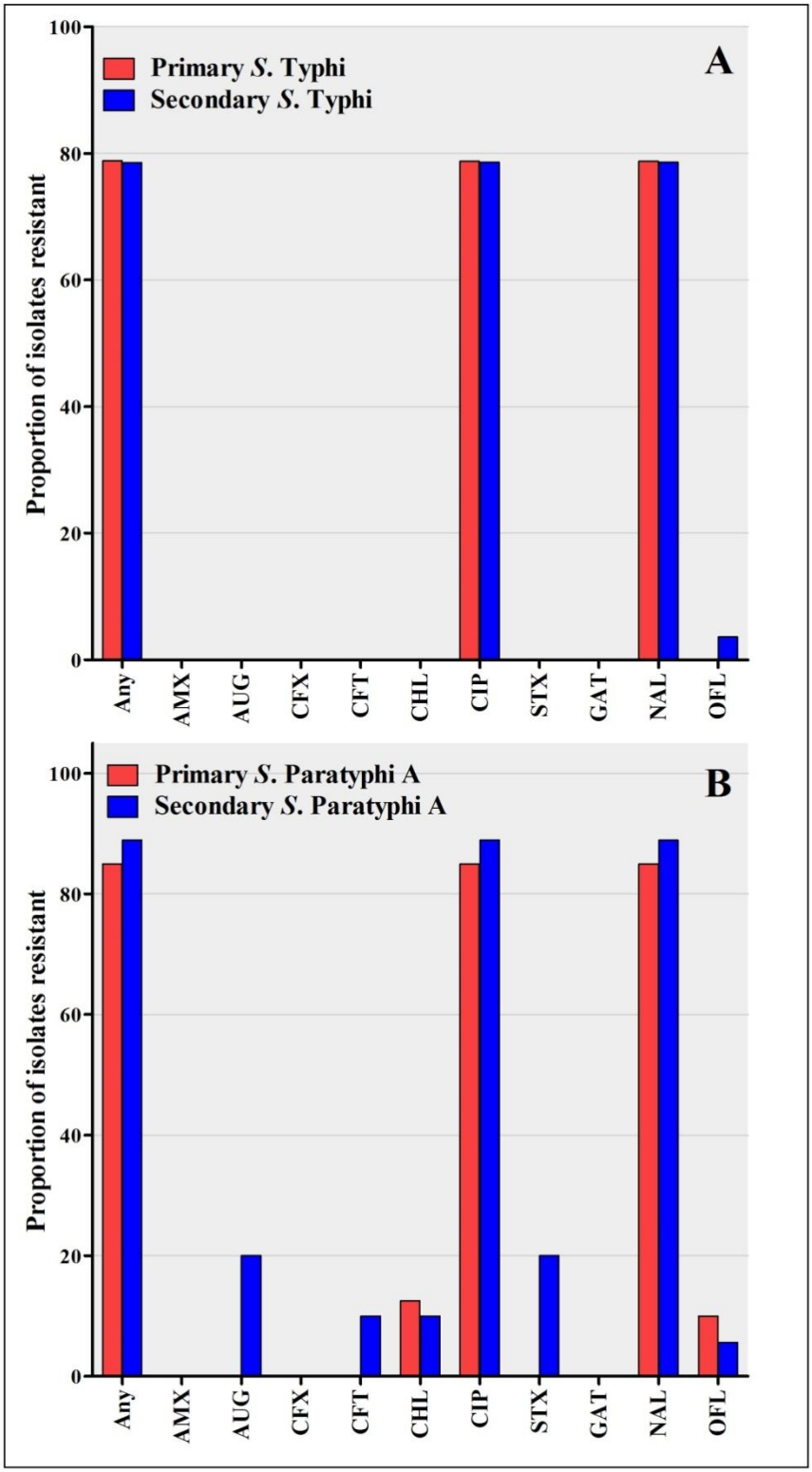


Figure 3.7: Proportion of primary and secondary isolates resistant or with a reduced susceptibility to a variety of antimicrobials from relapse cases culture-positive for *S*. Typhi (A) or *S*.Paratyphi A (B). Abbreviations – Any: any antimicrobial; AMX: Amoxicillin; AUG: Augmentin; CFX: Cefixime; CFT: Ceftriaxone; CHL: Chloramphenicol; CIP: Ciprofloxacin; STX: Cotrimoxazole; GAT: Gatifloxacin; NAL: Nalidixic acid; OFL: Ofloxacin

3.3.5 Genotying of *Salmonella* isolates

DNA was extracted as mentioned in chapter 2 from 33 paired *S*. Typhi relapse/re-infection isolates and subjected to single polymorphism genotyping at the Sanger Institute in the laboratory of Prof. Gordon Dougan. Initially, data from 26 paired *S*. Typhi isolates from the blood and the stool of patients with acute enteric fever (samples collected within 24 hours of one another) were compared in order to assess if it is likely that enteric fever patients may be infected with > 1 genotype. We found that 84.6 % (22/26) of paired *S.* Typhi isolates from the stool and blood from these patients were of identical haplotype (Table 3.12), demonstrating that patients are highly likely to be infected with one genotype. Additionally, to define relapse or re-infection we compared the serotypes and genotypes of 35 paired isolates of *S*. Typhi from those failing treatment (Table 3.13). We found that 2/35 (5.7%) had a different serotype on the secondary isolation, both patients were re-infected with *S*. Typhi after a primary *S.* Paratyphi A infection. Additionally, from those patients relapsing/re-infected with *S*. Typhi, 8/35 (22.9%) had different genotypes; the remainder were identical (71.4%, 25/35). Therefore, if we define relapse as isolation of the same organism within six months of primary infection and reinfection as infection with a different serotype or genotype within six months of primary infection, a total of 28.6 % (10/35) of patients had reinfection whereas 71.4 % (25/35) relapsed.

Table 3.12: Paired strains isolated from the blood and stool of enteric fever patients

	Blood Isolate		Stool Isolate	
Patient ID	**Serotype**	**Haplotype**	**Serotype**	**Haplotype**
DM052	Typhi	H58G-b4	Typhi	H58G-b4
DM106	Typhi	H42A	Typhi	H58G-b4
DM123	Typhi	H58G-b4	Typhi	H58G-b4
DM217	Typhi	H42A	Typhi	H42A
DM226	Typhi	H58G-b2	Typhi	H58G-b2
DM267	Typhi	H58G-b2	Typhi	H58G-b2
DM340	Typhi	H42A	Typhi	H42A
DM373	Typhi	H58G-b4	Typhi	H58G-b4
ED059	Typhi	H42A	Typhi	H42A
ED076	Typhi	H14	Typhi	H58G-b4
ED104	Typhi	H58G	Typhi	H58G
ED126	Typhi	H58G-b2	Typhi	H58G-b2
ED219	Typhi	H14	Typhi	H14
ED295	Typhi	H58G-c2	Typhi	H58G-c2
ED373	Typhi	H42A	Typhi	H82
ED401	Typhi	H42	Typhi	H42
ED448	Typhi	H42A	Typhi	H42A
ED471	Typhi	H58G-b2	Typhi	H58G-c2
ED504	Typhi	H42A	Typhi	H42A
ED570	Typhi	H58G-c1	Typhi	H58G-c1
ED571	Typhi	H42A	Typhi	H42A
ED603	Typhi	H58G-b2	Typhi	H58G-b2
ED672	Typhi	H42	Typhi	H42
ED694	Typhi	H58G-b2	Typhi	H58G-b2
ED698	Typhi	H58G-a2	Typhi	H58G-a2
ED754	Typhi	H58G-b2	Typhi	H58G-b2

Table 3.13: Paired strains isolated from the enteric fever patients to define relapse/reinfection

Patient ID	Primary isolate (blood)		Secondary isolate	
	Serotype	Haplotype	Serotype	Haplotype
DM091	Typhi	H58b	Typhi	H58b
DM097	Typhi	H58b	Typhi	H58b
DM147	Typhi	H58G	Typhi	H58G
DM217	Typhi	H42A	Typhi	H42A
DM223	Typhi	H58b	Typhi	H58b
DM226	Typhi	H58b	Typhi	H58b
DM 267	Typhi	H58G	Typhi	H58b
DM320	Paratyphi A	-	Typhi	H50
DM340	Typhi	H42A	Typhi	H42A
DM373	Typhi	H58b	Typhi	H58b
ED028	Typhi	H82	Typhi	H58b
ED030	Typhi	Ancestral	Typhi	Ancestral
ED140	Typhi	H42A	Typhi	H42A
ED177	Typhi	H58b	Typhi	H58b
ED188	Typhi	H58b	Typhi	H52
ED237	Typhi	H85	Typhi	Unknown
ED416	Paratyphi A	-	Typhi	H58G
ED455	Typhi	H42A	Typhi	Unknown
ED473	Typhi	H58b	Typhi	H58b
ED479	Typhi	H16	Typhi	H16
ED557	Typhi	H58b	Typhi	H58b
ED568	Typhi	Ancestral	Typhi	H58b
ED585	Typhi	H58b	Typhi	H58b
ED590	Typhi	H58b	Typhi	H58b
ED612	Typhi	H58b	Typhi	H16
ED614	Typhi	H58b	Typhi	H58b
ED765	Typhi	Ancestral	Typhi	Ancestral
ED777	Typhi	H58b	Typhi	H58b
ED831	Typhi	H58b	Typhi	H58b
ED835	Typhi	H58b	Typhi	H58b
ED838	Typhi	H58b	Typhi	H58b
ED841	Typhi	H50	Typhi	H50
ED846	Typhi	H58b	Typhi	H58b
ED850	Typhi	H50	Typhi	Ancestral
ED851	Typhi	H58b	Typhi	H58b

3.4 Discussion

Typhoid induces systemic and local humoral and cellular immune responses, but these confer incomplete protection against relapse and reinfection [48]. Relapses are known to occur in 10 to 15% of patients, usually after 2 to 3 weeks after the initial attack [48, 229]. Results here show that 5% of the patients enrolled in one of three trials relapsed and were either blood culture positive or had fever within 14 days of enrolment. Relapse cases were on average slightly younger than non-relapse cases and a majority of them were males. Patients who relapsed were also more likely to have a history of diarrhoea than non-relapsed patients. In contrast, constipation was found to be associated with relapse in the patients infected with MDR strains in Pakistan [228]. Patients who would eventually relapse had a higher median count of individuals living in the same household (20 people) and reported eating in a common mess more frequently than non-relapse cases, which may indicate consistent exposure to infection through crowding or infected food handlers. Similar proportions of relapse and non-relapse cases reported consuming food frequently from a street, which has shown to be a risk factor for acquiring the infection in this setting previously. Relapse cases were much more likely to report a household member with an illness during follow up. Additionally, household members of relapse cases were much more likely to be admitted to hospital, with enteric and to subsequently enter an enteric fever clinical treatment trial run at Patan Hospital. This may be due to the fact that the patients who are sick for a longer period of time are transmitting the disease within a household. Our previous

study has revealed that intra-household transmission may occur during acute typhoid fever [78].

Differences in relapse rates are partly dependent on the antimicrobials used for the therapy of the initial infection [137, 139].Relapse can occur without a history of clinical intervention but more often follows antimicrobial treatment. The incidence of relapse following treatment with new antibacterial drugs, including fluoroquinolones (1.5%) or broad-spectrum cephalosporins (5%), is much lower than that normally observed after treatment with traditional antibiotics (chloramphenicol, trimethoprim- sulfamethoxazole, and ampicillin) [103, 229, 232-235]. A large proportion of both relapse and non-relapse patients had taken some form of treatment in the two weeks prior to enrolment, indicating that self prescription of antimicrobials in the community is common and may contribute to observed resistance patterns in hosptial. Almost half of the relapse cases in this study originated from the DM study (cefixime v. gatifloxacin), and specifically on the treatment regime of cefixime compared to other antimicrobials chloramphenicol, gatifloxacin and ofloxacin. The reason for high failure rates associated with cefixime, a third generation cephalosporin, may be due to the poor intracellular penetration into macrophages and reticuloendothelial tissues which *Salmonella* colonise [236].

Secondary isolates of relapse cases exhibited largely similar antimicrobial resistance patterns to the primary isolates. *S*. Paratyphi A isolates were more likely to show antimicrobial resistance or intermediate susceptibility than *S.* Typhi isolates. Median MIC values of ciprofloxacin and nalidixic acid for the relapse *S*. Typhi

isolates were higher than non-relapse isolates. Additionally, *S.* Typhi and *S.* Paratyphi A relapse isolates showed a reduced susceptibility to ofloxacin and chloramphenicol than non-relapse cases respectively. The lower range of MICs against gatifloxacin was observed in the *S.* Paratyphi A relapse strains in comparison to the non-relapse strains. Infections with *S.* Typhi strains with reduced susceptibilities to ciprofloxacin and ofloxacin have been associated with the failure of treatment with these antimicrobials and increased disease severity [129, 148, 237, 238]. Fluoroquinolones, such as ciprofloxacin and ofloxacin, were well tolerated and early randomized trials found that these antimicrobials were very effective for the treatment of typhoid fever. However, *S*. Typhi and *S*. Paratyphi A isolates have been reported with reduced susceptibility to fluoroquinolones from Asia and Africa [63, 163, 239].

Data also suggest that enteric fever patients are highly likely to be infected with one genotype. Paired *S.* Typhi isolates from the stool and blood from these patients were of identical haplotype. Paired *S*. Typhi isolates were genotyped to differentiate between the relapse and reinfection. A majority of the relapse isolates were identical to the primary isolate (mostly H58G and H42A) indicating relapse is a major complication and may occur even in antimicrobial treated patients. Few patients were reinfected with either different *Salmonella* serotype or genotype. Recurrent typhoid fever due to reinfection with different Vi-phage types has been documented [227, 240]

3.5 Conclusion

Approximately 5% of the patients who were enrolled in one of the three clinical trials at Patan Hospital experienced relapse of enteric fever after an initial infection. Relapse rates were partly dependent on the antimicrobial used for treatment of the initial infection as majority of relapse cases were associated with antimicrobial cefixime. Demographic, socioeconomic and clinical information in addition to antimicrobial susceptibility patterns were compared between relapse and non-relapse cases of enteric fever. Isolates of *S*. Paratyphi A were more likely to show antimicrobial resistance than *S.* Typhi isolates. The antimicrobial resistance patterns of secondary isolates of relapse were largely similar to primary isolates. Furthermore, genotying of paired isolates of *S*. Typhi revealed that majority of the relapse strains were identical to the primary isolate. The results show that relapse is more common complication in enteric fever than reinfection in Kathmandu which may be associated with the treatment antimicrobial and its resistance pattern.

4 The Microbiological and Clinical Characteristics of Invasive *Salmonella* in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal

4.1 Abstract

Gallbladder carriage of invasive *Salmonella* is considered fundamental in sustaining enteric fever transmission. I investigated the prevalence, characteristics and relevance of invasive *Salmonella* in the gallbladder by examining their bile and tissue obtained from 1,377 individuals who underwent cholecystectomy in Kathmandu. Twenty percent of bile samples contained a Gram-negative organism, with *S*. Typhi and *S*. Paratyphi A isolated from 24 and 22 individuals, respectively. Gallbladders that contained *Salmonella* were more likely to show evidence of acute inflammation with extensive neutrophil infiltrate than those without *Salmonella*, corresponding with higher neutrophil and lower lymphocyte counts in the blood of *Salmonella* positive individuals. Antimicrobial resistance in the invasive *Salmonella* isolates was limited, indicating that gallbladder colonization is unlikely to be driven by antimicrobial resistance. The overall role of invasive *Salmonella* carriage in the gallbladder is not understood. A total of 3.5% of individuals who underwent cholecystectomy in this setting have a high concentration of antimicrobial sensitive, invasive *Salmonella* in their bile. Such individuals will become increasingly important if current transmission mechanisms are disturbed. Therefore,

prospectively identifying these individuals is paramount for rapid local and regional elimination.

4.2 Introduction

With adequate treatment the majority of patients recover completely from enteric fever [48]. However, an estimated 5% of individuals infected with *S*. Typhi develop a sustained infection of the gallbladder [183]. These individuals are referred to as 'carriers', and like the infamous 'typhoid Mary' [241], are outwardly asymptomatic, continue to intermittently shed organisms for a prolonged period and often have no recollection of an acute episodes of enteric fever [48]. In a subset of individuals infected with *S*. Typhi, the organisms chronically colonise the gallbladder and are shed intermittently into the intestinal lumen and thus in the faeces. It is gallbladder colonisation and faecal shedding that form a central dogma for the transmission and persistence of enteric fever. As a consequence of the internal localisation of organisms, this dogma is difficult to challenge in humans and the host-restricted nature of the relevant pathogens make carriage difficult to replicate precisely in non-mutant mouse models [84]. As a result, data regarding the prevalence, bacteriology and mechanisms of carriage are sparse.

The only population-based study estimating chronic *Salmonella* carriage in an endemic setting is from Chile where investigators gathered data from autopsies, calculating a carriage rate of 694 per 100,000 [183]. Investigations of *Salmonella* carriage suggest that the propensity to become a chronic carrier follows the typical epidemiology of gallbladder disease. Thus, the likelihood of carriage increases with

age and is more common in females [211]. Existing data also imply that individuals with gallstones or other gallbladder abnormalities are at increased risk of carriage[225]. These epidemiological theories are supported by laboratory-based investigations, which have shown that *Salmonella* can form biofilms and survive for prolonged period on gallstones [225, 242]. There remains a significant burden of enteric fever across Asia, yet the understanding of *Salmonella* carriage in these populations is limited. *S*. Paratyphi A has also been isolated from the gallbladders of patients undergoing cholecystectomy and it has been suggested that carriage of invasive *Salmonella* are likely to play a pivotal role in the persistence of these pathogens in Kathmandu, Nepal [202]. The purpose of the work presented in this chapter is to define the microbiology and epidemiology of invasive *Salmonella* carriage in Kathmandu. To address this aim, haematological and biochemical characteristics of the patients who underwent cholecystectomy were performed. Microbiological and histopathological examination of bile samples and extracted gallbladder tissues obtained from those patients were conducted.

4.3 Results

4.3.1 Microbiological examination of bile from cholecystectomy patients

From June 2007 until October 2010, a total of 1,496 patients underwent cholecystectomy for acute or chronic cholecystitis at Patan Hospital in Kathmandu. From these patients, bile samples from 1,377 individuals were obtained and

subjected to microbiological examination; 119 (8%) patients either denied consent or were unavailable for recruitment. A Gram-negative organism was isolated from 20% (274/1,377) of the bile samples. *E. coli*, *Salmonella* spp. and *Klebsiella* spp. were the most commonly isolated organisms, found in 78 (5.7%), 48 (3.5%) and 41 (3.0%) of the bile samples, respectively (Figure 4.1). The remainder of the culture positive bile samples contained a range of organisms including *Psuedomonas* spp., *Acinetobacter* spp., *Enterobacter* spp., *Citrobacter freundii, Vibrio* spp. and *Serratia marcescens* (Figure 4.1). Of the 48 *Salmonella* isolated, 24 (50%) were *S*. Typhi, 22 (46%) were *S.* Paratyphi A and two (4%) were *S. enterica* group C. Baseline data, stratified by microbiological culture result are shown in Table 4.1. Notably, fitting with the typical epidemiological characteristics of cholelithiasis, 77% (1,066/1,377) of the patients were female and the median age was 39 years (range: 16 to 76 years). The median age of those with *Salmonella* in their bile was 35 years (range: 18 to 67 years) and 73% were female. It is noteworthy that none of the pre-surgical stool cultures from any patients were *Salmonella* positive and, when questioned, only 15% (7/48) of the *Salmonella* bile-positive patients had a memorable history of enteric fever, none of which had been confirmed by microbiological culture. From available records, 16% (7/43) of *Salmonella* bile-positive patients reported 0.5 days of fever on entry, 7% (3/46) were admitted with jaundice, 5% (2/41) had a palpable gallbladder and 4% (2/45) were admitted with pancreatitis.

Forty-six *Salmonella* isolates were available for antimicrobial susceptibility testing by disc diffusion. Fifty-nine percent (27/46) of the *Salmonella* isolates were resistant to NA, and a single *S*. Paratyphi A isolate was resistant to both NA and

ciprofloxacin. All *S*. Typhi and *S*. Paratyphi A strains were susceptible to ceftriaxone, chloramphenicol, gatifloxacin and ofloxacin and no MDR was detected (Table 4.2 and Table 4.3).

4. The microbiological and clinical characteristics of invasive *Salmonella* in gallbladders from cholecystectomy patients in Kathmandu, Nepal

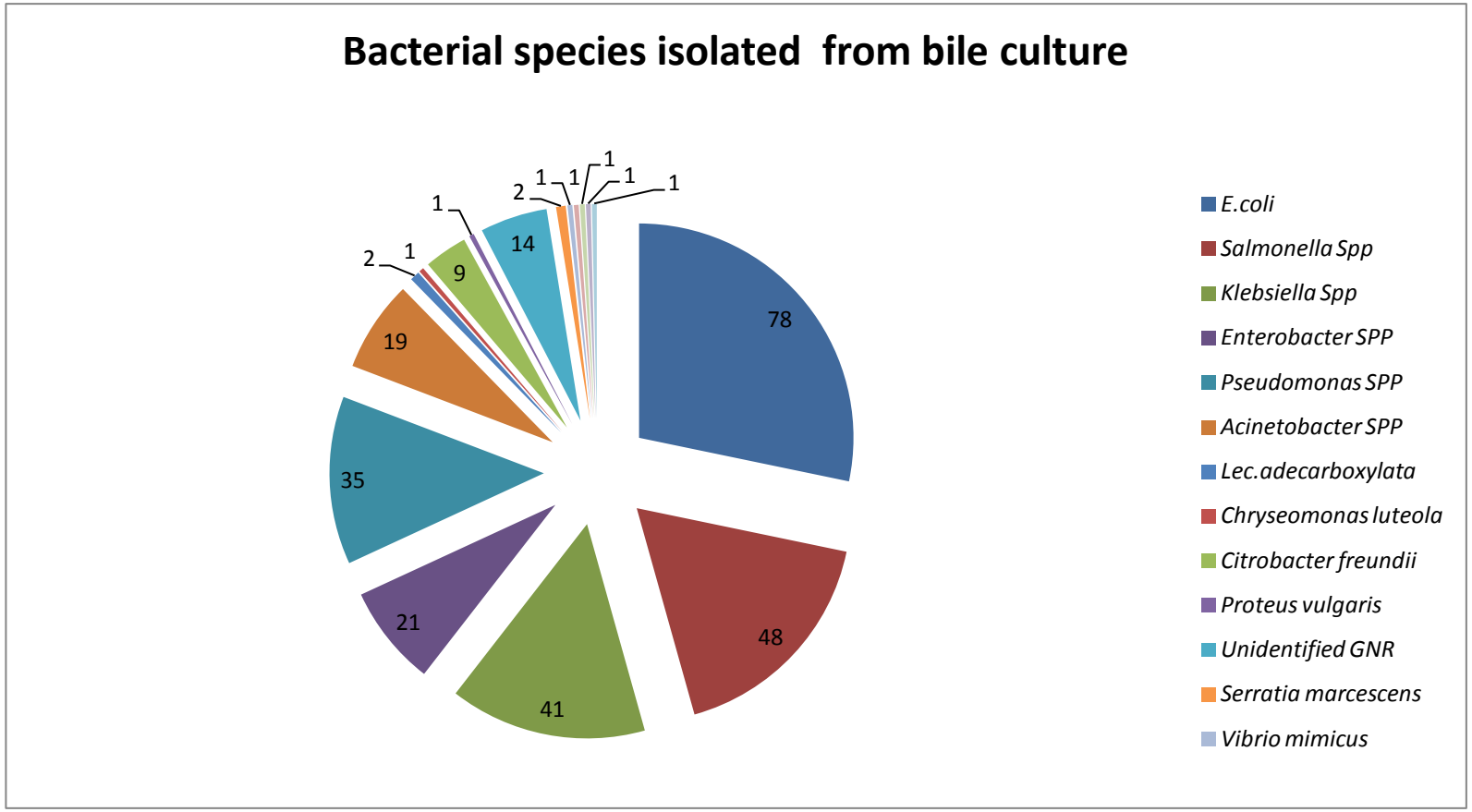


Figure 4.1: Different species of Gram-negative bacteria isolated from bile culture. Various lactose fermenting as well as non lactose fermenting bacteria were isolated. The proportion of isolates is shown by the figure in each section.

4. The microbiological and clinical characteristics of invasive *Salmonella* in gallbladders from cholecystectomy patients in Kathmandu, Nepal

Table 4.1: The baseline characteristics of the *Salmonella* positive, culture negative and the culture positive non-*Salmonella* bile culture groups

Bacterial isolate	Women		Men		Enteric fever history			Surgery	
	Patients	Age	Patients	Age	Patients	Febrile illness	*Salmonella*[*]	Elective	Acute
	n (%)	Median (range)	n (%)	Median (range)	n (%)	n (%)	n (%)	n (%)	n (%)
Salmonella									
S. Typhi	24 (1.7)	33.5 (20-60)	18 (75.0)	39 (30-49)	6 (25.0)	3 (12.5)	0 (0)	17 (70.8)	3 (12.5)
S. Paratyphi A	22 (1.6)	36.5 (24-67)	17 (77.3)	28 (18-57)	5 (22.7)	4 (18.2)	0 (0)	14 (63.6)	7 (31.8)
Salmonella Group C	2 (0.1)	-	0 (0)	35.5 (21-50)	2 (100)	0 (0)	0 (0)	2 (100)	0 (0)
Total	48 (3.5)	34.5 (20-67)	35 (72.9)	38 (18-57)	13 (27.1)	7 (14.6)	0 (0)	33 (68.8)	10 (20.8)
Non-*Salmonella*									
Escherichia coli	78 (5.7)	50 (21-84)	53 (67.9)	57 (26-76)	23 (29.5)	7 (9.0)	2 (2.6)	69 (88.5)	5 (6.4)
Klebsiella spp	41 (3)	52 (25-90)	35 (85.4)	73.5 (42-80)	5 (12.2)	5 (12.2)	1 (2.4)	36 (87.8)	2 (4.9)
Pseudomonas spp.	33 (2.4)	36 (14-74)	30 (90.9)	62.5 (53-72)	2 (6.1)	8 (24.2)	2 (6.1)	30 (90.9)	1 (3.0)
Acinetobacter spp.	19 (1.4)	42.5 (21-61)	14 (73.7)	37 (25-51)	5 (26.3)	6 (31.6)	3 (15.8)	16 (84.2)	1 (5.3)
Enterobacter spp.	21 (1.5)	53 (20-70)	13 (61.9)	55.5 (13-67)	6 (28.6)	3 (14.3)	2 (9.5)	17 (81.0)	1 (4.8)
Other	34 (2.5)	35 (21-84)	22 (64.7)	50 (33-80)	9 (26.5)	6 (17.6)	2 (5.9)	32 (94.1)	0 (0)
Total	226 (16.4)	39 (16-76)	167 (73.9)	46 (11-80)	50 (22.1)	35 (15.5)	12 (5.3)	200 (88.5)	10 (4.4)
Culture negative	1103 (80.1)	38 (16-76)	864 (78.3)	44 (11-75)	214 (19.4)	176 (16.0)	29 (2.6)	983 (89.1)	61 (5.5)
Total	1377 (100)	39 (16-76)	1066 (77.4)	45 (11-80)	277 (20.1)	218 (15.8)	41 (3.0)	1216 (88.3)	81 (5.9)

[*]Blood culture confirmed

Table 4.2: Antimicrobial resistance patterns of Gram-negative organisms from the bile of patients undergoing cholecystectomy for selected antimicrobials

Organism	Patients n (%)	Antimicrobial resistance n (%)			
		AMX	CFT	CIP	OFL
S. Typhi	24 (1.7)	0/23 (0)	0/24 (0)	0/24 (0)	0/24 (0)
S. Paratyphi A	22 (1.6)	0/22 (0)	0/22 (0)	1/22 (4.5)	1/22 (4.5)
Other	2 (0.1)	1/2 (50.0)	0/2 (0)	0/2 (0)	0/2 (0)
Total *Salmonella*	48 (3.5)	1/47 (2.1)	0/48 (0)	1/48 (2.1)	1/48 (2.1)
Escherichia coli	78 (5.7)	32/76 (42.1)	12/76 (15.8)	23/77 (29.9)	21/77 (27.3)
Klebsiella spp.	41 (3.0)	36/39 (92.3)	9/39 (23.1)	6/39 (15.4)	6/38 (15.8)
Pseudomonas spp.	33 (2.4)	23/31 (74.2)	4/31 (12.9)	4/31 (12.9)	4/31 (12.9)
Acinetobacter spp.	19 (1.4)	14/19 (73.7)	7/19 (36.8)	6/19 (31.6)	4/19 (21.1)
Enterobacter spp.	21 (1.5)	16/19 (84.2)	6/20 (30.0)	2/20 (10.0)	1/20 (5.0)
Other	34 (2.5)	14/18 (77.8)	5/20 (25.0)	3/20 (15.0)	2/17 (11.8)
Total Non-*Salmonella*	226 (16.4)	135/202 (66.8)	43/205 (21.0)	44/206 (21.4)	38/202 (18.9)

AMX: Amoxicillin; CFT: Ceftriaxone; CIP: Ciprofloxacin; OFL: Ofloxacin

Table 4.3: Antimicrobial resistance patterns of Gram-negative organisms from bile of patients undergoing cholecystectomy for selected antimicrobials

Organism	Antimicrobial resistance n (%)				
	CHL	**STX**	**GEN**	**AMI**	**NAL**
S. Typhi	0/22 (0)	4/23 (17.4)	0/11 (0)	0/0 (0)	11/22 (50.0)
S. Paratyphi A	2/22 (9.1)	0/22 (0)	0/16 (0)	0/1 (0)	16/19 (84.2)
Other	1/2 (50)	0/2 (0)	0/1 (0)	0/0 (0)	1/2 (50.0)
Total *Salmonella*	3/46 (6.5)	4/47 (8.5)	0/28 (0)	0/1 (0)	28/43 (65.1)
Escherichia coli	14/69 (20.3)	22/76 (28.9)	4/40 (10.0)	0/39 (0)	20/42 (47.6)
Klebsiella spp.	12/38 (31.6)	11/39 (28.2)	4/24 (16.7)	1/25 (4.0)	9/18 (50.0)
Pseudomonas spp.	21/28 (75.0)	21/30 (70.0)	2/20 (10.0)	1/18 (5.6)	9/13 (69.2)
Acinetobacter spp.	11/19 (57.9)	9/19 (47.4)	5/9 (55.6)	4/10 (40.0)	6/11 (54.5)
Enterobacter spp.	3/19 (15.8)	3/20 (15.0)	1/8 (12.5)	0/5 (0)	2/14 (14.3)
Other	5/18 (27.8)	3/18 (16.7)	1/4 (25.0)	1/3 (33.3)	5/19 (26.3)
Total Non-*Salmonella*	66/191 (34.6)	69/202 (34.2)	17/105 (16.2)	7/100 (7.0)	51/117 (43.6)

CHL: Chloramphenicol, STX: Co-trimoxazole, GEN: Gentamycin; AMI: Amikacin; NAL: Nalidixic acid

4.3.2 Bacterial load of *Salmonella* in bile

To quantify the bacterial load in the bile, real-time PCR was performed on total nucleic extracted from the bile of six *S*. Paratyphi A positive individuals and 12 *S*. Typhi positive individuals. All qualitative serovar specific PCR data corresponded precisely with the culture data. The median target copy numbers/ bacterial loads were 9.3×10^4 (IQR 5×10^4–2.3×10^5) CFU/ml^{-1} for *S*. Paratyphi A and 5.2×10^4 (IQR 2×10^4–7.28×10^5) CFU/ ml^{-1} for *S*. Typhi. The difference in bacterial load between the two organisms was non-significant (p=0.93; Mann-Whitney U test), yet, were approximately two and three orders of magnitude greater than those previously reported in bone marrow and blood, respectively [103, 128].

4.3.3 Signs and symptoms of all cholecystectomy patients

Among overall patients who visited to Patan Hospital for cholecystectomy, 80% were female, 73% in non-*Salmonella* group and 80% in *Salmonella* group (Table 4.3). *Salmonella* carriers were on average younger (36.5 years) than non-*Salmonella* carriers (42.1 years).

Right upper quadrant (RUQ) pain was one of the commonest complaints upon entry. Patients reported feeling RUQ pain for periods of time ranging from less than one week to more than 5 years. *Salmonella* positive patients were more likely to feel continuous

RUQ pain than culture-negative patients (p=0.038) or culture-positive, non-*Salmonella* patients (p=0.029, Chi-squared test) (Table 4.4).

4.3.4 Haematological and biochemical characteristics

The 1,377 individuals undergoing cholecystectomy were divided into three groups on the basis of their bile culture results: *Salmonella* positive, culture negative, and culture positive for non- *Salmonella*. Individuals that were *Salmonella* positive were more likely to have experienced continuous right upper-quadrant pain (10%, 5/48) compared to those that were culture negative (3%, 37/1,151) (p=0.008, chi squared test) and those that were culture positive for non-*Salmonella* (2%, 5/214) (p=0.008, chi squared test). Haematology and biochemistry data from the patients were compared using the Mann-Whitney U test (Table 4.5). There was no significant difference in liver enzyme or bilirubin levels between the *Salmonella* positive group and the other two groups. Yet, the *Salmonella* positive group had a higher median neutrophil count and a lower median lymphocyte count than the culture negative group and the culture positive non-*Salmonella* group (Table 4.5).

Table 4.4: Right upper quadrant (RUQ) pain characteristics of all patients, culture negative, culture-positive, *Salmonella*-negative and *Salmonella* positive, n (%)

Characteristic	Culture negative n=1030	Culture positive , non-*Salmonella* n=201	*Salmonella* positive n=46	p1*	p2*
RUQ Pain	932 (81)	179 (83.6)	39 (81.3)	0.202	0.417
Duration of pain					
<1 week	70 (6.1)	15 (7)	6 (12.5)		
1-4 week	989 (85.9)	22 (10.3)	6 (12.5)		
1-6 month	245 (21.3)	49 (22.9)	7 (14.6)		
6-12 month	162 (14.1)	19 (8.9)	5 (10.4)	0.317	0.733
1-2 year	121 (10.5)	16 (7.5)	3 (6.3)		
2-5 year	105 (9.1)	24 (11.2)	6 (12.5)		
>5 year	77 (6.7)	23 (10.7)	4 (8.3)		
Timing					
After fatty food	21 (1.8)	3 (1.4)	3 (6.3)	0.087	0.089
After spicy food	8 (0.7)	2 (0.9)	1 (2.1)	0.330	0.466
On & Off	546 (47.4)	94 (43.9)	16 (33.3)	0.173	0.369
Continuous	37 (3.2)	5 (2.3)	5 (10.4)	**0.038**	**0.029**

* Chi-square test, boldface indicates p≤0.05
p1: Comparing culture-negative to *Salmonella*-positive patients
p2: Comparing culture positive for non-*Salmonella* to *Salmonella*-positive patients

Table 4.5: The haematological and the biochemical characteristics of the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups

Haematology	**Culture negative**			**Culture positive non-*Salmonella***			***Salmonella* positive**			**p1***	**p2***
	n	**median**	**IQR**	**n**	**median**	**IQR**	**n**	**median**	**IQR**		
Total cell (x 10^3/µL)	953	7.9	6.6-9.5	188	7.85	6.45-10.15	42	9.45	6.4-14	**0.025**	0.058
Neutrophil (x 10^3/µL)	917	66	58-74	177	65	58-75	41	72	60-82	**0.012**	**0.042**
Lymphocyte (x 10^3/µL)	914	31	24-38	175	31	23-38	40	24.5	17-36	**0.007**	**0.040**
Monocyte (x 10^3/µL)	270	1	1-2	70	1.5	1-2	9	1	1-2	0.676	0.848
Eosinophil (x 10^3/µL)	641	2	1-4	131	2	1-4	22	2	2-4	0.999	0.515
Basophil (x 10^3/µL)	54	0	0-1	14	0	0-0	1	6	-	0.058	**0.020**
Total bilirubin (mg/mL)	965	0.8	0.68-1	190	0.8	0.7-1	42	0.86	0.7-1.1	0.119	0.280
Conjugated bilirubin (mg/mL)	950	0.2	0.19-0.26	186	0.2	0.18-0.24	41	0.2	0.2-0.28	0.414	0.419
AST (u/L)	961	30	23-41	190	29.5	23-40	42	28	24-38.9	0.878	0.986
ALT (u/L)	960	30	21.9-43	188	29.5	21-43.5	42	30.5	24-41	0.898	0.953
ALP (u/L)	941	122	82-209	188	150.5	94.5-232.5	42	124.5	96-191	0.622	0.276
Amylase (u/L)	136	61.5	41.5-252.5	20	57.5	38-86.5	9	87	34-190	0.867	0.437

* Mann-Whitney U test, boldface indicates p≤0.05
p1: Comparing culture-negative to *Salmonella*-positive patients
p2: Comparing culture positive for non-*Salmonella* to *Salmonella*-positive patients
IQR: Interquartile range

4.3.5 Surgical and histopathological characteristics

The major surgical and post-surgical characteristics of the gallbladders from the three groups were compared using Fisher's exact test (Table 4.6). The majority of *Salmonella* positive individuals had gallstones (96%, 46/48); yet, there was no significant difference in the proportion of individuals with gallstones between the three groups. We did, however, identify several gallbladder characteristics that were associated the presence of *Salmonella*. Namely, gallbladder distension and inflammation was more frequently observed in the *Salmonella* positive group than the culture negative group and the non-*Salmonella* culture positive group (Table 4.6). Furthermore, the presence of an empyema (pus within the gallbladder cavity) was also more common in the *Salmonella* positive group than the other two groups. Inflammation was more likely to be due to polymorphonuclear infiltration than lymphocytic infiltration in the *Salmonella* infected gallbladder tissue, with 13% (6/48) of the *Salmonella* positive gallbladder specimens having massive neutrophil infiltrate near the lumen, compared to 4% (51/1,151) and 5% (10/214) of the culture negatives and the non-*Salmonella* culture positives, respectively. Furthermore, an additional 15% (7/46) of the *Salmonella* positive gallbladder specimens had acute-on-chronic cholecystitis (neutrophil infiltrate near the lumen with lymphocyte infiltrate and dysplasia in the mucosa) compared to 5% (10/214) and 7% (14/214) of the culture negatives and the non-*Salmonella* culture positives, respectively (Table 4.7). Correspondingly, chronic

inflammation without large neutrophil infiltrate was not observed in gallbladder tissue from the *Salmonella* positive group.

Table 4.6: The gallbladder characteristics within the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups

Characteristic	Culture negative n=1,103	Culture positive non-*Salmonella* n=214	*Salmonella* positive n=48	p1*	p2*
Gallbladder tissue thickness					
Thick (> 4mm)	173 (15.7)	36 (16.8)	10 (20.8)	0.35	0.602
Normal (4mm)	493 (44.7)	86 (40.0)	21 (43.8)		
Thin (< 4mm)	74 (6.7)	12 (5.6)	1 (2.1)		
Gallbladder					
Contracted	108 (9.8)	25 (11.7)	1 (2.1)	**0.026**	**0.035**
Distended	221 (20.0)	52 (24.3)	15 (31.3)		
Gall stones					
None	19 (1.7)	6 (2.8)	3 (6.3)	0.101	0.481
Single	344 (31.2)	62 (29.0)	14 (29.2)		
Multiple	684 (62.0)	133 (62.1)	28 (58.3)		
Pathology					
Inflammation	93 (8.4)	17 (7.9)	8 (16.7)	**0.046**	0.060
Empyema	90 (8.2)	21 (9.8)	10 (20.8)	**0.003**	**0.033**
Sludge	57 (5.2)	8 (3.7)	1 (2.1)	0.338	0.581
Mucocele	50 (4.5)	7 (3.3)	1 (2.1)	0.427	0.664

* Fisher's exact test, boldface indicates $p \leq 0.05$
p1: Comparing culture-negative to *Salmonella*-positive patients
p2: Comparing culture-positive, *Salmonella*-negative to *Salmonella*-positive patients

Table 4.7: Histopathological (post- cholecystectomy) evaluation within the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups, n (%)

Type inflammation	**Culture negative** **n=1038**	**Culture positive, non- *Salmonella*** **n=201**	***Salmonella* positive** **n=46**	**p1***	**p2***
Acute	51 (4.9)	10 (5.0)	6 (13.0)	**0.001**	**0.008**
Acute on chronic	59 (5.7)	14 (7.0)	7 (15.2)		
Chronic	872 (84.0)	163 (81.1)	25 (54.3)		
Subacute	17 (1.6)	1 (0.5)	0 (0)		

* Fisher's exact test, boldface indicates $p \leq 0.05$
p1: Comparing culture-negative to *Salmonella*-positive patients
p2: Comparing culture-positive, *Salmonella*-negative to *Salmonella*-positive patients

As shown in Table 4.7, *Salmonella* positive patients were more likely to have acute inflammation recorded from the post-cholecystectomy histopathological report than either culture negative (p=0.001) or culture positive, non-*Salmonella* patients (p=0.008). Both non-*Salmonella* groups were much more likely to have evidence of chronic inflammation instead.

Finally, surgical patients were followed up on month 3 and month 6 after the surgery. A small questionnaire was administered requesting information on complaints and symptoms since discharge. A total of 21 patients visited the hospital for follow up on month 3, 17 patients on month 6, and 19 patients visited both 3 and 6 months. A majority (90%) of the patients were feeling well (36/40). and none of the patients had any significant clinical complaints. Stool samples were collected from 28 patients on month 3 follow up and 20 patients on month 6 follow up. None of the stool collected was found to be culture positive for *Salmonella*.

4.4 Discussion

The mechanism of gallbladder infection/colonization remains contentious, and it is unknown if *Salmonella* promote gallbladder damage during chronic infection or if the organisms exploit existing gallbladder damage to stimulate colonization [181]. Bile is

typically sterile, and consists of organic and inorganic compounds, bile acids, cholesterol, phospholipids and the pigment biliverdin. Sterility is partially maintained by the secretion of IgA and mucus, preventing bacterial survival and adhesion to the surface of the lumen and the major bile duct, respectively [243]. Here, I found a wide array of organisms in the bile of individuals undergoing cholecystectomy, some of which have been previously isolated from the gallbladder [184, 244, 245]. Again, whether these organisms functionally stimulate cholecystitis or cholelithiasis, or merely have the ability to colonise damaged gallbladders, remains unclear. Data presented here confirm that non-*Salmonellae* organisms, with a spectrum of pathogenic potential, are as equally adept at colonising the gallbladder and surviving within the bile as typhoidal *Salmonella*. Yet, non-*Salmonellae* appear not to stimulate the same pathology as *Salmonella*; *Salmonella* infected tissue was more commonly associated with systemic and local acute inflammatory responses. Mouse experiments, utilising *Salmonella* Typhimurium, have shown that *Salmonella* can replicate within the epithelial cells of the gallbladder [246], and that colonised gallbladders displayed evidence of the epithelial destruction and local neutrophil infiltrate. Here, extensive neutrophil infiltrate was found, yet I am unable to confirm if the bacteria are damaging the tissue or colonising previously damaged tissue. However, as shown by an increased prevalence of gallbladder distension, right upper quadrant pain, empyema and a raised systemic neutrophil count, there is an evident association of invasive *Salmonella* in the gallbladder with an acute inflammatory response. Previously, the presence of

individuals in Kathmandu with *Salmonella* in their gallbladder has been noted, highlighting the presence of *S*. Paratyphi A [202]. The role of chronic carriage of *S.* Paratyphi A has received much less attention than that of S. Typhi and it is unknown as to what extent chronic gallbladder carriage is contributing to the increasing burden of *S*. Paratyphi A across many parts of Asia [62]. Enteric fever caused by *S.* Paratyphi A increased from 17.5% (155/885) in 1993 to 34% (926/2,718) in 2003 in the location of this study [69]. Furthermore, we found an almost equal ratio of *S.* Typhi and *S*. Paratyphi A (1:0.9) isolated from bile, yet the isolates from blood from acutely infected patients over the same period is lower (1:0.4) [78]. This disparity may result from a multitude of factors, but may predict that *S*. Paratyphi A is more adept at inducing carriage in this population, or, once in the gallbladder, may be more likely to induce an acute inflammatory response, requiring a surgical intervention, than *S.* Typhi. We found that 3.5% of the individuals undergoing gallbladder surgery had invasive *Salmonella* in their bile in this area with a high incidence of enteric fever [75]. A report from a similar patient demographic in India suggest an equivalent rate of 5%, and in Chile, 7.3% of bile cultures were found to be positive for *Salmonella* [211]. The long-term carriage of invasive *Salmonella* in the gallbladder is thought to be central to the maintenance and transmission of these human-restricted pathogens [181]. However, data from our previous work in Kathmandu suggests that direct transmission plays a negligible role in acute infections, and we have hypothesized that carriers merely act as a reservoir for maintaining local strain diversity in areas of high endemicity [78]. Here, we found

antimicrobial resistance to only nalidixic acid in the *Salmonella* from the gallbladder. Although nalidixic acid resistance often precedes resistance to other fluoroquinolones, these isolates were susceptible to gatifloxacin and ofloxacin. Firstly, these data show that infection with an antimicrobial resistant organism is not likely to be associated with *Salmonella* carriage. Secondly, if one considers nalidixic acid resistance as a proxy marker of contemporary strains, the organisms in the gallbladder have probably been there for some time (i.e. from a period when nalidixic acid resistance was less prevalent) [67, 69]. NA resistance is a growing problem in Kathmandu. From an ongoing clinical trial enrolling enteric fever patients over the last two years at Patan Hospital, 80% (171/214) of invasive *Salmonella* isolates demonstrated resistance to nalidixic acid, which is greater than the proportion (59%) found from bile isolates in the current study (unpublished data). This evidence supports our current hypothesis of gallbladder carriage playing a limited role in the acute transmission of enteric fever in Kathmandu. Whilst we argue that in locations such as Kathmandu, the role of carriers in enteric fever transmission may be negligible, it is reasonable to suggest that those shedding invasive *Salmonella* play a vital important role in low transmission setting. In the USA, up to 30% of enteric fever infections are anticipated to result from contact with a chronic carrier [195]. Therefore, these individuals will become increasingly important as indirect transmission this area begins to subside after the introduction of an effective intervention strategy. However, currently there is no appropriate diagnostic test for the detection of long-term carriers. Bile cultures from string devices

are considered effective [195], but are impractical for screening large cohorts [111]. The presence of gallbladder disease is, perhaps, currently the best clinical predictor of carriage of invasive *Salmonella* [211]. However, it remains unclear as to why some patients progress to become chronic shedders and others do not. The development of a rapid diagnostic for the detection of invasive *Salmonella* carriage should accelerate regional elimination of enteric fever and add insight into the epidemiological role of these individuals. One of the major caveats of this study is the fact that it is a passively acquired patient population, which may not accurately reflect the general population of Kathmandu. Additionally, all patients in the study had some form of gallbladder abnormality, although it is unclear whether the infecting organisms had induced such abnormalities. Nevertheless, in the absence of an alternative methodology, I have attempted to estimate the burden and mechanism of invasive *Salmonella* carriage.

4.5 Conclusion

A total of 3.5% of individuals undergoing gallbladder surgery had invasive *Salmonella* in their bile in this highly populated enteric fever endemic region. Data presented here demonstrate that *S.* Paratyphi A is almost as prevalent as *S*. Typhi in the gallbladder in this population and that carriage is not driven by antimicrobial resistance. The overall role of invasive *Salmonella* carriage in endemic settings such as Kathmandu is not understood, and in this location *Salmonella* in the gallbladder may not play a dominant

role in the transmission of acute enteric fever. However, those carriers will become more important if current transmission mechanisms are disturbed. Therefore, prospectively identifying these individuals is paramount for rapid local and regional elimination.

5 The immunological responses to acute and chronic infections of *Salmonella* Typhi and *Salmonella* Paratyphi A in Kathmandu, Nepal

5.1 Abstract

Antibody responses to the Vi capsular polysaccharide, the O-antigen and the H flagellar antigen can be detected throughout an acute typhoid fever infection and for prolonged periods post infection. Furthermore, high anti-Vi antibody titres have also been recorded in individuals resident in highly endemic typhoid areas with no clinical history of typhoid fever and chronic carriers. I aimed to understand the nature of the immune response to chronic carriage by comparing antibody levels to O:2 antigen and Vi antigen in the plasma from patients with *Salmonella* in their gallbladder, members of the general population and individuals with acute typhoid infections. I performed a series of ELISAs on plasma from these patient groups and followed up these assays by measuring the bactericidal capabilities of the plasma and the pro-inflammatory cytokine response. I found that individuals with *S.* Typhi and *S*. Paratyphi A in their gallbladder have dramatically elevated levels of IgG to O:2 and Vi antigens. Furthermore, these plasma samples have high bactericidal activity yet low pro-inflammatory cytokine levels. These data suggest that *S*. Typhi and *S*. Paratyphi A are stimulating a constant immunological response, in the form of antibody. However, I

suggest that low levels of pro-inflammatory cytokines predict that the organisms in the gallbladder are regulating and dampening the inflammatory response. Furthermore, combining cytokine profiles and antibody levels may be a method of prospectively detecting carriers in the general population.

5.2 Introduction

Antibody responses (IgA and IgM) to Vi capsular polysaccharide, LPS antigen and the H flagellar antigen can be detected throughout an acute typhoid fever infection and for prolonged periods post infection (IgG) [121, 247]. However, high anti-Vi antibody titres (IgG) have also been recorded in individuals resident in highly endemic typhoid areas with no clinical history of typhoid fever [194, 198]. The seroprevalence of typhoid (the prevalence of individuals testing positive for anti-Vi antibody in a population) is highest in endemic countries, although comparative titres can vary according to geographic location, age, and exposure rates [120]. The Vi antigen is present on the surface of *S.* Typhi and a limited number of other *Salmonella* serovars; therefore, antibody against Vi has been studied as a potential screening tool for typhoid carriers as carriers often produce higher levels of anti-Vi antibody (IgG) than is observed in the general population of acute infections [37, 184, 199, 200]. Similarly, anti-Vi antibody that is produced during the earlier stage of an infection (IgM) has been targeted for the

developing of rapid detection tests for typhoid fever, although due to the nature of IgM, these tests lack specificity [119].

Various assays have previously been used for the purpose of serological identification of acute typhoid fever as well as chronic carriers, but have often shown poor results. The ELISA for typhoid is more sensitive than other methods like passive hemagglutination assay for detecting those that have been exposed in one way or another to the organism [36, 37, 122]. However, the majority of ELISAs that have been developed use Vi-antigen and, therefore, can only detect exposure to *S.* Typhi and no research has, as yet, been performed on investigating exposure to *S*. Paratyphi A. This is highly relevant, as increasing incidence rates of enteric fever caused by *S.* Paratyphi A have been reported across South and Southeast Asia. Furthermore, work in our research group in Kathmandu, Nepal has shown the *S*. Paratyphi A infections are clinically indistinguishable from *S*. Typhi infections, are more drug resistant than *S*. Typhi (Chapter 3) and, like *S.* Typhi is capable of prolonged survival in the gallbladder [202]. I aimed to address this by comparing antibody responses between Vi antigen and O:2 antigen (O:2 is the *S*. Paratyphi A O-antigen (LPS) in the general population, patients with acute enteric fever and individuals with *S*. Typhi and *S*. Paratyphi A in their gallbladder.

5. The immunological responses to acute and chronic infections of *Salmonella* Typhi and *Salmonella* Paratyphi A in Kathmandu, Nepal

Cytokines are small cell-signaling protein molecules that are secreted by numerous cells within the immune system that permit cellular communication and are synonymous with the response to infection. Therefore, cytokines are thought to play a major role in both pathogenesis and control of systemic *Salmonella* infections, such as typhoid fever. Again there are limited data on how *S*. Typhi and *S*. Paratyphi A directly interact with the immune system and cytokines probably play a key role in both acute and chronic infections. Some immunological investigations have shown high-level production of pro inflammatory cytokines (cytokines that promotes a systemic inflammatory response) in patients with acute *S*. Typhi infections [51, 248, 249]. The role of cytokines responses in individuals with acute *S*. Paratyphi A infections of those with chronic biliary carriage of invasive *Salmonella* have not been elucidated.

I aimed to investigate the roles of anti-O:2 and anti-Vi antibody during acute and chronic invasive *Salmonella* infections. I hypothesized that, firstly, there would be significant exposure to both O:2 and Vi antigens in the general population and secondly that carriers of *S*. Typhi and *S*. Paratyphi A would have elevated IgG against Vi and O:2 antigens, respectively. Both these populations are key for understanding typhoid fever in this area, with respect to local vaccine implementation and eventual disease elimination. In this chapter I present a description of the distribution of antibody responses against *S*. Typhi Vi antigen and *S*. Paratyphi A O:2 antigen in acute enteric fever patients, chronic biliary carriers and the general hospital population. To

understand the functionality of the antibody response in the same patient groups, a series of SBA were performed on plasma samples collected from these patients. The SBAs were performed to examine if the groups that had been exposed to the two pathogens had differential functional antibody responses; ELISAs permit the calculation of antibody levels, SBAs estimate functionality through activation of the complement cascade and the demonstration of bactericidal activity. Additionally, plasma from acute enteric fever patients, chronic carriers of *Salmonella* and bile culture negative patients was tested against a number of pro-inflammatory cytokines to investigate any potential associations between cytokines production and the various forms of enteric fever.

5.3 Results

5.3.1 Antibody response to Vi and O:2 antigens in the general hospital population in Kathmandu, Nepal

To understand "seroconversion" and to interpret serology data in individuals that have been exposed to a particular pathogen it is important to appreciate the background exposure levels to the antigens. This is particularly important with respect to the Vi antigen (*S*. Typhi) and the O:2-antigen (*S*. Paratyphi A) as they may cross react with other Gram-negative organisms or antibody responses in acutely infected individuals may be induced by prolonged exposure. Therefore, a total of 795 plasma samples collected from patients visiting emergency Department of Patan Hospital between

January 2010 and September 2010, were measured for IgG against Vi and O:2 in age stratified (0-65 years) plasma samples using the indirect ELISA method as described in chapter 2.

The resulting data demonstrated a consistently high level of IgG against Vi and O:2 in all age groups. Anti-O:2 IgG was lowest at birth then steeply increased towards the peak at the age of 11-12 years and subsequently declined; yet persisted into old age (Figure 5.1). In contrast, anti-Vi IgG was highest at birth (less than 1 year) then gradually declined until secondarily peaks at the age of 17-18 years.

To investigate if there was an association between anti-Vi IgG and anti-O:2 IgG and age, the data were stratified into four age groups (0-10 years, 11-20 years, 21-40 years and 41-65 years) and the IgG values were plotted against each other and Spearman's values were calculated (Figure 5.2). There was a weak correlation between levels of IgG to Vi and to O:2 (Spearman's ρ: 0.30, $P < 0.001$) across all age groups. This correlation was most apparent within the group aged 11-20 years (Spearman's ρ: 0.50, $P < 0.001$) and then declined with age (Figure 5. 2).

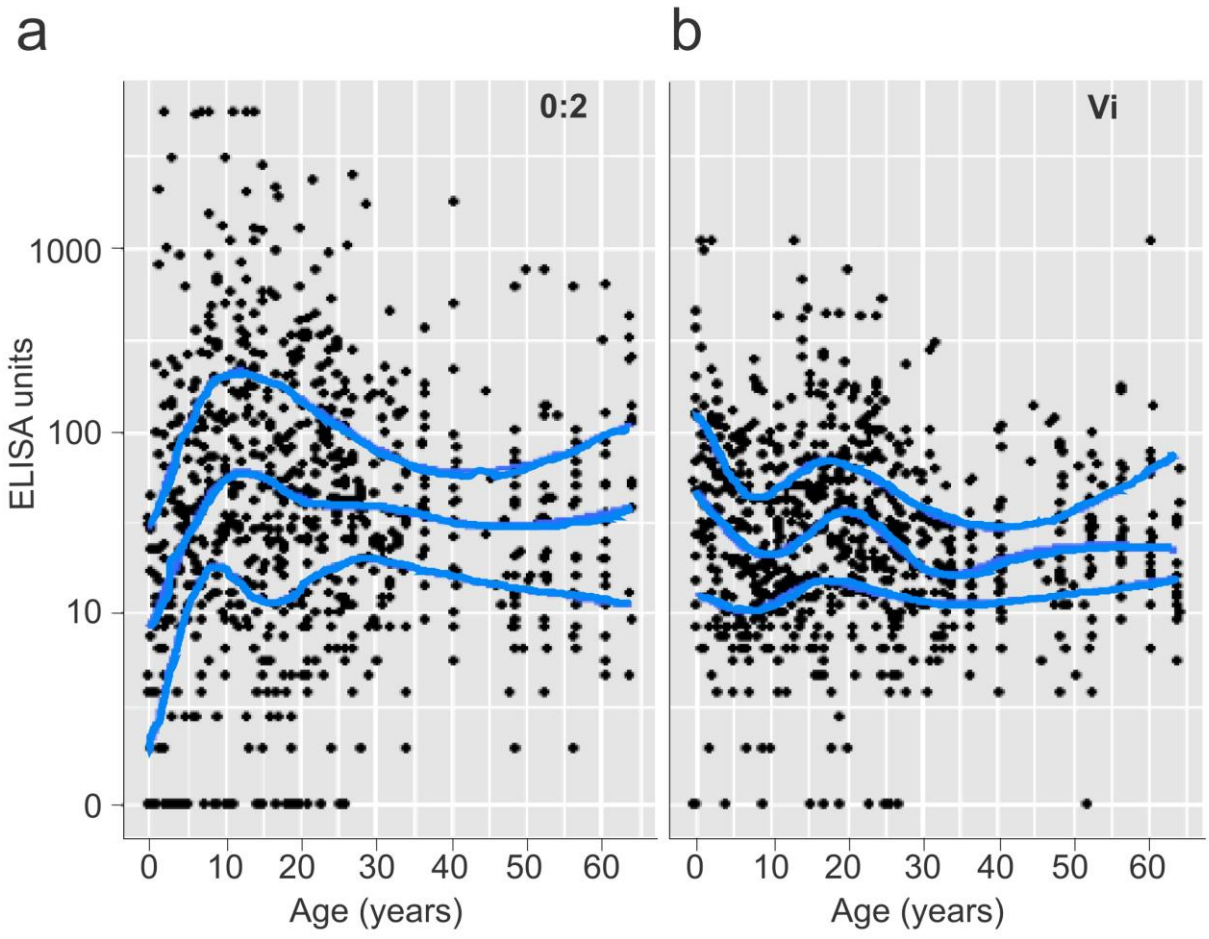


Figure 5.1: IgG serology against Vi antigen (*S*. Typhi) and O:2 antigen (*S*. Paratyphi A) in an age-stratified cross-section of the population of Kathmandu

Scatter plots showing antibody (IgG) levels against O:2-antigen **(a)** and Vi-antigen **(b)** in an age stratified population of Kathmandu, Nepal. Smoothed lines correspond to age-dependent median and quartiles which were estimated based on quantile regression with age included as a natural cubic spline function with 5 degrees of freedom.

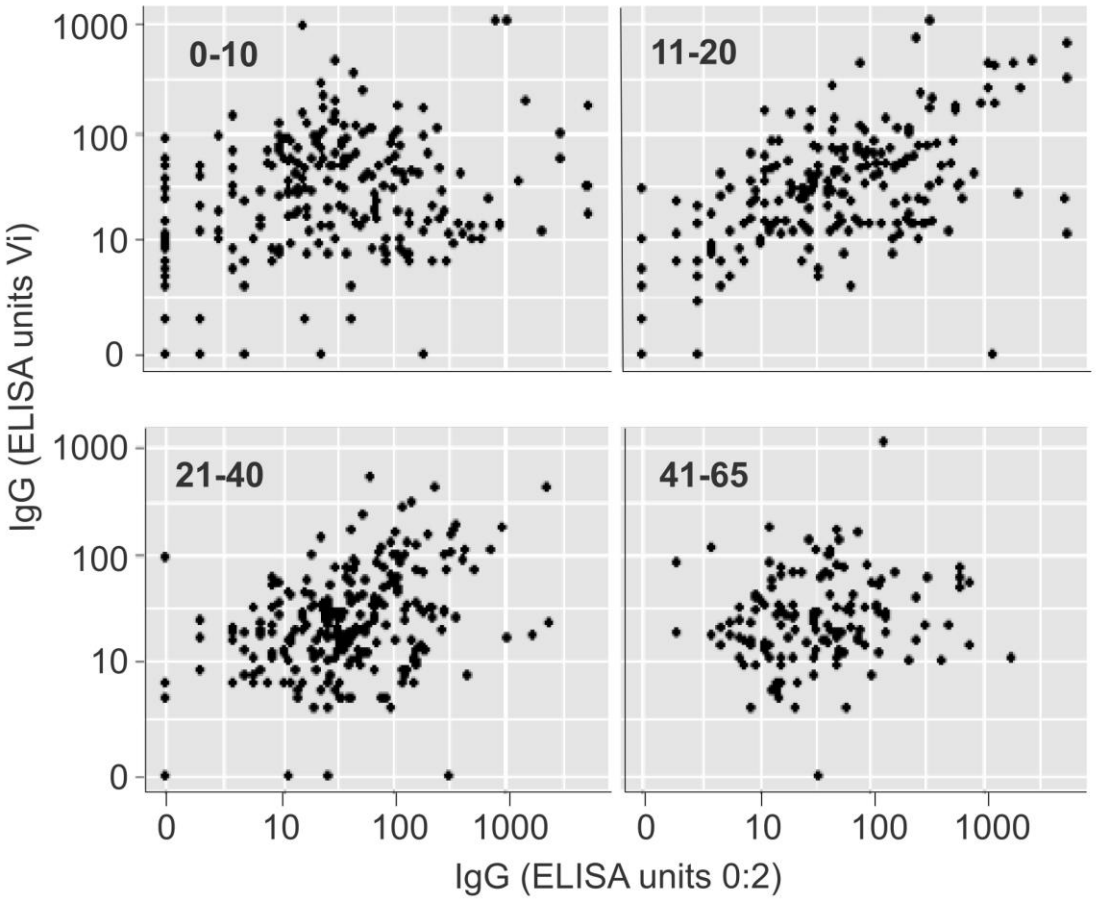


Figure 5.2: IgG serology against Vi-antigen (*S*. Typhi) and O:2-antigen (*S.* Paratyphi A) in an age-stratified cross-section of the population of Kathmandu Age stratified scatter plots scatterplots (clockwise, 0-10 years, 11-20 years, 21-40 years and ≥ 40 years) of IgG O:2 (x axis) and IgG Vi (y axis).

5.3.2 Antibody response to Vi and O:2 antigens in patients with culture confirmed typhoid fever in Kathmandu, Nepal

Plasma samples collected from 33 *S*. Typhi culture positive and 25 *S*. Paratyphi A culture confirmed acute enteric fever patients were subjected to O:2 and Vi ELISAs to describe the natural history acute and convalescent antibody response to *S*. Typhi and *S*. Paratyphi A infections. Anti-O:2 and anti-Vi IgM and IgG were measured in the plasma of *S*. Typhi and *S*. Paratyphi A culture confirmed patients at four different time intervals (Day 1, Day 8, Month 1, and Month 3). The resulting data over the time course are shown in Figure 5.3 (O:2) and Figure 5.4 (Vi). Notably, IgM and IgG against both antigens was detectable in all plasma samples, i.e. plasma from those with a culture confirmed *S*. Typhi infection was additionally used to measure anti-O:2 antibody, and vice versa.

The IgG antibody responses against O:2 antigen in the *S*. Typhi and the *S*. Paratyphi A patients were comparable over the time series, with both peaking on Day 8 of infection and then declining, suggesting a non-specific polyclonal response to O-antigen during infection (Figure 5.3). The IgM response to O:2-antigen was also similar between the two groups, peaking on Day 1 and Day 8 of infection and then declining. A series of pairwise comparisons were performed on the antibody titres at all time points (Table 5.1). *S.* Typhi and *S.* Paratyphi A patients had significantly higher anti-O:2 IgG antibody titres on day 8 compared to those on day 1, month 1 ($P<0.001$ for all groups) (Table 5.1). The variation in antibody titres at the other time points was non-significant, suggesting that the IgG returns to baseline (Day 1) by day 28 post infection.

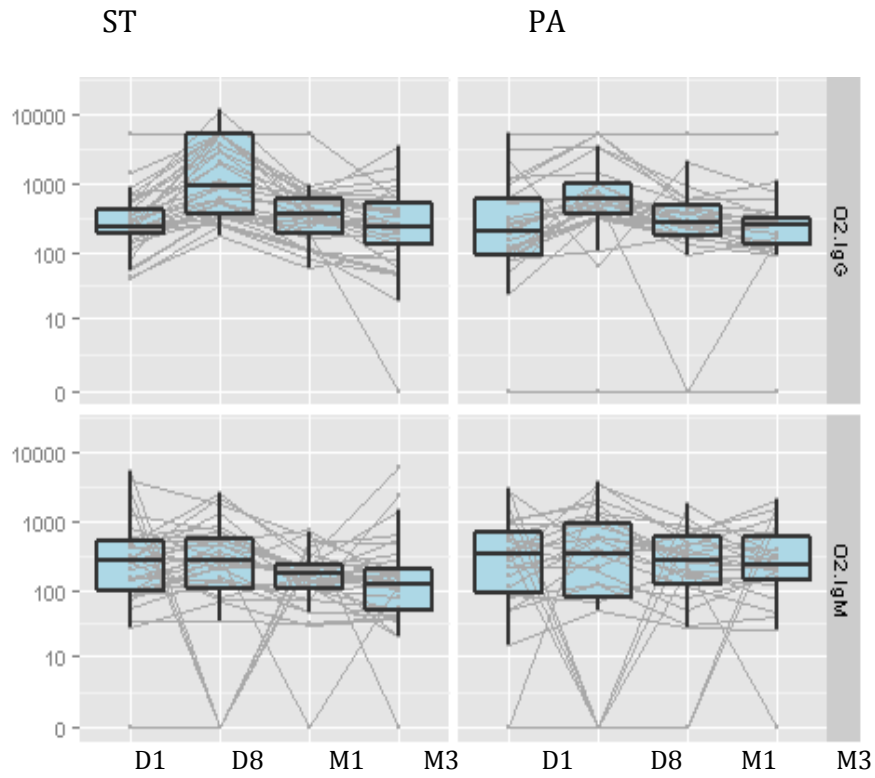


Figure 5.3: IgG and IgM serology against O:2-antigen (S. Paratyphi A) over three months in patients with culture confirmed enteric fever

Boxplots showing the changing patterns over time (D1: Day 1; Day 8: Day 8; M1: Month 1; M3: Month 3) (x axis) against antibody titers in ELISA Units (log10) of IgG (upper) and IgM (lower) against O:2 antigen in patients with culture confirmed enteric fever caused by *S.* Typhi (ST) (n=33) or *S.* Paratyphi A (PA) (n=25). Each box shows the upper and lower quartiles of the data at each time point, the horizontal black lines within the boxes represent the median and grey lines linking between boxes signify the natural time series of IgM or IgG in individual patients.

Table 5.1: Pairwise comparisions of anti-O:2 antibody titres over time

	O:2 IgG (all)			**O:2 IgG (ST)**			**O:2 IgG (PA)**		
	D1	D8	M1	D1	D8	M1	D1	D8	M1
D8	<0.0001	-	-	<0.0001	-	-	0.0002	-	-
M1	0.1863	<0.0001	-	0.0425	<0.0001	-	0.8527	0.0003	-
M3	0.8706	<0.0001	0.0139	0.775	<0.0001	0.0697	0.539	0.0003	0.1096

	O:2 IgM (all)			**O:2 IgM (ST)**			**O:2 IgM (PA)**		
	D1	D8	M1	D1	D8	M1	D1	D8	M1
D8	0.2592	-	-	0.5434	-	-	0.294	-	-
M1	0.0089	0.0023	-	0.0082	0.0123	-	0.3276	0.0613	-
M3	0.005	0.0342	0.6808	0.013	0.0551	0.8601	0.1574	0.2578	0.7454

Table shows the p values for the various pairwise comparisons comparing median antibody titers (IgG upper and IgM lower) against O:2 antigen in plasma throughout an infection time series. Data taken from Figure 5.3, highlighted boxes indicate statistical significance.

The IgM response to O:2 antigen was markedly different to that of the IgG response. Notably, there was no significant difference in IgM antibody titres in patients with S. Paratyphi A infections throughout the course of the infection and the period afterwards (Table 5.1). However, anti-O:2 IgM in *S*. Typhi patients peaked on Days 1 and 8 and then declined over the three months post infection.

The IgG and IgM antibody response patterns against Vi antigen over the time series were comparable and, indeed, comparable between those with *S*. Typhi and *S*. Paratyphi A infections (Figure 5.4). These data, again, indicate prolonged exposure and/or cross-reactivity of the antigen. IgG and IgM anti-Vi antibody peaked on Day 8, declined at month 1 and then peaked again after 3 months.

Again, a series of pairwise comparisons was performed on the antibody titres at all time points (Table 5.2). *S.* Typhi and *S.* Paratyphi A patients had significantly higher anti-Vi IgG antibody titres on day 8 and 3 months post infection compared to those on day 1 and 28 days ($P<0.001$ for all groups) (Table5.1). The variation in antibody titres at the other time points was significant, suggesting a natural fluctuation in IgG and IgM against Vi after an infection. The secondary peak in both *S*. Typhi and *S*. Paratyphi A patients suggests secondary exposure and natural antibody boosting within 3 months of the primary symptomatic infection.

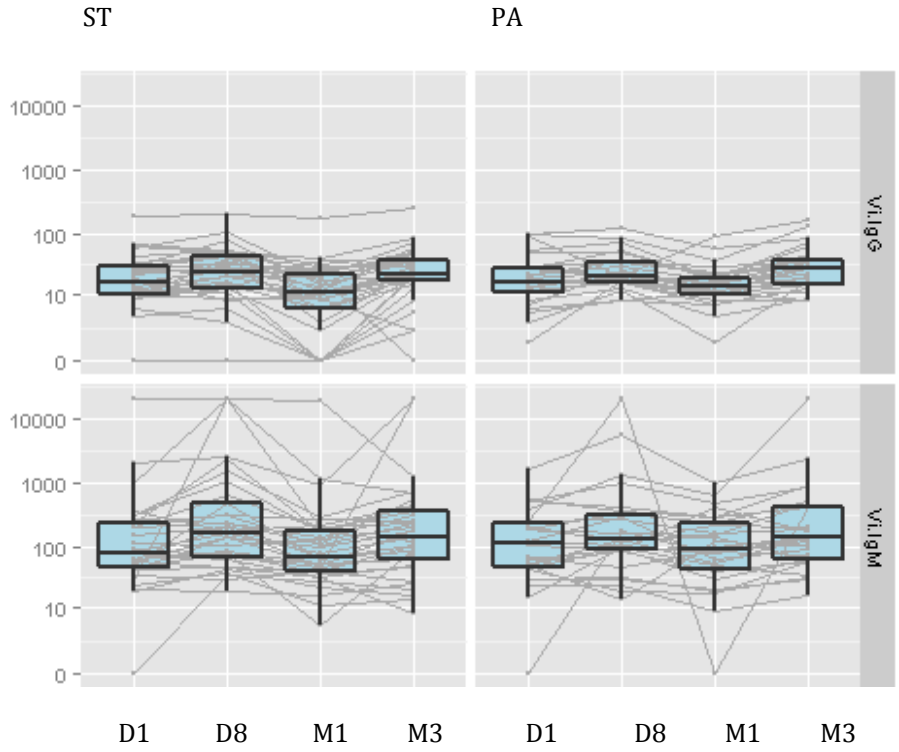


Figure 5.4: IgG and IgM serology against Vi-antigen (*S*. Typhi) over three months in patients with culture confirmed enteric

Boxplots showing the changing patterns over time (D1: Day 1; Day 8: Day 8; M1: Month 1; M3: Month 3) (x axis) against antibody titers in ELISA Units (log10) of IgG (upper) and IgM (lower) against Vi antigen in patients with culture confirmed enteric fever caused by *S.* Typhi (ST) (n=33) or *S.* Paratyphi A (PA) (n=25). Each box shows the upper and lower quartiles of the data at each time point, the horizontal black lines within the boxes represent the median and grey lines linking between boxes signify the natural time series of IgM or IgG in individual patients.

Table 5.2: Pairwise comparisions of anti-Vi antibody titres over time

	Vi IgG (all)			**Vi IgG (ST)**			**Vi IgG (PA)**		
	D1	D8	M1	D1	D8	M1	D1	D8	M1
D8	<0.0001	-	-	0.0009	-	-	0.0063	-	-
M1	<0.0001	<0.0001	-	0.0011	<0.0001	-	0.0105	0.0003	-
M3	<0.0001	0.4431	<0.0001	0.0027	0.7862	0.0001	0.0006	0.5016	<0.0001

	Vi IgM (all)			**Vi IgM (ST)**			**Vi IgM (PA)**		
	D1	D8	M1	D1	D8	M1	D1	D8	M1
D8	0.0003	-	-	0.0005	-	-	0.1213	-	-
M1	0.0139	<0.0001	-	0.0202	<0.0001	-	0.3345	0.0077	-
M3	0.0002	0.3284	<0.0001	0.008	0.1404	0.0026	0.0063	0.8303	0.0027

Table shows the p values for the various pairwise comparisons comparing median antibody titres (IgG upper and IgM lower) against Vi antigen in plasma throughout an infection time series. Data taken from Figure 5.3, highlighted boxes indicate statistical significance.

5.3.3 Antibody responses in *S.* Typhi and *S.* Paratyphi A carriers

It has been shown that *S*. Typhi carriers have elevated IgG against Vi antigen and this may be used a method to prospectively detect carriers in an endemic region. However, a suitable potential marker for *S*. Paratyphi A carriage has never been identified. The collection of plasma from patients with acute and chronic *S*. Typhi and *S*. Paratyphi A infections permitted a unique opportunity to compare antibody responses between these groups. The O:2 and Vi ELISAs were performed on plasma from 10 individuals with *S*. Typhi positive bile, 5 individuals with *S*. Paratyphi A positive bile and 30 individuals with culture negative bile. The boxplots of the resulting antibody titres, grouped by culture result, are shown in Figure 5.5 and the pairwise comparisons are shown in Table 5.3.

Culture confirmed carriers (*S*. Typhi and *S*. Paratyphi A) were found to have significantly higher levels of anti-Vi IgG compared with the bile culture negative control group (Figure 5.5, Table 5.3). This was most apparent in the *S*. Typhi group, which had the highest median antibody titres of the three groups. IgG antibody titres against O:2 could also distinguish between the bile positive *S*. Paratyphi A cases, the bile positive *S*. Typhi cases and the bile culture negative controls. Interestingly, no such pattern was observed with respect to IgM against either of these antigens, as there was no significant difference in IgM antibody titres against O:2 or Vi between any of the three groups. Despite the sample sizes being small in all cases, these data suggest that screening for IgG antibody against the O:2 and Vi

antigens could be used to prospectively detect carriers in a typhoid endemic location.

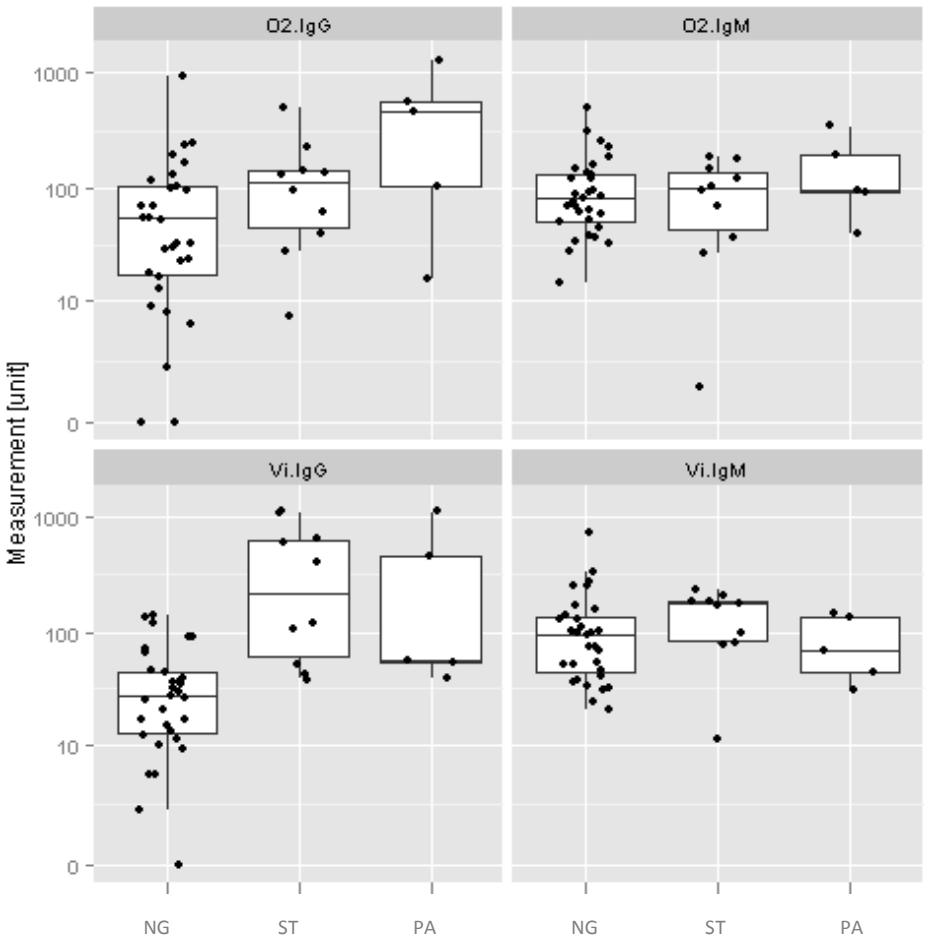


Figure 5.5: IgG and IgM serology against Vi-antigen and O:2 antigen in bile culture positive and culture negative patients

Boxplots showing the antibody titers in ELISA Units of IgG (left) and IgM (right) against Vi antigen (lower) and O:2 antigen (upper) in patients with a negative bile culture (NG) (n=30) and positive bile culture for *S.* Typhi (ST) (n=10) or *S.* Paratyphi A (PA) (n=5). Each box shows the upper and lower quartiles of the data, the horizontal black lines within the boxes represent the median.

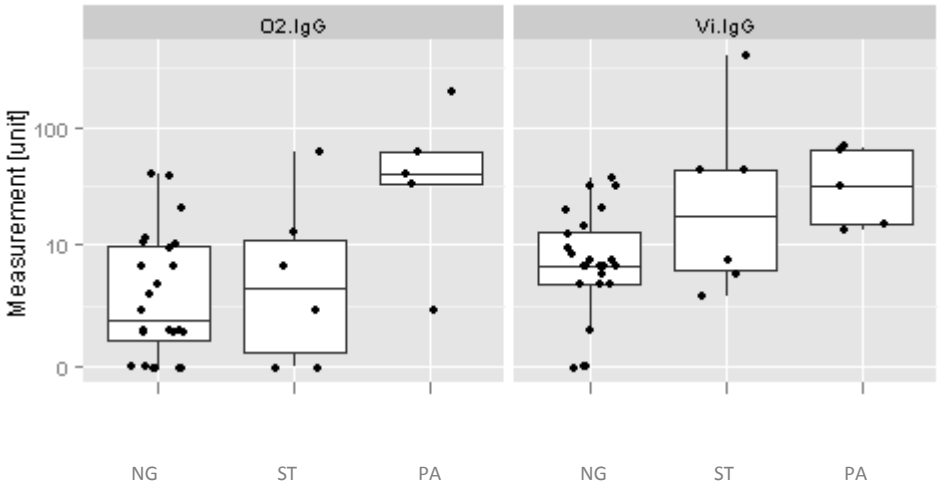
Table 5.3: Pairwise comparisions of anti-Vi antibody and anti-O:2 antibody titres in bile culture positive and culture negative patients

	O:2 IgG		**O:2 IgM**	
	NG	PA	NG	PA
PA	0.047	-	0.31	-
ST	0.141	0.254	0.98	0.44
	Vi IgG		**Vi IgM**	
	NG	PA	NG	PA
PA	0.0153	-	0.749	-
ST	0.0002	0.7121	0.154	0.099

Table shows the p values for the various pairwise comparisons comparing median antibody titers (O:2 upper and Vi lower) in patients with a negative bile culture (NG) (n=30) and positive bile culture for *S.* Typhi (ST) (n=10) or *S.* Paratyphi A (PA) (n=5). Data taken from Figure 5.5, highlighted boxes indicate statistical significance.

However, taking blood from large cohorts of people for the purposes of serological screening is not always feasible and we proposed that saliva (or crevicular fluid from the base of the gum) could be used to detect antibody. To investigate this, saliva samples were collected from 35 individuals (6 individuals with S. Typhi positive bile, 5 individuals with S. Paratyphi A positive bile and 24 individuals with culture negative bile) from surgical patients as described in chapter 2 and subjected to IgG ELISAs against O:2 and Vi (Figure 5.6). Saliva samples from chronic carriers colonized with both *S*. Typhi and *S*. Paratyphi A had higher levels of IgG against both Vi and O2 antigens than the control group. However, only the *S*. Paratyphi A carriers saliva antibody titres reached statistical significance compared to the other groups.

Next, I compared the IgG responses to Vi and O:2 antigens between these various groups, the age stratified plasma bank samples (0-10 years, 11-20 years, 21-40 years and 41-65 years), acute infection at Day 1 and Day 8, chronic infection and no growth gallbladder controls. These analyses were serovar specific, i.e. acute and chronic *S*. Typhi and *S*. Paratyphi A infections were compared independently and performed to identify different titres in the various groups. The boxplots of these data are shown in Figure 5.6 (O:2 antigen) and Figure 5.7 (Vi-antigen). Elevated IgG antibody titres against O:2 can be clearly seen in those with *S*. Paratyphi A carriage compared to the gallbladder controls and the general population ($p < 0.001$, in all cases). Anti-O:2 IgG antibody titres were highest in the carriers with the exception of individuals with acute *S*. Paratyphi A infections on Day 8.



	O:2 IgG		**Vi IgG**	
	NG	PA	NG	PA
PA	0.008	-	0.007	-
ST	0.713	0.118	0.193	0.537

Figure 5.6: IgG serology against Vi-antigen and O:2 antigen in bile positive and culture negative patients with saliva

Boxplots showing the antibody titers in ELISA Units (log10) of anti-O:2 IgG (left) and anti-Vi IgG (right) in the saliva of patients with a negative bile culture (NG) (n=24) and positive bile culture for *S.* Typhi (ST) (n=6) or *S.* Paratyphi A (PA) (n=5). Each box shows the upper and lower quartiles of the data, the horizontal black lines within the boxes represent the median. Table shows the p values for the various pairwise comparisons, highlighted boxes indicate statistical significance.

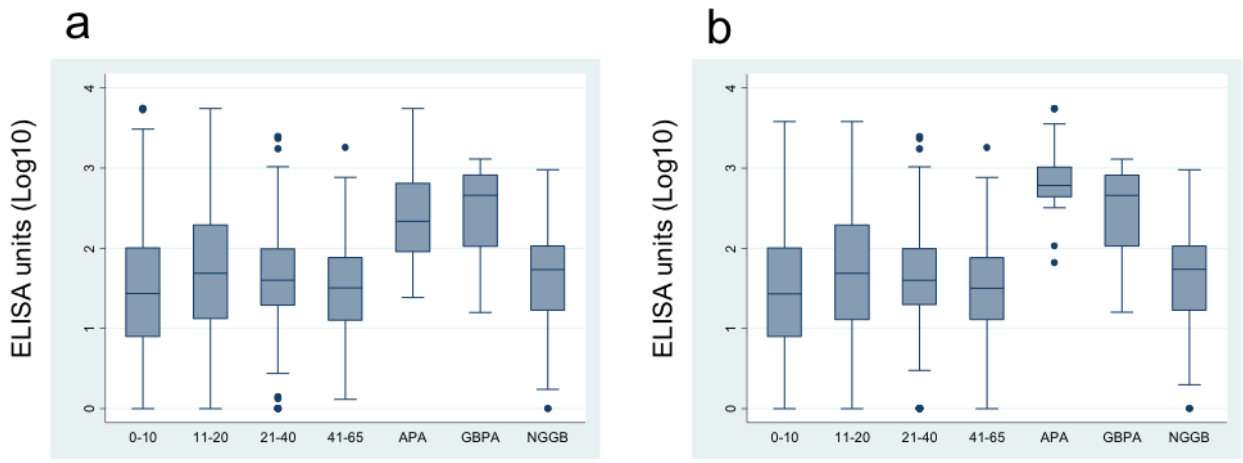


Figure 5.7: A comparision of anti-O:2 IgG between *S*. Paratyphi A carriers, acute *S*. Paratyphi A patients and the general population

Box plot of ELISA Units (log10) of IgG against O:2 in the general hospital population stratified by age groups (0-10, 11-20, 21-40, 41-65 years) compared to acute confirmed *S*. Paratyphi A (APA) on Day 1 **(a)** and Day 8 **(b),** gallbladder carriage confirmed *S*. Paratyphi A (GBPA) and non-growth gallbladder controls (NGGB). The boxes show the upper and lower quartiles of the data from each group. The horizontal short blue lines within the boxes represent the median.

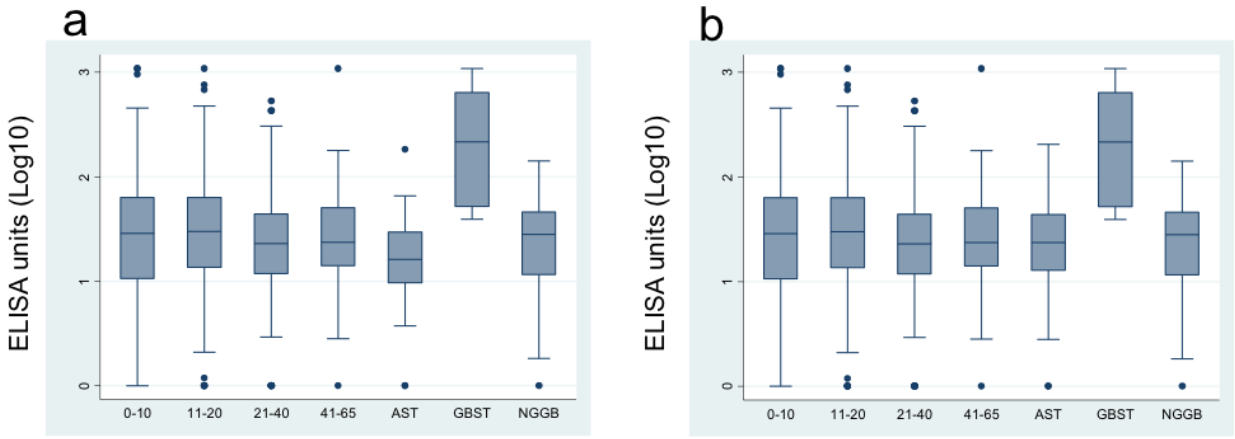


Figure 5.8: A comparision of anti-Vi IgG between *S*. Typhi carriers, acute *S*. Typhi patients and the general population

Box plot of ELISA Units (log10) of IgG against Vi in the general hospital population stratified by age groups (0-10, 11-20, 21-40, 41-65 years) compared to acute confirmed *S*. Typhi (AST) on Day 1 **(a)** and Day 8 **(b)**, gallbladder carriage confirmed *S*. Typhi (GBST) and non-growth gallbladder controls (NGGB). The boxes show the upper and lower quartiles of the data from each group. The horizontal short blue lines within the boxes represent the median.

The anti-Vi IgG responses were similar, with carriers demonstrating the highest antibody titres in comparison to general hospital population and those with acute *S.* Typhi infections on Day 0 and Day 8. There was little variation observed between the general population, the acute typhoid cases and the no growth gallbladder controls.

5.3.4 Serum bactericidal activity

To examine the functionality of the antibody response to O:2 and Vi, a series of serum bactericidal assays (SBA) were performed. The SBA permits a measurement of the ability of the serum to induce complement-mediated killing of the bacteria, this is induced by antibody specific to the bacteria, which activates the complement cascade. SBA was performed using Day 1 plasma samples from 33 *S*. Typhi and 25 *S*. Paratyphi A confirmed acute patients, 10 *S.* Typhi carriers, 7 *S*. Paratyphi A carriers, 31 bile culture negative patients and 44 plasma bank samples. The assay was performed by exposing mid-log phase *S*. Paratyphi A to plasma and baby rabbit complement; bacterial colony forming units were enumerated at time points 0, 1.5 hours and 3 hours. Mid-log phase *S*. Paratyphi A, plasma and inactivated baby rabbit complement were used as a control. The bactericidal ability of the plasma samples were scored from 1 to 8 according the dilution of the plasma at which 50% of the bacteria were killed (compared to the control). A score of 1 corresponded with 50% bacterial killing with a 1:40 plasma dilution after 1.5 and 3 hours, a score of 2 corresponded with 50% bacterial killing with a 1:120 plasma dilution, and so on in

3-fold serial dilutions, finally, a score of 8 corresponded with 50% bacterial killing with a 1:87,840 plasma dilution. The resulting data are shown in Figure 5.9. All plasma samples demonstrated significant bactericidal activity against *S*. Paratyphi A; this was irrelevant with respect to the organism they were infected with. The scoring system permitted us to calculate significance in bactericidal activity between the various groups. The highest bactericidal activity was observed in the plasma samples from the carriers, which all demonstrated functionality down to a 1:87,840 plasma dilution in both the *S*. Typhi carriers and the *S*. Paratyphi A carriers after 1.5 and 3 hours, these were significantly lower plasma dilutions than the non-growth gallbladder controls and the general population ($p<0.0001$, two sided T-test). Overall, the carriers had marginally significantly higher bactericidal activity than the patients with acute *S*. Typhi and acute *S*. Paratyphi A infections ($p=0.04$, two sided T-test) (Figure 5.9b). These data show that individuals living in Nepal have functional antibody against *S*. Paratyphi A, which is increased during acute infection and again increased during chronic carriage. Furthermore, as the assays were performed with *S*. Paratyphi A, the results show that this response is not serotype specific as *S*. Typhi acute cases and carriers all demonstrated high bactericidal titres against *S*. Paratyphi A. Bactericidal assays were not performed with S. Typhi due to time constraints of the project, these will be performed prior to publication.

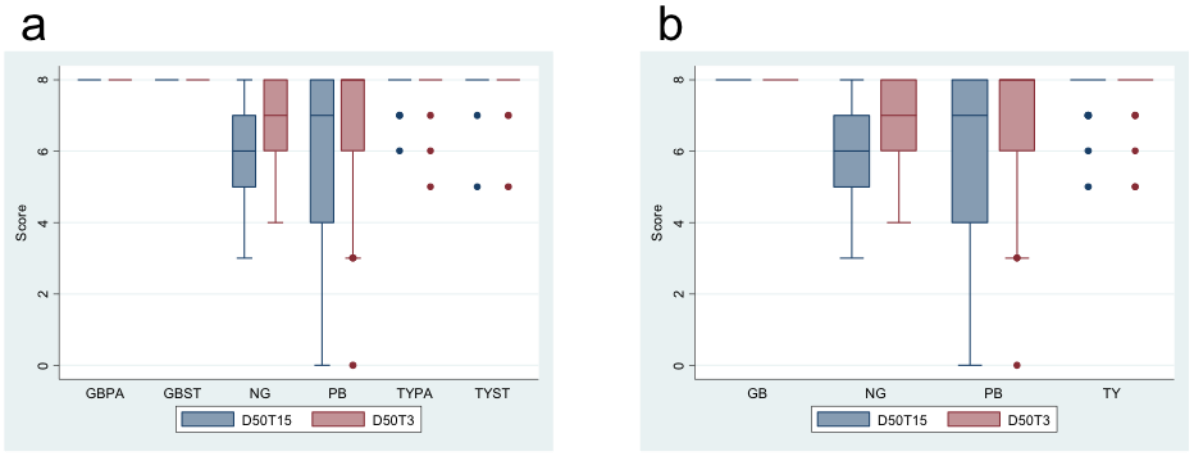


Figure 5.9: Serum bactericidal killing of *S*. Paratyphi A

Boxplots showing the bactericidal score, equating with 50% bacteria killing ability at 1.5 hours (blue) and 3 hours (red) with **(a)** 3 fold serially diluted plasma from *S*. Paratyphi A chronic carriers (GBPA), *S*. Typhi chronic carriers (GBST), non-growth bile culture controls (NG), plasma derived from general hospital population (PB), *S*. Paratyphi A confirmed acute enteric fever patient (TYPA), *S*. Typhi confirmed acute enteric fever patient (TYST), and **(b)** patients grouped by chronic carriage (GB), non-growth bile culture controls (NG), plasma derived from general hospital population (PB), and TY (acute enteric fever patients). Boxplots show lower and upper quartiles. Horizontal line within a box shows a median value.

5.3.5 Cytokines responses during acute and chronic typhoid infections

Our data suggested that the antibody response might play a critical role in carriage, predicting that carriers were immune from systemic infection by having high antibody titres that have high bactericidal killing potential. To understand the role of immune response in greater detail I compared the levels of a range of pro-inflammatory comparing acute and chronic *S*. Typhi and *S*. Paratyphi A infections. Cytokines are important for regulating the early inflammatory response, for example, TNF is produced and released by macrophages and epithelial cells in response to bacterial antigens, including lipopolysaccharide, and has an important role in controlling infection caused by intracellular infections. IL-1b, IL-2, IL-4, IL-10, IL-5, IL-6, IL-12, IL-13, TNF-α and INF-γ were measured in the plasma of 10 *S*. Typhi carriers, 7 *S*. Paratyphi A carriers, 17 age-matched growth negative gallbladder patients (age matched) 12 *S*. Typhi confirmed acute patients and 17 *S*. Paratyphi A confirmed acute enteric fever patients. The resulting data are shown in Figure 5.10.

There was a consistent pattern between all pro-inflammatory cytokines tested. Higher levels of all cytokines were detected in patients with acute disease compared to patients with carriage, indicating that *S*. Typhi and *S*. Paratyphi A stimulate a strong systemic pro-inflammatory response during an acute infection, yet not during a chronic infection (Figure 5.10, Table 5.4). Furthermore, the systemic cytokine levels were higher in the culture negative gallbladder patients than in those with *Salmonella* carriage, suggesting that either the culture negative patients either had a weak inflammatory response or those with *Salmonella* in the

gallbladder have a below baseline inflammatory response. I additionally compared the ratio of INF-γ to IL-10 and TNF-α to IL-10 between the three groups as a lower ratio signifies repression of the pro-inflammatory response. The ratio INF-γ to IL-10 was significantly lower (P=0.02, two sided T-test, Table 5.4) in the chronic carriers when compared to the acute typhoid patients and the no growth gallbladder controls. These data suggest that the anti inflammatory effect of IL-10 may suppresses the development of the disease in chronic carriers.

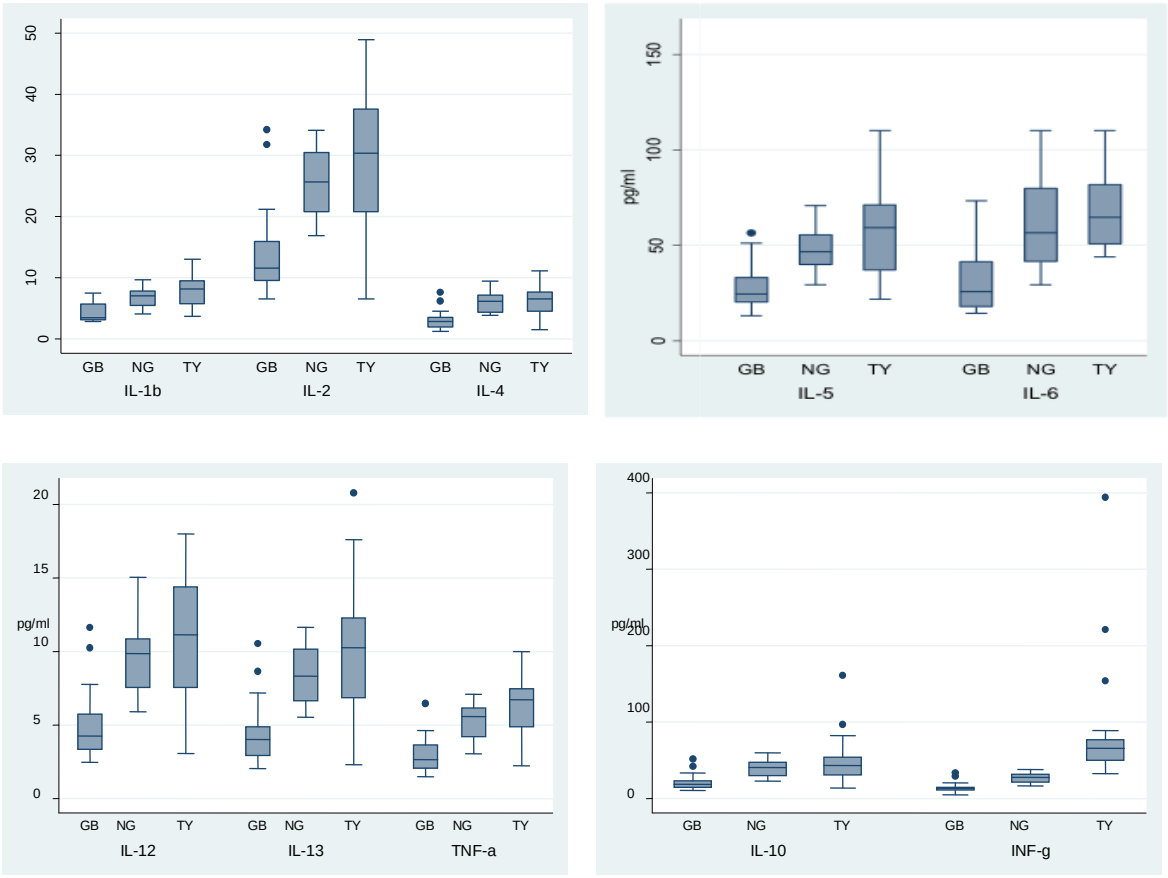


Figure 5.10: Systemic pro-inflammatory cytokine responses in acute and chronic *Salmonella* infections

Boxplots of cytokine (IL-1b, IL-2, IL-4, IL-10, IL-5, IL-6, IL-12, IL-13, TNF-α and INF-γ) levels (pg/ml) in the plasma of 17 *S*. Typhi/Paratyphi A carriers (GB), 17 age-matched growth negative gallbladder patients (NG) and 29 *S*. Typhi/ Paratyphi A confirmed acute patients (TY).

Table 5.4: Cytokine T- test p-values

	IL-1b		IL-2		IL-4		IL-5		IL-6		IL-10	
	GB	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**
TY	<0.0001	0.07	<0.0001	0.11	<0.0001	0.5	<0.0001	0.13	<0.0001	0.17	<0.0001	0.15
NG	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-

	IL-12		IL-13		INF-γ		TNF-α		INF-γ /IL-10		TNF-α/IL-10	
	GB	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**	**GB**	**NG**
TY	<0.0001	0.16	<0.0001	0.09	<0.0001	<0.0001	<0.0001	0.12	0.02	0.02	0.58	0.78
NG	<0.0001	-	<0.0001	-	<0.0001	-	<0.0001	-	0.69	-	0.68	-

Two sides T-test p values comparing cytokine levels between *S*. Typhi/Paratyphi A carriers (GB), age-matched growth negative gallbladder patients (NG) and *S*. Typhi/ Paratyphi A confirmed acute patients (TY). Significant p values are highlighted in yellow.

5.4 Discussion

This chapter provides the first description of anti-Vi and anti-O:2 antibodies expressed by acute enteric fever patients at different time points, chronic *Salmonella* carriers, and in an age stratified general population.

Higher levels of anti-Vi IgG have been detected in the general population, particularly among 20-40 year old in an endemic region who have no known history of typhoid fever [120, 194, 198]. However, little is known about the antibodies produced against *S*. Paratyphi A antigen O:2. Once thought to be milder than the *S*. Typhi enteric fever, enteric fever caused by *S*. Partyphi A, is undistinguishable from typhoid fever and *S.* Parayphi A is more resistant than *S.* Typhi and the results in Chapter 4 demonstrated that *S*. Paratyphi A carriers are almost in equal number as of *S*. Typhi.

High levels of IgG were expressed against Vi as well as O:2 antigens by the general population. However, different patterns were observed. Anti-Vi IgG was observed highest at birth (less than 1 year group) which may reflect the transplacental transfer of maternal antibody. The gradual decline during infancy perhaps reflects the loss of maternal antibody. The secondary rise is seen at around age 10 which peaks at age 17 to 18 years. The rise in antibody titre at age around 10 may indicate the beginning of subclinical exposure to *S*. Typhi at early school age. In contrast, anti-O:2 IgG was lowest at birth then increased peaking at the age 11 to 12 years , then gradually declined and again persisted into old age. This difference may be due

to different exposure routes as *S*. Paratyphi A is acquired mainly through contaminated food.

Next I aimed to describe the natural history of acute and convalescent antibody responses to *S*. Typhi and *S*. Paratyphi A infections at different time intervals. Both *S*. Typhi and *S*. Paratyphi A confirmed patients expressed antibodies against both Vi and O:2 antigens. This suggests that these antigens are cross-reactive between bacterial species. This may be due to the antibodies produced in both typhoid and paratyphoid A patients to a common antigen, O:12 which is present in *S*. Typhi and *S*. Paratyphi A LPS [250]. The IgM and IgG expressed by both typhoid and paratyphoid A confirmed groups against O:2 had significantly higher antibody titres on day 8 compared to those on day 1, and month 1. Additionally, IgM response to O:2-antigen also peaked on day 1 of infection. This may be because IgM is expressed early in an infection and patients enrolled in the clinical trials had fever for at least 3 days. The IgM response to successfully treated bacterial infections generally persists for only a few weeks or months and detection of IgM antibodies might be of more diagnostic significance than detecting IgG [251]. Likewise, *S*. Typhi and *S.* Paratyphi A patients had significantly higher anti-Vi IgG antibody titres on day 8 and 3 months post infection compared to those on day 1 and month 1. It is generally believed that IgG is produced only in the late stages of the disease. However, studies have reported the expression of anti-Vi antibody in acute typhoid fever patients by the end of the second week [119, 198]. This suggests the natural fluctuation of IgG and IgM. The secondary peak in both *S.* Typhi and *S*. Paratyphi A patients suggests

secondary exposure and natural antibody boosting within 3 months of the primary symptomatic infection.

IgG can persist for more than 2 years after typhoid infection, therefore, the detection of specific IgG cannot differentiate between acute and convalescent cases. However, more than 85% of plasma samples expressed more than one antibody isotypes detected on culture confirmed typhoid fever patients [36].

High titres of serum IgG against the capsular Vi antigen are found in approximately 90% of chronic biliary *S.* Typhi carriers [37, 199]. However, antibody responses regarding *S*. Paratyphi A chronic carriers are not known. Results in this chapter show that culture confirmed carriers of *S*. Typhi and *S*. Paratyphi A were found to have significantly higher levels of anti-Vi IgG compared with the bile culture negative control group. Interestingly, no such pattern was observed with respect to IgM against either of these antigens, as there was no significant difference in IgM antibody titres against 0:2 or Vi between any of the three groups. This suggests that Vi screening for the detection of chronic carriers in the community could be useful method. However, collecting blood samples for community screening method is an invasive method and may not be feasible. Therefore, I collected saliva samples from chronic carriers using Oracol swabs. Saliva has been shown to be an useful method for the detection of antibodies in acute typhoid case [252, 253]. Although we found higher levels of anti-Vi IgG expressed by *S*. Typhi and *S*. Paratyphi A confirmed chronic carriers in saliva than the control group (bile culture negative), it was not statistically significant. Furthermore, anti O:2 IgG expressed by *S*. Paratyphi A carriers had higher antibody titres when compared with bile culture negative

group and general population. *S*. Typhi carriers demonstrated the highest antibody titres against Vi, when compared to other groups, little difference was observed between general population, the acute typhoid cases and the no growth bile culture group.

SBA was performed on plasma samples of acute culture confirmed enteric fever patients, chronic carriers, bile culture negative group and general population in order to examine if these antibodies expressed were functional. All plasma samples showed bactericidal activity against *S*. Paratyphi A indicating that individuals living in a typhoid endemic region have functional antibody against *S*. Paratyphi A. However, bactericidal activity increased during an acute infection and during chronic carriage. A recent work in Nepal demonstrated that individuals had serum antibodies with bactericidal activity against *S*. Typhi [120]. The exact role of serum antibodies in protection against typhoid fever remains unclear as bactericidal activity is increased during an acute infection.

Cytokines play a major role in both pathogenesis and control of systemic *Salmonella* infections. During invasive *Salmonella* infection, pro-inflammatory cytokines, most notably IL-6, IL-1β, TNF-α, and IFN-γ are activated [50, 51, 173, 254]. IL-18 is important for IFN-γ release and early host resistance to *Salmonella* infections [173]. Individuals genetically deficient in immunity mediated by IL-12 or IFN γ are highly susceptible to *Salmonella* infections [255, 256]. Reports show that there is an association between circulating TNF-α levels and typhoid fever severity [249] and that low *ex vivo* production of TNF-α was associated with a delayed recovery. However, cytokine levels in chronic carriers have not been investigated. We

examined a series of pro-inflammatory cytokines expressed by acute enteric patients and chronic carriers. The results show that higher levels of cytokines were detected in patients with acute disease compared to patients with chronic carriage. The consistent patterns between all pro-inflammatory cytokines indicate that *S*. Typhi and *S*. Paratyphi A stimulate a strong systemic pro-inflammatory response during an acute infection but not during a chronic infection. Furthermore, the systemic cytokine levels were lower in *Salmonella* carriers than bile culture negative group, suggesting that either the culture negative patients either had a weak inflammatory response or those with *Salmonella* in the gallbladder have a below baseline inflammatory response. I additionally compared the ratio of INF-γ to IL-10 and TNF-α to IL-10 between the three groups as a lower ratio signifies repression of the pro-inflammatory response. The ratio INF-γ to IL-10 was significantly lower in the chronic carriers when compared to the acute typhoid patients and the bile culture negative controls. These data suggest that the anti inflammatory effect of IL-10 may suppresses the development of the disease in chronic carriers.

5.5 Conclusion

The results show that the individuals carrying *S.* Typhi and *S*. Paratyphi A in their gallbladder have significantly elevated levels of IgG to Vi and O:2 antigens. This suggests that these organisms are stimulating a constant immunological response in the host. Serum bactericidal assay performed on the plasma samples on such individuals showed high bactericidal activity suggesting antibodies expressed are

functional. Low pro-inflammatory cytokine levels in their plasma samples may be an indication why these individuals do not develop the active infection. In combination with cytokine profiles, antibody levels may be exploited for the method of detection of chronic carriers in the general population.

6 Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

6.1 Abstract

The mechanism of *S.* Typhi carriage is poorly understood and remains controversial. Yet, all the proposed hypothesises of gallbladder carriage fall under two major mechanisms. Either the bacteria form biofilms on gallstones and do not invade the local tissue *or* the organism invade the epithelial layer of the gallbladder and survive and replicate intracellularly. In chapter four, I found that the majority of individuals with *Salmonella* in their gallbladder had gallstones; therefore, I hypothesize that this is most likely mechanism and that gallstones and non-invading *Salmonella* organisms were causing the acute inflammation. In this chapter, I investigated the microscopic and microbiological characteristics of invasive *Salmonella* in the gallbladder. Using immuno-fluorescence microscopy, electron microscopy and genome sequencing I show that *S*. Typhi may be controlling the inflammatory process through the expression of the Vi capsule in the gallbladder and that the organisms may be a different population of organisms to those causing acute disease in the same geographical area. These data question the current dogmas surrounding the carriage of *S*. Typhi in gallbladder and predict a pivotal role of Vi capsule and gallstones in maintaining carriage.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

6.2 Introduction

The results in chapter four demonstrated that both *S.* Typhi and *S.* Paratyphi A have the ability to colonise the gallbladder, therefore, one can assume that this ability is not unique to *S*. Typhi, as was once thought. Furthermore, serological testing for IgG against the O:2-antigen (*S*. Paratyphi A) and the Vi-antigen (*S*. Typhi) demonstrated that carriers or those with invasive *Salmonella* inside their gallbladder have exceptionally high antibody titres, which is cross-reactive and may be cross protective against multiple *Salmonella* serotypes (Chapter 5). High antibody titres could be used to detect carriers prospectively [37, 199, 200] (potentially even using saliva) and suggest that such individuals have continual boosting of their immune response during prolonged colonisation. However, the mechanism of this constant immune boosting and the nature of the exposure to the organisms to the immune system are unknown and is a dichotomy. Firstly, it could be predicted that the organisms are in a privileged environment and sheltered from the immune system within the gallbladder. This remains a solid dogma, as if carriage is essential for maintenance and transmission of the organisms then over stimulation of the host immune system is not in the long-term interests of the colonising organisms. However, high antibody titres to these pathogens may suggest that the organism is frequently recognised by the immune system and the individuals are immune to systemic infection (bacteraemia) but obviously not colonisation of the gallbladder.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

The basic histopathological and haematological characterisations described in chapter four suggested that those with invasive *Salmonella* in their gallbladder had signs of acute but not chronic inflammation. Again this is contradictory, as carriage is believed to be a chronic condition, yet acute inflammation is not compatible with long term colonisation. However, it is unclear if the resident *Salmonella* or some secondary damage, such as gallstones, additional infecting bacteria, malignant cells or some other form or biological insult, is stimulating acute inflammation. This question is almost impossible to answer and limited by access to a population with *Salmonella* in their gallbladder, as individuals only present when there is some form of right upper quadrant or abdominal pain requiring clinical investigation. From previous laboratory studies and clinical observations two major hypothesises for the mechanism of prolonged colonisation of the gallbladder by invasive *Salmonella* have been proposed (Figure 6.1). Either the bacteria form biofilms on gallstones and do not invade the local tissue [181, 225, 242] or the organism invade the epithelial layer of the gallbladder and survive and replicate intracellularly [225, 246]. In chapter four, I found that the majority of individuals with *Salmonella* in their gallbladder had gallstones; therefore, I hypothesize that this is most likely mechanism and that gallstones and not invading *Salmonella* organisms were causing the acute inflammation. This would be the most compatible with long-term survival in the gallbladder. If this were the case, one would predict that the organisms within the gallbladder were extracellular and as *S*. Typhi plays an immunomodulatory role in the gallbladder by somehow suppressing the immune response within the gallbladder and the immune boosting occurs at the mucosal surface of

gastrointestinal tract when the organisms enter and travel through the gastrointestinal tract.

To add insight into the mechanism of carriage and to address this carriage hypothesis, I performed a series of experiments and collaborated with researchers based at the Wellcome Trust Sanger Institute in Cambridge, UK, and the Ohio State University in Columbus, USA, to study tissue and the organisms taken from individuals that had undergone cholecystectomy in Kathmandu, Nepal (Chapter 4). In this chapter I outline the microscopic characteristics of invasive *Salmonella* in the gallbladder, demonstrate that *S*. Typhi may be controlling the inflammatory process through the expression of the Vi capsule and may be a different population of organisms to those causing acute disease in the same geographical area.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

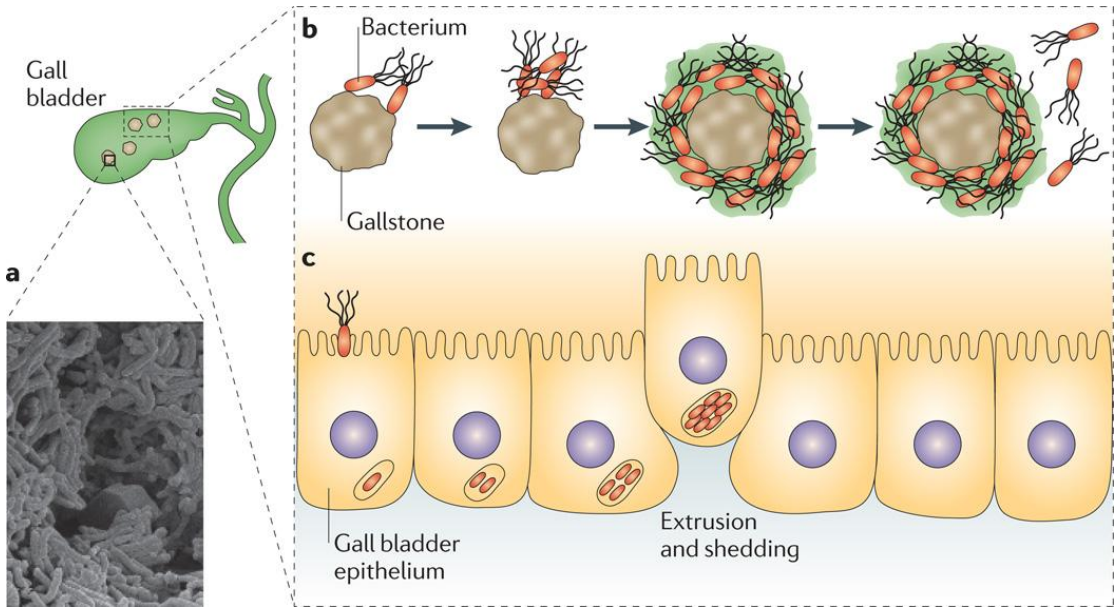


Figure 6.1: Model of *S.* Typhi biofilm formation on cholesterol gallstones [181]

a) Electron micrograph of *S.* Typhi in a biofilm on the surface of a human gallstone. **b)** *S.* Typhi probably gains access to the gall bladder during the acute phase of infection and initially attaches to gallstone surfaces through a specific interaction between flagellin and cholesterol. On cholesterol, biofilm formation is dependent on the presence of exopolysaccharide (green), probably including the O antigen capsule. Detachment of bacteria from the biofilm would allow entry into the intestine via bile, followed by shedding in the faeces and urine. **c)** A possible alternative strategy by which *S.* Typhi persists in the gall bladder is through invasion of gall bladder epithelial cells. In this model, invasive bacteria replicate intracellularly, and shedding could occur as a part of epithelial regeneration, wherein gall bladder epithelial cells containing *S.* Typhi would be extruded to the lumen, and released bacteria could infect new cells or be shed into the intestine via bile.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

6.3 Results

6.3.1 Haematoxylin and Eosin staining of gallbladder tissue

I performed Haematoxylin and Eosin (H&E) staining on sections of gallbladder cut from tissue samples that were culture positive and culture negative for invasive *Salmonella*, as described in Chapter four. Examples of the resulting H&E stained sections are shown in Figure 6.2. H&E staining permits the definition of inflammatory cells within the tissue by defining the nuclear detail (Haematoxylin) (blue/purple), and counterstaining of the tissue (Eosin) (red). A "healthy" section (without acute or chronic inflammation) of gallbladder (Figure 6.2a) shows the various layers of the organ, intact gallbladder tissue architecture, the luminal surface and only limited cellular infiltrate in the tissue. The tissue can be subdivided into four distinct areas: the lumen, the mucosa, the smooth muscle layer and the subserosal connective tissue (Figure 6.2a). The current hypotheses for maintenance of *Salmonella* in the gallbladder are for the presence of *Salmonella* on the luminal surface (and gallstones) and/or within the mucosal layer after invasion (Figure 6.1)

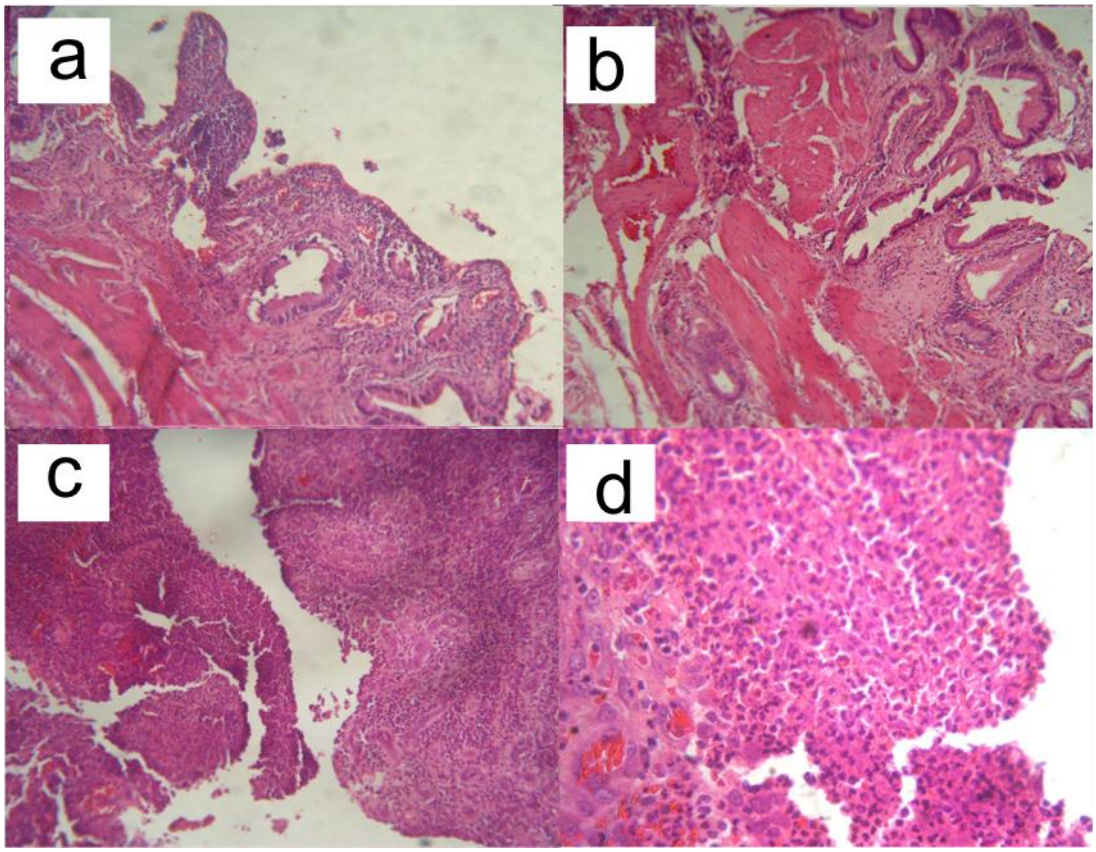


Figure 6.2: Haematoxylin and Eosin staining of gallbladder tissue

a) H&E staining of healthy gallbladder tissue at a magnification of 100x. The non-stained area is the lumen, the next area in with cellular definition is the mucosa and the smooth muscle layer is beneath the mucosa. **b)** H&E staining of a typical gallbladder section from a patient with chronic cholecystitis at a magnification of 100x. **c)** H&E staining of a typical gallbladder section from a patient with acute cholecystitis that was *Salmonella* culture positive at a magnification of 100x. **d)** H&E staining of the same gallbladder section seen in c but at a magnification of 400x.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

As described in Chapter four, histopathological signs of chronic inflammation within the tissue were not commonly observed in sections taken from culture positive *Salmonella* gallbladder samples. An example of chronic inflammation, taken from a culture negative bile sample, within the gallbladder tissue is shown in Figure 6.2 b. This tissue sections shows a significant loss of tissue architecture in comparison to the "normal" tissue. Signs of chronic inflammation can be clearly observed by heavy staining and elongation of the crypts at the luminal surface, and heavy lymphocyte infiltration near the luminal surface and even within the deeper tissue layers. The H&E staining suggests that prolonged presence of *Salmonella* in the gallbladder does not induce aggressive lymphocyte infiltrate or elongation of the crypts within at the luminal surface Figure 6.2c and 6.2d.

Confirming the histopathology report described in Chapter four, the majority of gallbladder sections associated with invasive *Salmonella* demonstrated a staining pattern indicative of acute inflammation. This acute inflammation can be clearly observed in Figure 6.2c and at a higher magnification in Figure 6.2d, these sections show that the tissue architecture has greater integrity in comparison to the section with chronic inflammation and there is no obvious cellular dysregulation in the deeper tissue. However, there is a large density of neutrophils (blue/purple stained cells in Figure 6.2c and 6.2d) and there appears to be a lack of obvious crypts and some dysregulation of the luminal surface. As all tissue samples were taken from individuals with some form of gallbladder abnormality it is difficult to assess the overall cause of the inflammation and the tissue dysregulation at the mucosal surface. However, the majority of individuals had one or more gallstones and these

may cause mechanical damage to the surface and induce an acute inflammatory response.

6.3.2 Immuno-fluorescence of *S.* Typhi within the gallbladder

To understand the mechanism of *S.* Typhi colonisation within the gallbladder, sections from *S*. Typhi positive (and negative as a control) were cut and subjected to immuno-fluorescence using two stains, Dapi and Phalloidin. Dapi is a nuclear stain that fluoresces blue when bound to double-stranded DNA and permits the definition of tissue and inflammatory cell infiltrate. Phalloidin binds to F-actin and, therefore, can be used to identify tissue structure and locations of actin reassortment (green). Additionally, the sections were incubated with an anti O:9 O-antigen antibody (and Cy5 as a red secondary antibody) to detect the outer membrane of *S*. Typhi. Examples of the immuno-fluorescence staining are shown in Figure 6.3 and 6.4.

The immuno-fluorescence staining was performed on several *S*. Typhi positive and *S.* Typhi negative sections and all the positive/ negative staining results were consistent. Firstly, the Dapi stain confirmed that there was significant cellular infiltrate near the luminal surface and also within the deeper mucosal layers of the tissue, in the form of neutrophils and other polymorphonuclear inflammatory cells, as shown by the H&E staining (Figure 6.3a and 6.4a). Secondly, it could be observed that there was extensive F-actin staining in the tissue near the luminal surface, indicative of actin rearrangement (Figure 6.3b).

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

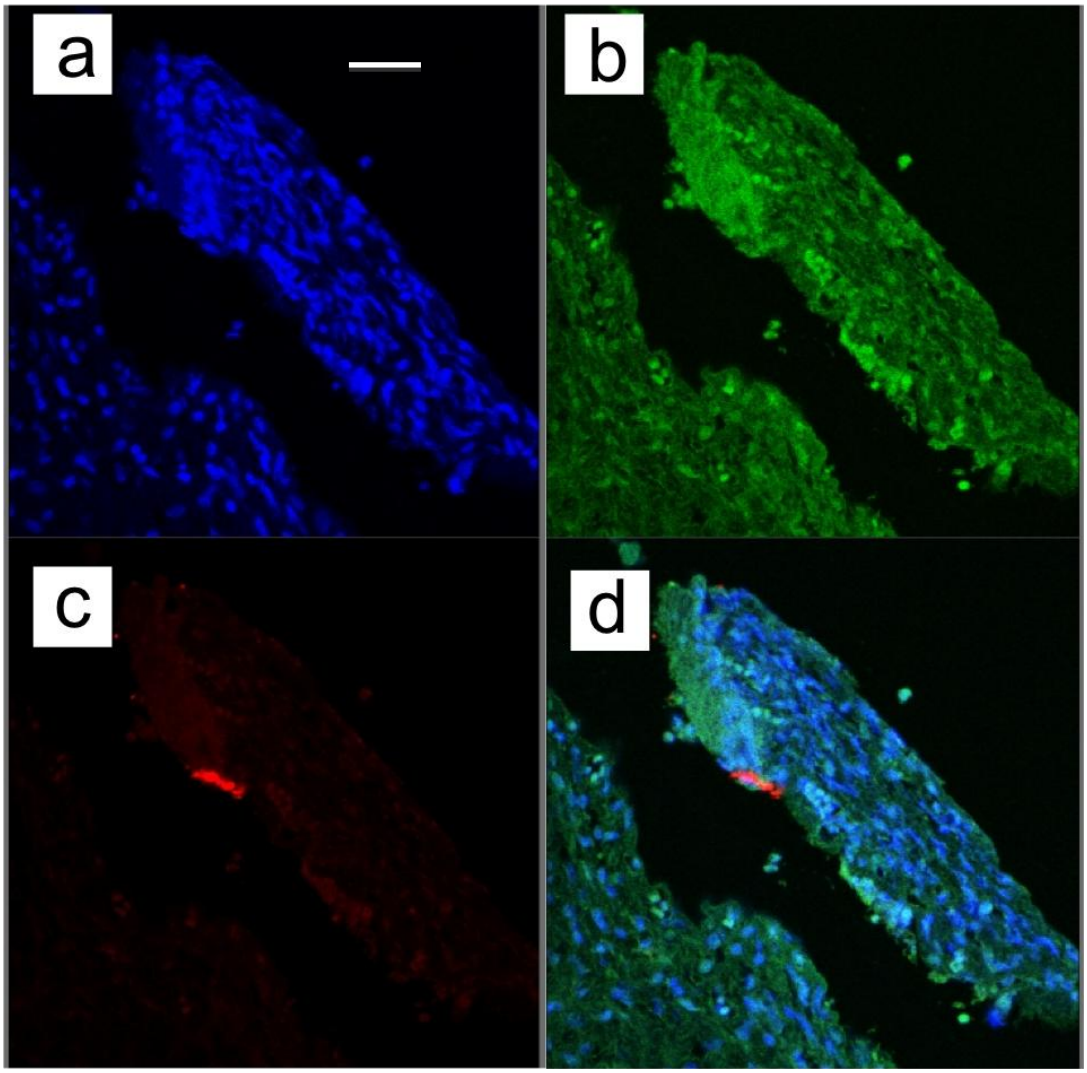


Figure 6.3: Immunofluorescence of *S*. Typhi in the gallbladder

a) Dapi (blue) staining of a section of *S.* Typhi culture positive gallbladder showing the location of the cellular nuclei. **b)** Phalloidin staining of a section of *S.* Typhi culture positive gallbladder showing the action structure within the tissue. **c)** O:9 O-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section. **d)** Three colour overlay of all stains. The white line in panel a) depicts 100μm and is consistent for all images.

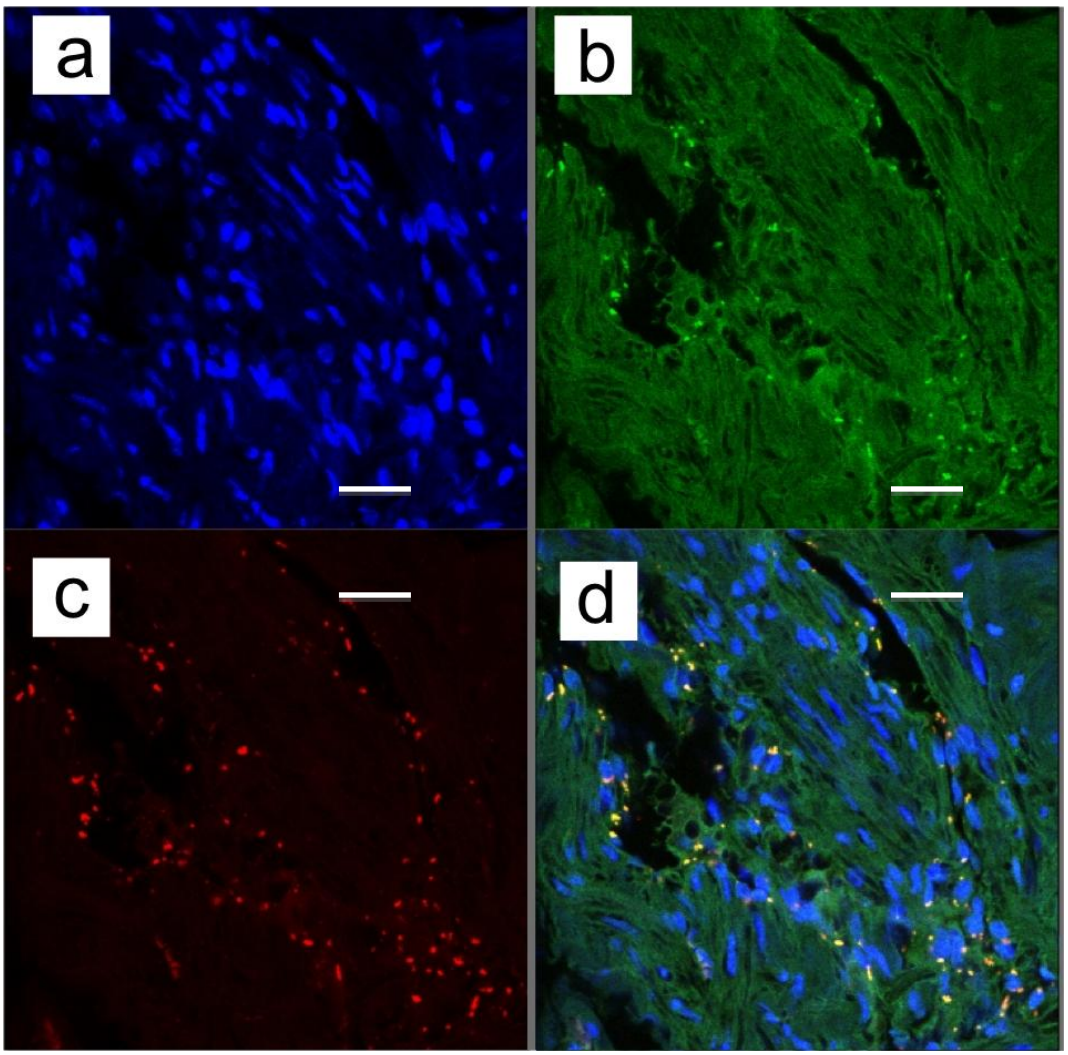


Figure 6.4: Immunofluorescence of *S.* Typhi inside the epithelial layer of the gallbladder

a) Dapi (blue) staining of a section of *S.* Typhi culture positive gallbladder showing the location of the cellular nuclei**. b)** Phalloidin staining of a section of *S.* Typhi culture positive gallbladder showing the action structure within the tissue. **c)** O:9 O-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section. **d)** Three colour overlay of all stains. The white line in panel a) depicts 50μm, and 100μm in b, c and d.

Thirdly, it could be observed that the organisms were present in two locations, both near the mucosal surface and within the mucosal tissue (Figure 6.3c and 6.4c, respectively). It appears that the organisms near the external surface are in a cluster and associated with a region of actin reassortment and neutrophil infiltrate (Figure 6.3b). Whereas, the organisms within the tissue are mainly singular and more sporadically distributed. Furthermore, the overlaid image of all three stains shows that the organisms in proximity to mucosal surface appear to be extracellular (stained red) and on the outer surface of the tissue (Figure 6.3d). However, the organisms within the deeper part of the mucosa are yellow in the overlaid image, suggesting that the organisms are potentially intracellular. These data suggest that both hypotheses for the maintenance of *S*. Typhi within the gallbladder may be important.

Next, I performed additional staining with the same sections and the same primary stains, but I replaced anti-O:9 antibody with a Vi antibody. Vi is a capsular polysaccaride antigen, a linear polymer of α-1,4 2-deoxy-2-*N*-acetylgalacturonic acid that is variably O acetylated at the C-3 position [12]. The Vi capsular antigen is a significant virulence factor for typhoid fever, as strains positive for Vi production have higher rates of infection [21]. The Vi capsule is regulated by two-component-positive regulatory systems *rcsB*, *rcsC* and *ompR-envZ,* in addition to the promoter of the *viaB* region, and modulated by osmolarity [17,20, 257]. Therefore, the Vi capsule is highly susceptible to changes in environment and may be important for modulating the immune response during carriage. Immuno-fluorescence staining suggested that the organisms within the gallbladder are Vi positive, both on the

mucosal surface and within the deeper tissue (Figure 6.5 and 6.6). However, this staining was most apparent on the organisms on the extracellular surface than those found within the mucosal layer. The same secondary antibody (Cy5, red) was used for O:9 and Vi (with different sections) and there is clear co-localisation of the two antigens. Furthermore, the organisms within the mucosal layer can again be observed in yellow, suggesting they are intracellular and expressing the Vi capsule.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

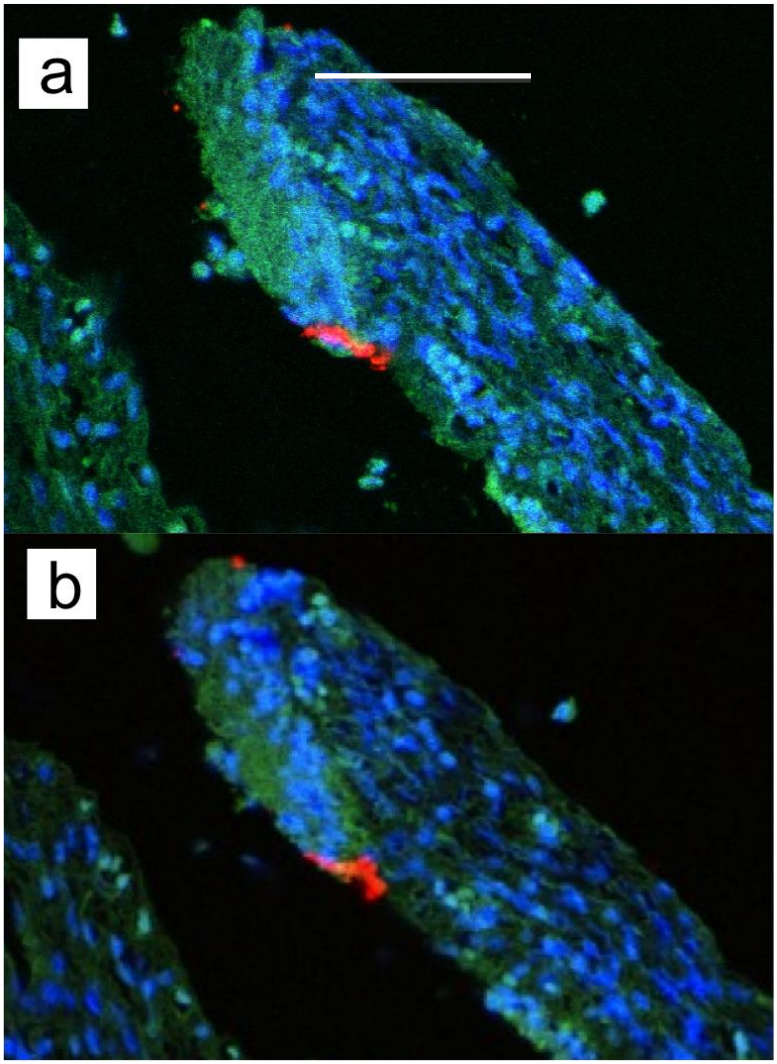


Figure 6.5: Immunofluorescence of Vi positive *S*. Typhi on the surface of the epithelial layer of the gallbladder

a) O:9 O-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section in a three colour overlay as before. **b)** Vi-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section in a three-colour overlay as before. The white line in panel a) depicts 100μm and is consistent for all images.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

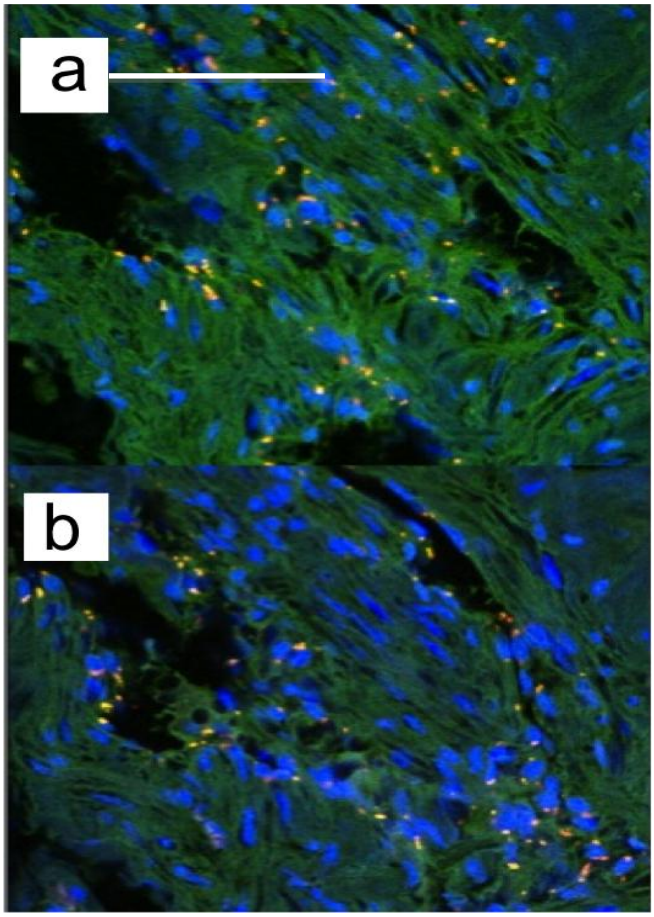


Figure 6.6: Immunofluorescence of Vi positive *S*. Typhi within the mucosal layer of the gallbladder

a) O:9 O-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section in a three colour overlay as before. **b)** Vi-antigen staining, with a secondary Cy5 antibody (red), highlighting the location of *S.* Typhi within the tissue section in a three-colour overlay as before. NB the organisms appear yellow as they are below the green layer and potentially intracellular. The white line in panel a) depicts 100μm and is consistent for all images.

6.3.3 Electron microscopy of *S.* Typhi within the gallbladder and *S.* Typhi on gallstones

In collaboration with Mr David Goulding at the Wellcome Trust Sanger Institute in Cambridge in the UK, we performed transmission electron microscopy (TEM) on gallbladder samples containing *Salmonella* Typhi. TEM was performed to obtain a high-resolution impression of the organisms with the gallbladder and to confirm, or otherwise, the intracellular/extracellular nature of the organisms observed within the mucosal tissue layers. Examples of the resulting TEM images are shown in Figure 6.7.

TEM was performed on the same sections used for immuno-fluorescence and focussed on the areas of the tissue samples that were positive for *S*. Typhi. TEM revealed two main findings. Firstly, we were unable to confirm the presence of *S*. Typhi on the mucosal surface. An area of tissue corresponding with the area that was positive for *S*. Typhi by immune-fluorescence in Figure 6.6 can be seen under TEM in Figure 6.7a. The area shows a region of fatty deposits and no areas that contained *S.* Typhi were observed. We suggest that the area of tissue positive for *S.* Typhi in Figure 6.6 may be antibody bound to fatty deposits and demonstrating non-specific hybridisation. However, we were able to confirm the presence of extracellular organisms on the mucosal surface. Organisms can be seen in loose contact with the surface of the tissue in Figure 6.7b and 6.7c. Notably, the organisms are associated with areas of the mucosal damage and appear be sloughing off the

surface (Figure 6.7d). The Vi capsule can also be observed on the outer surface of the organism.

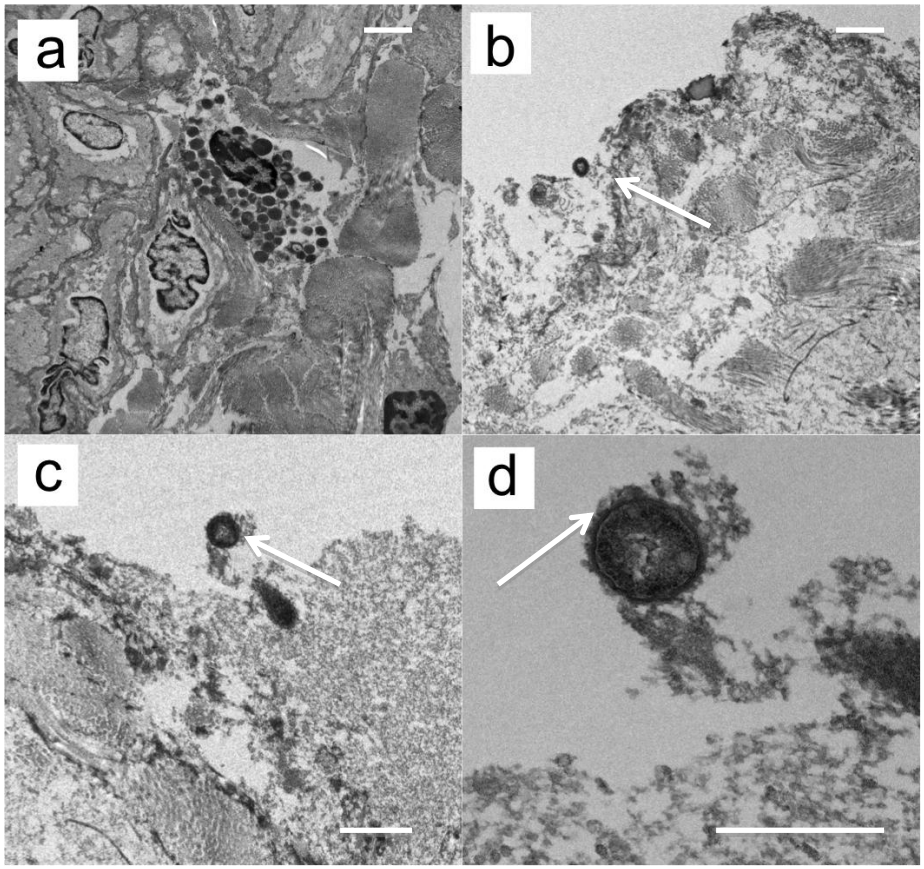


Figure 6.7: Transmission electron microscopy images of *S*. Typhi inside the gallbladder

a) Mucosal section of *S.* Typhi positive gallbladder negatively staining and viewed with TEM. The cells in the centre are thought to be fat droplets, the white line corresponds with a size of 1µm. **b)** Luminal epithelial section of *S.* Typhi positive gallbladder negatively staining and viewed with TEM. *S.* Typhi is clearly visible on the surface (arrow), the white line corresponds with a size of 1µm. **c)** Luminal epithelial section of *S.* Typhi positive gallbladder negatively staining and viewed with TEM. *S.* Typhi is clearly visible on the surface (arrow), the white line corresponds with a size of 1µm. **d)** Magnified image of c) showing *S.* Typhi in loose contact with the epithelial layer and the presence of the Vi capsule (arrow), the white line corresponds with a size of 1µm.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

Next, in collaboration with Professor John Gunn at the University of Ohio, we aimed to investigate the nature of gallstones taken from individuals with and without *S.* Typhi in their gallbladder. Gallstones are crystalline concretions formed within the gallbladder by accretion of bile components, typically these stones are made of cholesterol. Cholesterol stones vary from light yellow to dark green or brown and are oval, between 2 and 3 cm long and consist of approximately 80% cholesterol. It is hypothesised that biofilm formation on gallstones is a pivotal step in *Salmonella* carriage and that exopolysaccharide, probably involving O:antigen [242] and, potentially, Vi is a major biofilm component. Gallstones from individuals without *S*. Typhi in their gallbladder and from individuals with *S.* Typhi in their gallbladder was subject to scanning electron microscopy (SEM) to investigate biofilm formation. SEM produces a magnified image by using electrons instead of light. A beam of electrons is produced at the top of the microscope; the electron beam follows a vertical path through the microscope, which is held within a vacuum. Once the beam hits the sample, electrons and X-rays are ejected from the sample. This produces the final image. The resulting three-dimensional images of the gallstones are shown in Figure 6.8.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

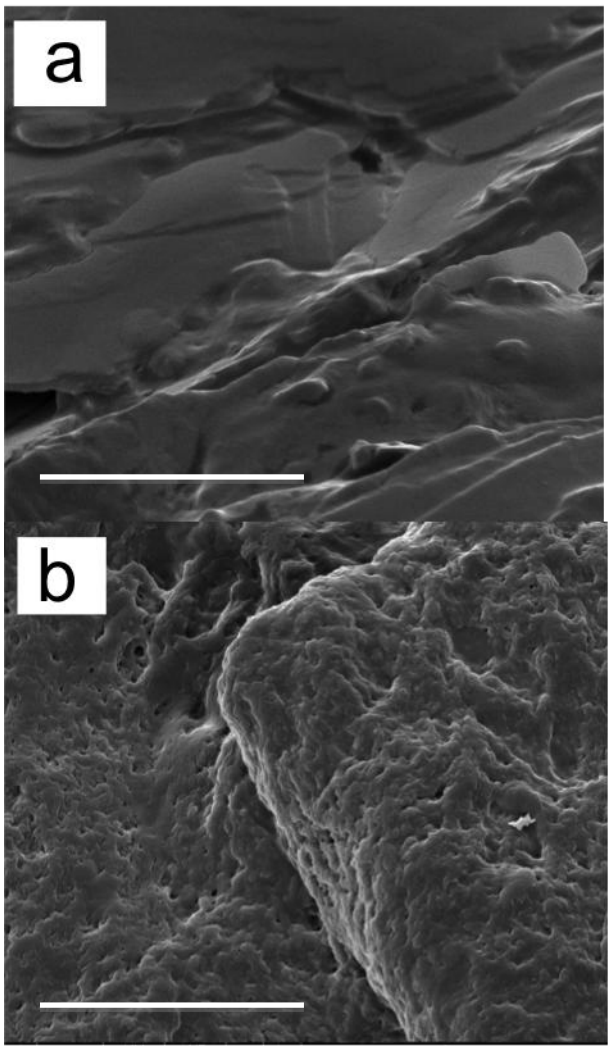


Figure 6.8: Scanning electron microscopy image of the surface of gallstone

a) SEM image of the surface of "normal" cholesterol gallstone at a magnification of 108,000x, line measures 10μm. The gallstone is smooth containing ripple of cholesterol layers. **b)** SEM image of the surface of cholesterol gallstone taken from an individual with *S.* Typhi in the gallbladder at a magnification of 10,000x, line measures 10μm. The gallstone is pitted and covered in a mucoid layer, presumably caused by bacterial biofilm formation.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

The SEM image of gallstone taken from an individual without *S.* Typhi in the gallbladder shows a smooth, non-pitted surface with exposed cholesterol. The SEM image of gallstone taken from an individual with *S.* Typhi in the gallbladder shows a different morphology. The gallstone is pitted and shows complete coverage by a smooth mucoid layer (Figure 6.8b). These images and the other microscopy images suggest that the organisms are extracellular and in association with the mucosal surface and form a biofilm on the surface of gallstones.

6.3.4 *S.* Typhi from carriers are genetically distinct from strains causing acute disease in the same population

There are multiple outstanding questions regarding carriage, microscopy was performed to gain a better understanding of the mechanism of carriage within the gallbladder. Carriage of invasive *Salmonella* remains an anomaly and whilst we can show the organism appears to be stable within the privileged compartment of the gallbladder we are unsure of the role of the organism in transmission and maintenance of the disease in a highly endemic setting. Our previous work has suggested that the majority of enteric fever in Kathmandu is attributable to contaminated food and water, and genotyping of paired isolates in the same household suggests that acute or chronic shedding from associated household members is not a major factor in disease transmission[78].

I hypothesised that organisms in the gallbladder are phenotypically and genotypically distinct from those causing acute disease in the same population. To

test this hypothesis I compared the antimicrobial susceptibilities from *S*. Typhi and *S.* Paratyphi A isolated from gallbladders (see Chapter 4) to the antimicrobial susceptibilities from *S*. Typhi and *S*. Paratyphi A isolated from acute infections over the same time period. Antimicrobial susceptibilities against azithromycin, nalidixic acid, ofloxacin and ciprofloxacin were compared by MIC between *S.* Typhi and *S*. Paratyphi A isolated from the bile, presumed to have arisen from carriage, to all *S.* Typhi and *S*. Paratyphi A isolated from the blood of patients with acute enteric fever over a similar time period presenting at the same location (Chapter 3 and 4). The MIC results were interpreted using CLSI guidelines 2012 [214]. Fifty-seven percent (25/44) of the *Salmonella* isolates from bile exhibited resistant to nalidixic acid, whereas 76 % (387/508) of *Salmonella* from blood over the same time period were resistant to nalidixic acid. Sixty-one percent (28/46) of the *Salmonella* from bile were resistant to both ciprofloxacin and ofloxacin, whilst a higher proportion of isolates from blood were resistant to both ciprofloxacin (74 %, 259/349) and ofloxacin (73 %, 254/349).

The MIC distributions to azithromycin and ofloxacin were stratified by serovar (*S*. Typhi and *S*. Paratyphi A) and by isolation site (bile or blood) and compared using the Mann-Whitney U test (Figure 6.9). The isolates from blood had a higher median MIC to azithromycin (8 μg/ml) than isolates from bile (6 μg/ml) (p = 0.039). Furthermore, the *S*. Paratyphi A and *S*. Typhi blood isolates had higher median MICs to azithromycin than the corresponding serovar from bile (median *S*. Paratyphi A MIC to azithromycin from blood = 12μg/ml; median *S*. Paratyphi A MIC to azithromycin from bile = 8μg/ml (p = 0.015), median *S*. Typhi MIC to azithromycin

from blood = 6μg/ml; median *S*. Typhi MIC to azithromycin from bile = 5μg/ml (p = 0.006)). The MIC distribution to azithromycin was mirrored by ofloxacin, as the median MICs to ofloxacin were higher in the bile isolates than the blood isolates, yet this distinction was only significant within the *S*. Typhi isolates (median ofloxacin MIC *S*. Typhi from blood = 0.38μg/ml; median ofloxacin MIC *S*. Typhi from bile = 0.064μg/ml (p = 0.011).

In an attempt to understand the role that carriage strains of *S.* Typhi may play on transmission in Kathmandu and to place these isolates into a broader context 14 *S*. Typhi isolates were whole genome sequenced at the Wellcome Trust Sanger Institute in the UK. The genome sequences of carriage isolates were then compared to a random cross-section of *S*. Typhi strains isolates from individuals over the same time period with acute typhoid fever. A phylogenetic tree resulting from the chromosomal single nucleotide polymorphisms in the chromosomal sequences is shown in Figure 6.10.

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

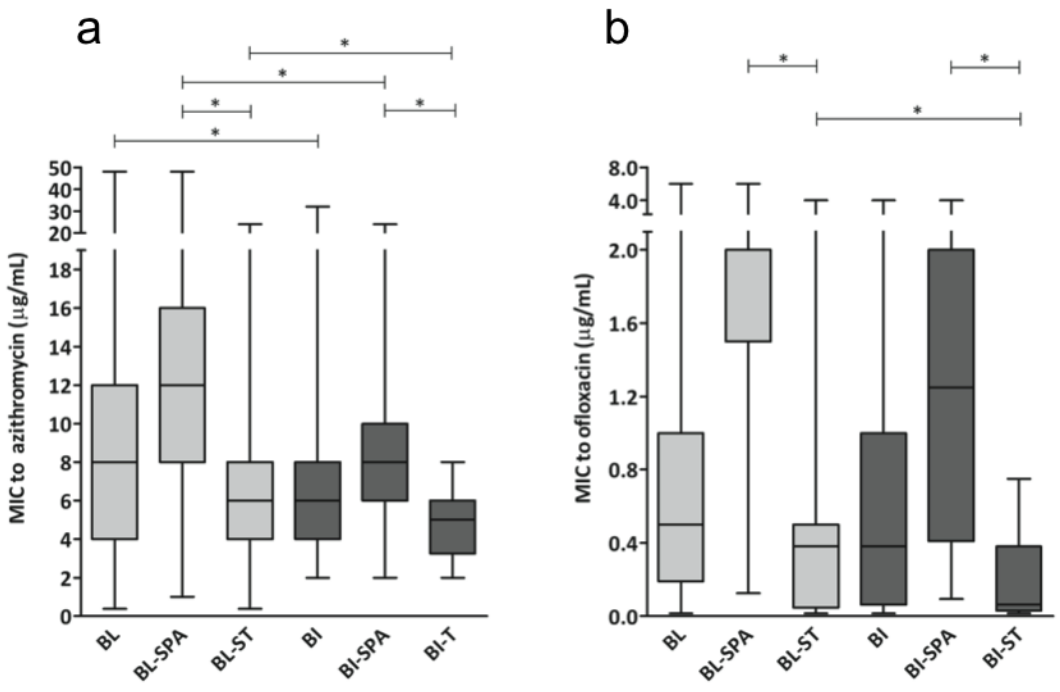


Figure 6.9: Antimicrobial susceptibility patterns of *S*. Typhi and *S*. Paratyphi A from acute infections and gallbladders in Kathmandu, Nepal

Boxplots showing the median and interquartile ranges of the MICs to **a)** azithromycin and **b)** ofloxacin from *S*. Typhi and *S*. Paratyphi A isolates from blood and bile. Blood isolates are shown in light gray and bile isolates are shown in dark gray, boxes as coded as follows; all *Salmonella* isolates from blood; BL, *S*. Paratyphi A from blood; BL-SPA, *S*. Typhi from blood; BL-ST, all *Salmonella* isolates from bile; BI, *S*. Paratyphi A from bile; BI-SPA and *S*. Typhi from bile; BI-ST. Asterisk indicates a significant difference in median MIC value between indicated groups (Mann-Whitney U test).

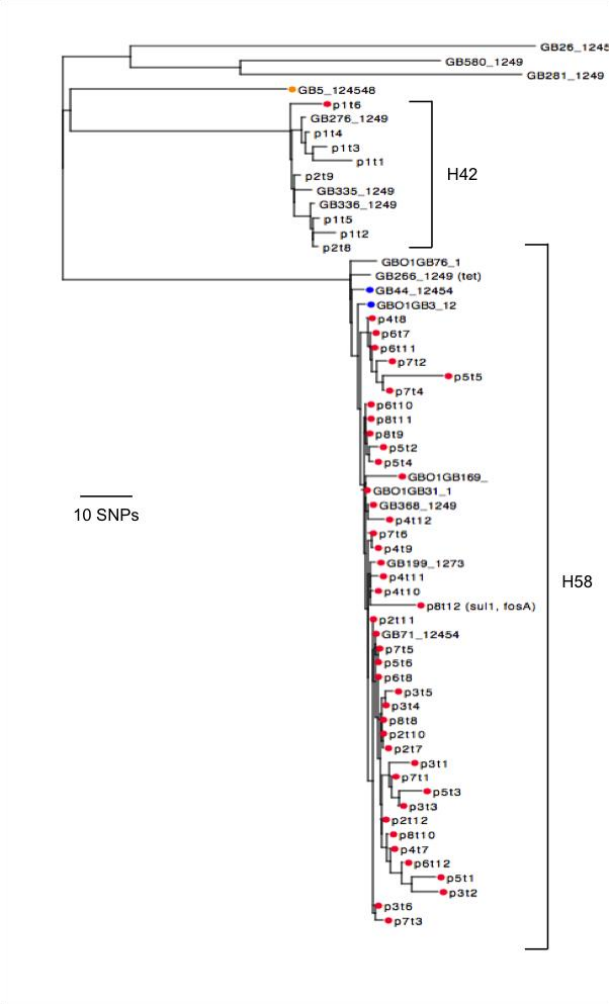


Figure 6.10: Phylogenetic tree of *S.* Typhi acute and carriage isolates from Kathmandu

Phylogenetic tree constructed from concatenated SNPs indentified (approximately 1,500 in total) in the core genome (accessory removed) sequences of *S.* Typhi isolated from acute typhoid patients and gallbladders (coded GB) in Kathmandu, Nepal. This tree is a result of maximum likelihood phylogeny estimated in RAxML. The scale of 10 snps is shown at the left of the tree and the H42 andH58 subgroups are highlighted. Mutations in the *gyrA* are coloured; serine-83-tyrosine (blue), serine-83-phenylalanine (red) and Asparagine-87-Tyrosine (orange). Additional resistance genes are labeled (sul1+fosA, and tet).

6. Interactions between *Salmonella* Typhi and *Salmonella* Paratyphi A and the gallbladder

The phylogenetic tree separates the sequenced strains into two major groups. The largest is at the base of the tree and consists of strains belonging to the H58 group. The smallest lies towards the top of the tree and contains strains belonging to the H42 group. Both H58 and H42 are known to predominate in Kathmandu [78, 82]and have been shown to freely circulate within the city (67 H42 acute cases and 259 H58 acute cases [78]). There are also three additional H14 strains at the top of three, which is less common in acute cases of typhoid fever in Kathmandu caused only five infections from our work from 2005-2009 [78].

The tree can tell us several things about the carriage isolates, firstly, and with the exception of the isolates at the top of the tree, the gallbladder strains fall within the same phylogenetic structure of those causing acute disease in the same population (gallbladder isolates are labelled GB in Figure 6.10). Secondly, H58 is the predominant strain in Kathmandu and we can clearly observe H58 carriage isolates, hence these may be important for conserving this reservoir. Thirdly, we were unable to detect any isolates from carriage that had an identical SNP structure to those causing acute disease in the same population. Lastly, there were several carriage strains that demonstrated an atypical genotype compared to strains from acute infections. Unsurprisingly, there were no additional genomic regions or loci associated with carriage that were shared between the isolates. However, when information regarding antimicrobial resistance loci or drug mutations was overlaid on tree (coloured and labelled accordingly in Figure 6.10) there were several anomalies. The typical mutation in the DNA gyrase gene *gyrA* inducing reduced susceptibility to flouroquinolones is Serine-83- Phenylalanine (labelled red in Figure

6.10) and several gallbladder strains exhibited this mutation. However, one strain contained an Asparagine-87-Tyrosine and two contained a Serine-83-Tyrosine. Both these mutations are rare in the *S*. Typhi population and neither mutations were identified in the isolates causing acute infections on Kathmandu. Furthermore, we identified two H58 gallbladder isolates without a *gyrA* mutation, one of which contained an additional tetracycline resistance gene that was not detected in any additional isolates. Finally, there is no robust molecular clock for *S*. Typhi; therefore, it is difficult to estimates rate and dates of existence and evolution. Notwithstanding this limitation on evolutionary rates, it is apparent from the tree that the many of the gallbladder isolates lay towards the base of the tree, suggesting that they have been in existence for some time.

6.4 Discussion

In this chapter, the microscopic characteristics of invasive *Salmonella* in the gallbladder were investigated to understand the mechanism of carriage within the gallbladder. Phenotypes and genotypes of *Salmonella* strains isolated from chronic carriers were compared with *Salmonella* causing acute typhoid infection to investigate if these strains were similar.

Long term *Salmonella* carriage is a chronic condition and carriers are known to be asymptomatic. Therefore, acute inflammation is not compatible with long-term colonisation. However, histopathological examination (H and E staining) of *Salmonella* infected gallbladder tissues showed that typhoid carriers are more likely

to have acute inflammation with polymorphonuclear infiltration. This was further confirmed by immuno-fluorescence staining which showed neutrophil infiltrate near the luminal surface. Previously in Chapter 4, haematological characteristics of chronic carriers showed higher neutrophil count, and increased prevalence of gallbladder distension, right upper quadrant pain, empyema, all of these indicated acute inflammatory response. In contrast, chronic inflammation of gallbladder tissues were seen in majority of patients (non *Salmonella* positive group) who underwent cholecystectomy (79%, n=1024/1301) with heavy lymphocytic infiltration near the luminal surface which extended up to the deeper tissue layers. These data support our hypothesis that the gallstone formation and not invading *Salmonella* organisms were causing the acute inflammation. This would be the most compatible with long-term survival in the gallbladder.

Chronic *Salmonella* carriers are of special concern from a public -health point of view as they are reservoirs for the spread of disease. *Salmonella* are known to utilize multiple strategies to evade and modulate host innate and adaptive immune responses in order to persist in the presence of a robust immune response [178, 258]. Chronic carriers are known to have high levels of antibodies to the Vi antigen [184, 196, 200]. Carriers may receive continuous natural boosting by organisms being reseeded, potentially in high numbers back into the intestinal tract [49]. Results from chapter 5 revealed that chronic carriers had high levels of antibody against Vi and had high bactericidal activity but low levels of pro inflammatory cytokine levels. These data suggest that *S*. Typhi is constantly stimulating immunological response, in the form of antibody. Low levels of cytokines predict

that the organisms in the gallbladder are regulating and dampening the inflammatory response. Thus, the detection of antibody against Vi could be used for the identification of chronic carriers in the community, however, this should be interpreted with caution as population in an endemic areas may have high levels of anti Vi antibody titers despite a low prevalence of chronic carriers [194].

Gallbladder alterations as a result of typhoid fever are poorly characterized and largely unknown. *S.* Typhi upon reaching the gallbladder can establish an acute, active infection accompanied by inflammation or persist in the organ long after symptoms subside, suggesting unique mechanisms used by the organism to mediate colonization in a bile rich environment [245, 259]. A study using *S*. Typhimurium in a mouse model demonstrated that intracellular replication of *Salmonella* occurs in gallbladder epithelial cells during an acute infection [246]. However, it is not understood how *Salmonella* survive within a gallbladder in chronic carriers. In order to study the characteristics of *Salmonella* within the gallbladder; tissues collected from chronic carriers were examined through Transmission Electron Microscopy (TEM). TEM revealed that *Salmonella* were primarily extracellular in the gallbladder and were associated with area of the mucosal damage, which appeared to be sloughing off the surface. However, we were unable to confirm the presence of *S*. Typhi on the mucosal surface. Immunofluorescence microscopy and TEM confirmed the expression of Vi on the outer surface of the organism. This may suggest that Vi expression is vital for the survival of *Salmonella* in the gallbladder. Persistent colonization of the gallbladder depends upon additional factors like bile resistance. Bile is a lipid rich, biological detergent which emulsifies and solubilizes fats, has

antimicrobial properties [243]. Bile has been shown to promote the formation of biofilm on human gallstones *in vitro* in a bile dependent manner [242]. A biofilm is defined as a population of one or more organisms attached to each other and a surface by means of a bacterium initiated matrix and has been associated with medical as well as industrial settings and have been implicated as the cause of many antibiotic resistant, persistent infections in humans [190, 260]. The majority of *Salmonella* positive individuals had gallstones (96%, 46/48). It has been previously suggested that gallstones play a major role in carrier state development and has been demonstrated that individuals with gallstones are more likely to become carriers than individuals without gallbladder abnormalities via the formation of biofilms on the gallstone surface [186, 225, 242]. *In vitro* studies have demonstrated that mice fed with a high cholesterol diet developed gallstones and exhibited increased gallbladder colonization after *S*. Typhimurium infection in comparison to controls [225].

Studying the chronic carriage of invasive *Salmonella* is difficult as these are human restricted pathogens, this is further complicated by the fact that gallstone formation occurs in patients for many years without symptoms and is attributed to a combination of environmental and genetic causes, typically inked to cholesterol super saturation from bile [261]. SEM examination supports our hypothesis that *Salmonella* carriage is the result of biofilm formation on the gallbladder surfaces. Gallstone taken from an individual with *S.* Typhi in the gallbladder showed a pitted, and covered in a mucoid layer, presumably caused by biofilm formation. Bacterial biofilms were also visualized on cholesterol gallstones from carriers in Mexico

strongly suggesting biofilm formation is an important step for the development of chronic carrier state [225]. Although chronic carriage of *Salmonella* is frequently associated with the presence of gallstones, the progression from infection to carrier state remains unclear.

Salmonella isolates from bile culture were less commonly resistant to NA, ofloxacin, cirpofloxacin and azithromycin than those isolated from acute enteric fever patients. These two groups of isolates may represent differing subsets of the same bacterial population. Using NA resistance as a proxy marker of contemporary strains, isolates from the gallbladders were less commonly resistant then those from the blood. The organisms in the gallbladder have probably been there for some time that is from a period when NA resistance was less prevalent. We concluded that antimicrobial resistant organism is not a risk factor for carriage and that the strains isolated in the same time from blood and bile from those residing in the same population are distinct.

In order to examine if the strains from carriers were different from acute strains, genome sequences from carriage were compared with to a random cross section of *S*. Typhi strains isolated from acute typhoid fever. Majority of strains causing typhoid fever in Kathmandu were found to be H58 [78, 82]. The H58 group prevails globally as a result of recent clonal expansion in parts of Asia and Africa [221, 262, 263]. The presence of H58 among carriage strains indicates that it is becoming an important reservoir. SNP typing result showed that gallbladder stains and acute strains were not identical. All the genotypes labeled as red showed a typical

mutation in the DNA gyrase, which conferred reduced susceptibility to fluoroquinolone, is serine-83-phenylalanine. One strain contained an Asparagine-87-Tyrosine and two contained a Serine-83-Tyrosine. Both these mutations are rare in the *S*. Typhi population and neither mutations were identified in the isolates causing acute infections on Kathmandu. Furthermore, we identified two H58 gallbladder isolates without a *gyrA* mutation, one of which contained an additional tetracycline resistance gene that was not detected in any additional isolates. Many of the gallbladder stains were at the base of the tree indicating that they have been in existence for some time. A study from Indonesia also showed that strains from chronic carriers were genetically different from strains causing acute infection [264]. Thus, the role of chronic carriers in disease transmission may be limited as the strains causing acute typhoid fever are genetically different. However, such carriers are important reservoirs, less invasive diagnostic methods are needed in order to identify them.

6.5 Conclusion

It is not known why some individuals develop chronic carrier state. We found that majority of chronic carriers had gallstones and biofilm was observed on the surfaces of cholesterol gallstone surfaces, which may have enhanced the chronic carrier state. We also found that *Salmonella* were primarily extracellular within the gallbladder and the expression of Vi antigen may indicate a major role it plays in its persistence and survival mechanism. Infection with an antimicrobial resistant organism is not

likely to be associated with *Salmonella* carriage. Genotyping of strains isolated from chronic carriers show that these strains are different from acute circulating strains, which indicates that their role in disease transmission may be limited. However, investigating the chronic *Salmonella* carrier state should provide an insight into bacterial survival strategies, as well as information that could be used for the treatment of chronic *Salmonella* carriers.

7 General Discussion

Enteric fever remains the most common clinical and blood culture confirmed diagnosis among patients with febrile illness in urban Nepal [67, 68, 70], where the disease is endemic and drinking water quality and sewage treatment facilities are poor. With adequate antimicrobial treatment therapy, majority of patients completely recover [48]. However, some individuals may have recurrent infection and some may even progress to become long-term chronic carriers. Relapse is one of the most common cause of recurrent enteric fever [48, 228] and despite a large relapse burden, factors influencing a predisposition for relapse are not well understood. The findings of my work highlighted that the differences in relapse rates are partly dependent on the antimicrobial used for therapy of the initial infection [139]. Further work should be focussed on more sensitive aspects of relapse/reinfection, specifically, the immunology and host genetics. I speculate that some individuals are more prone to relapse than others, which may also be examined by drug uptake and pharmacokinetics.

Some individuals infected with *S*. Typhi become life-long carriers, periodically shedding the bacteria in their faeces. Such carriers serve as an asymptomatic reservoir of diverse *S*. Typhi and *S*. Paratyphi A strains, and the carrier state is an essential feature that is required for survival of these *Salmonella* within a restricted host population. This situation is further complicated by the fact that some of these chronic carriers often have no recollection of an acute episodes of typhoid fever [48]. Gallbladder colonisation and faecal shedding form a central dogma for the

transmission and persistence of typhoid fever. Epidemiological data regarding the prevalence, bacteriology and mechanisms of carriage are limited because of the internal localisation and the host-restricted nature of the bacteria. As a result, the precise role of chronic carriers in disease transmission remains unclear. Furthermore, the role of chronic carriage of *S.* Paratyphi A has received lesser attention than that of *S*. Typhi and it is unknown as to what extent chronic gallbladder carriage is contributing to the increasing burden of *S*. Paratyphi A across many parts of Asia [62].

Additionally, carriers are preseumably, unaffected by currently available typhoid vaccines, which poses a challenge to a control programme involving immunisation [193, 194]. Such carriers are of special concern from public health point of view however, identifying them is extremely difficult as chronic carriers of *Salmonella* are asymptomatic and there are no gold standard tests available. Monitoring *Salmonella* in the stool is one option for identification of chronic carriers but is hampered by low level or sporadic shedding of the organism. Studies, including work in this thesis, have shown that typhoid carriers may produce higher levels of Vi antibodies over extended periods of time compared to acutely infected typhoid patients [196]. Sero-screening studies have revealed that background levels of anti-Vi antibody titres in typhoid endemic areas appear to be high despite a low prevalence of true chronic carriers [194]. Therefore, the results of antibody detection techniques should be interpreted in the context of the background antibody levels in the local population [120, 184]. Furthermore, Vi antigen is not expressed by *S*. Paratyphi A and results from this thesis have demonstrated that carriage of *S*. Paratyphi A in the

gallbladder is almost as common as *S*. Typhi. Thus, there is a clear need for the development of techniques that are less invasive that can identify chronic *S.* Paratyphi A as well as *S.* Typhi carriers.

The work presented in my thesis adds insight into bacterial survival strategies within the gallbladder, as well as information that could be used for the identification of chronic *Salmonella* carriers in general population. In combination with cytokine profiles, and antibody levels may be utilised for the method of detection of chronic carriers in the general population. Future work in this area has to be foccussed on developing similar serological assays to prospecitively detect carriers. Several methods are currently available, including antigen arrays, and in vitro induced antigen technology are being explored to detect more specific antigen/antibody associations for invasive *Salmonella* carriage. These future tests should be available for screening large populations to indentify carriers. This should help reduce the burden of enteric fever more rapidly in places where disease incidence is reducing. Additional future studies should include RNA sequenceing of the colonising organisms in the gallbladder and perhaps some cellular immunology to further grasp how the organism is manipulating the host immune system to induce prolonged colonisation.

The biggest finding from this study was the difference in strains causing colonisation with respect to strains causing acute enteric fever. This, perhaps, is the area that requires future research; the impact of carriage is a firm dogma in enteric fever and additional strains need to be recovered prospectively for cohorts of indidivuals after an acute enteric fever infection. Clearly this is both laborious and costly and

propspectively identifying carriers is still a hurdle, yet with some focussed investment it may be possible to gain a greater understanding of the role of carriage in areas with high, medium and low enteric fever incidence.

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9 Appendices

9.1 Consent form for Gallbladder study

CONSENT FORM

Investigation of the carriage of invasive *Salmonellae* in gall bladders in Kathmandu, Nepal.

Typhoid is endemic in Nepal. Kathmandu is known worldwide as the typhoid capital of the world. In view of this fact, it is important to know what proportion of our Nepalese population carries the bacterium *Salmonella* Typhi/ *Salmonella* Paratyphi in their bodies. This population is important because it is because of them that typhoid keeps circulating in the community and remains endemic. A person can carry the typhoid bacteria in the stool, or in the bile. Moreover, a person can be a typhoid carrier without ever having suffered from typhoid at all! Very little is known about the carriage of Salmonella in bile and how gallstones favour the existence of the bacteria in bile if it does. This study is done to find out what factors in the gallbladder predispose a patient to carry the typhoid bacteria in it and what percentage of our community are typhoid carriers?

By participating in this study, we will be saving the gallbladder tissue as well as the gallstones that the surgeons will be taking out at operation. We will also be culturing some of the bile for *Salmonella* and other bacteria. We will also be taking some of your blood sample for analysis to determine whether you are a carrier. We will be saving your stool sample before the operation and at month three and six postoperatively.

By taking part in this study, you will be helping us to help you and other people in the community to safeguard themselves from being typhoid carriers and to be successfully treated.

All the investigation charges required for the above mentioned investigations regarding the study will be free of cost to you. You will be managed in the same manner that other patients coming for cholecystectomy will if they are not enrolled in the study.

To participate in this study is your own will and you are free to exit from the study at your own will at any time later. On doing so, you will not be penalised and you will receive the same care that you did when you were enrolled.

All the details of your investigations will be kept completely confidential.

If you have any queries regarding the above mentioned facts or anything else regarding the study, please contact:

Dr. Pukar Maskey Dr. Nely Khatri Dr. Samir Koirala Dr. Buddha Basnyat

Patan Hospital.

Phone No. 5521034, 5522278, 5522266

CONSENT FORM

Investigation of the carriage of invasive *Salmonella* in gall bladders in Kathmandu, Nepal.

☐ I have read the information sheet and I understand the implications of this study and hereby agree to take part in the study. I understand that some of the samples may have to be sent abroad for further investigations and agree to it as well.

☐ I agree that the samples may be stored and further tests undertaken (including genetic tests) in future studies of susceptibility to typhoid and also to other infectious diseases.

Name of the patient:……

Signature:……..

Date:……

Name of the physician:…….. .

Signature:……

Date:……

9.2 Consent form or patients enrolled in clinical trials

INFORMATION SHEET/CONSENT FORM

Many people in Nepal are infected by typhoid fever. The treatment of this disease is becoming difficult over the past few years. Hence, we are conducting this study in a joint venture of Patan Hospital, Oxford University, UK and Oxford University Clinical Research Unit, Vietnam to obtain more information about the treatment of typhoid fever. This study has been approved by Nepal Health Research Council and Oxford Tropical Research Ethics Committee (OXTREC).

In this study, we are comparing two drugs that has been used in the Patan Hospital for the treatment of typhoid fever. One is Ceftriaxone which is given intravenously and the other Gatifloxacin which is given orally. Both durgs are effective in the treatment of typhoid fever. We are trying to determine the duration of illness to get cured and the relapse rates for both the drugs.

We request all the patients with typhoid fever visiting Patan Hospital to take part in the study. You will receive either one of the two drugs mentioned above if you agree to take part in the study.

You will have to take medicine for 7days. One of our CMAs (Community Medical Assistant) from the hospital will visit you twice daily at your home for 10-15 days to see your progress. If you do not get better then we might give you additional medication. You will be asked to come for follow up in the hospital on Day 8, Day 15, Month 1, Month 3 and Month 6 after completion of your treatment.

During each visit, your blood and stool examination will be done. During the first 7days, a finger-prick test will be done each day to determine the blood sugar level. Additional blood test will also be done to determine the genetic susceptibility. Your blood sample might be sent abroad for further analysis.

All your identity and information will be kept highly confidential.

All the medications and investigations will be free of charge if you choose to participate in this study.

Like any other treatment regimens, there might be some adverse events to the above drugs. For example, pain at the injection site, nausea, and vomiting. You may ask your treating doctor about the treatment process.

Taking part in the study is entirely your decision and is voluntary. You may

discontinue at any time even if you had initially given consent to participate in the study. Even if you do not participate in the study or drop out from the study, you will be given standard care of treatment.

If you have any questions, please feel free to ask your treating doctor or any of the doctors mentioned below.

Dr Samir Koirala, Dr Amit Arjyal, Dr Buddha Basnyat

Patan Hospital.

Phone No. 5521034, 5522278, 5522266

Have you understood everything mentioned above? Yes
No

Have you understood the advantages and disadvantages

of taking part in this study? Yes No

Did you get chance to clarify your doubts? Yes No

Consent for enrolling into the study (Adults)

I have understood the advantages and disadvantages of participating in the study and I am willing to participate in the study.

Patient's name :_____ Signature:_____

Doctor's name:_____ Signature:_____

Date:_____

Consent for enrolling into the study (Child)

I have understood the advantages and disadvantages of participating in the study. I give consent on behalf of my _____ to participate in the study.

Guardian's name:_____ Signature:_____

Relation to the patient:_____

Doctor's name:_____ Signature:_____

Date:_____

9.3 Clinical record form of Gallbladder study

Investigation of the carriage of invasive *Salmonella* in gallbladders in Kathmandu, Nepal

HOSP NO: _ _ _ _ _ _ STUDY NO:_ _ _ DATE OF SURGERY: _ _ _ / _ _ / _ _

Name	Sex	Age
Address District		Occupation

Chief Symptoms

- **RUQ Pain** : Yes / No Describe:_____Duration_____Timing_____
- **Fever** : Yes / No Describe:_____
- **Other** :

Major Signs

- **RUQ Tenderness :** Yes / No if yes, details:_____
- **Jaundice :** Yes / No if yes, details:_____
- **Palpable Gall Bladder :** Yes / No if yes, details:_____
- **Others :**

Significant Past/ Present Medical History

● **Acute Cholecystitis** : Yes / No. When : _____ Was hospitalization needed? Yes / No ● **Jaundice** : Yes / No. When : _____ Was hospitalization needed? Yes / No ● **Pancreatitis** : Yes / No. When : _____ Was hospitalization needed? Yes / No ● Details:
● **Diabetes Mellitus**: Yes / No. If yes, Type:_____ ● Details of what medications taken: ● **Other**:

Has the patient ever had a febrile illness of >=5days?

Yes / No / not known **When,** if yes:_____ years back **Is the diagnosis known?** Yes / No if yes, **what?_____** **Was it typhoid?** Yes / No **Was it culture confirmed?** Yes / No **Has the patient been vaccinated against typhoid?** Yes / No if yes, **Type:_____**

Antibiotics used within the past 15 days:

Yes / No. If yes, drug, dose, duration:

Investigations:

Total WBC: _____ /mm^3 Differential: N __ L __ M __ E __ B __ Others _____
Bilirubin: Total ____ mg/dL Conjugated ____ mg/dL
AST : _____U/L ALT : _____U/L Alk Phos: _____U/L Amylase:_____ U/L
Ultrasound Findings: Gall Bladder Wall Thickness: _____mm Number of Stones: None / Single / Multiple Common Bile Duct: Diameter_____mm Stones: Yes / No Other:

Other investigations:

Operation Findings:

Surgery: Acute / Elective , Give details of indication:_____
Post OP Diagnosis:-
Gall Bladder: Inflamed / Contracted / Distended / Normal
GB Wall: Thickened / Normal
Stones: None / Single / Multiple If single, diameter : <1cm / >1cm
CBD: Normal / Dilated Diameter : <1cm / >1cm
Other details:

Pre-induction Antibiotic:

Drug:_____Dose:_____

Bile Culture

Histopathology Report

Cholecystitis / Carcinoma / Other
Specify if other:

9.4 Clinical record form of a clinical trial

….. STUDY NOTES

Please Circle the appropriate answer for all questions

1. PATIENT DETAILS

PATIENT NAME:	

FATHER NAME:	
MOTHER NAME:	
…. STUDY NO:	
TREATMENT ARM:	Gatifloxacin / Ceftriaxone (circle as appropriate)
DATE OF ENTRY TO STUDY:	
HOSPITAL NUMBER:	
SEX:	MALE / FEMALE
AGE:	**_____ YEARS.**
OCCUPATION:	
ETHNIC GROUP:	
RELIGION:	

ADDRESS DETAILS:	House number: Ward No. Road: Area: Town / District:	Telephone number: Mobile number:

Inclusion/Exclusion Criteria

Inclusion Criteria		
The patient must meet the inclusion criteria to enter the study: The answers to **all** the following questions must be **YES**		
Do you suspect Enteric Fever?	Yes	No
Is the oral temperature ≥ 39^0 C?	Yes	No
Is the duration of fever ≥ 4 days?	Yes	No
Is the patient ≥ 10 kilos?	Yes	No
Exclusion criteria The patient **CANNOT** enter the trial if the answers to any of the following are **YES**:		
Is the patient diabetic?	Yes	No
Is the patient > 60 years of age?	Yes	No
Is the patient obese (BMI >…….)	Yes	No
Does the patient have history of cardiac arrhythmia?	Yes	No

Does the patient have other concurrent chronic illnesses?		
Chronic Obstructive Pulmonary Disease	Yes	No
Cirrhosis	Yes	No
Chronic Renal Failure	Yes	No
Is the patient on treatment with steroids?	Yes	No
Is the patient pregnant?	Yes	No
Is the patient a lactating mother?	Yes	No
Does the patient have epilepsy or a history of fits?	Yes	No
Is the patient allergic to any of the study drugs?	Yes	No
Has the patient received a quinolone, fluroquinolone, chloramphenicol antibiotic within the previous week? (Patients who have received a cephalosporin, macrolide, ampicillin, or co-trimoxazole can be included as long as they have **not** shown evidence of clinical response).	Yes	No

History

Reasons for coming to hospital:		**Duration of illness (days):**
Duration of symptoms (record days, 0 = not reported)		
Fever:	Cough:	Constipation:
Headache:	Diarrhoea:	Vomiting:
Abdominal pain:	Anorexia:	Nausea:
Any other significant symptoms**:**		

<table>
<tr><td colspan="2">Past History</td></tr>
<tr><td>Previous typhoid: Y / N

When, if Yes:</td><td>Hypertension: Y / N

Duration, if yes_____</td></tr>
<tr><td>Family member with typhoid: Y / N

When, if Yes:</td><td>TB: Y / N

When, if Yes:_____</td></tr>
<tr><td>Typhoid vaccination: Y / N

When, if Yes:

Type if known:</td><td>Viral Hepatitis: Y / N

When, if Yes:

Type if Known: _____</td></tr>
<tr><td colspan="2">Any daily medication necessary: Y / N (If yes:)

Drug:_____Dose_____ Drug:_____Dose_____ Drug:_____Dose_____
Drug:_____Dose_____</td></tr>
<tr><td colspan="2">Known allergy to drugs: Y / N If Yes: drug:_____</td></tr>
<tr><td colspan="2">Treatment in the last 2 weeks

Yes No Not known (If Yes fill in Name / Dose / Duration / Date Begun)

Antibiotics:_____ where prescribed _____

Other treatment:_____</td></tr>
</table>

Patient's definition of Cure: 1. Resolution of symptoms ----a. fever

(if appropriate) b. Headache

c. Anorexia

d. others-specify _____

2. Able to get back to work

3. Others / Specify _____

Household Details

How many people sleep in the house in which the patient lives?_____ Specify:	How many in your family sharing the same kitchen ? _____ Specify:
Water source: Source of water used: 1. Tap water (piped supply): Well tube well stone sprout others: _____	
Drinking water Is the same source mentioned above used for drinking ? Yes No If no, mention the source of drinking water:	**How is drinking water treated ?** 1. untreated 2. filtered 3. boiled 4. boiled and filtered 5. chlorinated 6. others _____

Eating place / habits:	
Where does the patient eat most often? home common mess restaurants / small hotels others _____	Where has the patient eaten in past 3 weeks? restaurants / small hotels street vendors party none other than the usual place others _____

General General appearance: Fair sick looking Pallor : Yes No Icterus: Yes No Roseolae: Yes No Stiff neck: Yes No Enlarged lymph nodes Yes No (details if yes) Site _____number_____size_____ Site _____number_____size_____	**Vitals** **Oral Temperature _____ C** Pulse _____beats/min Blood Pressure _____mm/Hg Respiratory Rate _____ /Min Weight _____ KG Height _____ cms BMI (Body Mass Index) _____ **Oral cavity / Throat:** (record any abnormal finding)
Chest / CVS (record any abnormal finding)	**Musculoskeletal**: (record any abnormal finding)

Abdomen: Tenderness: YES NO Splenomegaly YES NO If yes:___cm Hepatomegaly: YES NO If yes:___cm Others:	**CNS**: Mental State: Normal Agitiated Confused Glasgow Coma Score (GCS): _____ / 15 Eye____ Verbal____Motor____ Any other abnormal CNS finding
Any other findings:	

	DAY	**1**	**2**	**3**	**4**	**5**	**6**	**7**
	Date							
	Time Seen							
History	Feeling better	See Admission History and examination	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Fever		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Headache		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Anorexia		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Pain abdomen		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Cough		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Constipation		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Diarrhoea		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Vomiting		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Nausea		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Black stool		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Confusion		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO

	Sweating / dizziness		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Fainting / blackouts		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Nocturia / polyuria		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Others							
Examination	**RBG**	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
	Pulse rate		/min	/min	/min	/min	/min	/min
	Bld Pres / Cap ref		mmHg	mmHg	mmHg	mmHg	mmHg	mmHg
	Resp rate		/min	/min	/min	/min	/min	/min
	Jaundice		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO

Temperature record and Medication	Abd Tenderness		YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	DAY	**1**	**2**	**3**	**4**	**5**	**6**	**7**
AM	**Oral Temperature**	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs
	Paracetamol Use since last visit	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs

	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____
	Oral Temperature	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs
	Paracetamol Use since last visit	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs

	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____

	DAY	**8**	**9**	**10**	**11**	**12**	**13**	**14**
	Date							
	Time Seen							
History	Feeling better	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Fever	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Headache	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Anorexia	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Pain abdmn.	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Cough	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Constipation	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Diarrhoea	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Vomiting	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Nausea	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Black stool	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Confusion	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO

	Sweating / dizziness	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Fainting / blackouts	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Nocturia / polyuria	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Others							
Examination	**RBG**	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
	Pulse rate	/min	/min	/min	/min	/min	/min	/min
	Bld Pres / Cap ref	mmHg	mmHg	mmHg	mmHg	mmHg	mmHg	mmHg
	Resp rate	/min	/min	/min	/min	/min	/min	/min
	Jaundice	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
Temperature record and	AbdTenderness	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO

Medication	**DAY**	**8**	**9**	**10**	**11**	**12**	**13**	**14**
AM	**Oral Temperature**	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs
	Paracetamol Use since last visit	Total ____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs

	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____
PM	**Oral Temperature**	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs
	Paracetamol Use since last visit	Total ____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs

	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____

	DAY	**15**	**16**	**17**	**18**	**19**	**20**	**21**
	Date							
	Time Seen							
History	Feeling better	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Fever	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Headache	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Anorexia	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Pain abdomen.	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	Cough	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO

	Constipation	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Diarrhea	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Vomiting	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Nausea	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Black stool	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Confusion	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Sweating / dizziness	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Fainting / blackouts	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Nocturia / polyuria	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO	YES	NO
	Others												

Examination	**RBG**	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL	mg/dL
	Pulse rate	/min	/min	/min	/min	/min	/min	/min
	Bld Pres / Cap ref	mmHg	mmHg	mmHg	mmHg	mmHg	mmHg	mmHg
	Resp rate	/min	/min	/min	/min	/min	/min	/min
	Jaundice	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
Temperature record and Medication	AbdTenderness	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO	YES NO
	DAY	**15**	**16**	**17**	**18**	**19**	**20**	**21**
	Oral Temperature	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs

AM	Paracetamol Use since last visit	Total ____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs
	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____
	Oral Temperature	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs	_____C _____Hrs

PM	Paracetamol Use since last visit	Total ____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs	Total _____mg Last dose _____hrs
	Record drug dispensed and Dose	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____	Drug:_____ Dose1:_____

Day 8 Follow Up

Is the patient feeling better? Yes No If no, what are the complaints?	Does the patient feel completely cured? Yes after _____ days of treatment, When a. fever started to decrease b. fever completely subsided c. headache resolved d. anorexia resolved e. others _____ No still does not feel cured Complaints_____
Is the follow up maintained yes no	Reason if no
Has there been any febrile illness in the family since patient's enrolment in the study ? **Yes No** **Specify if Yes:**	
Examination: **Temperature:_____ C** Other findings if present	**Abdomen**: Tenderness: YES NO Splenomegaly YES NO If yes:____cm Hepatomegaly: YES NO If yes:____cm

SIDE-EFFECTS	**Yes**	**No**		
SIDE EFFECTS	**Date**	**Duration outcome**	**management**	
Allergy				
Nausea				
Vomiting				
Diarrhoea / Constipation				
Dizziness				
Excessive Thirst				
Sweating				
Increased urinary fq.				

15 DAY OUTCOME ASSESSMENT

<table>
<tr><td>uncomplicated recovery?

YES NO

If No, describe the complications:</td><td>Fever Clearance time in hours:</td></tr>
<tr><td>Treatment failure:

Clinical Yes No

_____

Microbiological Yes No</td><td>Did the patient die: Yes No

Date of death (if yes)

_____</td></tr>
<tr><td colspan="2">Patient's subjective assessment of illness:
When did the patient feel cured ? after _____ days of treatment,
When a. fever started to decrease
b. fever completely subsided
c. headache resolved
d. anorexia resolved
e. others _____
f. still does not feel cured. _____</td></tr>
<tr><td colspan="2">Examination:
Temperature:_____ C
Other findings if present</td></tr>
</table>

Abdomen: Tenderness: YES NO Splenomegaly YES NO If yes:___cm Hepatomegaly: YES NO If yes:___cm
Has there been any febrile illness in the family since patient's enrolment in the study ? **Yes No** **Specify if Yes:**

SIDE-EFFECTS	**Yes**	**No**		
SIDE EFFECTS	**Date**	**Duration outcome**	**management**	
Allergy				
Nausea				
Vomiting				
Diarrhoea / Constipation				
Dizziness				
Excessive Thirst				
Sweating				
Increased urinary fq.				

_____ DAY ASSESSMENT

<table>
<tr><td colspan="4">Reason for this visit</td></tr>
<tr><td colspan="4">Any Specific complaints ? Yes No

Specify if Yes</td></tr>
<tr><td colspan="4">Examination</td></tr>
<tr><td>General : Fair Sick</td><td>Pulse</td><td>Temperature</td><td>BP</td></tr>
<tr><td>RR</td><td>Icterus</td><td>Pallor</td><td>Cyanosis</td></tr>
<tr><td colspan="4">Other Findings</td></tr>
<tr><td colspan="4">Has there been any febrile illness in the family since patient’s enrolment in the study ?

Yes No

Specify if Yes:</td></tr>
</table>

1 Month Follow – up: Record Date _____

Study Patient			
Is the patient well today?	YES NO	If 'NO', do you suspect enteric fever?	YES NO
Has the patient been unwell since last seen?	YES NO Date:_____	Did they attend a doctor/hospital?	YES NO Specify
What was the diagnosis?	Specify:	What treatment did they receive	Specify:
Has the patient had a relapse/further enteric fever?	YES NO	Has *S.enterica* Typhi or Paratyphi been isolated from blood since completing the study medication?	YES NO

If the patient is unwell please record the temperature: _____C

Investigations due at 1 month follow – up:

All patients must provide a stool sample for culture **Collected:** YES NO

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever ≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____

	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			

Check the investigation schedule!

1 Month Follow-Up continued

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever ≥ 3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		

	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			
Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their	Fever ≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		

	symptoms?	Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES/NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			
3 Month Follow – up: Record Date _____					
Study Patient					

Is the patient well today?	YES NO	If 'NO', do you suspect enteric fever?	YES NO
Has the patient been unwell since last seen?	YES NO Date:_____	Did they attend a doctor/hospital?	YES NO Specify
What was the diagnosis?	Specify:	What treatment did they receive	Specify:
Has the patient had a relapse/further enteric fever?	YES NO	Has *S.enterica* Typhi or Paratyphi been isolated from blood since completing the study medication?	YES NO
If the patient is unwell please record the temperature: _____C			
Investigations due at 3 month follow – up:			
All patients must provide a stool sample for culture **Collected: YES NO**			

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
		Fever ≥3/7	YES NO		

	What were their symptoms?	Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			

Check the investigation schedule!

3 Month Follow-Up continued

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever ≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____

	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			
Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		

6 Month Follow – up: Record Date _____

Study Patient					
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			

Is the patient well today?	YES NO	If 'NO', do you suspect enteric fever?	YES NO
Has the patient been unwell since last seen?	YES NO Date:_____	Did they attend a doctor/hospital?	YES NO Specify
What was the diagnosis?	Specify:	What treatment did they receive	Specify:
Has the patient had a relapse/further enteric fever?	YES NO	Has *S.enterica* Typhi or Paratyphi been isolated from blood since completing the study medication?	YES NO
If the patient is unwell please record the temperature: _____C			
Investigations due at 6 month follow – up:			
All patients must provide a stool sample for culture **Collected: YES NO**			

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____

	What were their symptoms?	Fever ≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			

Month Follow-Up continued

Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever ≥ 3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		
	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____

	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			
Household Members	Have any other members of the household been unwell since the patient was last seen?	YES NO	Specify relationship to the study patient _____ _____	Name of affected relative _____	Age of affected relative _____
	What were their symptoms?	Fever ≥3/7	YES NO		
		Headache	YES NO		
		Abdominal Pain	YES NO		
		Constipation	YES NO		
		Diarrhoea	YES NO		
		Vomiting	YES NO		
		Other	Specify		

	Did they attend hospital?	YES NO Specify which: _____	Admitted: YES NO How many days? _____	Did they receive antibiotics YES NO	Specify type _____
	Was the diagnosis Enteric Fever?	YES NO	If NO Specify	What treatment did they receive	Specify _____
	Did the patient enter the ED study?	YES NO	If Yes, record ED study number: _____		
	What was the outcome?	Recovered? YES NO Died? YES NO			

CULTURE Results

Blood culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Blood culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Stool culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Stool culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Urine culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Urine culture	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

ANTIBIOGRAM

Bacteria isolated: ***S.typhi*** ***S.paratyphi*** **other (specify):** **From blood/ bone marrow/ stool (please indicate)** **Date:_____**									
Sens	NA	Oflox	Cipro	Gati	Azm	Chlor	Amp	Cotrim	Cefotaxime
R or S									
MIC									

BLOOD TEST Results

	Date	Date	Date	Date	Date
	Day	Day	Day	Day	Day
WBC					
Haematocrit					
Platelets					
Stabs					

Neutrophils					
Lymphocytes					
Monocytes					
Eosinophils					
Basophils					
RBG					
Creatinine					
Bilirubin TT					
AST/ SGOT					
ALT / SGPT					
Others					

Chest X-Ray	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

OTHER TESTS

ECG, keep with notes	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
Result			

Other tests	Date: _____(D___)	Date: _____ (D___)	Date:_____ (D___)
USG			
CSF			

Study Investigation Schedule

		Study Day					
Investigation - Tick when completed		**Day 1/Study entry, before starting treatment**	**Day 8**	**Day 15**	**1 Month**	**3 Months**	6 Months
Haematocrit		YES	Yes	Yes	REPEAT IF PREVIOUSLY ABNORMAL	REPEAT IF PREVIOUSLY ABNORMAL	REPEAT IF PREVIOUSLY ABNORMAL
White Cell count and differential		YES	Yes	Yes			
Platelet count		YES	Yes	Yes			
Blood Glucose		Yes	Yes	Yes	Yes	HbA1c	
Creatinine		Yes	REPEAT IF PREVIOUSLY ABNORMAL				
Bilirubin		Yes					
SGOT		YES					
SGPT		YES					
Blood culture (5 –8 mls)		YES	YES (in +ve)				
3ml EDTA * from	patient	YES	YES				
	Mother						
	Father						
Stool culture		YES	YES (in +ve)		YES (in +ve)	YES (in +ve)	YES (in +ve)
Urine culture		If clinically indicate or if symptoms/signs suggest further/unresolved infection					
CXR							

Abdominal Ultrasound	

***Please do not collect more than 3 mls or the blood will clot!**

9.5 Serotyping and MIC results of *Salmonella* isolates from bile culture

		Serotyping			MIC						
GB No	**Culture result**	**O9**	**Vi**	**O2**	**NA**	**CI**	**TX**	**GA**	**CL**	**OF**	**AZ**
5	S.Typhi	+	+	-	64	4	0.75	0.064	0.125	0.25	2
18	Salmonella Gr C	-	-	-	16	32	12	1.5	0.38	3	32
26	S.Typhi	+	+	-	1	2	0.64	0.006	0.006	0.023	4
44	S.Typhi	+	+	-	96	3	0.19	0.094	0.25	0.38	4
63	S.Paratyphi A	-	-	+	>256	4	0.25	0.25	0.38	1	8
58	S.Paratyphi A	-	-	+	>256	4	0.25	0.25	0.38	1	8
71	S.Typhi	+	+	-	>256	3	0.19	0.094	0.19	0.25	3
113	S.Paratyphi A	-	-	+	1.5	3	0.25	0.032	0.023	0.094	6
193	S.Paratyphi A	-	-	+	1.5	4	0.19	0.032	0.023	0.094	24
199	S.Typhi	+	+	-	128	3	0.19	0.094	0.25	0.38	6
214	S.Paratyphi A	-	-	+	2	4	0.25	0.064	0.032	0.19	6
224	S.Paratyphi A	-	-	+	12	4	0.25	0.125	0.125	0.38	6
245	S.Paratyphi A	-	-	+	>256	8	0.38	0.75	1	4	16
266	S.Typhi	+	+	-	1.5	2	0.094	0.008	0.006	0.023	4
276	S.Typhi	+	+	-	1.5	3	0.064	0.008	0.004	0.023	4
280	S.Paratyphi A	-	-	+	>256	4	0.38	0.25	0.38	0.75	8
281	S.Typhi	+	-	-	1.5	3	0.094	0.012	0.016	0.047	6
335	S.Typhi	+	-	-	1	3	0.125	0.006	0.006	0.023	6
336	S.Typhi	+	-	-	1	2	0.094	0.006	0.008	0.023	2
368	S.Typhi	+	+	-	>256	2	0.19	0.094	0.25	0.25	6
493	S.Paratyphi A	-	-	+	>256	4	0.38	0.38	0.38	1	16
580	S.Typhi	+	+	-	1.5	3	0.125	0.012	0.012	0.023	4
624	S.Paratyphi A	-	-	+	1.5	3	0.38	0.032	0.032	0.094	8
625	S.Paratyphi A	-	-	+	>256	4	0.25	0.25	0.38	0.75	8
637	S.Paratyphi A	-	-	+	>256	4	0.25	0.38	0.5	1	6
640	S.Paratyphi A	-	-	+	>256	4	0.5	0.38	0.5	1	6
672	S.Paratyphi A	-	-	+	1.5	4	0.125	0.006	0.008	0.023	8
726	S.Paratyphi A	-	-	+	>256	4	0.19	0.38	0.38	1.5	6
O1GB 3	S.Typhi	+	-	-	>256	3	0.25	0.125	0.25	0.5	6
O1GB 4	Salmonella Gr C	-	-	-	2	4	0.38	0.023	0.016	0.064	6
O1GB20	S.Paratyphi A	-	-	+	>256	6	0.19	0.25	0.5	1	12
O1GB 31	S.Typhi	+	+	-	>256	3	0.19	0.094	0.19	0.38	8
O1GB 75	S.Paratyphi A	-	-	+	>256	12	0.5	0.75	1	3	16

O1GB 76	S.Typhi	+	+	-	1	3	0.094	0.008	0.008	0.023	4
O1GB 125	S.Paratyphi A	+	+	-	128	3	0.125	0.064	0.19	0.25	4
O1GB 169	S.Typhi	+	+	-	128	3	0.25	0.094	0.25	0.25	6
O1GB387	S.Typhi	+	+	-	1	2	0.19	0.006	0.016	0.023	6
O1GB428	S.Typhi	+	-	-	1.5	2	0.094	0.008	0.008	0.032	2
O1GB436	S.Typhi	+	+	-	1.5	3	0.094	0.008	0.008	0.023	6
01GB441	S.Typhi	+	+	-	1	2	0.047	0.006	0.004	0.016	3
01GB496	S.Typhi	+	+	-	>256	3	0.19	0.064	0.25	0.25	8
01GB620	S.Paratyphi A	-	-	+	>256	4	0.19	0.38	0.38	1.5	6
O1GB650	S.Typhi	+	+	-	>256	4	0.19	0.094	0.25	0.25	6
01GB686	S.Paratyphi A	-	-	+	>256	6	0.25	0.38	0.5	1	8
01GB705	S.Typhi	+	+	-	1.5	3	0.19	0.008	0.012	0.032	2
01GB710	S.Typhi	+	+	-	>256	4	0.19	0.125	0.25	0.38	8

9.6 Serotyping and MICs of *Salmonella* isolates from DM (Gati v. Cefixime) study

			Serotyping			MIC							
DM No.	**Day**	**Culture result**	**O:2**	**O:9**	**Vi**	**GA**	**IX**	**TX**	**CL**	**NA**	**CI**	**OF**	**AZ**
1	1	*S*.Typhi	-	+	+	0.006	0.023	0.064	8.0	2.0	0.006	0.016	8.0
4	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.250	0.380	4.0
6	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.380	4.0
7	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.500	6.0
8	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.500	1.500	48.0
13	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.190	0.380	4.0
15	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.190	0.250	6.0
17	1	*S*.Typhi	-	+	+	0.012	0.023	0.094	4.0	2.0	0.500	1.000	6.0
26	1	*S*.Typhi	-	+	+	0.190	0.250	0.190	4.0	>256	0.250	0.380	6.0
29	1	*S*.Typhi	-	+	+	0.250	0.250	0.094	4.0	>256	0.250	0.380	4.0
32	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.500	0.750	16.0
34	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	8.0	>256	0.250	0.380	6.0
35	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.500	8.0
38	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.750	2.000	8.0
41	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.500	4.0
42	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.500	8.0
45	1	*S*.Typhi	-	+	+	0.125	0.250	0.190	6.0	>256	0.250	0.380	16.0
49	1	*S*.Paratyphi A	+	-	-	0.032	0.190	0.125	6.0	4.0	0.016	0.094	8.0
52	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.250	0.380	6.0
53	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	12.0	>256	0.250	0.380	4.0
56	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.500	8.0
58	1	*S*.Typhi	-	+	+	0.008	0.047	0.125	4.0	2.0	0.008	0.023	2.0
59	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	6.0	2.0	0.380	1.000	6.0
67	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.190	8.0	>256	0.500	1.500	12.0
68	1	*S*.Typhi				0.125	0.250	0.190	8.0	>256	0.190	0.250	8.0
71	1	*S*.Typhi	-	+	+	0.125	0.500	0.125	8.0	>256	0.380	0.500	8.0
76	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.380	0.380	3.0
77	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.380	4.0

80	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.500	6.0
82	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.750	2.000	8.0
84	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.500	6.0
85	1	*S*.Typhi	-	+	+	0.125	0.125	0.190	8.0	>256	0.380	1.000	12.0
86	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.380	8.0
87	1	*S*.Typhi	-	+	+	0.008	0.023	0.190	6.0	2.0	0.006	0.023	3.0
89	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.250	12.0	>256	0.500	1.000	8.0
91	1	*S*.Typhi	-	+	+	0.125	0.500	0.125	8.0	>256	0.190	0.250	6.0
92	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	6.0	2.0	0.006	0.016	3.0
93	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.094	8.0	>256	0.380	1.500	8.0
94	2	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.250	0.380	6.0
97	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.190	0.250	24.0
98	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.500	8.0	>256	0.500	1.500	8.0
102	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.250	0.500	6.0
103	1	*S*.Typhi	-	+	+	0.094	0.380	0.190	8.0	>256	0.250	0.500	8.0
104	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.250	0.380	4.0
105	1	*S*.Typhi	-	+	+	0.094	0.032	0.094	6.0	>256	0.190	0.380	3.0
106	1	*S*.Typhi	-	+	+	0.094	0.380	0.190	6.0	>256	0.190	0.380	4.0
107	1	*S*.Typhi	-	+	+	0.008	0.023	0.064	8.0	2.0	0.008	0.023	3.0
111	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.500	2.000	8.0
116	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.190	8.0	>256	0.500	2.000	8.0
118	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	1.000	1.500	8.0
121	1	*S*.Typhi	-	+	+	0.094	0.032	0.064	6.0	>256	0.064	0.380	4.0
123	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.190	0.380	8.0
138	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.250	0.380	6.0
141	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.250	0.380	8.0
144	1	*S*.Typhi	-	+	+	0.094	0.023	0.064	6.0	>256	0.125	0.250	4.0
145	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	8.0	2.0	0.012	0.047	8.0
147	1	*S*.Typhi	-	+	+	0.008	0.380	0.125	6.0	2.0	0.008	0.023	3.0
149	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.250	0.380	24.0
156	1	*S*.Typhi	-	+	+	0.094	0.032	0.094	6.0	>256	0.190	0.500	3.0
157	1	*S*.Typhi	-	+	+	0.094	0.380	0.190	8.0	>256	0.500	1.000	6.0
158	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.250	8.0	>256	1.000	1.500	8.0
159	1	*S*.Typhi	-	+	+	0.094	0.380	0.250	8.0	>256	0.380	0.500	6.0
161	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.500	0.500	8.0

166	1	*S*.Typhi	-	+	+	0.125	0.023	0.094	6.0	>256	0.250	0.380	4.0
168	1	*S*.Typhi	-	+	+	0.094	0.500	0.125	8.0	>256	0.190	0.380	4.0
169	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.380	0.380	8.0
171	1	*S*.Typhi	-	+	+	0.094	0.380	0.190	8.0	>256	0.190	0.190	4.0
172	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.500	0.500	8.0
173	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	12.0	>256	0.250	0.250	8.0
174	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.750	0.500	6.0
175	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	6.0	>256	1.500	3.000	16.0
176	1	*S*.Typhi	-	+	+	0.094	0.380	0.190	8.0	>256	0.500	0.750	8.0
178	1	*S*.Paratyphi A	+	-	-	0.125	0.380	0.190	8.0	>256	1.000	1.500	6.0
179	1	*S*.Typhi	-	+	+	0.064	0.023	0.094	6.0	8.0	0.125	0.190	1.5
180	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	6.0	>256	0.500	0.500	8.0
181	1	*S*.Typhi	-	+	+	0.008	0.023	0.064	8.0	1.5	0.008	0.023	12.0
183	1	*S*.Paratyphi A	+	-	-	0.125	0.380	0.190	8.0	>256	0.380	1.000	8.0
186	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	1.500	4.000	16.0
188	1	*S*.Paratyphi A	+	-	-	0.125	0.250	0.190	8.0	>256	0.500	1.500	16.0
189	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.380	0.380	6.0
192	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	1.000	1.500	12.0
197	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.500	0.500	4.0
199	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.380	0.380	8.0
202	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	6.0	>256	0.500	0.500	8.0
203	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	>256	2.0	0.250	0.380	4.0
206	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.750	0.750	8.0
207	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.380	0.500	6.0
210	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.380	0.750	8.0
211	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.750	1.500	12.0
213	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.380	0.380	8.0
215	1	*S*.Typhi	-	+	+	0.094	0.250	0.190	8.0	>256	0.380	0.380	12.0
216	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.380	0.750	8.0
217	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	8.0	1.5	0.016	0.032	8.0
218	1	*S*.Typhi	-	+	+	0.006	0.125	0.064	6.0	2.0	0.006	0.032	4.0
219	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	1.000	2.000	16.0
221	1	*S*.Paratyphi A	-	+	+	0.006	0.023	0.064	6.0	2.0	0.003	0.012	4.0
223	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	6.0	>256	0.250	0.380	4.0
224	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.380	0.500	6.0

226	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	6.0	>256	0.380	0.380	6.0
227	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.250	12.0	>256	0.750	3.000	24.0
230	1	*S*.Typhi	-	+	+	0.094	0.047	0.125	6.0	8.0	0.380	0.250	8.0
231	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	8.0	>256	0.500	0.750	8.0
233	1	*S*.Paratyphi A	+	-	-	0.500	0.190	0.125	6.0	>256	0.500	1.500	32.0
237	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.380	0.380	6.0
239	1	*S*.Typhi	-	+	+	0.008	0.023	0.094	8.0	2.0	0.012	0.023	6.0
240	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.380	0.250	4.0
242	1	*S*.Paratyphi A	+	-	-	0.250	2.000	0.190	8.0	>256	1.000	3.000	16.0
243	1	*S*.Typhi	-	+	+	0.006	0.023	0.064	4.0	2.0	0.012	0.032	3.0
247	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.750	3.000	24.0
253	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.250	8.0	>256	1.000	2.000	32.0
254	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	12.0	>256	0.750	1.500	12.0
255	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	8.0	>256	0.500	1.000	4.0
256	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	0.500	1.000	8.0
257	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.500	0.500	4.0
258	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.750	0.500	12.0
260	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.190	8.0	>256	1.000	2.000	24.0
262	1	*S*.Typhi	-	+	+	0.008	0.016	0.064	4.0	2.0	0.012	0.023	8.0
265	1	*S*.Paratyphi A	+	-	-	0.380	0.500	0.190	8.0	>256	1.000	2.000	24.0
267	1	*S*.Typhi	-	+	+	0.125	0.125	0.094	3.0	>256	0.380	0.380	8.0
268	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.380	0.500	6.0
274	1	*S*.Paratyphi A	+	-	-	0.500	0.380	0.190	6.0	>256	1.000	1.500	16.0
282	1	*S*.Typhi	-	+	+	0.190	0.250	0.047	1.5	>256	0.250	0.380	4.0
283	1	*S*.Typhi	-	+	+	0.190	0.250	0.125	4.0	>256	0.250	0.380	4.0
288	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	8.0	>256	0.250	0.380	6.0
290	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	6.0	>256	0.380	0.500	8.0
295	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.190	8.0	>256	1.000	1.500	8.0
298	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	6.0	>256	0.250	0.380	12.0
299	1	*S*.Typhi	-	+	+	0.032	0.190	0.125	8.0	>256	0.380	0.500	8.0
301	1	*S*.Typhi	-	+	+	0.190	0.250	0.094	3.0	>256	0.380	0.500	8.0
302	1	*S*.Typhi	-	+	+	0.125	0.125	0.094	3.0	>256	0.380	0.380	6.0
304	1	*S*.Typhi	-	+	+	0.094	0.190	0.094	3.0	>256	0.380	0.380	4.0
305	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.250	8.0	>256	0.500	1.500	12.0
306	1	*S*.Typhi	-	+	+	0.008	0.016	0.047	2.0	1.0	0.003	0.008	6.0

307	1	*S*.Typhi	-	+	+	0.125	0.190	0.094	3.0	>256	0.380	0.500	6.0
309	1	*S*.Typhi	-	+	+	0.013	0.500	0.190	8.0	>256	0.250	0.380	8.0
312	1	*S*.Typhi	-	+	+	0.125	0.125	0.094	4.0	>256	0.250	0.380	8.0
313	1	*S*.Paratyphi A	+	-	-	0.047	0.380	0.125	8.0	3.0	0.023	0.125	8.0
314	1	*S*.Typhi	-	+	+	0.006	0.023	0.064	6.0	2.0	0.004	0.023	4.0
316	1	*S*.Typhi	-	+	+	0.125	0.500	0.125	4.0	>256	0.250	0.250	6.0
317	1	*S*.Typhi	-	+	+	0.125	0.190	0.094	3.0	>256	0.500	0.380	4.0
320	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	12.0	>256	0.750	2.000	24.0
323	1	*S*.Paratyphi A	+	-	-	0.380	0.500	0.250	8.0	>256	0.750	1.500	8.0
329	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.094	6.0	>256	0.750	1.000	8.0
330	1	*S*.Typhi	-	+	+	0.125	0.380	0.125	8.0	>256	0.250	0.380	6.0
331	1	*S*.Typhi	-	+	+	0.125	0.250	0.125	6.0	>256	0.380	0.500	12.0
340	1	*S*.Typhi	-	+	+	0.012	0.023	0.094	8.0	2.0	0.008	0.023	2.0
345	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.500	0.500	8.0
348	1	*S*.Typhi	-	+	+	0.125	0.380	0.190	8.0	>256	0.380	0.380	6.0
349	1	*S*.Typhi	-	+	+	0.125	0.500	0.125	8.0	>256	0.380	0.380	4.0
353	1	*S*.Typhi	-	+	+	0.094	0.380	0.125	8.0	>256	0.250	0.380	6.0
356	1	*S*.Paratyphi A	+	-	-	0.250	0.250	0.190	8.0	>256	0.750	1.500	6.0
358	1	*S*.Paratyphi A	+	-	-	0.380	0.380	0.190	4.0	>256	1.000	1.500	12.0
363	1	*S*.Paratyphi A	+	-	-	0.047	0.380	0.250	8.0	4.0	0.047	0.190	8.0
368	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	6.0	>256	0.250	0.380	6.0
373	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	8.0	>256	0.380	0.500	8.0
377	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	6.0	>256	0.190	0.250	12.0
383	1	*S*.Typhi	-	+	+	0.008	0.023	0.064	4.0	2.0	0.006	0.008	8.0
386	1	*S*.Typhi	-	+	+	0.125	0.500	0.190	6.0	>256	0.380	0.380	8.0
387	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.250	6.0	>256	0.750	1.500	12.0
390	1	*S*.Paratyphi A	+	-	-	0.250	0.380	0.250	8.0	>256	0.750	1.500	12.0
30	20	*S*.Typhi	-	+	+	0.125	0.190	0.064	2.0	>256	0.38	0.38	6.0
56	24	*S*.Paratyphi A	+	-	-	0.380	0.250	0.125	4.0	>256	0.38	1	48.0
72	23	*S*.Paratyphi A	+	-	-	0.380	0.380	0.125	8.0	>256	1	2	8.0
91	17	*S*.Typhi	-	+	+	0.125	0.190	0.094	4.0	>256	0.25	0.38	24.0
93	24	*S*.Paratyphi A	+	-	-	0.064	0.380	0.125	8.0	4.0	0.047	0.19	16.0
97	30	*S*.Typhi	-	+	+	0.125	0.125	0.047	4.0	>256	0.38	0.5	6.0
223	21	*S*.Typhi	-	+	+	0.125	0.190	0.094	3.0	64.0	0.19	0.38	6.0
313	30	*S*.Paratyphi A	+	-	-	0.023	0.125	0.125	6.0	3.0	0.023	0.125	12.0

387	20	*S*.Paratyphi A	+	-	-	0.500	0.250	0.064	4.0	>256	0.38	1	8.0
183	10	*S*.Paratyphi A	+	-	-	0.380	0.190	0.125	4.0	>256	0.5	1.5	32.0
Stool Isolates													
52	1	*S*.Typhi	-	+	+	0.094	0.125	0.047	3.0	>256	0.19	0.25	4.0
72	1	*S*.Paratyphi A	+	-	-	0.250	0.190	0.094	4.0	>256	0.38	1	8.0
106	1	*S*.Typhi	-	+	+	0.125	0.125	0.094	3.0	>256	0.19	0.25	4.0
116	1	*S*.Paratyphi A	+	-	-	0.190	0.190	0.125	4.0	4.0	0.023	0.19	6.0
123	1	*S*.Typhi	-	+	+	0.125	0.125	0.094	3.0	>256	0.19	0.25	16.0
217	1	*S*.Typhi	-	+	+	0.750	0.190	0.094	1.5	>256	0.008	0.016	4.0
226	1	*S*.Typhi	-	+	+	0.125	0.125	0.125	3.0	>256	0.19	0.25	4.0
249	1	*S*.Typhi	-	+	+	0.125	0.016	0.064	2.0	>256	0.125	0.19	6.0
266	1	*S*.Paratyphi C	-	-	+	0.023	0.064	0.032	3.0	2.0	0.016	0.094	3.0
267	1	*S*.Typhi	-	+	+	0.094	0.125	0.125	6.0	>256	0.19	0.38	4.0
340	1	*S*.Typhi	-	+	+	0.012	0.016	0.047	1.5	1.5	0.006	0.023	3.0
373	10	*S*.Typhi	-	+	+	0.094	0.190	0.064	2.0	>256	0.19	0.25	4.0
320	30	*S*.Typhi	-	+	+	0.016	0.047	0.047	2.0	2.0	0.008	0.064	4.0

9.7 Serotyping and MICs of *Salmonella* isolates from ED (Gati v.Chloro) study

Blood isolates

ED		**Culture**	**Serotyping**			**MIC**								
No	**DAY**	**Result**	**O:2**	**O:9**	**Vi**	**GA**	**TX**	**CL**	**NA**	**CI**	**OF**	**AC**	**TS**	**AZ**
3	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.75	2	2	0.125	16
4	1	*S*.Typhi	-	+	+	0.016	0.094	6	2	0.012	0.047	0.75	0.064	6
6	1	*S*.Typhi	-	+	+	0.19	0.094	4	8	0.125	0.25	0.75	0.032	6
7	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.012	0.047	0.75	0.047	4
8	1	*S*.Typhi	-	+	+	0.19	0.125	4	8	0.19	0.25	0.75	0.032	6
12	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.012	0.047	0.75	0.047	4
20	1	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.38	0.5	1	0.032	8
21	1	*S*.Paratyphi A	+	-	-	0.38	0.25	8	>256	0.5	1.5	2	0.125	16
22	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.008	0.047	0.75	0.032	6
24	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.125	0.047	0.5	0.032	4

27	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	2	0.19	12
28	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.75	0.023	6
30	1	*S*.Typhi	-	+	+	0.012	0.125	4	2	0.012	0.064	0.75	0.047	6
31	1	*S*.Typhi	-	+	+	0.012	0.094	4	1.5	0.008	0.032	0.5	0.032	6
32	1	*S*.Typhi	-	+	+	0.016	0.094	4	3	0.012	0.064	0.5	0.032	6
33	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	0.5	2	1.5	0.125	16
34	1	*S*.Paratyphi A	+	-	-	0.5	0.25	6	>256	0.5	1.5	2	0.125	16
35	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	1.5	0.094	16
37	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	1.5	0.125	16
38	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.008	0.047	0.5	0.047	6
39	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	1.5	0.125	12
41	1	*S*.Typhi	-	+	+	0.023	0.125	4	3	0.008	0.047	0.75	0.064	4
43	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	1.5	0.125	16
44	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.75	2	2	0.19	16
46	1	*S*.Typhi	-	+	+	0.023	0.094	4	3	0.016	0.064	0.5	0.032	4
47	1	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.38	0.75	1	0.094	8
49	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	2	0.19	16
50	1	*S*.Typhi	-	+	+	0.19	0.19	4	>256	0.38	0.75	1	0.032	12
51	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	2	0.25	16
52	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	2	0.125	16
53	1	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	2	0.125	16
59	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.008	0.047	0.5	0.047	6
65	1	*S*.Paratyphi A	+	-	-	0.75	0.25	16	>256	0.75	2	2	0.19	12
68	1	*S*.Paratyphi A	+	-	-	0.5	0.25	16	>256	0.75	1.5	2	0.19	16
69	1	*S*.Typhi	-	+	+	0.25	0.25	12	>256	0.38	0.5	1	0.032	12
70	1	*S*.Paratyphi A	+	-	-	0.5	0.25	12	>256	0.75	1.5	2	0.125	12
71	1	*S*.Paratyphi A	+	-	-	0.25	0.19	12	>256	0.5	1.5	1.5	0.125	12
72	1	*S*.Typhi	-	+	+	0.19	0.094	12	>256	0.38	0.38	0.5	0.012	16
74	1	*S*.Paratyphi A	+	-	-	0.064	0.25	12	4	0.032	0.19	1.5	0.19	16
76	1	*S*.Typhi	-	+	+	0.023	0.19	12	3	0.023	0.094	0.75	0.032	6
79	1	*S*.Paratyphi A	+	-	-	0.5	0.25	12	>256	0.75	1.5	2	0.19	16
82	1	*S*.Typhi	-	+	+	0.012	0.094	8	2	0.012	0.047	0.75	0.032	6
83	1	*S*.Typhi	-	+	+	0.012	0.094	8	3	0.012	0.047	0.75	0.047	8
89	1	*S*.Typhi	-	+	+	0.19	0.19	12	>256	0.38	0.5	1	0.032	12
90	1	*S*.Typhi	-	+	+	0.19	0.125	8	8	0.19	0.25	0.75	0.032	6

91	1	*S*.Typhi	-	+	+	0.25	0.19	12	>256	0.38	0.5	1	0.032	8
92	1	*S*.Typhi	-	+	+	0.016	0.125	12	4	0.012	0.047	0.75	0.064	4
93	1	*S*.Typhi	-	+	+	0.25	0.19	12	>256	0.38	0.5	1	0.047	8
94	1	*S*.Typhi	-	+	+	0.012	0.094	4	1.5	0.012	0.047	0.5	0.032	6
97	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.023	8
99	1	*S*.Paratyphi A	+	-	-	0.064	0.125	8	4	0.047	0.19	1.5	0.125	16
102	1	*S*.Typhi	-	+	+	0.008	0.064	4	0.38	0.012	0.047	0.5	0.032	6
104	1	*S*.Typhi	-	+	+	0.094	0.094	4	6	0.125	0.25	0.5	0.023	6
106	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.75	0.023	8
108	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.012	0.047	0.5	0.032	4
109	1	*S*.Typhi	-	+	+	0.012	0.064	4	2	0.012	0.047	0.5	0.047	4
110	1	*S*.Typhi	-	+	+	0.008	0.064	4	1.5	0.012	0.047	0.5	0.047	6
111	1	*S*.Typhi	-	+	+	0.094	0.094	4	4	0.125	0.19	0.5	0.047	6
114	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	8
117	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
119	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	8
126	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.5	0.5	0.023	8
130	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.012	0.047	0.5	0.047	6
134	1	*S*.Typhi	-	+	+	0.012	0.064	4	2	0.012	0.047	0.5	0.032	8
138	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.012	0.047	0.5	0.032	6
139	1	*S*.Typhi	-	+	+	0.008	0.064	3	0.75	0.008	0.032	0.5	0.016	6
140	1	*S*.Typhi	-	+	+	0.125	0.094	4	>256	0.19	0.5	0.5	0.032	6
142	1	*S*.Typhi	-	+	+	0.094	0.064	4	6	0.125	0.25	0.38	0.016	8
143	1	*S*.Typhi	-	+	+	0.5	0.125	8	>256	0.5	2	1.5	0.094	6
145	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.75	2	1.5	0.125	12
147	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
148	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
149	1	*S*.Typhi	-	+	+	0.012	0.064	6	1.5	0.012	0.047	0.5	0.047	6
150	1	*S*.Typhi	-	+	+	0.125	0.064	4	>256	0.25	0.5	0.38	0.016	8
152	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
156	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	12
158	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.5	0.75	0.032	8
161	1	*S*.Paratyphi A	+	-	-	0.064	0.19	8	4	0.047	0.19	1.5	0.125	16
164	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	1	2	1.5	0.125	16
168	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	1	2	1.5	0.125	16

169	1	*S*.Typhi	-	+	+	0.5	0.125	8	>256	1	2	1.5	0.125	16
171	1	*S*.Typhi	-	+	+	0.006	0.064	4	1.5	0.006	0.023	0.5	0.032	4
174	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	12
176	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.016	0.047	0.5	0.032	6
177	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.75	0.75	0.032	8
179	1	*S*.Paratyphi A	+	-	-	0.75	0.19	8	>256	0.75	2	1.5	0.094	16
181	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	12
182	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	1	2	1.5	0.125	12
188	1	*S*.Paratyphi A	+	-	-	0.75	0.19	12	>256	1	3	1.5	0.125	8
190	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.75	0.032	4
191	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.125	16
192	1	*S*.Paratyphi A	+	-	-	0.5	0.19	6	>256	0.5	2	1.5	0.094	24
193	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.094	16
194	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	24
195	1	*S*.Typhi	-	+	+	0.012	0.094	4	1.5	0.016	0.047	0.75	0.094	8
197	1	*S*.Typhi	-	+	+	0.125	0.094	4	256	0.19	0.5	0.38	0.023	16
199	1	*S*.Paratyphi A	+	-	-	0.064	0.19	8	3	0.032	0.19	1.5	0.125	16
203	1	*S*.Typhi	-	+	+	0.016	0.094	3	2	0.012	0.047	0.38	0.032	4
206	1	*S*.Typhi	-	+	+	0.012	0.064	4	2	0.012	0.047	0.38	0.047	6
214	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.023	8
215	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
216	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
219	1	*S*.Typhi	-	+	+	0.016	0.064	4	2	0.125	0.047	0.38	0.047	4
220	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	4
223	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.032	16
224	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.023	12
226	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.008	0.047	0.5	0.032	8
227	1	*S*.Typhi	-	+	+	0.012	0.064	3	2	0.008	0.047	0.038	0.032	6
231	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.032	12
232	1	*S*.Typhi	-	+	+	0.012	0.094	4	1.5	0.012	0.047	0.5	0.032	6
233	1	*S*.Paratyphi A	+	-	-	0.5	0.19	6	>256	0.5	2	1	0.094	16
234	1	*S*.Typhi	-	+	+	0.006	0.047	4	1.5	0.006	0.023	0.25	0.023	6
236	1	*S*.Typhi	-	+	+	0.012	0.094	4	1.5	0.012	0.047	0.5	0.064	8
237	1	*S*.Typhi	-	+	+	0.016	0.064	3	1.5	0.012	0.047	0.38	0.032	8
240	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.25	0.38	0.5	0.032	12

243	1	*S*.Typhi	-	+	+	0.016	0.064	3	1.5	0.012	0.047	0.5	0.047	6
247	1	*S*.Typhi	-	+	+	0.125	0.125	4	>256	0.25	0.38	0.5	0.023	8
248	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	16
249	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	12
254	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.5	0.032	12
261	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	12
262	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	1.5	1	0.125	16
265	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	12
267	1	*S*.Paratyphi A	+	-	-	0.38	0.125	8	>256	0.38	1.5	1	0.125	16
269	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.5	0.032	12
272	1	*S*.Paratyphi A	+	-	-	0.38	0.125	6	>256	0.5	1	1	0.094	16
274	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	1.5	1.5	0.125	16
275	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	1.5	1.5	0.125	16
282	1	*S*.Typhi	-	+	+	0.094	0.064	4	6	0.125	0.19	0.38	0.023	8
285	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.008	0.032	0.38	0.047	8
286	1	*S*.Paratyphi A	+	-	-	0.38	0.125	6	>256	0.5	1.5	1	0.094	24
292	1	*S*.Typhi	-	+	+	0.125	0.094	4	>256	0.25	0.38	0.5	0.023	12
293	1	*S*.Paratyphi A	+	-	-	0.5	0.125	6	>256	0.5	1.5	1	0.094	16
294	1	*S*.Typhi	-	+	+	0.012	0.064	3	2	0.012	0.047	0.5	0.016	12
295	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	75	0.032	16
296	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	24
297	1	*S*.Typhi	-	+	+	0.016	0.064	6	2	0.012	0.032	0.5	0.032	12
302	1	*S*.Typhi	-	+	+	0.5	0.19	8	>256	0.5	1.5	1	0.125	24
307	1	*S*.Typhi	-	+	+	0.25	0.125	8	>256	0.25	0.5	0.75	0.032	16
317	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.125	32
318	1	*S*.Paratyphi A	+	-	-	0.5	0.19	12	>256	0.25	1.5	1.5	0.125	16
320	1	*S*.Paratyphi A	+	-	-	0.5	0.19	12	1.5	0.016	1.5	1.5	0.125	24
321	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	24
325	1	*S*.Typhi	-	+	+	0.016	0.064	6	>256	0.5	0.094	0.5	0.047	8
327	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	16
329	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.5	0.5	0.75	0.032	16
330	1	*S*.Paratyphi A	+	-	-	0.38	0.125	8	>256	0.5	1.5	1	0.125	12
335	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	12
337	1	*S*.Paratyphi A	+	-	-	0.047	0.125	6	4	0.032	0.19	1	0.125	12
338	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	2	1	0.125	12

340	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.125	12
341	1	*S*.Typhi	-	+	+	0.5	0.125	6	>256	0.5	2	1	0.094	8
343	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.032	6
345	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	0.5	2	1	0.125	8
347	1	*S*.Paratyphi A	+	-	-	0.5	0.125	6	>256	0.5	2	1	0.125	12
348	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	0.5	2	1	0.125	12
350	1	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.012	0.047	0.38	0.032	4
353	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.75	0.75	0.032	6
356	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	2	1.5	0.125	8
359	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.75	0.032	6
361	1	*S*.Paratyphi A	+	-	-	0.38	0.125	8	>256	0.5	1.5	1	0.125	12
362	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	12
363	1	*S*.Paratyphi A	+	-	-	0.38	0.125	8	>256	0.5	1.5	1	0.125	12
364	1	*S*.Paratyphi A	+	-	-	0.38	0.19	8	>256	0.5	1.5	1.5	0.125	4
366	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.125	12
373	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.008	0.047	0.38	0.047	8
375	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	16
383	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1	0.125	16
384	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.125	12
390	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.012	0.047	0.5	0.047	4
393	1	*S*.Paratyphi A	+	-	-	0.064	0.125	6	3	0.032	0.19	1	0.064	8
400	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.5	0.023	6
401	1	*S*.Typhi	-	+	+	0.012	0.064	2	2	0.008	0.032	0.38	0.023	6
402	1	*S*.Typhi	-	+	+	0.094	0.094	6	96	0.125	0.25	0.5	0.032	6
405	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.5	0.38	0.023	6
408	1	*S*.Paratyphi A	+	-	-	0.5	0.19	6	>256	0.5	2	1	0.094	6
417	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	0.38	1.5	1	0.094	8
425	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.19	0.5	0.38	0.023	4
429	1	*S*.Typhi	-	+	+	0.012	0.094	3	1.5	0.008	0.047	0.38	0.047	4
432	1	*S*.Paratyphi A	+	-	-	0.5	0.19	6	>256	0.75	2	1.5	0.25	8
440	1	*S*.Typhi	-	+	+	0.125	0.064	3	>256	0.19	0.38	0.38	0.023	4
443	1	*S*.Paratyphi A	+	-	-	0.38	0.125	8	>256	0.5	1.5	1	0.125	12
446	1	*S*.Paratyphi A	+	-	-	0.25	0.125	8	>256	0.38	1	1	0.094	8
448	1	*S*.Typhi	-	+	+	0.012	0.064	3	1.5	0.008	0.032	0.38	0.032	4
455	1	*S*.Typhi	-	+	+	0.008	0.094	4	1	0.008	0.032	0.38	0.023	6

459	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1	0.094	12
461	1	*S*.Typhi	-	+	+	0.016	0.064	4	2	0.008	0.047	0.38	0.032	4
464	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.5	0.38	0.023	4
471	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.75	0.032	6
473	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.25	0.5	0.5	0.032	8
478	1	*S*.Typhi	-	+	+	0.016	0.094	3	2	0.016	0.064	0.38	0.032	3
482	1	*S*.Typhi	-	+	+	0.008	0.064	3	1.5	0.008	0.032	0.38	0.023	4
483	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.19	0.38	0.38	0.023	4
484	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.5	0.032	12
492	1	*S*.Typhi	-	+	+	0.012	0.125	6	2	0.008	0.047	0.75	0.047	6
494	1	*S*.Paratyphi A	+	-	-	0.5	0.25	16	>256	0.75	1.5	2	0.125	12
497	1	*S*.Typhi	-	+	+	0.016	0.25	12	3	0.023	0.064	1	0.047	8
498	1	*S*.Typhi	-	+	+	0.25	0.25	8	>256	0.38	0.75	1	0.094	8
500	1	*S*.Typhi	-	+	+	0.023	0.125	6	3	0.012	0.047	0.75	0.032	6
503	1	*S*.Paratyphi A	+	-	-	0.5	0.25	12	>256	0.75	1.5	2	0.19	16
504	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.38	0.75	0.032	6
506	1	*S*.Typhi	-	+	+	0.25	0.19	>256	>256	0.38	0.5	>256	>32	8
508	1	*S*.Typhi	-	+	+	0.016	0.125	6	2	0.012	0.047	0.75	0.047	6
512	1	*S*.Typhi	-	+	+	0.25	0.19	8	>256	0.38	0.5	1	0.032	8
513	1	*S*.Typhi	-	+	+	0.19	0.19	8	>256	0.38	0.5	1	0.032	8
514	1	*S*.Typhi	-	+	+	0.023	0.19	8	1.5	0.032	0.064	1	0.064	8
516	1	*S*.Typhi	-	+	+	0.25	0.19	8	>256	0.38	0.75	1	0.032	8
518	1	*S*.Typhi	-	+	+	0.125	0.19	8	>256	0.38	0.5	0.75	0.064	6
519	1	*S*.Paratyphi A	+	-	-	1	0.25	64	>256	1.5	4	3	0.38	24
523	1	*S*.Paratyphi A	+	-	-	0.38	0.19	16	>256	0.5	1.5	1.5	0.125	12
530	1	*S*.Typhi	-	+	+	0.016	0.125	8	3	0.023	0.064	0.5	0.032	8
533	1	*S*.Typhi	-	+	+	0.008	0.094	6	2	0.008	0.032	0.75	0.023	8
538	1	*S*.Typhi	-	+	+	0.125	0.19	8	>256	0.38	0.38	1	0.032	6
540	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.38	0.75	0.032	8
541	1	*S*.Typhi	-	+	+	0.006	0.094	8	1.5	0.008	0.032	0.5	0.032	6
544	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.38	0.75	0.023	8
548	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	8
549	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	12
550	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.5	0.75	0.023	8
552	1	*S*.Typhi	-	+	+	0.125	0.19	8	>256	0.25	0.5	1	0.032	8

555	1	*S*.Typhi	-	+	+	0.19	0.094	8	>256	0.25	0.38	0.5	0.032	8
557	1	*S*.Paratyphi A	+	-	-	0.38	0.19	12	>256	0.5	1.5	1.5	0.125	12
558	1	*S*.Typhi	-	+	+	0.012	0.094	8	2	0.012	0.047	0.75	0.047	6
560	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.38	0.75	0.032	8
561	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	8
564	1	*S*.Typhi	-	+	+	0.125	0.19	8	>256	0.25	0.38	1	0.032	8
567	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.5	0.75	0.032	8
568	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	1	0.032	8
569	1	*S*.Typhi	-	+	+	0.016	0.125	8	3	0.016	0.047	0.5	0.032	8
570	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.5	0.75	0.032	8
571	1	*S*.Typhi	-	+	+	0.012	0.094	6	2	0.012	0.047	0.5	0.032	6
574	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	1	0.032	6
577	1	*S*.Typhi	-	+	+	0.125	0.125	12	>256	0.25	0.38	1	0.032	6
578	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.25	0.5	0.75	0.032	8
579	1	*S*.Paratyphi A	+	-	-	0.38	0.19	12	>256	0.5	1.5	1.5	0.125	12
581	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.38	0.5	1	0.032	8
585	1	*S*.Typhi	-	+	+	0.125	0.125	8	>256	0.38	0.5	0.75	0.032	8
587	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	8
589	1	*S*.Typhi	-	+	+	0.125	0.125	4	>256	0.38	0.5	0.75	0.032	8
593	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.5	0.5	0.023	4
603	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.032	8
607	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.023	12
610	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.38	0.5	0.75	0.023	8
611	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.38	0.5	0.75	0.032	16
612	1	*S*.Typhi	-	+	+	0.25	0.125	8	>256	0.38	0.5	0.75	0.032	16
614	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.25	0.5	0.5	0.032	12
619	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	12
621	1	*S*.Typhi	-	+	+	0.25	0.125	8	>256	0.25	0.5	0.75	0.032	16
622	1	*S*.Typhi	-	+	+	0.125	0.064	4	>256	0.19	0.38	0.38	0.016	8
624	1	*S*.Paratyphi A	+	-	-	0.5	0.125	8	>256	0.5	1.5	1	0.125	24
627	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	12
628	1	*S*.Typhi	-	+	+	0.19	0.094	6	>256	0.19	0.38	0.38	0.032	8
629	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	12
630	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.25	0.38	0.5	0.032	12
632	1	*S*.Paratyphi A	+	-	-	1.5	0.19	32	>256	1.5	6	2	0.19	48

633	1	*S*.Typhi	-	+	+	0.19	0.064	4	>256	0.25	0.38	0.5	0.023	6
634	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.25	0.5	0.5	0.032	8
637	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	12
638	1	*S*.Typhi	-	+	+	0.125	0.125	6	>256	0.25	0.38	0.75	0.023	8
639	1	*S*.Typhi	-	+	+	0.19	0.125	8	>256	0.25	0.5	0.75	0.032	12
643	1	*S*.Typhi	-	+	+	0.016	0.064	6	1.5	0.016	0.064	0.5	0.064	12
645	1	*S*.Typhi	-	+	+	0.19	0.094	3	>256	0.19	0.38	0.38	0.023	8
646	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	1.5	1.5	0.125	24
648	1	*S*.Typhi	-	+	+	0.125	0.094	3	>256	0.19	0.38	0.38	0.38	16
652	1	*S*.Typhi	-	+	+	0.012	0.047	3	1.5	0.012	0.032	0.38	0.032	12
659	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.5	0.75	0.032	24
661	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.25	0.5	0.5	0.032	16
662	1	*S*.Typhi	-	+	+	0.016	0.064	3	2	0.016	0.047	0.5	0.047	12
665	1	*S*.Typhi	-	+	+	0.064	0.125	2	>256	0.19	0.19	0.38	0.016	1.5
666	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.75	0.047	12
669	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.75	0.047	16
670	1	*S*.Typhi	-	+	+	0.19	0.19	4	>256	0.38	0.5	0.75	0.032	16
672	1	*S*.Typhi	-	+	+	0.125	0.064	4	>256	0.125	0.38	0.38	0.032	8
673	1	*S*.Typhi	-	+	+	0.094	0.064	3	>256	0.125	0.25	0.38	0.032	8
679	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.25	0.5	0.75	0.025	12
682	1	*S*.Typhi	-	+	+	0.19	0.094	3	>256	0.25	0.38	0.38	0.023	16
685	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.75	0.047	16
688	1	*S*.Typhi	-	+	+	0.064	0.125	2	>256	0.19	0.25	0.5	0.032	1.5
691	1	*S*.Paratyphi A	+	-	-	0.19	0.125	3	>256	0.25	0.75	1	0.19	3
692	1	*S*.Typhi	-	+	+	0.38	0.19	8	>256	0.5	1.5	0.75	0.094	4
693	1	*S*.Typhi	-	+	+	0.032	0.064	1.5	48	0.064	0.125	0.25	0.064	1
694	1	*S*.Typhi	-	+	+	0.064	0.094	2	>256	0.125	0.19	0.75	0.064	2
695	1	*S*.Typhi	-	+	+	0.064	0.094	3	64	0.25	0.25	0.5	0.047	2
697	1	*S*.Typhi	-	+	+	0.032	0.047	2	24	0.047	0.19	0.38	0.016	2
698	1	*S*.Typhi	-	+	+	0.047	0.125	2	48	0.38	0.25	0.4	0.032	3
705	1	*S*.Paratyphi A	+	-	-	0.38	0.19	4	>256	0.5	1.5	1	0.125	8
707	1	*S*.Typhi	-	+	+	1	0.25	16	>256	1	4	1.5	0.38	24
714	1	*S*.Typhi	-	+	+	0.003	0.094	2	0.75	0.006	0.023	0.5	0.047	1.5
722	1	*S*.Paratyphi A	+	-	-	0.25	0.125	3	>256	0.5	1	0.75	0.064	2
723	1	*S*.Paratyphi A	+	-	-	0.023	0.19	4	2	0.016	0.38	1	0.094	8

729	1	*S*.Typhi	-	+	+	0.047	0.094	3	>256	0.125	0.25	0.75	0.032	0.75
736	1	*S*.Paratyphi A	+	-	-	0.19	0.25	4	>256	0.25	1	1	0.094	1.5
737	1	*S*.Typhi	-	+	+	0.008	0.19	3	1.5	0.012	0.023	0.5	0.032	1
740	1	*S*.Typhi	-	+	+	0.125	0.064	3	>256	0.125	0.38	0.5	0.064	2
741	1	*S*.Paratyphi A	+	-	-	0.25	0.125	3	>256	0.38	0.75	0.75	0.064	1
746	1	*S*.Paratyphi A	+	-	-	0.25	0.19	4	>256	0.25	1	1.5	0.064	6
747	1	*S*.Typhi	-	+	+	0.023	0.094	2	96	0.19	0.25	0.5	0.023	1
748	1	*S*.Paratyphi A	+	-	-	0.38	0.19	4	>256	0.38	1.5	1	0.125	8
749	1	*S*.Paratyphi A	+	-	-	0.38	0.19	4	>256	0.5	1.5	1	0.125	6
750	1	*S*.Paratyphi A	+	-	-	0.19	0.19	3	>256	0.25	1	0.75	0.094	1.5
753	1	*S*.Typhi	-	+	+	0.006	0.047	4	1	0.004	0.016	0.5	0.016	0.75
754	1	*S*.Typhi	-	+	+	0.032	0.125	2	>256	0.19	0.19	0.38	0.047	1
757	1	*S*.Typhi	-	+	+	0.032	0.19	3	48	0.125	0.25	0.75	0.023	1.5
758	1	*S*.Typhi	-	+	+	0.047	0.125	2	192	0.125	0.25	0.38	0.023	1.5
760	1	*S*.Typhi	-	+	+	0.006	0.094	2	1	0.012	0.023	0.38	0.023	1.5
762	1	*S*.Typhi	-	+	+	0.064	0.064	2	>256	0.125	0.25	0.38	0.047	2
765	1	*S*.Typhi	-	+	+	0.003	0.064	1.5	0.5	0.006	0.023	0.38	0.023	2
766	1	*S*.Paratyphi A	+	-	-	0.38	0.25	4	>256	0.5	1.5	0.75	0.125	4
767	1	*S*.Typhi	-	+	+	0.032	0.094	2	32	0.25	0.25	0.5	0.023	6
768	1	*S*.Paratyphi A	+	-	-	0.5	0.25	6	>256	0.5	3	0.75	0.064	4
771	1	*S*.Paratyphi A	+	-	-	0.38	0.125	3	>256	0.25	1	0.75	0.047	1.5
772	1	*S*.Typhi	-	+	+	0.125	0.064	3	>256	0.125	0.38	0.5	0.094	2
774	1	*S*.Typhi	-	+	+	0.19	0.19	4	>256	0.25	0.5	0.75	0.064	3
775	1	*S*.Typhi	-	+	+	0.047	0.125	3	>256	0.19	0.38	0.5	0.047	3
777	1	*S*.Typhi	-	+	+	0.064	0.25	3	64	0.25	0.38	0.5	0.032	2
779	1	*S*.Paratyphi A	+	-	-	0.38	0.38	8	>256	0.75	1.5	1.5	0.094	6
782	1	*S*.Paratyphi A	+	-	-	0.38	0.19	3	>256	0.5	1.5	0.75	0.25	6
785	1	*S*.Paratyphi A	+	-	-	0.25	0.125	3	>256	0.5	1	0.75	0.064	2
786	1	*S*.Typhi	-	+	+	0.19	0.094	3	>256	0.125	0.38	0.5	0.032	2
787	1	*S*.Typhi	-	+	+	0.006	0.125	3	0.75	0.012	0.016	0.5	0.023	2
791	1	*S*.Typhi	-	+	+	0.064	0.19	6	96	0.19	0.38	0.75	0.064	2
793	1	*S*.Typhi	-	+	+	0.064	0.125	4	>256	0.19	0.25	0.5	0.032	2
794	1	*S*.Typhi	-	+	+	0.094	0.094	4	64	0.094	0.38	0.5	0.032	4
795	1	*S*.Typhi	-	+	+	0.064	0.094	3	48	0.25	0.38	0.5	0.047	4
796	1	*S*.Typhi	-	+	+	0.047	0.19	2	>256	0.19	0.25	0.38	0.047	1.5

805	1	*S*.Paratyphi A	+	-	-	0.19	0.25	3	>256	0.25	1.5	0.75	0.094	2
806	1	*S*.Typhi	-	+	+	0.003	0.064	1.5	0.75	0.004	0.016	0.5	0.032	1
808	1	*S*.Typhi	-	+	+	0.064	0.125	2	>256	0.25	0.5	0.75	0.023	2
809	1	*S*.Typhi	-	+	+	0.047	0.094	2	>256	0.25	0.25	0.5	0.016	1.5
810	1	*S*.Paratyphi A	+	-	-	0.25	0.125	3	>256	0.38	0.75	0.75	0.047	2
811	1	*S*.Typhi	-	+	+	0.004	0.064	2	0.75	0.008	0.023	0.38	0.023	2
821	1	*S*.Typhi	-	+	+	0.25	0.094	2	>256	0.25	0.25	0.5	0.047	2
822	1	*S*.Typhi	-	+	+	0.006	0.094	2	2	0.006	0.023	0.38	0.047	0.75
824	1	*S*.Typhi	-	+	+	0.047	0.19	2	>256	0.25	0.38	0.5	0.023	3
826	1	*S*.Typhi	-	+	+	0.047	0.125	2	>256	0.19	0.25	0.5	0.032	2
829	1	*S*.Typhi	-	+	+	0.004	0.047	1.5	0.75	0.006	0.032	0.25	0.023	3
831	1	*S*.Typhi	-	+	+	0.047	0.19	2	>256	0.25	0.25	0.5	0.064	0.75
833	1	*S*.Typhi	-	+	+	0.064	0.094	2	>256	0.19	0.25	0.5	0.032	1.5
835	1	*S*.Typhi	-	+	+	0.047	0.125	2	>256	0.19	0.25	0.25	0.032	2
838	1	*S*.Typhi	-	+	+	0.064	0.094	3	96	0.125	0.25	0.38	0.032	0.75
841	1	*S*.Typhi	-	+	+	0.004	0.094	2	0.75	0.006	0.023	0.25	0.023	0.38
843	1	*S*.Paratyphi A	+	-	-	0.032	0.19	4	3	0.047	0.125	0.75	0.094	3
846	1	*S*.Typhi	-	+	+	0.047	0.125	1.5	48	0.19	0.38	0.25	0.023	2
849	1	*S*.Paratyphi A	+	-	-	0.25	0.125	3	>256	0.38	0.75	0.75	0.064	2
850	1	*S*.Typhi	-	+	+	0.006	0.047	2	0.75	0.006	0.032	0.5	0.023	2
851	1	*S*.Paratyphi A	+	-	-	0.047	0.125	2	>256	0.5	0.25	0.5	0.023	1.5
18	30	*S*.Paratyphi A	+	-	-	0.38	0.38	8	>256	0.5	2	2	0.125	16
28	30	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.38	0.5	0.023	8
30	22	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	2	1.5	0.094	16
140	25	*S*.Typhi	-	+	+	0.125	0.125	4	>256	0.19	0.38	0.5	0.032	6
161	25	*S*.Paratyphi A	+	-	-	0.38	0.25	8	>256	0.5	1.5	1.5	0.094	16
182	90	*S*.Paratyphi A	+	-	-	0.38	0.19	6	>256	0.38	1.5	1.5	0.094	16
237	30	*S*.Typhi	-	+	+	0.016	0.125	4	2	0.016	0.064	0.5	0.032	6
368	15	*S*.Paratyphi A	+	-	-	0.5	0.25	8	>256	0.5	1.5	2	0.125	16
455	32	*S*.Typhi	-	+	+	0.012	0.094	4	2	0.008	0.032	0.5	0.047	8
455	90	*S*.Typhi	-	+	+	0.016	0.125	4	2	0.012	0.016	0.75	0.047	8
479	40	*S*.Typhi	-	+	+	0.008	0.125	4	1.5	0.008	0.023	0.5	0.032	8
554	40	*S*.Paratyphi A	+	-	-	0.38	0.25	6	>256	0.5	1.5	1.5	0.094	12
568	30	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.25	0.5	0.75	0.023	8
570	8	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.38	0.5	0.75	0.023	12

585	42	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.38	0.5	0.75	0.032	8
614	26	*S*.Typhi	-	+	+	0.19	0.19	6	>256	0.38	0.5	1	0.032	8
416	30	*S*.Typhi	-	+	+	0.047	0.25	8	4	0.032	0.19	1.5	0.125	16
692	33	*S*.Paratyphi A	+	-	-	0.5	0.25	6	>256	0.5	4	1	0.125	3
735	26	*S*.Typhi	-	+	+	0.094	2	3	>256	0.25	0.75	0.75	0.38	3
765	7	*S*.Paratyphi A	+	-	-	0.38	0.125	4	>256	0.25	1	1	0.094	2
774	42	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.25	0.5	0.75	0.047	2
Stool Isolates														
18	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	12
18	30	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.19	8
42	1	*S*.Typhi	-	+	+	0.016	0.064	4	2	0.012	0.047	0.75	0.047	4
59	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.008	0.047	0.38	0.047	6
76	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.25	0.5	0.5	0.032	6
82	1	*S*.Typhi	-	+	+	0.012	0.064	3	2	0.012	0.047	0.38	0.032	6
97	1	*S*.Typhi	-	+	+	0.19	0.094	4	>256	0.25	0.5	0.38	0.023	6
97	30	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.38	0.5	0.5	0.032	6
98	1	*S*.Typhi	-	+	+	0.016	0.064	4	2	0.08	0.047	0.38	0.032	4
104	1	*S*.Typhi	-	+	+	0.125	0.064	4	6	0.125	0.25	0.38	0.023	6
215	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.75	0.75	0.032	8
219	1	*S*.Typhi	-	+	+	0.016	0.064	6	2	0.012	0.047	0.38	0.032	8
224	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	4
224	30	*S*.Paratyphi A	+	-	-	0.75	0.5	128	>256	1	3	3	1.5	8
295	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.75	0.75	0.032	16
297	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.008	0.047	0.5	0.047	8
304	1	*S*.Typhi	-	+	+	0.064	0.094	6	3	0.012	0.125	1	0.094	4
320	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	8
321	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.38	2	1.5	0.125	16
338	1	*S*.Paratyphi A	+	-	-	0.5	0.19	8	>256	0.5	2	1.5	0.125	12
351	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.75	0.75	0.047	16
373	1	*S*.Typhi	-	+	+	0.012	0.064	4	1.5	0.008	0.047	0.38	0.047	8

401	1	*S*.Typhi	-	+	+	0.19	0.125	4	>256	0.125	0.5	1	0.125	6
434	1	*S*.Typhi	-	+	+	0.016	0.094	4	2	0.012	0.047	0.5	0.047	8
439	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.75	0.75	0.023	6
448	1	*S*.Typhi	-	+	+	0.016	0.064	4	2	0.008	0.047	0.5	0.047	8
471	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.75	0.5	0.032	6
472	1	*S*.Typhi	-	+	+	0.25	0.094	4	>256	0.25	0.5	0.38	0.032	8
504	1	*S*.Typhi	-	+	+	0.125	0.094	4	>256	0.125	0.38	0.5	0.032	8
509	1	*S*.Typhi	-	+	+	0.023	0.064	6	3	0.016	0.064	0.38	0.047	6
517	1	*S*.Typhi	-	+	+	0.023	0.094	6	3	0.012	0.064	0.5	0.064	4
570	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.75	0.75	0.047	6
571	1	*S*.Typhi	-	+	+	0.016	0.094	6	2	0.012	0.047	0.5	0.047	8
590	1	*S*.Typhi	-	+	+	0.25	0.125	6	>256	0.38	0.5	0.75	0.047	6
603	1	*S*.Typhi	-	+	+	0.19	0.125	6	>256	0.38	0.75	0.75	0.032	6
645	1	*S*.Typhi	-	+	+	0.19	0.094	3	>256	0.38	0.5	0.5	0.032	8
672	1	*S*.Typhi	-	+	+	0.094	0.064	3	64	0.125	0.25	0.38	0.032	8
694	1	*S*.Typhi	-	+	+	1.5	0.19	3	96	0.19	0.25	0.38	0.047	6
754	1	*S*.Typhi	-	+	+	1.5	0.19	2	>256	0.25	0.19	0.38	0.047	4
393	1	*S*.Typhi	-	+	+	0.004	0.094	1.5	0.5	0.008	0.023	0.19	0.023	2

9.8 Serotyping and MICs of *Salmonella* isolates from O1TY (Gati v Oflox) study

01TY			**Serology**			**MIC**							
No	**Day**	**Culture Result**	**O:9**	**Vi**	**O:2**	**NA**	**CI**	**OF**	**GA**	**CL**	**TX**	**AZ**	**AUG**
4	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	3	0.094	1.5	0.5
13	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	4	0.125	4	0.75
14	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	2	0.094	3	0.75
18	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.25	3	0.75
19	1	S.Typhi	+	+	-	>256	0.25	0.25	0.064	4	0.25	2	0.75
20	1	S.Typhi	+	+	-	1.5	0.012	0.023	0.008	4	0.094	1	0.5

22	1	S.Typhi	+	+	-	2	0.016	0.032	0.012	6	0.094	2	0.75
26	1	S.Typhi	+	+	-	0.75	0.008	0.016	0.006	3	0.047	1	0.38
28	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	3	0.19	3	0.5
29	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	4	0.19	2	0.5
31	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	3	0.19	3	0.75
35	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.38	3	1
36	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	3	0.75
37	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	6	0.25	3	1
41	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	3	0.75
42	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	4	0.19	2	0.75
45	1	S.Typhi	+	+	-	>256	0.25	0.5	0.094	4	0.25	4	0.75
46	1	S.Typhi	+	+	-	0.75	0.008	0.023	0.008	4	0.094	2	0.38
51	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	6	0.25	4	1
52	1	S.Typhi	+	+	-	6	0.19	0.19	0.064	4	0.19	3	0.75
61	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	3	0.094	2	0.5
65	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	4	0.75
66	1	S.Typhi	+	+	-	1.5	0.016	0.023	0.006	4	0.19	4	0.75
69	1	S.Typhi	+	+	-	96	0.19	0.75	0.19	4	0.19	3	0.75
72	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	6	0.25	3	1
73	1	S.Typhi	+	+	-	96	0.125	0.25	0.094	4	0.094	2	0.75
75	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	6	0.19	3	1
77	1	S.Typhi	+	+	-	>256	1.5	0.38	0.094	6	0.19	3	1
79	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	6	0.25	4	1.5
80	1	S.Typhi	+	+	-	2	0.012	0.023	0.008	4	0.094	1	0.5
83	1	S.Typhi	+	+	-	3	0.094	0.19	0.064	4	0.19	2	1
86	1	S.Typhi	+	+	-	1.5	0.006	0.32	0.012	2	0.094	1.5	0.75
88	1	S.Typhi	+	+	-	96	0.25	0.25	0.094	4	0.19	1.5	0.75
90	1	S.Paratyphi A	-	-	+	>256	0.25	1	0.25	4	0.125	6	1
92	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.19	1.5	3
95	1	S.Typhi	+	+	-	128	0.19	0.25	0.094	3	0.19	1.5	0.75
96	1	S.Typhi	+	+	-	64	0.19	0.25	0.094	2	0.125	1.5	1
97	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.25	3	0.25	2	1
98	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.25	4	0.25	2	1
99	1	S.Typhi	+	+	-	1.5	0.008	0.016	0.008	4	0.19	1	0.5
102	1	S.Typhi	+	+	-	>256	0.38	0.5	0.125	3	0.19	3	0.5

103	1	S.Paratyphi A	-	-	+	>256	0.19	0.5	0.19	4	0.19	6	1
105	1	S.Typhi	+	+	-	1	0.008	0.032	0.008	4	0.125	ND	0.5
107	1	S.Typhi	+	+	-	48	0.064	0.25	0.094	4	0.125	2	0.75
109	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.25	6	0.19	3	0.75
112	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.25	6	0.19	3	1
128	1	S.Typhi	+	+	-	64	0.19	0.25	0.094	4	0.19	1.5	0.75
132	1	S.Typhi	+	+	-	96	0.19	0.25	0.094	4	0.19	1.5	0.75
134	1	S.Typhi	+	+	-	0.75	0.004	0.012	0.004	3	0.094	1	0.75
139	1	S.Typhi	+	+	-	2	0.004	0.023	0.006	3	0.094	2	0.38
142	1	S.Typhi	+	+	-	128	0.19	0.19	0.094	3	0.094	1.5	0.75
149	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.38	6	0.25	3	1.5
150	1	S.Typhi	+	+	-	1.5	0.016	0.047	0.016	3	0.125	ND	0.5
151	1	S.Typhi	+	+	-	1.5	0.004	0.023	0.008	4	0.125	0.75	0.75
152	1	S.Typhi	+	+	-	96	0.19	0.25	0.094	3	0.125	1.5	0.5
154	1	S.Typhi	+	+	-	1	0.006	0.023	0.006	3	0.094	0.75	0.75
157	1	S.Typhi	+	+	-	96	0.19	0.25	0.94	3	0.125	1	0.5
161	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.19	3	1
163	1	S.Typhi	+	+	-	1	0.012	0.023	0.008	3	0.094	1.5	0.75
165	1	S.Paratyphi A	-	-	+	>256	0.19	0.5	0.19	3	0.19	2	1
170	1	S.Typhi	+	+	-	64	0.19	0.19	0.064	3	0.25	1.5	0.75
171	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	4	0.38	4	1.5
172	1	S.Typhi	+	+	-	>256	0.19	0.5	0.125	4	0.094	3	0.5
176	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	3	0.19	2	1.5
178	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	6	0.38	3	1.5
180	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.25	3	1
184	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	6	0.25	2	1
185	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.25	3	1.5
191	1	S.Typhi	+	+	-	64	0.125	0.25	0.094	3	0.19	1	0.75
205	1	S.Typhi	+	+	-	64	0.25	0.25	0.064	4	0.25	1.5	0.75
208	1	S.Typhi	+	+	-	64	0.25	0.25	0.094	1.5	0.75	3	0.25
211	1	S.Typhi	+	+	-	64	0.25	0.38	0.094	4	0.19	1.5	1
216	1	S.Typhi	+	+	-	64	0.25	0.25	0.094	6	0.19	2	1
218	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	3	0.75
222	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	8	0.25	6	1.5
223	1	S.Typhi	+	+	-	>256	0.19	0.25	0.094	4	0.25	4	0.75

226	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	4	0.19	3	0.75
227	1	S.Paratyphi A	-	-	+	>256	0.75	0.75	0.38	6	0.25	4	1
230	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	4	0.19	3	0.75
233	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	2	0.094	ND	0.5
242	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	4	0.75
250	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	4	0.25	ND	1.5
256	1	S.Typhi	+	+	-	>256	0.25	0.25	0.047	3	0.19	ND	0.75
257	1	S.Typhi	+	+	-	1	0.006	0.032	0.008	3	0.094	1.5	0.5
258	1	S.Typhi	+	+	-	1	0.008	0.016	0.012	4	0.064	3	0.75
261	1	S.Typhi	+	+	-	1	0.006	0.016	0.006	4	0.094	2	0.5
264	1	S.Typhi	+	+	-	0.75	0.006	0.023	0.008	4	0.094	2	0.75
267	1	S.Typhi	+	+	-	0.75	0.008	0.023	0.012	3	0.064	ND	0.5
270	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	8	0.19	4	1
271	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	6	0.19	4	1.5
274	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	3	0.19	4	0.75
276	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	8	0.19	6	1
279	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	3	0.19	2	1
283	1	S.Typhi	+	+	-	1.5	0.008	0.23	0.012	3	0.094	3	0.5
286	1	S.Typhi	+	+	-	1	0.006	0.023	0.008	4	0.094	1.5	0.75
301	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	3	0.5
305	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.25	2	0.75
311	1	S.Typhi	+	+	-	1	0.006	0.016	0.008	4	0.094	1.5	0.75
312	1	S.Typhi	+	+	-	1.5	0.012	0.023	0.012	3	0.125	0.5	1
313	1	S.Typhi	+	+	-	1.5	0.006	0.023	0.008	4	0.19	2	0.5
314	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.25	2	0.75
315	1	S.Typhi	+	+	-	1.5	0.006	0.016	0.008	4	0.125	1	0.38
316	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	4	0.25	3	1
326	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	6	0.25	3	1
327	1	S.Typhi	+	+	-	>256	0.38	0.25	0.094	6	0.25	6	0.75
332	1	S.Typhi	+	+	-	1.5	0.008	0.023	0.012	3	0.125	1.5	0.38
337	1	S.Typhi	+	+	-	1.5	0.006	0.023	0.038	3	0.19	1.5	0.38
339	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	6	0.25	4	1
341	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	6	0.19	6	1.5
342	1	S.Typhi	+	+	-	>256	0.125	0.19	0.094	6	0.125	2	0.5
347	1	S.Typhi	+	+	-	>256	0.25	0.38	0.125	4	0.19	3	0.75

348	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	3	0.125	4	1
349	1	S.Typhi	+	+	-	1.5	0.008	0.023	0.006	3	0.094	1.5	0.5
356	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	8	0.25	4	1.5
366	1	S.Typhi	+	+	-	1.5	0.008	0.023	0.006	4	0.125	2	0.5
369	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.19	4	1
370	1	S.Typhi	+	+	-	>256	0.25	0.25	0.047	4	0.19	4	1
371	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	4	0.19	6	0.75
374	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	6	0.19	4	0.75
375	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	6	0.19	4	0.75
380	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	4	0.19	4	1
386	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	6	0.19	4	1
388	1	S.Paratyphi A	-	-	+	>256	0.75	1.5	0.38	6	0.19	4	1
391	1	S.Typhi	+	+	-	1.5	0.008	0.023	0.006	4	0.094	4	0.5
394	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	4	0.19	3	0.75
396	1	S.Typhi	+	+	-	>256	0.5	0.75	0.38	6	0.25	3	0.75
397	1	S.Typhi	+	+	-	>256	0.25	0.38	0.064	2	0.25	4	0.75
398	1	S.Typhi	+	+	-	>256	0.5	1	0.38	4	0.25	2	1
403	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.25	3	1
405	1	S.Typhi	+	+	-	0.75	0.006	0.016	0.006	4	0.25	1	0.5
408	1	S.Typhi	+	+	-	1.5	0.012	0.023	0.008	1.5	0.064	1	0.5
410	1	S.Typhi	+	+	-	4	0.094	0.125	0.064	2	0.125	1.5	0.5
413	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.19	3	1
416	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.38	6	0.38	3	1.5
420	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.38	6	0.25	3	0.75
421	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.25	2	0.75
424	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.5	6	0.25	6	1.5
426	1	S.Typhi	+	+	-	>256	0.5	1	0.5	6	0.25	4	1
430	1	S.Paratyphi A	-	-	+	>256	0.75	0.38	0.38	6	0.25	4	1
431	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.5	4	0.19	3	0.75
432	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	4	0.19	3	0.75
433	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	4	0.125	1.5	0.5
440	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.25	4	0.19	2	0.75
441	1	S.Typhi	+	+	-	>256	0.25	0.38	0.125	4	0.19	3	0.75
443	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	3	0.19	ND	0.75
442	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.19	4	1

444	1	S.Typhi	+	+	-	>256	0.25	0.25	0.064	6	0.25	6	1
445	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	6	0.25	3	0.75
446	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	6	0.25	4	1.5
447	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	4	0.19	3	0.75
448	1	S.Typhi	+	+	-	1.5	0.012	0.032	0.008	4	0.094	3	0.75
454	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.38	6	0.25	3	1
467	1	S.Paratyphi A	-	-	+	>256	0.5	0.75	0.38	4	0.19	ND	1
472	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	6	0.38	3	1
476	1	S.Typhi	+	+	-	>256	0.38	0.38	0.094	4	0.19	3	1
479	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.25	4	1.5
481	1	S.Typhi	+	+	-	>256	0.25	0.38	0.125	3	0.25	3	1
483	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.38	6	0.25	4	1
484	1	S.Typhi	+	+	-	0.5	0.006	0.016	0.006	2	0.094	ND	0.5
492	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	4	0.25	3	0.75
496	1	S.Paratyphi A	-	-	+	>256	0.75	1.5	0.38	6	0.25	6	1
498	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	4	0.125	4	0.75
505	1	S.Paratyphi A	-	-	+	>256	0.5	1.5	0.38	6	0.19	4	1
515	1	S.Typhi	+	+	-	>256	0.38	0.5	0.125	6	0.19	4	0.75
516	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	4	0.25	4	0.75
518	1	S.Typhi	+	+	-	>256	0.25	0.5	0.19	6	0.19	3	0.75
519	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	8	0.25	3	0.75
520	1	S.Typhi	+	+	-	1.5	0.008	0.023	0.006	4	0.094	6	0.75
521	1	S.Paratyphi A	-	-	+	>256	0.5	1.5	0.5	8	0.25	6	1
523	1	S.Paratyphi A	-	-	+	>256	0.25	1	0.38	4	0.125	ND	0.75
524	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	3	0.25	ND	0.75
525	1	S.Typhi	+	+	-	>256	0.125	0.38	0.094	3	0.125	ND	0.5
528	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	3	0.19	4	0.75
529	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	4	0.19	6	1
530	1	S.Paratyphi A	-	-	+	>256	0.25	1	0.38	4	0.19	ND	0.75
532	1	S.Typhi	+	+	-	1	0.004	0.016	0.006	2	0.064	3	0.75
533	1	S.Paratyphi A	-	-	+	>256	0.5	1.5	0.38	4	0.25	4	0.75
534	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	6	0.19	4	1
536	1	S.Typhi	+	+	-	96	0.25	0.38	0.094	1.5	0.25	1.5	0.75
537	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.28	3	0.19	4	1.5

538	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	4	0.19	ND	0.75
539	1	S.Paratyphi A	-	-	+	>256	0.5	1.5	0.38	4	0.25	4	1.5
541	1	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.25	4	0.19	4	1
544	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	3	0.19	6	1.5
545	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	4	0.19	4	1.5
546	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	4	0.19	6	2
547	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	4	0.19	4	1.5
548	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.125	3	0.75
550	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	3	0.19	4	1.5
553	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	3	0.125	4	1
554	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	4	0.125	6	1.5
555	1	S.Typhi	+	+	-	>256	0.38	0.38	0.125	2	0.19	4	0.75
557	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	3	0.19	3	0.75
559	1	S.Typhi	+	+	-	128	0.125	0.25	0.064	3	0.094	3	0.75
561	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	4	0.75
562	1	S.Typhi	+	+	-	1	0.008	0.023	0.006	2	0.064	2	0.75
566	1	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	4	0.125	4	1
571	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	4	0.19	4	1
572	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.25	4	0.19	4	1
575	1	S.Typhi	+	+	-	96	0.25	0.38	0.094	3	0.25	4	0.75
582	1	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	3	0.25	4	0.75
585	1	S.Typhi	+	+	-	>256	0.25	0.25	0.094	3	0.19	3	0.5
592	1	S.Paratyphi A	-	-	+	>256	0.25	1.5	0.38	4	0.19	4	0.75
596	1	S.Typhi	+	+	-	>256	0.19	0.25	0.064	3	0.125	4	0.5
599	1	S.Paratyphi A	-	-	+	>256	0.25	0.75	0.38	3	0.125	4	0.75
600	1	S.Typhi	+	+	-	>256	0.125	0.19	0.094	2	0.125	3	0.5
610	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	3	0.19	3	0.5
612	1	S.Paratyphi A	-	-	+	>256	0.38	0.75	0.25	3	0.125	ND	1
616	1	S.Paratyphi A	-	-	+	>256	0.25	0.38	0.125	4	0.094	ND	0.5
621	1	S.Typhi	+	+	-	>256	0.25	0.38	0.125	4	0.19	6	0.75
628	1	S.Typhi	+	+	-	>256	0.19	0.38	0.094	3	0.19	4	0.5
56	22	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	ND	0.5
60	21	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	6	0.19	ND	0.75
60	51	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	6	0.19	ND	1
101	8	S.Paratyphi A	-	-	+	>256	0.38	1	0.38	6	0.19	ND	1

138	32	S.Typhi	+	+	-	>256	0.19	0.38	0.094	4	0.125	ND	0.5
160	15	S.Paratyphi A	-	-	+	>256	0.5	1	0.38	6	0.19	ND	1
172	20	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	6	0.25	ND	1
191	22	S.Typhi	+	+	-	>256	0.19	0.38	0.094	3	0.125	ND	0.5
203	29	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.25	ND	0.75
256	21	S.Typhi	+	+	-	>256	0.25	0.38	0.125	4	0.19	ND	ND
454	22	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	4	0.125	ND	0.75
564	29	S.Paratyphi A	-	-	+	>256	0.38	1.5	0.38	4	0.19	ND	0.5
549	180	S.Typhi	+	+	-	>256	0.19	0.38	0.094	3	0.125	ND	0.5
628	60	S.Typhi	+	+	-	>256	0.25	0.38	0.94	3	0.19	ND	0.75
Stool Isolates													
26	1	S.Typhi	+	+	-	1	0.012	0.032	0.008	3	0.064	ND	0.5
61	1	S.Typhi	+	+	-	>256	0.19	0.38	0.125	4	0.125	ND	0.38
84	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.94	ND	0.5
205	1	S.Typhi	+	+	-	>256	0.25	0.38	0.094	4	0.19	ND	0.75
242	30	S.Typhi	+	+	-	>256	0.125	0.38	0.094	4	0.125	ND	0.5
312	1	S.Paratyphi A	-	-	+	6	0.016	0.094	0.032	4	0.094	ND	0.75
525	1	S.Typhi	+	+	-	>256	0.19	0.38	0.125	4	0.19	ND	0.75
561	1	S.Typhi	+	+	-	>256	0.25	0.38	0.125	4	0.19	ND	0.5

9.9 The one hundred and thirteen Typhi chromosomal loci assayed in *S*. Typhi isolates using the iPLEX Gold assay at the Sanger Institute

Position in	Name	Ancestral	Derived	Source (reference)
6317	BiP1	C	T	Roumagnac 2006
955273	BiP10	G	A	Roumagnac 2006
2271858	BiP102	G	A	Roumagnac 2006
2463900	BiP103	G	A	Roumagnac 2006
4391261	BiP104	C	T	Roumagnac 2006

2221217	BiP106	G	A	Roumagnac 2006
2412815	BiP107	T	C	Roumagnac 2006
2947124	BiP108	G	A	Roumagnac 2006
4218089	BiP109	C	T	Roumagnac 2006
958002	BiP11	G	A	Roumagnac 2006
1076027	BiP12	C	T	Roumagnac 2006
1089517	BiP13	T	C	Roumagnac 2006
1240637	BiP14	G	A	Roumagnac 2006
1285129	BiP15	C	T	Roumagnac 2006
1509712	BiP16	C	T	Roumagnac 2006
1619556	BiP17	C	T	Roumagnac 2006
1641434	BiP18	T	C	Roumagnac 2006
6261	BiP2	C	T	Roumagnac 2006
1812993	BiP20	A	G	Roumagnac 2006
1838206	BiP23	C	T	Roumagnac 2006
1943523	BiP24	C	T	Roumagnac 2006
2048732	BiP25	G	A	Roumagnac 2006
2048739	BiP26	T	G	Roumagnac 2006
2108140	BiP27	C	T	Roumagnac 2006
2108322	BiP28	G	A	Roumagnac 2006
2221079	BiP29	C	T	Roumagnac 2006
138680	BiP3	C	T	Roumagnac 2006
2220955	BiP30	G	A	Roumagnac 2006
2348902	BiP33	C	T	Roumagnac 2006
2413077	BiP34	A	G	Roumagnac 2006
2456099	BiP35	C	T	Roumagnac 2006
2464185	BiP36	A	G	Roumagnac 2006
2463810	BiP37	C	A	Roumagnac 2006
2463831	BiP38	G	A	Roumagnac 2006

180029	BiP4	C	T	Roumagnac 2006
2544044	BiP40	G	A	Roumagnac 2006
2643935	BiP41	G	A	Roumagnac 2006
2697121	BiP42	G	A	Roumagnac 2006
2825733	BiP43	G	A	Roumagnac 2006
2847564	BiP44	G	A	Roumagnac 2006
2902990	BiP45	G	A	Roumagnac 2006
2947118	BiP47	G	A	Roumagnac 2006
3062270	BiP48	C	T	Roumagnac 2006
3287913	BiP49	C	T	Roumagnac 2006
3339598	BiP51	C	T	Roumagnac 2006
3469152	BiP54	T	C	Roumagnac 2006
3476189	BiP56	C	T	Roumagnac 2006
3731468	BiP57	G	A	Roumagnac 2006
3731453	BiP58	C	T	Roumagnac 2006
3806041	BiP59	G	A	Roumagnac 2006
332645	BiP6	G	A	Roumagnac 2006
4286222	BiP60	C	T	Roumagnac 2006
4391077	BiP61	C	T	Roumagnac 2006
4429843	BiP62	G	A	Roumagnac 2006
4443365	BiP63	G	A	Roumagnac 2006
4651341	BiP65	T	G	Roumagnac 2006
2757499	BiP67	T	G	Roumagnac 2006
546949	BiP68	T	C	Roumagnac 2006
343912	BiP7	C	T	Roumagnac 2006
3245128	BiP76	G	A	Roumagnac 2006
3415355	BiP77	C	T	Roumagnac 2006
4422904	BiP79	C	T	Roumagnac 2006
580916	BiP8	T	C	Roumagnac 2006

1808734	BiP81	G	A	Roumagnac 2006
138729	BiP89	C	A	Roumagnac 2006
332683	BiP90	G	A	Roumagnac 2006
830308	BiP91	C	T	Roumagnac 2006
976842	BiP92	G	A	Roumagnac 2006
1813023	BiP93	G	A	Roumagnac 2006
2221033	BiP94	C	T	Roumagnac 2006
2221015	BiP95	A	G	Roumagnac 2006
2220992	BiP96	C	A	Roumagnac 2006
2221084	BiP99	A	G	Roumagnac 2006
1057075	H42a	C	T	Holt 2008
1161982	H42b	C	T	Holt 2008
229722	H58.1	G	A	Holt 2008
241789	H58.2	A	G	Holt 2008
522174	H58.3	C	T	Holt 2008
789538	H58.4	C	T	Holt 2008
991913	H58.5	G	A	Holt 2008
1193220	H58.6	T	C	Holt 2008
1534019	H58.7	G	A	Holt 2008
1579791	H58.8	C	T	Holt 2008
1750900	H58.9	G	A	Holt 2008
2003906	H58.10	G	A	Holt 2008
2014424	H58.11	G	A	Holt 2008
2418082	H58.12	G	A	Holt 2008
2499777	H58.13	G	A	Holt 2008
2843437	H58.14	A	G	Holt 2008
2915916	H58.15	G	A	Holt 2008
2915943	H58.16	C	A	Holt 2008
2916079	H58.17	C	T	Holt 2008

3061270	H58.18	C	T	Holt 2008
3180740	H58.19	G	A	Holt 2008
3196458	H58.20	T	C	Holt 2008
3202099	H58.21	C	T	Holt 2008
3259213	H58.22	C	T	Holt 2008
3514815	H58.23	C	T	Holt 2008
3694947	H58.24	C	G	Holt 2008
3784470	H58.25	G	T	Holt 2008
3788492	H58.26	C	T	Holt 2008
3810322	H58.27	C	T	Holt 2008
3826375	H58.28	C	T	Holt 2008
4297511	H58.29	C	A	Holt 2008
4379937	H58.30	G	A	Holt 2008
4387832	H58.31	C	T	Holt 2008
4546085	H58.32	C	T	Holt 2008
4546132	H58.33	G	A	Holt 2008
4581043	H58.34	T	A	Holt 2008
4610837	H58.35	C	T	Holt 2008
4653894	H58.36	C	T	Holt 2008
4798685	H58.37	C	T	Holt 2008
35555	H58.38	G	A	Holt 2008
3126962	H58.39	G	A	This work
922111	H58.40	G	A	This work
2267090	H58.41	C	T	This work
4519292	H58.42	C	A	This work
2976460	H58.43	T	C	This work
4401419	H58.44	C	T	This work
2405223	H58.45	C	T	This work
3129638	H58.46	G	A	This work

2628741	H58.47	C	T	This work
2952	H58.48	G	T	This work
1592955	H58.49	G	A	This work
3238431	H58.50	G	A	This work
2531257	H58.51	G	A	This work

ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, Dec. 2010, p. 5201–5208 Vol. 54, No. 12
0066-4804/10/$12.00 doi:10.1128/AAC.00963-10

Suitable Disk Antimicrobial Susceptibility Breakpoints Defining *Salmonella enterica* Serovar Typhi Isolates with Reduced Susceptibility to Fluoroquinolones▽†‖

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Received 15 July 2010/Returned for modification 18 August 2010/Accepted 4 September 2010

Infections with *Salmonella enterica* serovar Typhi isolates that have reduced susceptibility to ofloxacin (MIC ≥ 0.25 μg/ml) or ciprofloxacin (MIC ≥ 0.125 μg/ml) have been associated with a delayed response or clinical failure following treatment with these antimicrobials. These isolates are not detected as resistant using current disk susceptibility breakpoints. We examined 816 isolates of *S.* Typhi from seven Asian countries. Screening for nalidixic acid resistance (MIC ≥ 16 μg/ml) identified isolates with an ofloxacin MIC of ≥0.25 μg/ml with a sensitivity of 97.3% (253/260) and specificity of 99.3% (552/556). For isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, the sensitivity was 92.9% (248/267) and specificity was 98.4% (540/549). A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disc detected strains with an ofloxacin MIC of ≥0.25 μg/ml with a sensitivity of 94.6% (246/260) and specificity of 94.2% (524/556). A zone of inhibition of ≤30 mm detected isolates with a ciprofloxacin MIC of ≥0.125 μg/ml with a sensitivity of 94.0% (251/267) and specificity of 94.2% (517/549). An ofloxacin MIC of ≥0.25 μg/ml and a ciprofloxacin MIC of ≥0.125 μg/ml detected 74.5% (341/460) of isolates with an identified quinolone resistance-inducing mutation and 81.5% (331/406) of the most common mutant (carrying a serine-to-phenylalanine mutation at codon 83 in the *gyrA* gene). Screening for nalidixic acid resistance or ciprofloxacin and ofloxacin disk inhibition zone are suitable for detecting *S.* Typhi isolates with reduced fluoroquinolone susceptibility.

Enteric fever is an infection caused by *Salmonella enterica* serovars Typhi and Paratyphi A. These human restricted pathogens are transmitted by the fecal-oral route, and enteric fever is common in regions with poor standards of hygiene and sanitation. There are 27 million new enteric fever infections each year, of which approximately 200,000 are fatal (16). Antimicrobials are essential for appropriate clinical management of enteric fever, but antimicrobial resistance in *S.* Typhi and *S.* Paratyphi A have become a problem in regions where they are endemic (6, 8). Multiple-drug-resistant (MDR) *S.* Typhi and *S.* Paratyphi A (resistant to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin) are particularly common in some locations in Asia and have led to large epidemics. An MDR *S.* Typhi strain was responsible for an outbreak in Tajikistan in the late 1990s, causing over 24,000 infections (39).

The occurrence of MDR strains limits the options for antimicrobial therapy of enteric fever. The current WHO guidelines suggest that the fluoroquinolones are the optimal group of antimicrobials for the treatment of uncomplicated typhoid fever in adults (44). The fluoroquinolones, such as ciprofloxacin and ofloxacin, are comparatively inexpensive and well tolerated and in early randomized clinical trials were very effective. However, *S.* Typhi and *S.* Paratyphi A isolates with reduced susceptibility to fluoroquinolones have become common in Asia and are increasingly common in Africa (6, 8, 13, 26, 32, 37). Infections with *S.* Typhi strains with elevated MICs to ciprofloxacin and ofloxacin have been

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† Supplemental material for this article may be found at http://aac.asm.org/.

▽ Published ahead of print on 13 September 2010.

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associated with the failure of treatment with these antimicrobials and increased disease severity (15, 30, 33, 36, 43).

Investigations of *S*. Typhi with reduced susceptibility to fluoroquinolones has shown the association of elevated MIC with several single-base-pair mutations in the DNA gyrase gene, *gyrA*, and the topoisomerase gene, *parC* (4, 6, 33, 42). Furthermore, extensive genome sequencing and single nucleotide polymorphism (SNP) investigation of *S*. Typhi strains have further shown the dramatic impact of strains with *gyrA* mutations on the population structure of this monophyletic organism (35). Genotyping studies identified at least 15 independent *gyrA* mutations that have occurred within a decade and stimulated clonal expansion in Asia and Africa (6, 35). These data suggest that such strains have evolved rapidly and are maintained by a strong selective pressure.

The laboratory detection and identification of strains with reduced susceptibility to fluoroquinolones are important for the treating clinician, but such strains are categorized as susceptible by the current interpretive guidelines for fluoroquinolone disk susceptibility testing (3, 11, 19). These isolates are invariably resistant to nalidixic acid, and susceptibility testing with a nalidixic acid disk has been suggested as a suitable screening method for reduced fluoroquinolone susceptibility (11, 19). The British Society for Antimicrobial Chemotherapy (BSAC) has recommended that for invasive isolates of *Salmonella*, an MIC for reduced susceptibility to fluoroquinolones should be determined (3).

Here we have examined the relationship between *gyrA* and *parC* mutations, nalidixic acid resistance, ofloxacin and ciprofloxacin disk inhibition zone sizes, and MIC for a large number of *S*. Typhi clinical isolates from multiple locations in Asia over a 16-year period. We suggest disk susceptibility breakpoints for strains with reduced susceptibility to ciprofloxacin and ofloxacin, which may permit the diagnostic laboratory to detect such isolates and aid the clinical management of enteric fever.

MATERIALS AND METHODS

***S*. Typhi strain collection.** The *S*. Typhi strains used in this study were comprised of isolates collected as part of several independent investigations. The majority of the strains (516 strains) were collected from randomized controlled trials conducted between 1992 and 2002 in southern Vietnam. These trials were conducted using a standard protocol, except for the treatment regimens used, described in detail elsewhere (5, 7, 28, 31, 38, 40, 41). One hundred and four *S*. Typhi strains were isolated as part of a randomized controlled trial (gatifloxacin versus chloramphenicol [ISRCTN53258327]) at Patan Hospital, Kathmandu, Nepal, for the treatment of uncomplicated enteric fever between 2006 and 2008. The remaining *S*. Typhi strains (a total of 196) were collected between 2002 and 2003 as part of population-based prospective surveillance studies conducted by multiple teams in Jakarta, Indonesia ($n = 27$), Dhaka, Bangladesh ($n = 40$), Hechi City, Guang Xi, China ($n = 51$), Kolkata, India ($n = 25$), and Karachi, Pakistan ($n = 53$) (6).

A subset of the strains described above ($n = 100$; from Vietnam, Indonesia, China, India, and Pakistan) and a collection of contemporary *S*. Typhi strains from Vietnam and India ($n = 375$) were additionally selected for screening for *gyrA*, *gyrB*, *parC*, and *parE* mutations. These strains are presented in the supplemental material.

Microbiological methods. The isolates were identified by standard biochemical tests and agglutination with *Salmonella*-specific antisera (Murex Diagnostics, Dartford, United Kingdom). Antimicrobial susceptibilities were tested at the time of isolation by the modified Bauer-Kirby disk diffusion method, with zone size interpretation based on CLSI guidelines (9, 11). Antimicrobial disks tested were chloramphenicol (CHL) (30 μg), ampicillin (AMP) (10 μg), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 μg), ceftriaxone (CRO) (30 μg), ofloxacin (OFX) (5 μg), and nalidixic acid (NAL) (30 μg). Mueller-Hinton agar and antimicrobial discs were purchased from Unipath, Basingstoke, United Kingdom.

Isolates were stored on Protect beads (Prolabs, Oxford, United Kingdom) at −20°C. The isolates were later subcultured, and the disk antimicrobial susceptibility tests were repeated on Mueller-Hinton agar by CLSI methods for NAL (30 μg), ciprofloxacin (CIP) (5 μg), and ofloxacin (OFX) (5 μg). The zone of inhibited growth for each antimicrobial was measured by three separate investigators blind to the result of the measurements of the others. The average zone size recorded by the three readers was calculated. The MICs for the isolates were determined by the standard agar plate dilution method according to CLSI guidelines or by Etest according to the manufacturer's recommendations (AB Biodisk, Sweden) (10).

The antimicrobials evaluated were CIP (0.008 μg/ml to 4 μg/ml), OFX (0.008 μg/ml to 4 μg/ml), and NAL (0.5 μg/ml to 512 μg/ml). Antimicrobial powders for the agar plate dilution MICs were purchased from Sigma, United Kingdom. The MIC end points were read by two independent investigators, each blind to the result determined by the other. Discrepancies were resolved by discussion. *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923 were used as control strains for these assays. The results were interpreted according to current CLSI guidelines, susceptible being values of ≤8 μg/ml for nalidixic acid, ≤2 μg/ml for ofloxacin, and ≤1 μg/ml for ciprofloxacin. An isolate was defined as MDR if it was resistant to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin by disk susceptibility testing.

PCR amplification and sequencing of *gyrA*, *gyrB*, *parC*, and *parE* genes in *S*. Typhi. DNA from the strains that were selected for PCR amplification of the *gyrA*, *gyrB*, *parC*, and *parE* genes was extracted using the Wizard genomic DNA purification kit (Promega) according to the manufacturer's recommendations. Briefly, a single colony was inoculated in 1.5 ml of Luria-Bertani broth and incubated overnight at 37°C with shaking at 300 rpm to reach 10^8 CFU/ml. One ml of the bacterial culture was transferred to a microcentrifuge tube and centrifuged in a microcentrifuge at 13,000 rpm for 2 min. The supernatant was removed, and the bacterial pellet was used for DNA extraction. The extracted DNA was stored at −20°C until required.

Oligonucleotide primers for the amplification of the quinolone resistance-determining regions in *gyrA*, *gyrB*, *parC*, and *parE* genes in *S*. Typhi were as follows (6): *gyrA*, GYRA/P1 (5′-TGTCCGAGATGGCCTGAAGC) and GYRA/P2 (5′-TACCGTCATAAGTTATCCACG) (annealing temperature, 55°C); *gyrB*, StygyrB1 (5′-CAAACTGGCGGACTGTCAGG) and StygyrB2 (5′-TTCCGGCATCTGACGATAGA) (annealing temperature, 62°C); *parC*, StmparC1 (5′-CTATGCGATGT CAGAGCTGG) and StmparC2 (5′ TAA CAGCAGCTCGGCGTATT) (annealing temperature; 62°C); and *parE*, StmparE1 (5′-TCTCTTCCGATGAAGTGCTG) and StmparE2 (5′ ATACGG TATAGCGGCGGTAG) (annealing temperature, 62°C).

Predicted PCR amplicon sizes were 347 bp (*gyrA*), 345 bp (*gyrB*), 270 bp (*parC*), and 240 bp (*parE*). PCRs were performed under the following conditions: 30 cycles of 92°C for 45 s, 55°C or 62°C (depending on the primers) for 45 s, and extension at 74°C for 1 min, followed by a final extension step at 74°C for 2 min.

The DNA sequencing reactions were performed using the CEQ DTCS Quick Start kit (Beckman Coulter) and was sequenced using a CEQ 8000 capillary sequencer, and the resulting DNA sequence was analyzed using CEQuence Investigator CEQ2000XL (Beckman Coulter). All sequences were verified, aligned, and manipulated using Bioedit software (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). All *gyrA*, *gyrB*, *parC*, and *parE* sequences were compared to other *gyrA*, *gyrB*, *parC*, and *parE* sequences by BLASTn at NCBI. The DNA sequence of the various *S.* Typhi sequences of *gyrA*, *gyrB*, *parC*, and *parE* were downloaded and aligned with the produced sequences.

Data analysis. Zone size interpretive criteria and interpretive discrepancy rates were calculated by the error rate-bounded method of Metzler and DeHaan (27). The MIC breakpoints for reduced susceptibility were ≥0.25 μg/ml for ofloxacin and ≥0.125 μg/ml for ciprofloxacin. The zone size breakpoints were adjusted until the number of false-susceptible disk diffusion test results (very major discrepancies) and false-resistant disk tests (major discrepancies) were held to a minimum. Guidelines for acceptable discrepancy rates were according to the CLSI recommendation (12). Normally distributed data were compared using the Student *t* test, nonnormally distributed data using the Mann-Whitney U test, and proportions by the chi-square test. Statistical analysis was performed using EpiInfo, version 6 (CDC, Atlanta, GA), and SPSS for Windows version 10.1 (SPSS, Inc., Chicago, IL).

RESULTS

Antimicrobial susceptibility testing of *S*. Typhi isolates. We investigated 816 *S*. Typhi isolates collected between 1992 and 2008 from seven Asian countries: Vietnam, Nepal, Indonesia, India, Bangladesh, Pakistan, and China. Only one isolate (the

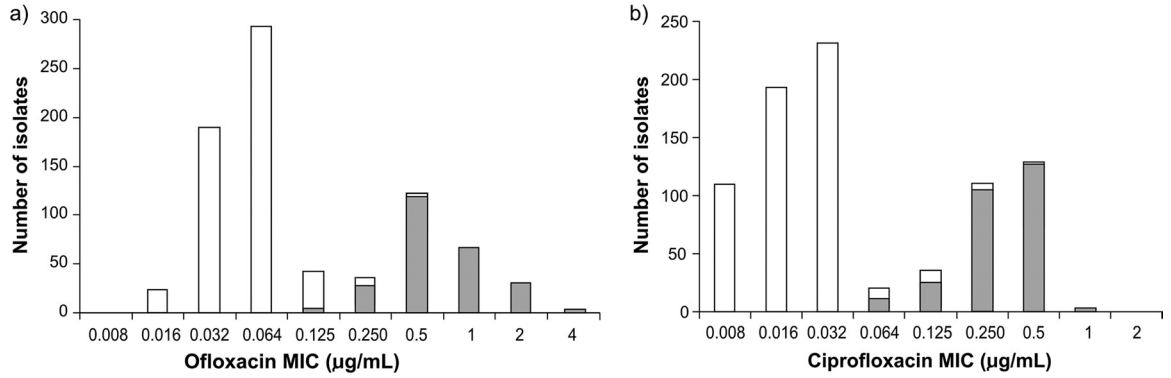

FIG. 1. Fluoroquinolone MIC histograms for 816 *S*. Typhi isolates from Asia. Histograms showing the distribution of MICs to ofloxacin (a) and ciprofloxacin (b) of 816 *S*. Typhi strains, isolated from patients with enteric fever. Each isolate used for analysis was isolated from an individual enteric fever patient. The MICs are plotted on the *x* axis, and the numbers of isolates corresponding with particular MICs are plotted on the *y* axis. The white proportion of the columns indicates the nalidixic acid-susceptible isolates ($n = 563$). The black proportion of the columns indicates the nalidixic acid-resistant isolates ($n = 253$). Both histograms show a bimodal distribution, which is partly differentiated by nalidixic acid resistance.

strain isolated on admission to the health care facility) from each patient was included for microbiological examination and analysis.

Of the 816 *S*. Typhi isolates tested, 466 (57.1%) were MDR (resistant to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole), while 303/816 (37%) were fully susceptible to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole. Two hundred fifty-three of the 816 isolates (31%) were resistant to nalidixic acid (MIC, ≥32 μg/ml), and 4 isolates had an MIC of 16 μg/ml (intermediate) to nalidixic acid but were classified as resistant according to the zone sizes from disk susceptibility testing (≤13 mm). Of the 466 MDR isolates, 145 (31.1%) were additionally resistant to nalidixic acid compared to 80/303 (26.4%) isolates that were fully susceptible to chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole ($P = 0.16$).

All 816 *S*. Typhi isolates were classified as susceptible to ciprofloxacin according to MIC testing (MIC ≤ 1 μg/ml), yet 12 gave a discrepant result with disk testing. These strains exhibited an inhibition zone size of ≤20 mm and were, therefore, classified as intermediate by disk testing. Two of the 816 *S*. Typhi strains were graded with intermediate resistance to ofloxacin with an MIC of 4 μg/ml but had inhibition zone sizes of ≥16 mm and were, therefore, classified as susceptible.

The distribution of the MIC levels to ciprofloxacin and ofloxacin for all 816 *S*. Typhi isolates is presented in Fig. 1. The histograms of the levels of MIC to ciprofloxacin and ofloxacin both demonstrate a bimodal distribution. The two distinct groups are partially divided by nalidixic acid susceptibility (Fig. 1, black shading denotes resistance to nalidixic acid). The 563 isolates that were susceptible to nalidixic acid had an MIC_{90} (range) to ciprofloxacin of 0.03 μg/ml (0.008 to 0.5 μg/ml) and of 0.06 μg/ml (0.016 to 0.5 μg/ml) to ofloxacin. The 253 isolates that were resistant to nalidixic acid had an MIC_{90} (range) to ciprofloxacin of 0.5 μg/ml (0.064 to 1 μg/ml) and to ofloxacin of 1.0 μg/ml (0.125 to 4 μg/ml).

Antimicrobial susceptibility test interpretive categories of *S*. Typhi to ciprofloxacin and ofloxacin. The current CLSI intermediate breakpoints are 2 μg/ml and 4 μg/ml, respectively, for ciprofloxacin and ofloxacin. Only 2 of the 816 strains tested had MIC levels greater than or equal to those of the current MIC breakpoints (Fig. 1). The MICs for nalidixic acid were compared with those of ofloxacin and ciprofloxacin in scatter plots (Fig. 2). The current interpretive breakpoints are shown in Fig. 2 as dark shading in red for ofloxacin and ciprofloxacin and in gray for nalidixic acid. The suggested interpretive breakpoints for reduced susceptibility are depicted by a broken line with an arrow (Fig. 2). As predicted, there was a linear relationship between the nalidixic acid MIC and the ofloxacin (Fig. 2a) and ciprofloxacin MICs (Fig. 2b).

Screening strains using nalidixic acid resistance (MIC ≥ 16 μg/ml) for the detection of isolates with an MIC of ≥0.25 μg/ml for ofloxacin had a sensitivity of 97.3% (253/260) and a specificity of 99.3% (552/556) (Fig. 2a). The number of very major discrepancies was 7/260 (2.7%), with none more than two dilutions above the breakpoint, and the number of major discrepancies was 4/556 (0.7%), with none more than two dilutions below the breakpoint. Screening for the detection of isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, using nalidixic acid resistance (MIC of ≥16 μg/ml), was not as reliable as that for ofloxacin, as it had a sensitivity of 92.9% (248/267) and a specificity of 98.4% (540/549). The number of very major discrepancies was 19/267 (7.1%), with 1/267 (0.4%) more than two dilutions above the breakpoint, and the number of major discrepancies was 9/549 (1.6%), with none more than two dilutions below the breakpoint.

We explored the relationship between the diameter of the zone of inhibition and the MICs for ciprofloxacin and ofloxacin, using 5-μg disks (Fig. 3). A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disk correlated with an MIC of ≥0.25 μg/ml, with the least number of discrepancies (Fig. 3a). The number of very major discrepancies was 14/260 (5.4%), with none more than two dilutions above the breakpoint, and the number of major discrepancies was 32/556 (5.7%), with 14/556 (2.5%) more than two dilutions below the breakpoint. A zone of inhibition of ≤28 mm around a 5-μg ofloxacin disc detected strains with an ofloxacin MIC of ≥0.25 μg/ml, with a sensitivity of 94.6% (246/260) and a specificity of 94.2% (524/556). A zone of inhibition of ≤30 mm around a 5-μg ciprofloxacin disk correlated with an MIC of ≥0.125 μg/ml, with the least num-

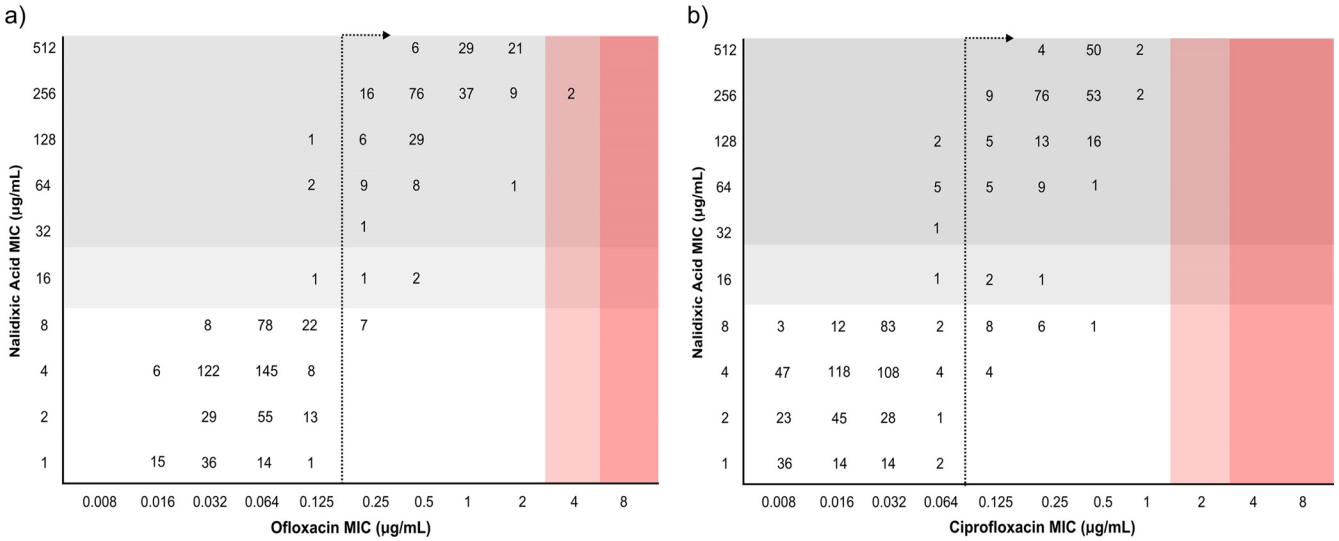

FIG. 2. Scatter plots relating ofloxacin and ciprofloxacin MICs to nalidixic acid MIC for 816 Asian *S*. Typhi isolates. Scatter plots comprised of MIC data from 816 *S*. Typhi isolates from Nepal ($n = 104$), India ($n = 25$), Indonesia ($n = 27$), Bangladesh ($n = 40$), Pakistan ($n = 53$), China ($n = 51$), and Vietnam ($n = 516$). Plots show the relationship between the MIC to nalidixic acid (*y* axis) and the MIC to ofloxacin (a) and ciprofloxacin (b) (*x* axis). The vertical and horizontal shading in each scatter plot indicates the current CLSI recommendations for breakpoints between susceptibility (white), intermediate (light gray, nalidixic acid; light red, ofloxacin and ciprofloxacin), and resistance (dark gray, nalidixic acid; dark red, ofloxacin and ciprofloxacin) (nalidixic acid MIC, ≤8 μg/ml and ≥32 μg/ml; ofloxacin MIC, ≤2 μg/ml and ≥8 μg/ml; and ciprofloxacin MIC, ≤1 μg/ml and ≥4 μg/ml). The red broken line corresponds to the proposed MIC breakpoint identifying strains with reduced susceptibility to fluoroquinolones (ofloxacin MIC of ≥0.25 μg/ml and ciprofloxacin MIC of ≥0.125 μg/ml).

ber of discrepancies (Fig. 3b). The number of very major discrepancies was 16/267 (6.0%), with 4/267 (1.5%) more than two dilutions above the breakpoint, and the number of major discrepancies was 32/549 (5.8%), with 22/549 (4.0%) more than two dilutions below the breakpoint. A zone of growth inhibition of ≤30 mm detected isolates with a ciprofloxacin MIC of ≥0.125 μg/ml, with a sensitivity of 94.0% (251/267) and a specificity of 94.2% (517/549).

Reduced susceptibility to fluoroquinolones and *gyrA*, *gyrB*, *parC*, and *parE* mutations. To further define the *S*. Typhi population with reduced susceptibility to fluoroquinolones, we produced PCR amplicons and then sequenced the quinolone resistance-determining region in the *gyrA*, *gyrB*, *parC*, and *parE* genes from a collection of 475 *S*. Typhi strains from Vietnam, China, India, Indonesia, and Pakistan. One hundred of these strains were described in the previous section, and 375 were more recent strains from Vietnam and India. The MIC range of these strains was 1 to 512 μg/ml to nalidixic acid, 0.008 to 6 μg/ml to ciprofloxacin, and 0.03 to 12 μg/ml to ofloxacin. These strains and the corresponding data from these strains are described in the supplemental material.

Fifteen of the 475 *S*. Typhi strains examined by PCR and sequencing of *gyrA*, *gyrB*, *parC*, and *parE* had no mutations in the quinolone resistance-determining regions of any gene. No strains had a mutation in the quinolone resistance-determining region of *gyrB* or *parE*. Four hundred sixty strains had either a single mutation or a combination of double or triple mutations in the *gyrA* and *parC* genes. DNA sequencing identified seven different amino acid substitutions: D87A, aspartic acid to asparagine at codon 87 in the *gyrA* gene; S83Y, serine to tyrosine at codon 83 in the *gyrA* gene; S83F, serine to phenylalanine at codon 83 in the *gyrA* gene; D87G, aspartic acid to glycine at codon 87 in the *gyrA* gene; S83F/D87N, serine to phenylalanine at codon 83 and aspartic acid to asparagine at codon 87 in the *gyrA* gene; S83F/D87G, serine to phenylalanine at codon 83 and aspartic acid to glycine at codon 87 in the *gyrA* gene; and S83F/D87G/S80I, serine to phenylalanine at codon 83 and aspartic acid to glycine at codon 87 in the *gyrA* gene and serine to isoleucine at codon 80 in the *parC* gene. The most commonly identified amino acid replacement was S83F, constituting (88%) 406/460 strains with a mutation, with S83Y the second most common mutant (10%) 46/460.

We compared the MICs to ofloxacin and ciprofloxacin of the 460 strains with the seven different mutation patterns and the 15 strains with no mutation detected (Fig. 4). When grouped into strains with and without a single mutation in the *gyrA* gene, the single mutation group had significantly higher MICs to ofloxacin (Fig. 4a) and ciprofloxacin (Fig. 4b) than those without a mutation. The most common amino acid substitution, S83F, had mean MICs of 0.75 μg/ml and 0.33 μg/ml to ofloxacin and ciprofloxacin, respectively. Figure 4 also shows the current CLSI breakpoints and the suggested ofloxacin breakpoint of 0.25 μg/ml and ciprofloxacin breakpoint of 0.125 μg/ml. An MIC of 0.25 μg/ml to ofloxacin and an MIC of 0.125 μg/ml to ciprofloxacin detected 74.5% (341/460) of the *S*. Typhi strains with an identified fluoroquinolone resistance mutation and 81.5% (331/406) of the most common *S*. Typhi mutant (S83F) with reduced susceptibility to fluoroquinolones.

DISCUSSION

The increasing recognition that *S*. Typhi isolates with reduced susceptibility to ofloxacin and ciprofloxacin may lead to treatment failure has led to calls for a revision of their break-

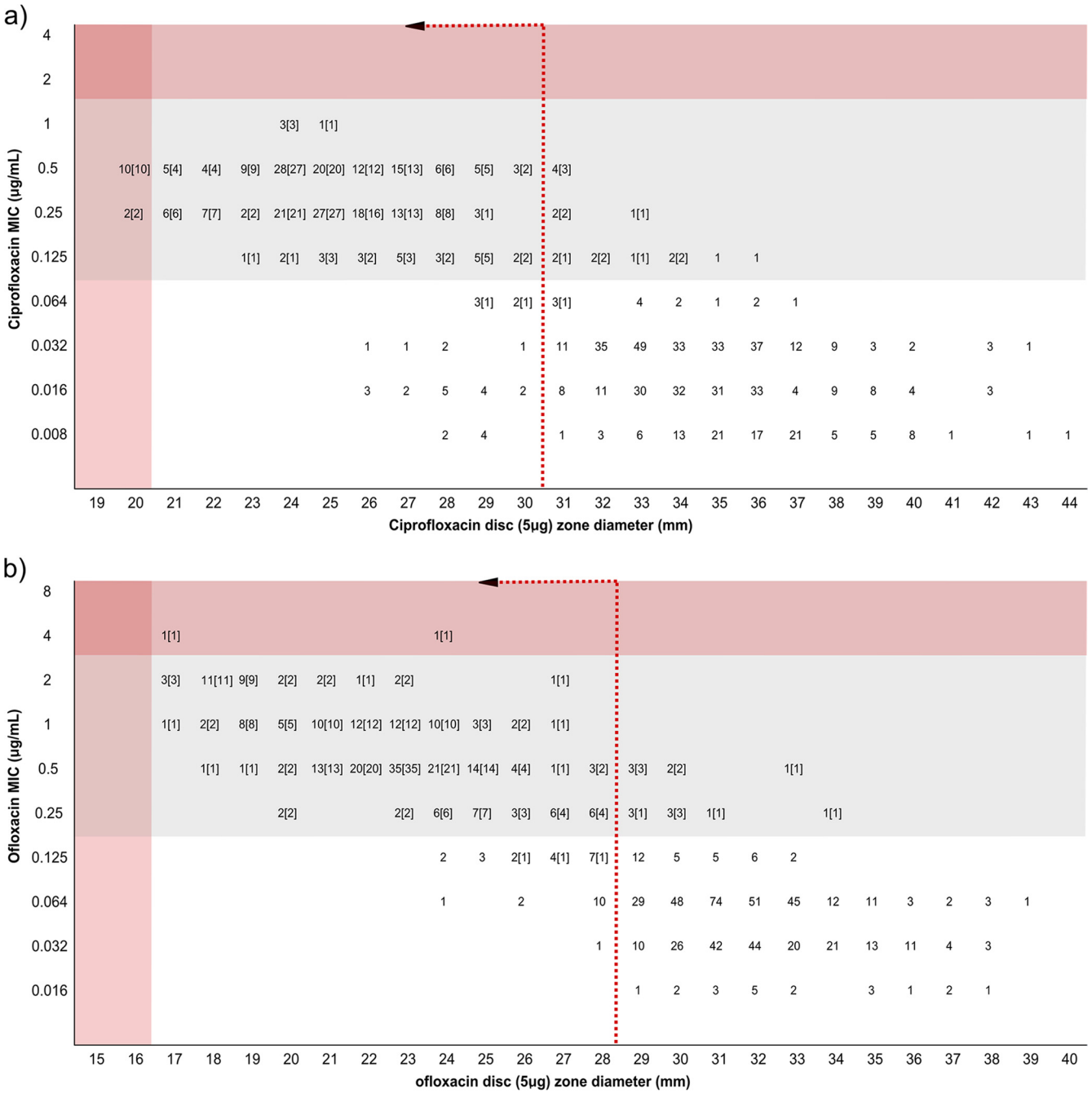

FIG. 3. Scatter plots relating ofloxacin and ciprofloxacin MIC to inhibition zone diameter for 816 Asian *S*. Typhi isolates. Scatter plots for 816 *S*. Typhi isolates comparing the inhibition zone diameters using a 5-μg ciprofloxacin disc (a) and a 5-μg ofloxacin disc (b) (*x* axis) and the corresponding MIC of ciprofloxacin (a) and ofloxacin (b) (*y* axis). The numbers in brackets relate to the 253 nalidixic acid-resistant isolates. The vertical red shading in each scatter plot is the current CLSI disc zone breakpoint for resistance (ofloxacin inhibition zone diameter, ≤16 mm; ciprofloxacin inhibition zone diameter, ≤21 mm). The horizontal red shading distinguishes strains with an MIC of ≥2 μg/ml for ofloxacin or an MIC of ≥1 μg/ml for ciprofloxacin. The gray shading is the proposed breakpoint for *S*. Typhi isolates with reduced susceptibility (ofloxacin MIC, ≥0.25 μg/ml; ciprofloxacin MIC, ≥0.125 μg/ml). The red broken line corresponds with the proposed breakpoints for strains with reduced susceptibility (ofloxacin inhibition zone diameter, ≤28 mm; ciprofloxacin inhibition zone diameter, ≤30 mm).

points. Breakpoints of ≥0.25 μg/ml for ofloxacin and levofloxacin and ≥0.125 μg/ml for ciprofloxacin and gatifloxacin have been suggested (1, 2, 14, 32). Nalidixic acid resistance and disk susceptibility testing have both been proposed as laboratory screening methods to detect such isolates. We have explored the performance of these methods with a large number of strains that are representative of *S*. Typhi isolates circulating in countries in Asia where it is endemic.

Nalidixic acid resistance had a sensitivity of 96.2% and 91.8% and a specificity of 99.5% and 98.5% for the detection

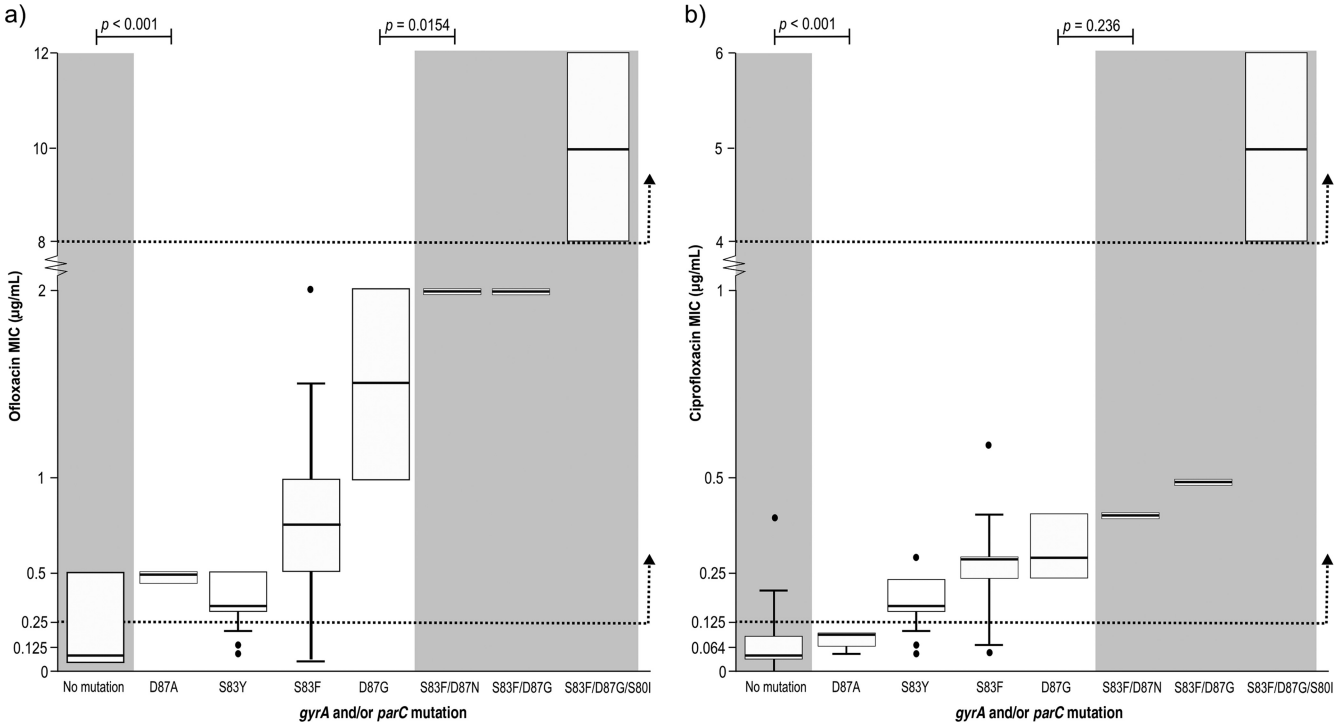

FIG. 4. The relationship of *gyrA* and *parC* mutations and the MICs to ofloxacin and ciprofloxacin in the *S*. Typhi strain isolated in Asia. Box plots (boxes relate to the 25th and 75th percentiles) relating mutations in the *gyrA* and the *parC* genes (the S80I mutation is in the *parC* gene, and the remainder are in the *gyrA* gene) to the MICs of ofloxacin (a) and ciprofloxacin (b) in 475 Asian clinical isolates of *S*. Typhi. The MICs to ofloxacin and ciprofloxacin are plotted on the *y* axis. The MICs to ofloxacin ranged from 0.016 to 12 μg/ml, and those to ciprofloxacin from 0.008 to 6 μg/ml. Median values for each mutant group are indentified by a solid line in the box. Bars demonstrate the 95% confidence interval for the groups with sufficient numbers; dots correspond to outliers. The *x* axis is subdivided into the eight different groups of *S*. Typhi strains identified and assayed, characterized as follows: no mutations in *gyrA* or *parC* ($n = 15$), D87A ($n = 2$), S83Y ($n = 46$), S83F ($n = 406$), D87G ($n = 2$), S83F/D87N ($n = 1$), S83F/D87G ($n = 1$), and S83F/D87G/S80I ($n = 2$). The upper broken lines indicate the current CLSI breakpoint recommendations for ofloxacin and ciprofloxacin. The lower broken lines correspond with the proposed breakpoints for strains with reduced susceptibility to ofloxacin and ciprofloxacin. Statistical significance was calculated between the nonmutant group and the single mutant group and between the single mutant group and the double/triple mutant group using the Student's *t* test.

of isolates with reduced susceptibility to ofloxacin and ciprofloxacin, respectively. Alternatively, using disk sensitivity testing, isolates with reduced susceptibility were detected by an ofloxacin (5-μg) disk inhibition zone diameter of ≤28 mm with a sensitivity of 94.6% and specificity of 94.2% and by a ciprofloxacin (5-μg) disk inhibition zone diameter of ≤30 mm with a sensitivity of 94.0% and specificity of 94.2%. Therefore, both methods had sufficiently high sensitivity for them to be used for screening and acceptably low levels of discrepancies (12). Disk inhibition zone size did, however, demonstrate a slightly lower specificity than nalidixic acid disk testing with this panel of isolates. Similar data for the relationship between nalidixic acid resistance and a decreased ciprofloxacin MIC have been presented for *S.* Typhi isolates in the United States (14) and India (23) and in non-*S*. Typhi *Salmonella* isolates in the United States (14) and Finland (21). For nalidixic acid-susceptible and -resistant *S*. Typhi isolates in India (23), the average disk inhibition zone sizes for ciprofloxacin were greater than those that we observed here. The non-*S*. Typhi study in Finland proposed a ciprofloxacin (5-μg) disk inhibition zone diameter of ≤37 mm as the breakpoint (21). The sensitivity of this approach was 100%, yet the specificity was only 51.9%.

In some isolates in this study, the nalidixic acid, ofloxacin, and ciprofloxacin MIC results were discrepant, in that isolates were nalidixic acid susceptible but with a reduced ofloxacin ($n = 10$) or ciprofloxacin susceptibility ($n = 22$). Similar results have been seen in other studies (13, 15, 22, 26). The clinical significance of these isolates is unclear, as there have been limited documented cases of infection with such strains treated with fluoroquinolones. It is likely that isolates that are nalidixic acid susceptible but with reduced ofloxacin and ciprofloxacin susceptibility contain resistance mechanisms other than mutations in the quinolone resistance-determining region of the *gyrA* gene. Possibilities include decreased permeability, an increase in active efflux, and the presence of plasmid-mediated genes, such as the *qnr* genes that encode a protein that protects the DNA gyrase from ciprofloxacin or *aac(6′)-Ib-cr*, an aminoglycoside-modifying enzyme with activity against ciprofloxacin (32).

The mutations that we detected in DNA gyrase genes and topoisomerase genes were consistent with previous reports (4, 6, 34, 42). The most common amino acid substitution detected was S83F, which has been found to be particularly associated with the H58 haplotype (35). This haplotype has become dominant in many areas of Asia in recent years and has also been found to have spread into Kenya in East Africa (24). Approx-

imately 20 to 25% of the isolates with a *gyrA* mutation had an MIC below the suggested breakpoints of 0.25 μg/ml for ofloxacin and 0.125 μg/ml for ciprofloxacin. The effect on the response to fluoroquinolone treatment of infection with isolates with a single *gyrA* mutation but with an MIC below the suggested breakpoints is not known. It is also possible that the isolates with a single *gyrA* mutation but an MIC above the suggested breakpoint have additional resistance mechanisms present (32).

The lack of universally observed guidelines for the detection of *S.* Typhi isolates with reduced susceptibility has meant that such isolates are frequently unrecognized by microbiology laboratories. Continued use of ciprofloxacin and ofloxacin for these infections may be driving the emergence of fully fluoroquinolone-resistant isolates of *S.* Typhi and *S.* Paratyphi A (20, 25, 34). Gatifloxacin, azithromycin, and ceftriaxone are better options for treating such infections, if the isolates also demonstrate resistance to first-line antimicrobials (7, 17, 18, 29, 31).

The use of nalidixic acid resistance as a surrogate screening test is often confusing because it is not used for the treatment of enteric fever. Furthermore, the emergence of nalidixic acid-susceptible isolates with reduced ofloxacin and ciprofloxacin susceptibility may mean that some isolates are missed. Therefore, a straightforward solution would be to modify the *S.* Typhi breakpoints to ≤30 mm and ≤28 mm for ciprofloxacin and ofloxacin, respectively. Interpretative breakpoints for the disk susceptibility tests with the antimicrobials actually used for treatment will better assist clinicians in the choice of therapy for enteric fever and will allow the collection of accurate surveillance data. Our data suggest disk breakpoints of ≤30 mm and ≤28 mm for ciprofloxacin and ofloxacin, respectively. These breakpoints have high specificity and sensitivity, permitting the detection of *S.* Typhi strains that have reduced susceptibility to ciprofloxacin and ofloxacin.

ACKNOWLEDGMENTS

We thank the following for their support of these studies: the directors and the clinical and microbiology staff of the Hospital for Tropical Diseases, Ho Chi Minh City, Vietnam; Vo Anh Ho and colleagues at Dong Thap Provincial Hospital, Dong Thap Province, Vietnam; the late Cao Xuan Thanh Phuong and colleagues at Dong Nai Pediatric Centre, Dong Nai Province, Vietnam; Nguyen Van Sach, Tran Thi Phi La, Nguyen Ngoc Rang, Nguyen Thi Be Bay, and colleagues at the An Giang Provincial Hospital, Long Xuyen, An Giang Province, Vietnam; and the International Vaccine Institute, Seoul, South Korea.

This work was supported by The Wellcome Trust, Euston Road, London, United Kingdom. S.B. is supported by an OAK Foundation Fellowship through Oxford University.

We declare that we have no competing interests.

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Gatifloxacin versus chloramphenicol for uncomplicated enteric fever: an open-label, randomised, controlled trial

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Summary

Background We aimed to investigate whether gatifloxacin, a new generation and affordable fluoroquinolone, is better than chloramphenicol for the treatment of uncomplicated enteric fever in children and adults.

Methods We did an open-label randomised superiority trial at Patan Hospital, Kathmandu, Nepal, to investigate whether gatifloxacin is more effective than chloramphenicol for treating uncomplicated enteric fever. Children and adults clinically diagnosed with enteric fever received either gatifloxacin (10 mg/kg) once a day for 7 days, or chloramphenicol (75 mg/kg per day) in four divided doses for 14 days. Patients were randomly allocated treatment (1:1) in blocks of 50, without stratification. Allocations were placed in sealed envelopes opened by the study physician once a patient was enrolled into the trial. Masking was not possible because of the different formulations and ways of giving the two drugs. The primary outcome measure was treatment failure, which consisted of at least one of the following: persistent fever at day 10, need for rescue treatment, microbiological failure, relapse until day 31, and enteric-fever-related complications. The primary outcome was assessed in all patients randomly allocated treatment and reported separately for culture-positive patients and for all patients. Secondary outcome measures were fever clearance time, late relapse, and faecal carriage. The trial is registered on controlled-trials.com, number ISRCTN 53258327.

Findings 844 patients with a median age of 16 (IQR 9–22) years were enrolled in the trial and randomly allocated a treatment. 352 patients had blood-culture-confirmed enteric fever: 175 were treated with chloramphenicol and 177 with gatifloxacin. 14 patients had treatment failure in the chloramphenicol group, compared with 12 in the gatifloxacin group (hazard ratio [HR] of time to failure 0·86, 95% CI 0·40–1·86, p=0·70). The median time to fever clearance was 3·95 days (95% CI 3·68–4·68) in the chloramphenicol group and 3·90 days (3·58–4·27) in the gatifloxacin group (HR 1·06, 0·86–1·32, p=0·59). At 1 month only, three of 148 patients were stool-culture positive in the chloramphenicol group and none in the gatifloxacin group. At the end of 3 months only one person had a positive stool culture in the chloramphenicol group. There were no other positive stool cultures even at the end of 6 months. Late relapses were noted in three of 175 patients in the culture-confirmed chloramphenicol group and two of 177 in the gatifloxacin group. There were no culture-positive relapses after day 62. 99 patients (24%) experienced 168 adverse events in the chloramphenicol group and 59 (14%) experienced 73 events in the gatifloxacin group.

Interpretation Although no more efficacious than chloramphenicol, gatifloxacin should be the preferred treatment for enteric fever in developing countries because of its shorter treatment duration and fewer adverse events.

Funding Wellcome Trust.

Lancet Infect Dis 2011; 11: 445–54

Published Online
April 29, 2011
DOI:10.1016/S1473-3099(11)70089-5

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Introduction

Enteric fever is a disease that predominantly affects children and is caused by the faecal–oral transmission[1] of *Salmonella enterica* serotype Typhi (*S typhi*) and *Salmonella enterica* Paratyphi A (*S paratyphi* A). There are an estimated 26 million infections and over 200 000 deaths caused by the disease worldwide each year.[2] In parts of south Asia, the incidence of enteric fever in children can be as high as 573 cases per 100 000 person years.[3]

Chloramphenicol was the standard treatment for enteric fever from the 1950s[1,4,5] until the development and spread of multidrug resistant (MDR; defined as resistance to all first-line antibiotics: chloramphenicol, amoxicillin, and co-trimoxazole) *S typhi* and *S paratyphi* A in the early 1990s. Subsequently, fluoroquinolones became first choice for the treatment of enteric fever. However, increased resistance to the older generation fluoroquinolones (ciprofloxacin and ofloxacin) has emerged. This reduces the options for treatment, and raises the spectre of fully resistant enteric fever.[1,6]

Conflicting reports have emerged from randomised controlled trials with relatively small sample sizes that assessed older fluoroquinolones (ciprofloxacin and ofloxacin) versus chloramphenicol for the treatment of enteric fever.[1,7] Additionally, no trials have been done to investigate the efficacy of chloramphenicol versus a newer fluoroquinolone, such as gatifloxacin, in the

treatment of enteric fever in children.[1,8] Recent reports suggest a general decline in the prevalence of MDR typhoid fever in Asia,[9–15] and two recent studies of patients with enteric fever in Kathmandu, Nepal reported a low prevalence of chloramphenicol resistance in *S typhi* and *S paratyphi* A isolates: nine (1·7%) in 522 strains of *S typhi*[16] and three (1·2%) of 247 strains of *S paratyphi* A.[10]

Gatifloxacin was effective in the treatment of nalidixic-acid-resistant enteric fever in two previous randomised trials done in Nepal[16] and Vietnam.[17] The drug targets both DNA gyrase and topoisomerase IV,[18,19] and hence is less inhibited by the common mutations of the *gyrA* gene of *S typhi* than are ciprofloxacin or ofloxacin.

We designed a randomised controlled trial to assess whether gatifloxacin had superior efficacy compared with chloramphenicol in adults and children with uncomplicated enteric fever in Nepal.

Methods

Patients

The study physicians enrolled patients who presented to the outpatient or emergency department of Patan Hospital, Lalitpur, Nepal from May 2, 2006, to August 30, 2008. Patients with fever for more than 3 days who were clinically diagnosed to have enteric fever (undifferentiated fever with no clear focus of infection on preliminary physical exam and laboratory tests) whose residence was in a predesignated area of about 20 km² in urban Lalitpur and who gave fully informed written consent were eligible for the study. Exclusion criteria were pregnancy or lactation, age under 2 years or weight

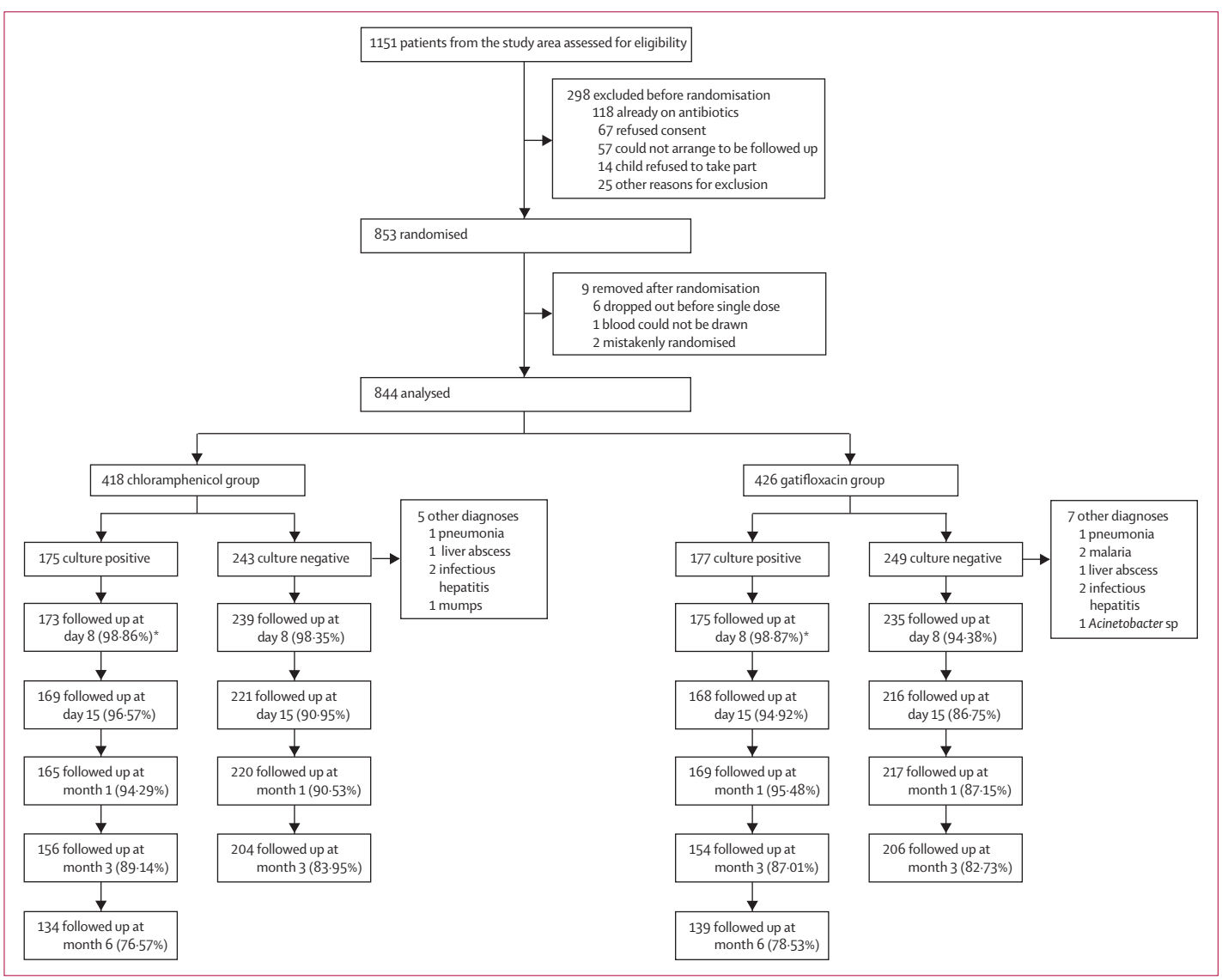

Figure 1: **Trial profile**
*Two culture-positive patients in both the chloramphenicol and glatifloxacin groups were lost to follow-up before day 8.

less than 10 kg, shock, jaundice, gastrointestinal bleeding, or any other signs of severe typhoid fever, previous history of hypersensitivity to either of the trial drugs, or known previous treatment with chloramphenicol, quinolone antibiotic, third generation cephalosporin, or macrolide within 1 week of hospital admission. Patients who had received amoxicillin or co-trimoxazole were included as long as they did not show evidence of clinical response. Ethical approval was granted by both Nepal Health Research Council and Oxford Tropical Research Ethics Committee.

Randomisation and masking

Randomisation was done in blocks of 50 without stratification by an administrator otherwise not involved in the trial. The random allocations were placed in sealed opaque envelopes, which were kept in a locked drawer and opened by the study physician once each patient was enrolled into the trial after meeting the inclusion and exclusion criteria. Patients were enrolled in the order they presented and the sealed envelopes were opened in strict numerical sequence. Masking was not possible because of the different formulations and ways of giving the two drugs.

Procedures

Each enrolled patient was randomly assigned to treatment with either gatifloxacin tablets (400 mg) 10 mg per kg per day in a single oral dose for 7 days or chloramphenicol capsules (250 mg or 500 mg) 75 mg per kg per day in four divided oral doses for 14 days. Gatifloxacin tablets were cut and weighed and the patients' daily doses were prepared in sealed plastic bags. The per-protocol planned duration of chloramphenicol treatment of 14 days was modified for blood-culture-negative patients, who received at least 8 days of chloramphenicol and stopped either on day 8 or 5 days after being afebrile, whichever came later. Gatifloxacin was given for 7 days in all patients.

After enrolment, patients were managed as outpatients and seen by trained community medical auxiliaries (CMAs), as described previously.[16] The CMAs made a visit to each patient's house every 12 h for either 10 days (gatifloxacin group), 14 days (chloramphenicol group), or until the patient was cured. The CMA directly observed each patient ingesting the single dose of gatifloxacin and two doses of chloramphenicol. The physicians re-examined the patients on days 8 and 15, and at 1, 3, and 6 months. All examinations were standardised and entered into case record forms.

Complete blood counts were done on days 1, 8, and 15. On day 1, serum creatinine, bilirubin, aspartate aminotransferase (AST), and alanine aminotransferase (ALT) were also checked. Random plasma glucose was measured on day 1, day 8, day 15, and 1 month. On days 2–7, during the evening visit, the blood glucose was measured by finger-prick testing (OneTouch SureStep, Johnson and Johnson, USA) by the CMAs. Haemoglobin A_{1C} was measured at 3 months.

Blood culture was done as described previously[16] in all patients at admission, in the culture-positive patients on day 8, and if symptoms and signs suggested further infection.

Stool cultures were done on admission in all patients, and in culture-positive patients after completion of treatment and at the 1 month, 3 month, and 6 month visits in 10 mL of Selenite F broth and incubated at 37°C. After the overnight incubation, the broth was subcultured onto MacConkey agar and xylose lysine decarboxylase agar media.

Isolates were screened using standard biochemical tests, and *S typhi* and *S paratyphi* A were identified using API20E (BioMerieux, Paris, France) and slide agglutination with specific antisera (MurexBiotech, Dartford, UK).

Minimum inhibitory concentrations (MICs) were calculated for amoxicillin, azithromycin, chloramphenicol, co-trimoxazole, nalidixic acid, ofloxacin, ciprofloxacin, tetracycline, gatifloxacin, and ceftriaxone by E-test (AB Biodisk, Solna, Sweden).

The primary endpoint of this study was the composite endpoint of treatment failure, which consisted of any one of the following: persistence of fever of more than 37·5°C at day 10 of treatment; the need for rescue treatment with

	Chloramphenicol (n=418)	Gatifloxacin (n=426)
Median (IQR) age (years)	15 (8–22)	16 (9–22)
Male sex	261 (62·4%)	279 (65·5%)
Median (IQR) weight (kg)	42 (20–51)	44 (23–53)
Median (IQR) duration of illness before admission (days)	5 (4–7)	5 (4–7)
Median (IQR) temperature at admission (°C)	38·95 (38·2–39·5)	38·90 (38·1–39·4)
Headache	375 (89·7%)	374 (87·8%)
Anorexia	323 (77·3%)	308 (72·5%)
Abdominal pain	181 (43·5%)	157 (37·1%)
Cough	145 (34·8%)	129 (30·4%)
Nausea	120 (28·7%)	136 (32·1%)
Vomiting	86 (20·7%)	81 (19·6%)
Diarrhoea	78 (18·8%)	79 (18·6%)
Constipation	60 (14·4%)	42 (9·9%)
Hepatomegaly	47 (11·2%)	66 (15·5%)
Splenomegaly	64 (15·3%)	55 (12·9%)
Median (IQR) haematocrit (%)	39 (36·0–43·5)	40 (36·0–43·0)
Median (IQR) leucocyte count (×10^9/L)	6·4 (5·0–8·1)	6·2 (5·1–8·1)
Median (IQR) platelet count (×10^9/L)	190 (162–219)	193 (165–232)
Median (IQR) AST (U/L)	46 (34–62)	44 (33–60)
Median (IQR) ALT (U/L)	29 (20–43)	30 (20–42)
Salmonella typhi isolated	125	124
Salmonella paratyphi A isolated	50	53
Positive pretreatment faecal cultures	20 (5·3%)	19 (5·1%)

AST=serum aspartate aminotransferase (normal range 12–30 U/L). ALT=serum alanine aminotransferase (normal range 13–40 U/L).

Table 1: **Baseline characteristics of patients according to treatment group**

	Chloramphenicol (n=175)	Gatifloxacin (n=177)	Comparison
Total number of treatment failures*	14	12	HR 0·86 (95% CI 0·40 to 1·86), p=0·70
Persistent fever at day 10	5	5	..
Need for rescue treatment	5	3	..
Microbiological failures	0	2	..
Relapse until day 31	7	4	..
Enteric fever related complications	0	0	..
Probability of treatment failure†	0·08 (95% CI 0·04 to 0·13)	0·07 (95% CI 0·03 to 0·11)	RD −0·01 (95% CI −0·07 to 0·04), p=0·64
Median time to fever clearance (days)†	3·95 (95% CI 3·68 to 4·68)	3·9 (95% CI 3·58 to 4·27)	HR 1·06 (95% CI 0·86–1·32), p=0·59
Microbiological failures‡	0/170 (0%)	2/167(1%)	§p=0·24
Relapses until day 31	7	4	HR 0·56 (95% CI 0·16–1·91), p=0·35
Number of culture confirmed relapses	5	3	..
Number of syndromic relapses	2	1	..
Probability of relapse until day 31†	0·04 (95% CI 0·01 to 0·07)	0·02 (95% CI 0·00 to 0·05)	..
Relapses until day 62	10	9	HR 0·87 (95% CI 0·35 to 2·15), p=0·77
Number of culture confirmed relapses	8	5	..
Number of syndromic relapses	2	4	..
Probability of relapse until day 62†	0·06 (95% CI 0·02 to 0·10)	0·06 (95% CI 0·02 to 0·09)	..
Relapses after day 62 (all of which were syndromic)	4	10	..

HR=hazard ratio (based on Cox regression). RD=absolute risk difference (based on Kaplan-Meier estimates). *Patients can have more than one type of treatment failure. †Kaplan-Meier estimates. ‡Only patients with a blood culture taken on day 8. §Based on Fisher's exact test.

Table 2: **Summary of primary and secondary outcomes for culture-positive patients (per-protocol analysis)**

ceftriaxone or ofloxacin as judged by the treating physician; microbiological failure, defined as a positive blood culture for *S typhi* or *S paratyphi* A on day 8; relapse, that is reappearance of culture-confirmed (including mismatch of serotypes [eg, day 1 blood culture positive for *S typhi* and relapse blood culture positive for *S paratyphi* A or vice versa]) or syndromic enteric fever on or after day 11 to day 31 in patients who were initially categorised as successfully treated; and occurrence of enteric-fever-related complications.[16] Time to treatment failure was defined as the time from the first dose of treatment until the date of the earliest failure event of that patient, and patients without an event were censored at the date of their last follow-up visit.

Secondary endpoints were fever clearance time (FCT: time from the first dose of treatment given until the temperature was ≤37·5°C and the patient remained afebrile for at least 48 h); time to relapse until day 31, day 62, or month 6 of follow-up; and faecal carriage at the follow-up visits at 1, 3, and 6 months. The patients' FCTs were calculated electronically on the basis of twice-daily recorded temperatures. Patients without recorded fever clearance or relapse were censored at the date of their last follow-up visit. To reduce possible bias, an investigator not involved in the recruitment of patients decided patients' final outcomes by use of a masked database.

Statistical analysis

The trial was designed as a superiority trial with the hypothesis that gatifloxacin was superior to chloramphenicol in patients with enteric fever. The sample size was calculated to detect a difference of 10% between the two groups in the proportion of patients reaching treatment failure at the two-sided 5% significance level with 80% power. We assumed treatment failure rates of 15% in the chloramphenicol and 5% in the gatifloxacin group, leading to a total required sample size of 160 patients with culture-confirmed enteric fever per group—320 patients in total. On the basis of results from a previous study,[10,16] we assumed that about 40% of patients who were randomly assigned treatment had culture-confirmed enteric fever. To allow for a loss to follow-up rate of about 5%, a total of 853 patients with suspected enteric fever were recruited to the trial.

Times to treatment failure, fever clearance, and relapse, were analysed by use of survival methods. The cumulative incidence of events was calculated with the Kaplan-Meier method, and comparisons were based on Cox regression models with the treatment group as the only covariate. For the primary endpoint (treatment failure), we also compared the absolute risk of treatment failure until day 31 on the basis of Kaplan-Meier estimates and standard errors according to Greenwood's formula.[20] Additionally, the time to treatment failure was analysed in the subgroups defined by culture result, pathogen (*S typhi* or *S paratyphi* A), and age (<16 years or ≥16 years), and heterogeneity of the treatment effect was tested with a Cox regression model that included an interaction between treatment and subgroup.

The per-protocol analysis population consisted of all patients with blood-culture-confirmed enteric fever.

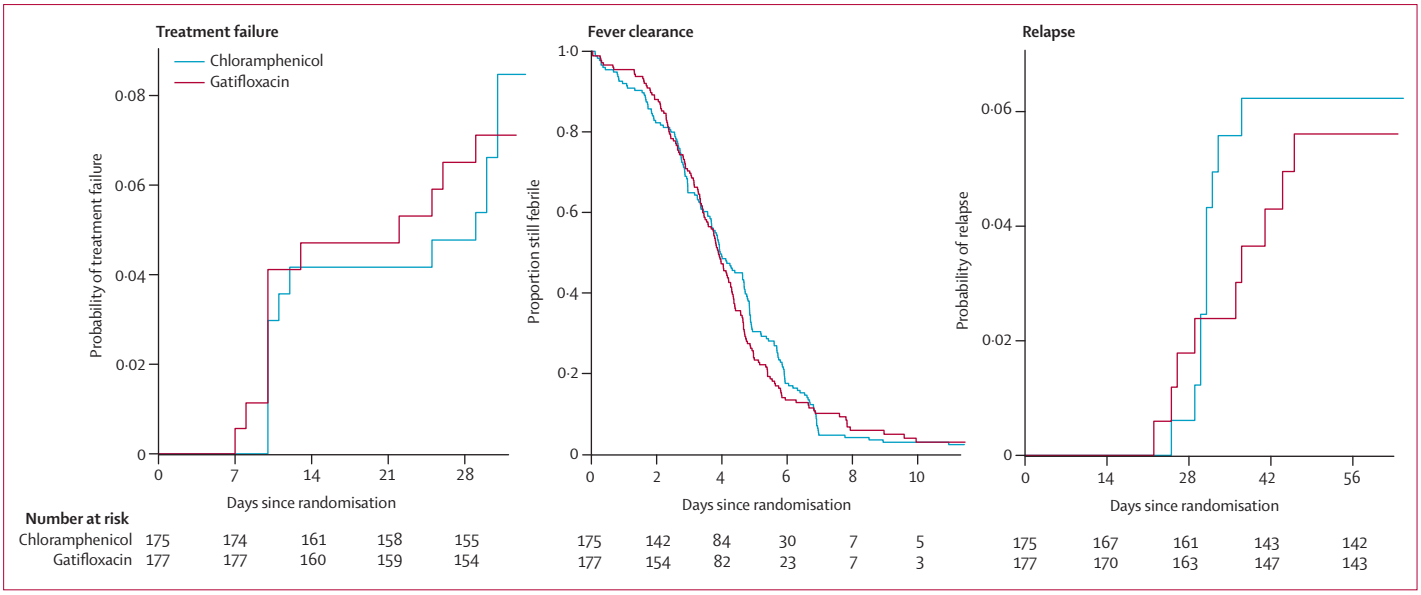

Figure 2: **Kaplan-Meier estimates for time to treatment failure, fever clearance, and relapse for culture-positive patients**

We also analysed all patients who were assigned treatment, with the exception of those patients who were mistakenly randomised or withdrew before the first dose of study treatment, for treatment failure and safety.

All reported tests were done at the two-sided 5% significance level, and 95% CIs are reported. All analyses were done with the statistical software R version 2.9.1.[21]

The trial is registered on controlled-trials.com, number ISRCTN 53258327.

Role of the funding source

The sponsor of the study had no role in study design, data collection, data analysis, data interpretation, or writing of the report. The corresponding author had full access to all the data in the study and had final responsibility for the decision to submit for publication.

Results

Of 1151 patients assessed, 853 were assigned treatment; 844 were analysed, 418 assigned chloramphenicol and

	Chloramphenicol (n=418)	Gatifloxacin (n=426)	Comparison
Total number of treatment failures*	26	15	HR 0·57 (95% CI 0·30–1·08), p=0·09
Persistent fever at day 10	7	6	..
Need for rescue treatment	6	4	..
Microbiological failures	0	2	..
Relapse until day 31	16	6	..
Enteric fever related complications	0	0	..
Probability of treatment failure†	0·07 (95% CI 0·04 to 0·09)	0·04 (95% CI 0·02 to 0·06)	RD −0·03 (95% CI −0·06 to 0·00); p=0·07
Median time to fever clearance (days)†	2·69 (95% CI 2·44 to 2·85)	2·69 (95% CI 2·41 to 2·88)	HR 0·99 (95% CI 0·87 to 1·14); p=0·93
Microbiological failures‡	0/185 (0%)	2/181(1%)	p§=0·24
Relapses until day 31	16	6	HR 0·37 (95% CI 0·14 to 0·94); p=0·04
Number of culture confirmed relapses	8	3	..
Number of syndromic relapses	8	3	..
Probability of relapse until day 31†	0·04 (95% CI 0·02 to 0·06)	0·02 (95% CI 0·00 to 0·03)	..
Relapses until day 62	23	12	HR 0·50 (95% CI 0·25 to 1·02); p=0·06
Number of culture confirmed relapses	12	5	..
Number of syndromic relapses	11	7	..
Probability of relapse until day 62†	0·06 (95% CI 0·04 to 0·08)	0·03 (95% CI 0·01 to 0·05)	..

HR=hazard ratio (based on Cox regression). RD=absolute risk difference (based on Kaplan-Meier estimates). *Patients can have more than one type of treatment failure. †Kaplan-Meier estimates. ‡Only patients with a blood culture taken on day 8. §Based on Fisher's exact test.

Table 3: **Summary of primary and secondary outcomes for all patients**

	Chloramphenicol	Gatifloxacin	HR (95%CI)	p for heterogeneity*
Population				
Culture positives	14/175	12/177	0·86 (0·40–1·86, p=0·70)	0·08
Culture negatives	12/243	3/249	0·25 (0·07–0·87, p=0·03)	..
Pathogen				
Salmonella typhi	11/125	8/124	0·73 (0·29–1·82, p=0·50)	0·51
Salmonella paratyphi A	3/50	4/53	1·32 (0·30–5·91, p=0·72)	..
Age				
Less than 16 years	18/222	10/217	0·58 (0·27–1·25, p=0·17)	0·98
16 years or older	8/196	5/209	0·59 (0·19–1·8, p=0·35)	..

*Heterogeneity was tested with a Cox regression model that included an interaction between treatment and subgroup.

Table 4: **Comparison of treatment failure in the culture-positive and culture-negative population and selected subgroups**

	Chloramphenicol (n=418)		Gatifloxacin (n=426)		p value*
	Number of patients with event (%)	Number of events	Number of patients with event (%)	Number of events	
Any adverse event	99 (24%)	168	59 (14%)	73	0·0003
Abdominal pain	11 (3%)	12	8 (2%)	8	0·5
Acne	2 (<1%)	2	0	0	0·2
Anorexia	9 (2%)	10	1 (<1%)	1	0·01
Diarrhoea	24 (6%)	26	5 (1%)	5	0·0002
Dizziness	11 (3%)	11	2 (<1%)	2	0·01
Nausea	26 (6%)	29	9 (2%)	9	0·003
Oral candidiasis	4 (1%)	4	0	0	0·06
Vomiting	36 (9%)	39	35 (8%)	35	0·9
Weakness	4 (1%)	4	0 (0%)	0	0·06

All adverse events in this list were non-severe (ie, grade 1 or grade 2) except for one grade 3 dehydration in the chloramphenicol group and one grade 3 abdominal pain in the gatifloxacin group. *Based on Fisher's exact test.

Table 5: **Adverse events: comparison of overall frequency and frequency of selected adverse events between the two treatment groups**

	Chloramphenicol (n=418)	Gatifloxacin* (n=426)
At baseline		
Grade 1	2/411 (0·5%)	1/414 (0·2%)
Grade 2	0/411 (0%)	2/414 (0·5%)
On day 8		
Grade 1	4/403 (1·0%)	1/188 (0·5%)
Grade 2	3/403 (0·7%)	1/188 (0·5%)
On day 15		
Grade 1	1/351 (0·3%)	1/166(0·6%)
Grade 2	0/351 (0%)	0/166 (0%)

Data are n (%) of patients tested. Grade 1 white blood cell (WBC) count 2000–2500×10⁶/L. Grade 2 WBC count 1500–1999×10⁶/L. No grade 3 or 4 leucopenia was recorded. *Not all patients who received gatifloxacin had haematological tests on day 8 and day 15.

Table 6: **Adverse events: leucopenia**

426 gatifloxacin (figure 1). The baseline characteristics of the patients were similar in the two treatment groups (table 1). The proportion of patients with treatment failure was similar in the two treatment groups in patients with culture-positive disease (table 2). Of the five patients with persistent fever on day 10 in the gatifloxacin group (table 2), two became afebrile on day 11 and did not require rescue treatment. The other three patients were effectively treated with intravenous ceftriaxone 50 mg/kg per day in a single dose for 7 days. The five patients in the chloramphenicol group who needed rescue treatment were successfully treated with ofloxacin 20 mg/kg per day in two divided doses per day for 7 days. In all cases, rescue treatment was initiated on either day 10 or day 11.

Two patients with microbiological failure in the gatifloxacin group also had persistent fever, and responded well to ceftriaxone 50 mg/kg per day in a single daily dose for 7 days. All relapse patients, consisting of seven (five of whom were culture confirmed) in the chloramphenicol group and four (three of whom were culture confirmed) in the gatifloxacin group, were also treated with ofloxacin 20 mg/kg per day, and recovered.

The secondary outcome measures, which included fever clearance time (median 3·95 days in the chloramphenicol group and 3·90 in the gatifloxacin group) and time to relapse until day 31 or day 62 also showed no significant difference between the groups (table 2). Only syndromic relapses were documented between day 62 and 6 months. Figure 2 shows the Kaplan-Meier estimates for the time to treatment failure, fever clearance, and relapse.

Stool samples at baseline were positive for *S typhi* or *S paratyphi* A in 16 (10%) of 157 patients in the chloramphenicol group and 14 (9%) of 160 patients in the gatifloxacin group. The proportion of positive stool samples at 1–6 months of follow-up was low in both groups: at 1 month, only three (2%) of 148 and none of 154 patients were stool-culture-positive in the chloramphenicol and gatifloxacin groups (p=0·12), respectively. At the end of 3 months, only one patient (in the chloramphenicol group) had a positive stool culture, and at 6 months no patients had a positive stool culture.

Table 3 shows the primary and secondary endpoints in all randomised patients, with the exception of patients who were mistakenly randomly allocated treatment or withdrew before the first dose of study treatment. There was a slightly greater risk of treatment failure in patients receiving chloramphenicol (p=0·09). Results in selected subgroups (table 4) suggest that this is primarily due to a higher failure rate of chloramphenicol in the culture-negative population, especially a higher rate of relapses until day 31 (nine [three confirmed, six syndromic] *vs* two [both syndromic]; HR of time to relapse=0·22, 95% CI 0·05–1·01, p=0·05). The median duration of chloramphenicol treatment was 9 days (IQR 8–11) in the culture-negative population, but there was not a significant association between the duration of treatment and the time to relapse (HR=0·93, 95% CI 0·66–1·30, p=0·66).

	Chloramphenicol (n=418)	Gatifloxacin (n=426)	p value*
Hyperglycaemia, grade 2†			
At baseline	1/414 (0·2%)	2/422 (0·5%)	1·00
On day 2 to day 7‡	25/407 (6·1%)	42/414 (10·1%)	0·04
On day 8	0/402 (0%)	1/400 (0·3%)	0·50
On day 15	1/366 (0·3%)	0/351 (0·%)	1·00
On month 1	1/375 (0·3%)	0/383 (0·0%)	0·50
Hypoglycaemia, grade 2 or worse§			
At baseline	4/414 (1.0%)	4/422 (1·0%)	1·00
On day 2 to day 7‡	1/407 (0·3%)	1/414 (0·2%)	1·00
On day 8	2/402 (0·5%)	2/400 (0·5%)	1·00
On day 15	4/366 (1·1%)	3/351 (0·9%)	1·00
On month 1	3/375 (0·8%)	4/383 (1·0%)	1·00
HbA_{1c}>6%			
On month 3	22/351 (6·3%)	20/359 (5·6%)	0·8

Data are n (%) of patients tested for abnormal blood glucose. *Based on Fisher's exact test. †Grade 2 non-fasting plasma glucose 161–250 mg/dL. No grade 3 or 4 hyperglycaemias were recorded. ‡On days 2 to 7, all patients were monitored with fingerstick glucose testing. §Grade 2 non-fasting plasma glucose 40–54 mg/dL. One grade 3 hypoglycaemia (30–39 mg/dL) was recorded at baseline, and two on day 15 (one in each group). No grade 4 hypoglycaemias were recorded.

Table 7: **Adverse events: dysglycaemia**

There was no indication of treatment effect heterogeneity in the subgroups defined by pathogen or age (table 4).

Most adverse events were mild (grade 1 and 2; table 5). Adverse events were slightly more common in the culture-positive patients than the culture-negative patients. In the chloramphenicol group, 44 (25%) of 175 culture-positive patients experienced at least one adverse event (81 events in total). In the gatifloxacin group, 30 (16·9%) of 177 culture-positive patients experienced at least one adverse event (38 events in total). Three patients in the chloramphenicol group had a white-blood-cell count between 1500 and 1999×10^6 cells per L on day 5–8, and had their chloramphenicol stopped. No grade 3 or 4 leucopenia was recorded (table 6). No grade 4 hypoglycaemias were recorded (table 7), and there were no life-threatening complications of enteric fever in this cohort.

Of all the strains of *S paratyphi* A and *S typhi* isolated, 251 (73%) of 345 were nalidixic acid resistant, and two (<1%) were multidrug resistant (table 8). Both MDR strains were *S typhi* isolated from patients in the gatifloxacin group. Two *S paratyphi* A isolates were resistant to chloramphenicol, one of which was isolated from a patient in the gatifloxacin group and one of which was isolated from a patient in the chloramphenicol group.

In culture-positive patients, nalidixic acid resistance was significantly associated with a slower rate of fever clearance (HR 0·57, 95% CI 0·40–0·81, p=0·002) for patients on gatifloxacin, but there was no significant difference in speed of fever clearance between patients with nalidixic-acid-resistant strains and those without in the chloramphenicol group (0·80, 0·56–1·14, p=0·21).

	Salmonella paratyphi A (n=103)	*Salmonella typhi* (n=249)	p value
Chloramphenicol			
MIC 50 (μg/mL)	8·00	4·00	..
MIC 90 (μg/mL)	12·00	8·00	<0·0001
Range	2·00–64·00	1·50 to >256·00	..
Amoxicillin			
MIC 50 (μg/mL)	1·00	0·50	..
MIC 90 (μg/mL)	2·00	1·00	<0·0001
Range	0·50–3·00	0·04 to >256·00	..
Cotrimoxazole			
MIC 50 (μg/mL)	0·12	0·03	..
MIC 90 (μg/mL)	0·19	0·06	<0·0001
Range	0·02–0·38	0·01 to >32·00	..
Tetracycline			
MIC 50 (μg/mL)	1·50	1·00	..
MIC 90 (μg/mL)	2·90	2·00	<0·0001
Range	0·50–8·00	0·38 to >256·00	..
Ceftriaxone			
MIC 50 (μg/mL)	0·19	0·12	..
MIC 90 (μg/mL)	0·25	0·19	<0·0001
Range	0·12–0·38	0·05–0·25	..
Azithromycin			
MIC 50 (μg/mL)	12·00	6·00	..
MIC 90 (μg/mL)	16·00	12·00	<0·0001
Range	1·00–48·00	0·38–24·00	..
Nalidixic acid			
MIC 50 (μg/mL)	>256·00	>256·00	..
MIC 90 (μg/mL)	>256·00	>256·00	<0·0001
Range	1·50 to >256·00	0·38 to >256·00	..
Ciprofloxacin			
MIC 50 (μg/mL)	0·50	0·25	..
MIC 90 (μg/mL)	0·75	0·38	<0·0001
Range	0·02–1·50	0·00–1·00	..
Ofloxacin			
MIC 50 (μg/mL)	1·50	0·38	..
MIC 90 (μg/mL)	2·00	0·50	<0·0001
Range	0·06–6·00	0·02–4·00	..
Gatifloxacin			
MIC 50 (μg/mL)	0·50	0·12	..
MIC 90 (μg/mL)	0·50	0·19	<0·0001
Range	0·02–1·50	0·00–1·00	..
Multidrug-resistant isolates	0 (0%)	2 (0·82%)	1·00
Nalidixic-acid-resistant isolates	92 (90·2%)	159 (65·43%)	<0·0001

*102 *S typhi* and 243 *S paratyphi* A were available for MIC testing. MIC50/90=concentration at which 50% and 90% of the organisms, respectively, are inhibited. Multidrug resistance is defined as resistance to chloramphenicol, ampicillin, and co-trimoxazole. Comparisons are based on Wilcoxon test for continuous data and Fisher's exact test for categorical data.

Table 8: **Antimicrobial susceptibility results: minimum inhibitory concentrations (MICs)* and resistance profile of *Salmonella paratyphi* A and *S typhi* isolates**

Discussion

Both chloramphenicol, which is a readily available drug in many resource-poor settings, and gatifloxacin, which is a newer generation fluoroquinolone, had excellent efficacy

in the treatment of culture-positive enteric fever, and both drugs had a favourable side-effect profile. Gatifloxacin did as well as, but was not superior to, chloramphenicol in an area with a high proportion (73%) of nalidixic-acid-resistant *S typhi* and *S paratyphi* A strains, but almost no chloramphenicol resistance.

With 844 patients analysed (figure 1), this is to our knowledge the largest randomised controlled trial in enteric fever, and the biggest trial comparing chloramphenicol with a fluoroquinolone. This is also the first trial to compare chloramphenicol to a fluoroquinolone in a predominantly paediatric population (table 1). We also assessed the—to our knowledge—largest population of blood-culture-negative patients with enteric fever. In patients who had blood-culture-negative syndromic enteric fever, both drugs were effective, but gatifloxacin was more effective in reducing syndromic clinical relapse.

There are underlying technical issues for typhoid and enteric fever treatment trials. One of the central limitations is the low sensitivity of the blood culture technique, which is estimated to be between 40% and 50%.[22] That most patients with enteric fever are categorised as syndromic, and treated empirically without a definitive diagnosis for enteric fever, is therefore not surprising. For the same reason, syndromic relapse was included as an outcome event in the a-priori defined analysis plan in this study.

The antibiotics used in this trial show different pharmacological properties. Gatifloxacin has important features likely to help with treatment adherence compared with chloramphenicol: gatifloxacin only needs to be taken once a day for 7 days, whereas chloramphenicol requires four doses per day for 14 days. There was no difference between the two drugs in terms of treatment failure and fever clearance time in the culture-positive group; however, the adverse effects profile showed that anorexia, nausea, diarrhoea, and dizziness, were significantly worse in the chloramphenicol group (table 5).

We monitored blood glucose levels closely in both treatment groups chiefly because of a recent Canadian, retrospective case-control study of 1·4 million elderly individuals (mean age 77) that showed that gatifloxacin was associated with dysglycaemia.[23] After this report, gatifloxacin was withdrawn from the US and Canadian markets. In our trial, between day 2 and day 7, the proportion of patients with a high (grade 2; 161–250 mg/dL) non-fasting blood glucose on finger-stick testing was higher in the gatifloxacin group versus the chloramphenicol group. However, there was no difference on days 15 and days 30. Similarly, at the end of 3 months, HbA_{1c} concentrations were not different in the two groups (table 7). Additionally, previous studies using gatifloxacin in a younger population have not reported clinically relevant dysglycaemia.[24] Finally, in another study comparing gatifloxacin with ofloxacin for the treatment of enteric fever that we are doing (ISRCTN63006567), we have not recorded any dysglycaemia. The gatifloxacin-associated dysglycaemia in the Canadian study might be attributed to an age-related decrease in renal function in elderly patients receiving gatifloxacin, and there might well be a pharmacokinetic or pharmacodynamic rationale for a potential age-related dose reduction.[25] Treatment options for enteric fever are clearly limited. Gatifloxacin is an efficacious drug for the treatment of enteric fever in young and otherwise healthy patients, and should be available for indication in this neglected disease. It would be prudent not to use gatifloxacin in patients over 50 years of age, or in patients with comorbidities such as diabetes or renal failure.

Most enteric fever trials are done in an inpatient setting, which does not reflect reality in developing countries, where most uncomplicated enteric fever treatment is done in an outpatient setting.[1,8] Our trial was completed

***Panel:* Research in context**

Systematic review

We searched Medline for the terms "gatifloxacin", "chloramphenicol", "clinical trial", and "typhoid/enteric fever". We also identified relevant articles from a recent Cochrane review,[7] WHO typhoid guidelines,[22] and a recent meta-analysis of fluoroquinolones versus other antibiotics in the treatment of typhoid fever.[26] There were ten trials[27–36] in the meta-analysis that compared fluoroquinolones with chloramphenicol. Multidrug-resistant strains were absent in all but one trial,[27] and nalidixic acid resistance was only reported in one trial[27] in which there were no nalidixic-acid-resistant strains. The meta-analysis concluded that fluoroquinolones were not significantly different from chloramphenicol for clinical failure or microbiological failure in an adult population. However, the sample sizes of the trials included in the analysis were small, and there was a paucity of paediatric data. There were only two previous trials of gatifloxacin for the treatment of uncomplicated enteric fever: one from Nepal and one from Vietnam.[16,17]

Interpretation

Gatifloxacin was not better than chloramphenicol in children and adults in Nepal with enteric fever. Both gatifloxacin and chloramphenicol showed similar efficacy in the treatment of blood-culture-positive enteric fever in a setting with strains of *S typhi* and *S paratyphi* A fully sensitive to chloramphenicol and resistant to nalidixic acid. Our trial showed that both in the adult and paediatric population gatifloxacin was not better than chloramphenicol. However, in a developing-country setting like Nepal in a young population where this disease predominates, gatifloxacin should be the preferred choice because of its shorter treatment duration, fewer adverse events, and lower cost in the treatment of enteric fever.

in an outpatient setting with the help of CMAs, as described in our earlier trial.[16] This model is more applicable to developing countries.

A very attractive feature, especially for resource-poor settings, is the inexpensiveness of the antibiotics studied here. The average price for a 14-day treatment course with chloramphenicol was US$7. The average price for a 7-day treatment with gatifloxacin was US$1·5.

A recent Cochrane review (panel) of fluoroquinolones for the treatment of enteric fever pointed out the weaknesses of typhoid fever treatment trials that have small sample sizes, inadequate randomisation and concealment, incomplete follow-up, and a lack of paediatric patients and standardised endpoints.[7] We tried to address these criticisms by recruiting a large sample of patients, by percisely defining our endpoints, and by attempting to reduce bias within the limits of an open trial.

Two other trials used gatifloxacin for the treatment of enteric fever (panel). [16,17] The first trial compared gatifloxacin to cefixime, and enrolled children and adult outpatients in Nepal.[16] This trial had to be prematurely stopped on the advice of the independent data safety monitoring committee because of the poor performance of cefixime. There was a high rate of overall treatment failure (persistent fever at day 7, relapse and death) with 29 (38%) of 70 patients failing in the cefixime group compared with three (3%) of 88 patients in the gatifloxacin group (HR 0·08, 0·03–0·28, p<0·001). There was one death in the cefixime group.

The second trial compared gatifloxacin with azithromycin, and was done in paediatric and adult inpatients in Vietnam.[17] There was no statistical difference between the two antibiotics, and both showed excellent efficacy. The median fever clearance times were 106 h in both groups. 13 (9%) of 145 patients in the gatifloxacin group had overall treatment failure as did 13 (9%) of 140 in the azithromycin group (HR 0·93, 0·43–2·0, p=0·85). Both trials were done in regions with high rates of nalidixic-acid-resistant strains: 83% in Nepal and 96% in Vietnam. In previous trials in Vietnam, patients treated with the older generation fluoroquinolone ofloxacin given at 20 mg/kg per day showed high clinical failure rates of 36% (23 of 63 patients) and prolonged mean fever clearance times of 8·2 days (95% CI 7·2–9·2 days).[37]

Gatifloxacin is not superior to chloramphenicol in terms of efficacy. However, on the basis of its shorter treatment duration, fewer adverse events, and lower cost, gatifloxacin should be the preferred treatment of enteric fever in developing countries.

Contributors

AA, BB, AK, SB, JF, and CD designed the study. AA, BB, SK, AK, SD, KA, NS, KS, MS, SL, KS, NK, US, JC, SB, JF, and CD participated in data collection. AA, BB, JF, MW, and CD analysed the data. AA, BB, JF, MW, and CD wrote the manuscript. All authors read and approved the final manuscript.

Conflicts of interest

BB, JF, and CD are supporting an application to the WHO Essential Medicines List (EMS) in support of *Gatifloxacin for treating enteric fever, Submission to the 18th Expert Committee on the Selection and Use of Essential Medicines*. All other authors declared no conflicts of interest.

Acknowledgments

We sincerely thank all our community medical auxiliary workers—namely, Surendra Shrestha, Randeep Tamrakar, Nabraj Regmi, Balmukunda Neupane, and Nabin Adhikari. Thanks also to Upendra Baral for looking after the office administration. We would also like to thank Krishna Prajapati, Rita Bajracharya, Nabaraj Dahal, and others from the laboratory at Patan Hospital. We are grateful to Bharat Yadav and his team of doctors, health assistants, and nursing staff at Patan Hospital Emergency and Outpatient Clinics. We would also like to thank the data and safety monitoring committee—namely, Zulfikar Bhutta, Phung Tuan, Keith P Klugman, Guy Thwaites, and Buddhi Paudyal. Finally, we are indebted to our patients and their families from Lalitpur. We thank the Wellcome Trust of Great Britain for funding the study.

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BMC Infectious Diseases

RESEARCH ARTICLE Open Access

The sensitivity of real-time PCR amplification targeting invasive *Salmonella* serovars in biological specimens

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Abstract

Background: PCR amplification for the detection of pathogens in biological material is generally considered a rapid and informative diagnostic technique. Invasive *Salmonella* serovars, which cause enteric fever, can be commonly cultured from the blood of infected patients. Yet, the isolation of invasive *Salmonella* serovars from blood is protracted and potentially insensitive.

Methods: We developed and optimised a novel multiplex three colour real-time PCR assay to detect specific target sequences in the genomes of *Salmonella* serovars Typhi and Paratyphi A. We performed the assay on DNA extracted from blood and bone marrow samples from culture positive and negative enteric fever patients.

Results: The assay was validated and demonstrated a high level of specificity and reproducibility under experimental conditions. All bone marrow samples tested positive for *Salmonella*, however, the sensitivity on blood samples was limited. The assay demonstrated an overall specificity of 100% (75/75) and sensitivity of 53.9% (69/128) on all biological samples. We then tested the PCR detection limit by performing bacterial counts after inoculation into blood culture bottles.

Conclusions: Our findings corroborate previous clinical findings, whereby the bacterial load of *S.* Typhi in peripheral blood is low, often below detection by culture and, consequently, below detection by PCR. Whilst the assay may be utilised for environmental sampling or on differing biological samples, our data suggest that PCR performed directly on blood samples may be an unsuitable methodology and a potentially unachievable target for the routine diagnosis of enteric fever.

Background

The detection of invasive *Salmonella* serovars such as *Salmonella* Typhi (*S.* Typhi) and *Salmonella* Paratyphi A (*S.* Paratyphi A) remains a challenging problem. Depending on the location, various different tests and clinical criteria are used to distinguish febrile disease of differing aetiology, many of which still may remain unsatisfactorily identified. In resource poor settings with a high disease burden, enteric fever is largely distinguished on the basis of clinical symptoms and syndromes [1-4]. Yet, clinical symptoms are not the most reliable assessment for enteric fever, as other conditions, such as typhus, malaria and leptospirosis have similar clinical manifestations and are also common in places such as Nepal [5,6].

The current WHO guidelines for typhoid fever states that "The definitive diagnosis of typhoid fever depends on the isolation of *S.* Typhi from blood, bone marrow or a specific anatomical lesion" and concludes "Blood culture is the mainstay of the diagnosis of this disease" [7]. However, in practice, neither blood or bone marrow culture is performed routinely. Many hospitals in resource limited settings do not have adequate microbiological laboratory facilities and personnel to perform such a technique. Our current unpublished data suggests that only 40% of

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patients with a clinical syndrome indicative of enteric fever attending Patan Hospital in Kathmandu are culture positive for invasive *Salmonellae*. The culturing of bone marrow biopsies from enteric fever patients has a higher sensitivity than blood culture (between 70% and 80% on clinically diagnosed cases [8,9]) but is seldom performed due to the aggressive nature of the investigation. Culturing biological specimens from patients can also not be considered rapid; it may take between one and three days for positive blood culture and a further one to two days for identification and antimicrobial resistance profiling.

We aimed to develop, initially for research purposes, a robust and rapid test for the identification of *S.* Typhi and *S.* Paratyphi A in biological specimens, with the possibility that it may form the basis of a suitable diagnostic test in the future. PCR offers a potentially attractive methodology for the detection of invasive *Salmonella* serovars. PCR amplification is commonly used in many clinical research laboratories for the detection of multiple pathogens. Furthermore, there are several publications demonstrating the utility of PCR for the detection of invasive *Salmonella* serovars in the blood [10-14]. Yet, the use of PCR for the definitive diagnosis of enteric fever is somewhat contentious, despite the method being previously referred to as "the gold standard for diagnosis" [15]. Our understanding is that PCR is not routinely performed in areas with endemic enteric fever and the invalidated methodology means PCR should be not considered a reliable method for diagnosis or for measuring disease burden.

Here we address some of the issues with the use of PCR for detection of invasive *Salmonella* serovars, and consider if this methodology could evolve into a standardised test that may be used as a complementary diagnostic tool in the future. Therefore, we developed a novel multiplex real-time PCR assay that would amplify specific DNA sequences from *S.* Typhi and *S.* Paratyphi A. We then tested the methodology on biological samples collected from enteric fever patients.

Methods

Patient selection, blood and bone marrow sampling

Blood samples were collected from patients presenting to Patan Hospital, Kathmandu, Nepal with suspected uncomplicated enteric fever that had not taken antimicrobials prior to admission. Bone marrow specimens were taken from patients admitted to the Hospital for tropical diseases in Ho Chi Minh City, Vietnam with suspected enteric fever. The study was approved by the scientific committees and ethical committees of the participating institutions. Written informed consent was obtained from all participants or guardians of participants. Samples of 10 ml of anti-coagulant blood were collected in EDTA tubes from febrile patients over the age of 12 years old; 6 ml was used for the isolation of *Salmonella* serovars by routine blood culture. The remaining 4 ml was centrifuged at 1,100 RCF for 10 minutes and the plasma and whole blood cell pellets were separated and stored at -80°C. Bone marrow biopsies were taken as previously described [16], bone marrow was cultured for the isolation of *Salmonella* serovars and 1 ml of tissue was stored at -80°C until DNA extraction.

Target sequence selection

Sequences unique to *S.* Typhi or *S.* Paratyphi A were identified using a whole-genome comparison of *S.* Typhi strain CT18 (GenBank AL513382) [17] and *S.* Paratyphi A strain AKU12601 (GenBank FM200053) [18], conducted using BLASTn and visualized using the Artemis Comparison Tool (ACT). To confirm whether these sequences were likely to discriminate more generally between members of the *S.* Typhi and *S.* Paratyphi A populations, we searched for sequences in all available *S.* Typhi (finished sequence for strain Ty2 (GenBank AE014613) and 17 additional 454 shotgun-sequenced strains (GenBank CAAV01000001-CAAV01003682)) [19] and *S.* Paratyphi A strains (finished sequence for strain ATCC9150 (GenBank CP000026)). Genomic data from the recent *S.* Typhi and *S.* Paratyphi A sequencing projects were mined to find genes that were specific for each serovar [18,19]. The criteria for selection were; a lack of homology with other genes in other pathogens or human sequences (to ensure no cross-reactivity) and the sequence was required to be conserved in all the re-sequenced and previously sequenced strains.

DNA manipulation, bacterial strains and construction of internal control

All bacterial strains used in this study are presented in Table 1. Strain *E. coli* VU1 was constructed by PCR amplifying the gB gene from Phocid herpes virus using the primers phHV-1 forward and reverse [20]. The gB gene amplicon was cloned into plasmid pCR 2.1-TOPO (Invitrogen). *E. coli* VU1 was to act as an internal control to monitor DNA extraction and amplification efficiency in all PCR reactions using primers phHV-1 forward and reverse and a specific probe [20]. PCR amplicons for all target sequences were produced by monoplex conventional PCR using the primer sequences outlined below. *E. coli* TOP10 cells (Invitrogen) were transformed with purified plasmid DNA containing target DNA sequence and PCR amplicons were sequenced (Applied Biosystems) to ensure accurate amplification. Purified plasmid DNA was used as template in all subsequent experiments which utilized a standard curve.

Total genomic and plasmid DNA extraction

Volumes of 200 μl to 2 ml of experimental blood samples (for laboratory assessment) were used for total DNA

Table 1: Bacterial strains used in this study

Strains	Number	Description/Source
Laboratory isolates		
Salmonella Typhi CT18	-	Sanger Institute collection
Salmonella Paratyphi A AKU12601	-	Sanger Institute collection
E. coli Vu 1	-	Cloned gB target sequence - this study
E. coli Vu 2	-	Cloned STY0201 target sequence - this study
E. coli Vu 3	-	Cloned SSPA2308 target sequence - this study
Clinical isolates		
Salmonella Typhi	80	OUCRU Nepal
Salmonella Paratyphi A	60	OUCRU Nepal
Staphylococcus. spp	3	OUCRU Vietnam
Streptococcus pneumoniae	1	OUCRU Vietnam
Streptococcus suis type 2	2	OUCRU Vietnam
Streptococcus group B	1	OUCRU Vietnam
Neisseria meningitidis	1	OUCRU Vietnam
Citrobacter freundii	4	OUCRU Vietnam
Klebsiella pneumoniae	1	OUCRU Vietnam
Salmonella serotypes	10	OUCRU Vietnam

extraction. From patient samples, we consistently used 2 ml of blood cell pellets and 1 ml of bone marrow biopsies spiked with 50 μl of *E. coli* VU1 for total DNA isolation. Extractions were performed under sterile conditions using the QIAamp DNA Blood Midi Kit (Qiagen) according to the manufacturer's recommendations. DNA was re-suspended in 300 μl of elution buffer, stored at 4°C and subjected to PCR within 24 hours of preparation. Plasmid DNA was purified from *E. coli* VU1 and from strains containing PCR target DNA using the QIAprep Spin Miniprep (Qiagen) according to the manufacturer's recommendations. In total, the PCR assay was performed on blood samples from 100 patients with blood culture confirmed enteric fever, 50 blood samples from patients with presumptive enteric fever (blood culture negative), 25 patients with bacteraemia caused by organisms other than *S.* Typhi or *S.* Paratyphi A and 28 bone marrow biopsies from patients with culture confirmed enteric fever cause by *S*. Typhi.

Primers and PCR conditions

Primers and probes specific A were designed using Primer Express Software (Applied biosystems) and manufactured by Sigma -Proligo (Singapore). Primers and probes sequences were as follows; *S.* Typhi; ST-Frt 5' CGCGAAGTCAGAGTCGACATAG 3', ST-Rrt 5' AAGACCTCAACGCCGATCAC 3', ST- Probe 5' FAM-CATTTGTTCTGGAGCAGGCTGACGG-TAMRA 3'; *S.* Paratyphi A; Pa-Frt 5'ACGATGATGACTGATTTATC-GAAC 3', Pa-Rrt 5' TGAAAAGATATCTCTCA-GAGCTGG 3', Pa-Probe 5' Cy5-CCCATACAATTTCAT TCTTATTGAGAATGCGC-BHQ5 3' and Phocid herpes virus; PhHV-Frt 5' GGGCGAATCACAGATTGAATC 3', PhHV-Frt 5' GCGGTTCCAAACGTACCAA 3', phHV-Probe-hex 5' Hex-TTTTTATGTGTCCGCCACCATCT-GGATC-TAMRA 3'.

PCR reactions were performed in 25 μl reaction volumes consisting of 5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 1 U of Hot start Taq DNA polymerase (Qiagen) and 5 μl of template DNA. Final reaction concentrations of the three primer and probe sets for internal control, *S.* Typhi and *S.* Paratyphi A were 0.4 μM of each primer and 0.15 μM of each probe. PCR was performed on a Bio-Rad Chromo 4 real-time PCR system and fluorescence was released via the TaqMan 5' to 3' exonuclease activity. All PCRs were cycled under the following conditions; 15 min at 95°C and 45 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C.

Real-time PCR, quantification, reproducibility and interpretation

Plasmid DNA with cloned target DNA sequences (*S.* Typhi and *S.* Paratyphi A) were purified and concentrations (μg/ml) were calculated by a NanoDrop spectro-

photometer (Thermo-Scientific). Concentrations were converted to copy number using the formula; mol/g × molecules/mol = molecules/g, via a DNA copy number calculator http://www.uri.edu/research/gsc/resources/cndna.html. Plasmid solutions were diluted in 10-fold serial dilutions ranging from 10^0 to 10^5 plasmid copies per μl. Serially diluted plasmid DNA was mixed in increasing (*S.* Typhi target) and decreasing (*S.* Paratyphi A target) concentrations and subjected to real-time PCR amplification. Standard curves for *S.* Typhi and *S.* Paratyphi A copy number were constructed by plotting the *Ct* value against the plasmid DNA copy number. The intra-assay co-efficient of variance was calculated by the assessing deviation in *Ct* values of a selected plasmid concentration. This was performed using four replicates all of which were amplified on the same day. Inter-assay variation was calculated by measuring the variation in *Ct* values of selected concentrations over a four day period. Accurate DNA extraction and amplification was confirmed in all experiments by production of a green signal from the internal control. A negative PCR result was concluded if negative controls were negative, the internal control showed an expected *Ct* value and the reporter signal for *S.* Typhi or *S.* Paratyphi A could not be detected. Data was deemed none-interpretable when the negative control demonstrated contamination and/or the internal control did not yield a sufficient *Ct* value. For each run of the real-time PCR assay, DNA from *S.* Typhi CT18 and *S.* Paratyphi A AKU12601 was included as a positive control in the assay plate. All statistical analyses were performed in R http://www.r-project.org/.

Laboratory assay for detection limits

The experimental detection limit was calculated using two methodologies and subsequent results were compared to assess variability. Initially, 10 ml cultures of *S.* Typhi and *S.* Paratyphi A were grown overnight with aeration at 37°C in Luria-Bertani media and 200 μl was used to inoculate one ml of whole blood. Serial dilutions of the inoculated blood samples were made and the bacterial suspensions were concurrently serially diluted in phosphate buffered saline. 200 μl of each dilution of saline and the corresponding blood specimen was used for total DNA extraction (as described above). DNA was re-suspended in 200 μl of elution buffer. Bacterial counts for each bacterial dilution in blood and saline were performed in triplicate and enumerated on Luria-Bertani media. The numbers of colony forming units were compared to the *Ct* value following real-time PCR amplification. Additionally, real-time PCR was performed on serial dilutions of isolated plasmid DNA containing cloned target sequences until a positive signal could no longer be detected from the assay.

Blood inoculation experiment

One ml of whole blood was inoculated with 200 μl of an overnight culture of *S.* Typhi (as above) and was equilibrated at 37°C with agitation for 16 hours. Serial dilutions of the inoculated blood sample were performed concurrently in phosphate buffered saline and whole blood. The resulting bacterial dilutions were enumerated on Luria-Bertani media and total DNA was extracted from 200 μl of diluted blood and PBS. Additionally, the remaining diluted blood samples were inoculated into 25 ml BACTEC Plus aerobic bottles (Becton - Dickinson) and incubated at 37°C in a BACTEC 9050 machine (Becton Dickinson) as per the manufacturers recommendations, until growth was detected. Any cultured organisms were sub-cultured to ensure no contamination. This experiment was also performed on inoculated whole blood individually treated with gentamycin and ciprofloxacin. Blood was inoculated as before and either gentamycin or ciprofloxacin (Sigma Aldrich) was added (to a final concentration of 100 μg/ml) and incubated at 37°C for two hours.

Results

Optimisation of a three color multiplex real-time PCR assay

We were able to identify several potential DNA sequence targets that were specific to *S.* Typhi or *S.* Paratyphi A and demonstrated no DNA homology to other sequences found in database searches. Ultimately, we selected an individual coding sequence target from each of the two serovars. These were; STY0201 from *S.* Typhi, (encoding a putative fimbrial-like adhesin protein located at position 210,264 in the *S.* Typhi CT18 chromosome genome sequence (Accession number NC_003198)) and SSPA2308 from *S.* Paratyphi A (encoding a hypothetical protein at position 2,572,177 in the *S.* Paratyphi A AKU_12601 chromosome (Accession number FM200053)).

The *S.* Typhi specific primers were predicted to produce a 131 bp amplicon from within gene STY0201 and the *S.* Paratyphi A primers were predicted to amplify a 104 bp fragment within the gene SSPA2308. PCR reactions were optimized and multiplexed. Strain *E. coli* incorporating the VU1 phocid virus gene was added to ensure accurate DNA extraction from all specimens and to act as a positive control during amplification. An *E. coli* or *Salmonella* gene target was deemed inappropriate due to obvious cross hybridization problems.

The serovar specific loci were present in all available genome sequences and we ensured the presence of the target sequences on DNA extracted from 140 *S.* Typhi and *S.* Paratyphi A strains by PCR (these strains included the 100 strains isolated from blood specimens used in later experiments). Prior to extraction, the bacterial cul-

tures were spiked with 200 μl of *E. coli* VU1. To control for potential cross reactivity, 10 other *Salmonella* serovars (including Enteritidis, Typhimurium and Paratyphi C) and 13 other bacterial pathogens commonly isolated during blood culture, including *Staphylococcus aureus* and *Streptococcus pneumoniae* (Table 1) were tested for the presence of the DNA sequences.

When the real-time PCR amplification was performed on DNA prepared from either *S.* Typhi or *S.* Paratyphi A the assay demonstrated serovar specific amplification on all tested DNA samples. We could detect a positive internal control signal in all amplifications and were unable to detect amplification of the *S.* Typhi and *S.* Paratyphi A target sequences in DNA from other *Salmonella* serovars or other bacterial pathogens (data not shown). Therefore, on extracted DNA, the real-time PCR assay demonstrated good specificity. The final assay conditions demonstrated no cross-hybridization when performed individually on DNA extracted from *E. coli* VU1, *S.* Typhi or *S.* Paratyphi A (Table 2). The addition of the internal control did not hinder detection of the target sequences from either *S.* Typhi or *S.* Paratyphi A over a range of DNA concentrations (Table 2).

Using serially diluted quantities of plasmid DNA containing *S.* Typhi and *S.* Paratyphi A (extracted from strains VU2 and VU3) target sequences, we assessed the detection limit, reproducibility and quantitative ability of the assay. Table 2 shows the results of consecutive standard curve experiments and demonstrates the overall performance, intra-assay variation and the inter-assay variation. The inter-assay co-efficient of variance ranged from 0.86 to 3.39% with copy number ranging from 5×10^1 to 5×10^5 copies per reaction. Repeat standard curve experiments were performed on DNA extracted from PBS and whole blood spiked with *S.* Typhi (Table 3). There was an insignificant variation ($p > 0.1$ with noneparametric student's t-test) in *Ct* value when the PCR assay was performed on DNA extracted from whole blood or PBS inoculated with a known quantity of bacterial cells. The detection limit of the assay ranged from between 1 to 5 target copies per reaction. Therefore, in spiked samples, the real-time PCR method, was specific, sensitive and not influenced by potential inhibitors in blood or by the addition of the *E. coli* internal control.

Performance of PCR assay on biological specimens

We performed the multiplex PCR assay on blood samples taken from 100 culture confirmed enteric fever patients. Fifty four of the 100 blood samples were culture positive for *S.* Typhi and 46 blood samples were culture positive for *S.* Paratyphi A. Both the *S.* Typhi and the *S.* Paratyphi A clinical isolates from these blood samples were verified for the real-time PCR target and all the *S.* Typhi and the *S.* Paratyphi A strains isolated from the corresponding blood samples had the appropriate DNA targets.

The real-time PCR was performed on DNA extracted from blood taken for microbiological culture at the time of clinical diagnosis, prior to the administration of antimicrobials. All samples were inoculated with *E. coli* VU1 before DNA extraction to ensure reliable DNA isolation and amplification. PCR was performed using 5 μl of DNA taken from a 300 μl re-suspension volume, which correlated with a 4 ml of whole blood; we calculated that the final PCR amplification was performed on an equivalent volume of 75 μl of whole blood. Data resulting from the positive amplicons is shown in Figure 1.

Reliable amplification was obtained from the *E. coli* VU1 internal control strain in all 100 tested samples and no samples produced an amplicon which indicated co-infection with both *S.* Typhi and *S.* Paratyphi A. Serovar specific amplification for *S.* Typhi and for *S.* Paratyphi A was observed in 23 and 18 samples respectively. The multiplex real-time PCR assay, consequently, had a sensitivity of 42% (23/54) for *S.* Typhi and 39% (18/46) for *S.* Paratyphi A. We were unable to amplify *S.* Typhi or *S.* Paratyphi A target DNA from any of the 50 blood samples from enteric fever patients that were culture negative, or from DNA extracted from blood taken from 25 patients with other known causes of bacteraemia; specificity 100% (75/75).

The assay was also performed on DNA extracted from 28 bone marrow biopsies which had been cultured and were known to be positive for *S.* Typhi. Specific amplification of the *S.* Typhi target sequence was detected in DNA extracted from all 28 biopsies, thus giving a sensitivity of 100% (28/28) in these specimens (Figure 1).

Quantitative assessment of the resulting *Ct* values showed that the number of copies of target DNA was lowest for *S.* Paratyphi A, ranging from 5 to 2,000 with a median of 39 copies per ml of blood (Figure 1). The *S.* Typhi positive amplifications ranged from 8 to 6,000 copies per ml, with a median of 60 copies per ml of whole blood. There was a statistically significant increase (noneparametric student's t-test) in target copies per ml in bone marrow samples when compared to blood samples (Figure 1). The number of *S.* Typhi target sequence in bone marrow ranged from 6 to 10,000 with a median of 633 copies per ml.

Real-time PCR detection limit

We demonstrated that an equivalent *Ct* value could be generated on DNA extracted from bacteria in PBS and whole blood, thus inhibition was not the limiting factor in poor sensitivity on blood specimens. Our data suggested that the lack of positive PCR amplification was due to the low number of organisms in the blood sample, which were below the detection limit of the PCR assay. We compared blood culturing and PCR under experimental conditions. A known quantity of colony forming units of *S.* Typhi were inoculated into whole blood. The sample was

Table 2: Assessment of the reproducibility of the multiplex real-time PCR assay on diluted plasmid DNA containing cloned target sequences

Amplification target	Result	Target copies				
		5×10^4	5×10^3	5×10^2	5×10^1	5×10^0
S. Typhi without internal control	*Ct* value*	22.76	25.61	27.92	31.22	-
S. Typhi with internal control	*Ct* value	22.07	25.56	27.76	31.81	-
Intra-assay variation†	CV (%)	0.45	0.25	0.41	0.67	1.01
Inter-assay variation‡	CV (%)	1.86	1	1.97	3.39	1.19
S. Paratyphi A without internal control	*Ct* value	21.27	24.78	27.77	31.16	-
S. Paratyphi A with internal control	*Ct* value	21.42	24.66	28.28	31.84	-
Intra-assay variation	CV (%)	0.41	0.7	0.67	0.86	1.34
Inter-assay variation	CV (%)	1.34	1.34	0.86	1.25	1.83

* Mean *Ct* value calculated from 4 individual replicates on 4 separate days, n = 16. †Intra-assay variation was calculated by measuring the coefficient of variance of *Ct* value on 4 concurrently run assays. ‡ Inter-assay variation was calculated by comparing variation in *Ct* value on experiments on 4 individual occasions.

equilibrated and 10 fold serial dilutions were performed in whole blood. The diluted blood samples were cultured in order to enumerate organisms, inoculated into BACTEC bottles and incubated. Additionally, total DNA was extracted from all samples and real-time PCR was performed as before. To assess the effect of antimicrobials (PCR may detect dead organisms) we also performed an matching experiment, yet the inoculated blood samples were exposed to gentamicin or ciprofloxacin for 2 hours. Results are presented in Table 4.

Target DNA was consistently amplified in samples up to the sixth 10 fold dilution, which corresponded to 2.5×10^2 DNA copies per ml of blood (Table 4). Amplification was not prevented when samples were treated with antimicrobials. Culturing of the inoculated blood samples in BACTEC bottles was consistently more sensitive than PCR amplification, both in the presence or absence of antimicrobials (Table 4).

Discussion

A molecular method for the detection of invasive *Salmonella* serovars in biological specimens appears to be an attractive addition to current methods. However, PCR is not commonly reported for the routine identification of invasive *Salmonellae*, this is in spite of a number of publications demonstrating its potential use in the clinical setting for diagnostic testing and bacterial identification [12,21-23]. It is assumed that PCR amplification may be a suitable test where blood culturing is not routinely performed. A potential advantage of PCR is that if it had a high level of sensitivity it may be performed on smaller volumes of blood than required for culture and may have

Table 3: Detection limit and *Ct* value comparison of PCR amplification on nucleic acid extractions from inoculated blood, inoculated PBS and purified plasmid DNA

	Amplification targets and *Ct* value					
		***S.* Typhi**			***S.* Paratyphi A**	
Equivalent cfu/ml	**PBS**	**Blood**	**Plasmid**	**PBS**	**Blood**	**Plasmid**
1×10^3	*Ct* value	21.27	24.78	27.77	31.16	-
1×10^4	*Ct* value	21.42	24.66	28.28	31.84	-
1×10^4	CV (%)	0.41	0.7	0.67	0.86	1.34
1×10^6	CV (%)	1.34	1.34	0.86	1.25	1.83

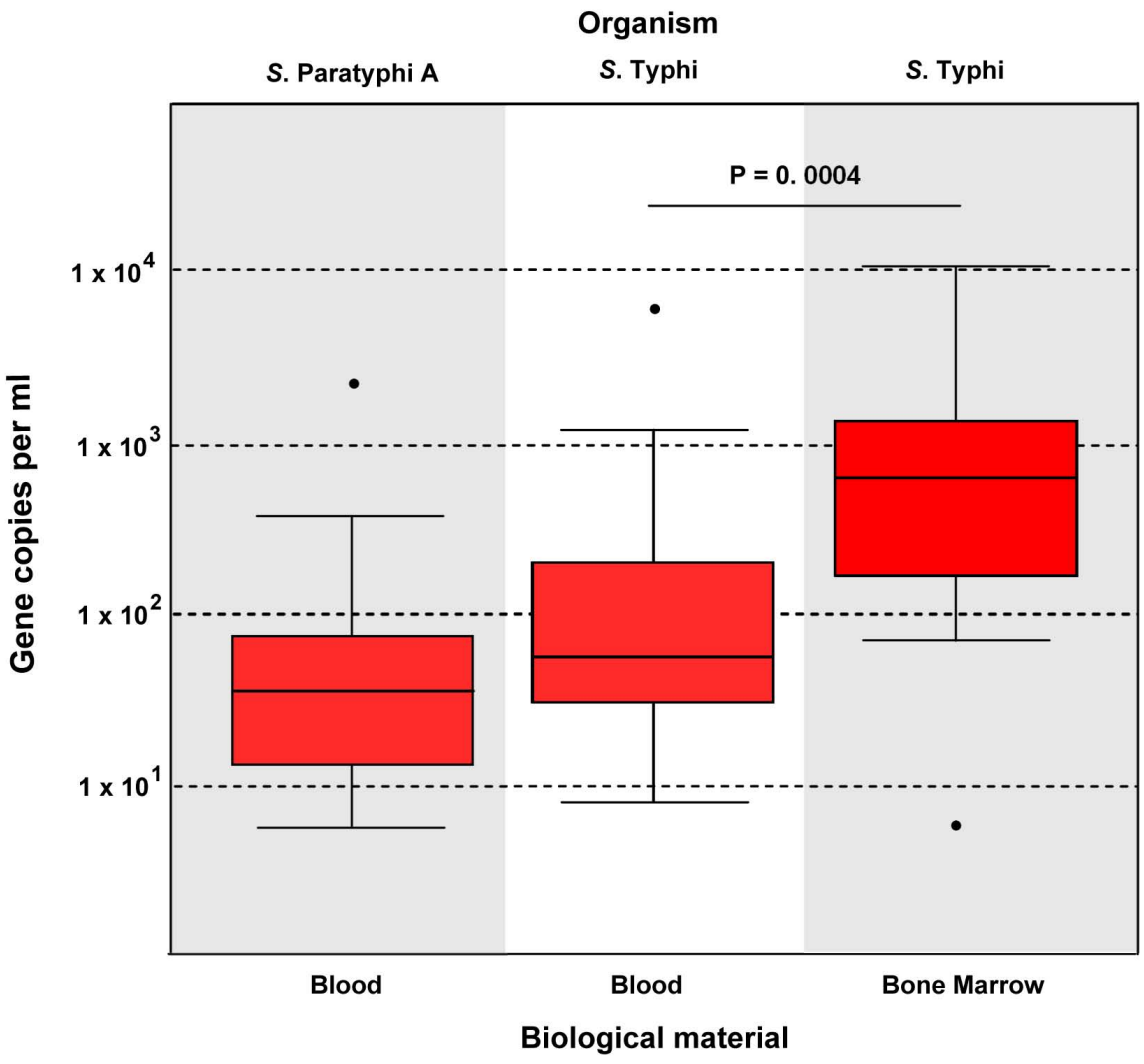

Figure 1 Real-time PCR amplification of *S.* Typhi and *S.* Paratyphi A in blood and bone marrow specimens from patients with culture confirmed enteric fever. The *Ct* values of amplification positive blood and bone marrow specimens have been converted by dilution factor to copies per ml of biological material (y axis). The median and quartile ranges of the number of copies per ml of biological sample are shown for amplification positive blood samples with *S.* Paratyphi A (n = 18), amplification positive blood samples with *S.* Typhi (n = 23) and amplification positive bone marrow samples with *S.* Typhi (n = 28). Statistical significance was calculated using a none-parametric student's t-test.

the added ability of detecting dead organisms. Such permutations may be probable in an endemic setting when taking blood from young children and when patients have access to none-prescribed antimicrobials.

Many of the previously published *S.* Typhi nucleic acid detection studies harbour limitations; the methodology is often inappropriately validated, equivalent blood volumes are not specified, the primers are nested and target the flagellin (*fliC*) gene and detection is via conventional agarose gel electrophoresis [13,15]. All these limitations may cause results with may not be reproducible and hinder the accurate amplification of target sequences in biological samples. Furthermore, many such reports suggest the usefulness of the technique in patients where enteric fever cannot be confirmed by other methods. Whilst the rapid nature of a PCR assay may compensate for many potential limitations, a balanced assessment of PCR sensitivity in a clinical setting was required. Real-time PCR addresses many of the limitations that can occur with conventional PCR. The system is sensitive, stringent and less prone to contamination with DNA from other organisms.

The data presented here may also have some limitations, including, the volume of nucleic acid used in the experimental procedure, the blood samples originating from one location and a period of storage prior to DNA

Table 4: Comparison of real-time PCR detection to blood culture with known inoculants of *S.* Typhi into blood samples

	Experimental condition and detection method								
	No antimicrobial			Gentamicin			Ciprofloxacin		
Dilution factor	cfu/ml	*Ct* value	Blood culture	cfu/ml	*Ct* value	Blood culture	cfu/ml	*Ct* value	Blood culture
10^{-4}	2.5×10^4	30.18	+	1×10^4	30.44	+	3×10^3	30.31	+
10^{-5}	2.5×10^3	33.53	+	1×10^3	33.45	+	3×10^2	33.44	+
10^{-6}	2.5×10^2	37.77	+	1×10^2	37.07	+	3×10^1	36.95	+
10^{-7}	2.5×10^1	-	+	1×10^1	-	+	3×10^0	-	+
10^{-8}	$<2.5 \times 10^1$	-	+	$<1 \times 10^1$	-	+	$<3 \times 10^3$	-	-

extraction. Nonetheless, this work represents an unbiased assessment of PCR in the identification of *S.* Typhi and *S.* Paratyphi A in biological specimens. The blood inoculation and bacterial quantification experiments support our findings on biological specimens and address some of the limitations from biological samples.

We attribute a lack of sensitivity of the assay to the low physiological level of invasive *Salmonella* organisms in the blood. The detection limits of the real-time PCR were comparable with cfu/ml in both inoculated blood and saline samples, demonstrating that human DNA or potential PCR inhibitors found in blood may not hinder amplification. We additionally found that a realistic detection limit of the assay was between 100 to 200 organisms per ml of whole blood. This may be increased by extracting DNA from a greater blood volume or by precipitation of the extracted DNA. Both improvements would be technically challenging and even if these limitations are taken into account, PCR may still fail to reach the sensitivity level of a standard blood culture.

Our quantitative data demonstrated median copies of target sequence of 39 and 60 per ml of blood for *S.* Paratyphi A and *S.* Typhi respectively and 600 copies of *S.* Typhi target per ml of bone marrow. These data are somewhat incomparable with a previous real-time PCR detection assay for *S.* Typhi in peripheral blood [10]. Massi *et al*. found a statistically significant difference between hypothetical loads of bacteria in blood between culture negative and culture positive blood specimens. Patients that were culture positive had between 1,010 and 4,350 target copies per ml of blood, whereas, patients that were culture negative had between 3.9 and 990 copies per ml of blood. Even taking into account dead organisms, these figures correspond with substantial bacterial loads in the blood of enteric fever patients. This discrepancy is an important observation as it has been shown that *S.* Typhi induces febrile disease with a nominal number of organisms circulating in the blood. Using quantitative counts of bacteria in blood, Wain *et al.* demonstrated that 25% of all acute typhoid patients had less than 0.1 cfu/ml and only 1% tested had a cfu/ml of greater than 100 organisms per ml of blood [24]. Our PCR results concur with these data and supports our understanding that the lack of sensitivity is dependent on the low number of invasive *Salmonellae* in the blood. Therefore, to detect a living organism, the PCR would have to be performed directly on DNA extracted from 10 ml of blood. A lack of detectable organisms is a potential consideration for other bacterial pathogens, such as *Mycobacterium tuberculosis*; meta-analyses suggest that PCR detection of this organism in biological material may also pose a similar challenge [25,26].

It is of note, however, that the PCR assay demonstrated a sensitivity of 100% on culture positive bone marrow biopsies. Bacterial loads in bone marrow biopsies from enteric fever patients have been shown to be significantly higher than bacterial loads in peripheral blood [16]. Additionally, the tenfold increase in copies per ml in bone marrow, when compared to blood, may be explained not only by the organisms surviving within macrophages in the bone marrow, but also the potential ability of the assay to detect DNA from dead organisms within cells. A combination of culturing, either from blood or bone marrow, with PCR amplification may improve sensitivity and time to diagnosis. It is clear that typhoid diagnostics requires the use of some new approaches and fresh considerations [27].

Conclusions

Our data demonstrates that a low level of bacteria in the blood makes PCR amplification of specific *S.* Typhi and *S.* Paratyphi A sequences on biological samples technically challenging. Whilst specificity for the technique is indisputably high, the sensitivity when compared to blood culturing is low. Further assessment of the use of PCR amplification for the detection of invasive *Salmonellae* in blood is required. Previous publications have demonstrated that PCR is both a specific and highly sensitive method for detection of *S.* Typhi in blood. Our study questions the use of PCR for the diagnosis of enteric fever

and suggests that the number of organisms and the volume of blood required for accurate identification using PCR on biological samples may be un-physiological and impractical.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
TVTN, AK, SD1, HNT, LTPT performed the experiments. SK, SD2, AA, BB and CD provided biological material and experimental input. KH performed the bioinformatic analysis. TTC and JIC cultured the micro-organisms used. NVVC, JF and SB conceived the study and prepared the manuscript. All authors have read and approved the final manuscript.

Acknowledgements
The authors wishing to acknowledge the ongoing efforts of the clinical staff, the microbiology department and the typhoid research group at Patan Hospital, Kathmandu and John Wain for supplying bone marrow samples. This work was funded by the Wellcome Trust, 215 Euston Road, London NW1 2BE, United Kingdom. SB is funded by an OAK foundation fellowship through Oxford University.

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Received: 22 June 2009 Accepted: 21 May 2010
Published: 21 May 2010

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Pre-publication history
The pre-publication history for this paper can be accessed here:
http://www.biomedcentral.com/1471-2334/10/125/prepub

doi: 10.1186/1471-2334-10-125
Cite this article as: Nga *et al.*, The sensitivity of real-time PCR amplification targeting invasive Salmonella serovars in biological specimens *BMC Infectious Diseases* 2010, **10**:125

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Highly Resistant *Salmonella enterica* Serovar Typhi with a Novel *gyrA* Mutation Raises Questions about the Long-Term Efficacy of Older Fluoroquinolones for Treating Typhoid Fever

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As a consequence of multidrug resistance, clinicians are highly dependent on fluoroquinolones for treating the serious systemic infection typhoid fever. While reduced susceptibility to fluoroquinolones, which lessens clinical efficacy, is becoming ubiquitous, comprehensive resistance is exceptional. Here we report ofloxacin treatment failure in typhoidal patient infected with a novel, highly fluoroquinolone-resistant isolate of *Salmonella enterica* serovar Typhi. The isolation of this organism has serious implications for the long-term efficacy of ciprofloxacin and ofloxacin for typhoid treatment.

Antimicrobial therapy is critical for typhoid treatment, but the circulation of antimicrobial-resistant organisms has become ubiquitous in many regions of endemicity, and the presence of multidrug-resistant *Salmonella enterica* serovars Typhi and Paratyphi A (showing resistance to chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin) precludes treatment with these antimicrobials (6). Clinicians are now highly dependent on the fluoroquinolones for typhoid therapy. Yet, predictably, widespread fluoroquinolone usage has been followed by the emergence of isolates with elevated MICs (1). These isolates are characterized by point mutations within the *gyrA* (DNA gyrase) gene and occasionally an additional nucleotide substitution in the *parC* gene (7). The propagation of these organisms is particularly concerning when one considers a lack of feasible alternatives and an explicit correlation between increasing MICs to fluoroquinolones and treatment failure (8). Yet, despite the widespread dissemination of such organisms, the isolation of organisms that exhibit MICs of >1.0 μg/ml to ofloxacin has been, until now, negligible.

In June 2011, a 13-year-old male presented to the outpatient department at Patan Hospital in Kathmandu, Nepal, with a fever that had started 10 days previously, peaking at around 39°C daily. The patient also developed a headache and mild abdominal discomfort with nausea. On the eighth day of fever, the patient was taken to a local medical store, where it was recommended that he be treated with 200 mg of ofloxacin to be taken twice daily. After 2 days of ofloxacin treatment, the patient's symptoms became more pronounced, and he showed increased restlessness and a temperature in excess of 40°C. He presented to the outpatient department on the tenth day of illness but had no signs of icterus, anemia, lymphadenopathy, cyanosis, edema, or dehydration and no rash on the trunk. His temperature was recorded at 38.8°C, with a pulse of 104/min and a respiratory rate of 24/min. A clinical diagnosis of typhoid was made, and a complete blood count and culture and sensitivity were requested. The treating clinician increased the ofloxacin dosage to 300 mg (20 mg/kg of body weight/day) twice daily for 7 days. However, the patient returned to the outpatient department after the additional 7 days of ofloxacin treatment without improvement. In the intervening period, his blood culture had yielded *Salmonella* Typhi, which was highly resistant to ofloxacin (zone size, 11 mm), ciprofloxacin (zone size, 11 mm), and nalidixic acid (zone size, 0 mm); a secondary blood culture was not taken. On the basis of these findings, he was prescribed oral azithromycin (20 mg/kg/day) once daily for 7 days. The fever declined on the third day of the azithromycin treatment (the twentieth day of fever), and ultimately, the patient made a fortuitous and uneventful recovery.

After the patient had recovered from the infection, and with written consent, we investigated the *Salmonella* Typhi isolate. The resulting fluoroquinolone MICs were exceptional; in excess of 256 μg/ml against nalidixic acid, greater than 32 μg/ml against ofloxacin and ciprofloxacin, 6 μg/ml against levofloxacin, and 2 μg/ml against gatifloxacin. The *Salmonella* Typhi isolate, was not, however, multidrug resistant. We purified DNA from the *Salmonella* Typhi isolate, aiming to define the molecular basis of the fluoroquinolone resistance. We PCR amplified the *gyrA*, *gyrB*, and *parC* genes and sequenced the resultant PCR amplicons (1). We also attempted to amplify the common Gram-negative plasmid-mediated quinolone resistance (PMQR) determinants: *qnrA*, *qnrB*, *qnrS*, *aac(6)lb-cr*, and *qepA* (5). We were unable to detect any of the five common PMQR genes. However, DNA sequencing of the three fluoroquinolone target loci identified a single previously described mutation in the *parC* gene, changing serine to isoleucine at codon 80 (S80I), and a double mutation in the *gyrA* gene (Fig. 1). The two *gyrA* mutations were both within the quinolone resistance-determining region (QRDR). The primary *gyrA* substitution was common, inducing a replacement of serine with phenylalanine at codon 83. However, the second mutation was novel, the substitution of cytosine for thymine at nucleotide 248 had the

Received 21 December 2011 **Returned for modification** 25 January 2012
Accepted 16 February 2012
Published ahead of print 27 February 2012
Address correspondence to Buddha Basnyat, rishibas@wlink.com.np.

doi:10.1128/AAC.06414-11
The authors have paid a fee to allow immediate free access to this article.

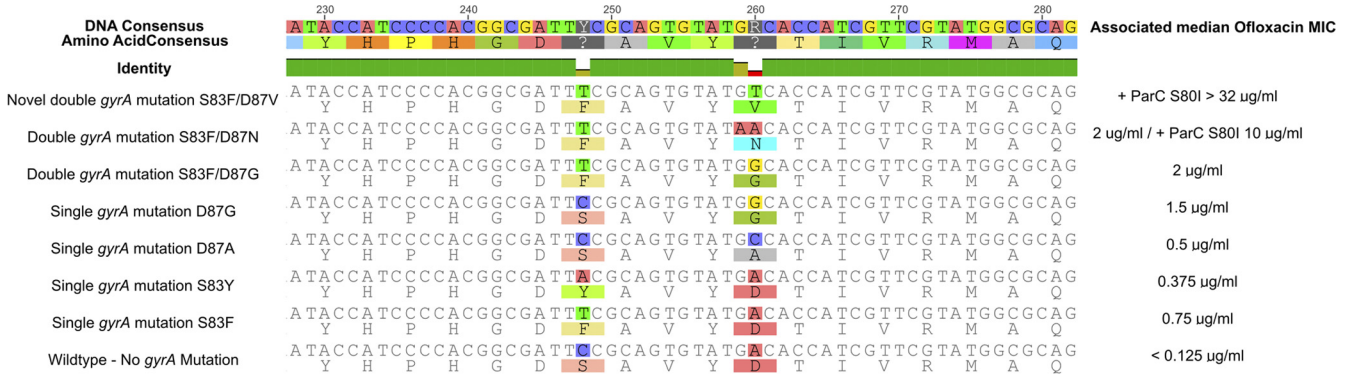

FIG 1 DNA and predicted amino acid alignments of the DNA gyrase gene (*gyrA*) from *Salmonella* Typhi isolates with reduced susceptibility to fluoroquinolones. Shown is an outline of seven identified conformations of the QRDR of the DNA gyrase *gyrA* gene in clinical *Salmonella* Typhi isolates based on the 2010 study by Parry et al. (7) and the novel S83F D87V mutation isolated here. The DNA and the corresponding amino acid consensus are shown in the first and second rows of the figure, respectively, with the DNA identity between sequences shown beneath. For each conformation, the DNA sequence is shown at the top and the predicted amino acid sequence is shown beneath. The median MIC of ofloxacin for each of the mutations (with and without a tertiary S80I mutation in the ParC topoisomerase) is shown to the right, based on data from this report and reference 3.

effect of changing aspartic acid to valine at codon 87 (Fig. 1). The resulting DNA sequence was submitted to EMBL.

Antimicrobials are crucial for treating typhoid, and resistance is the main constraint that compels antimicrobial therapy preferences to change with time. The current WHO guidelines suggest that the fluoroquinolones are the optimal group of antimicrobials for uncomplicated typhoid treatment in adults (10). However, *Salmonella* Typhi and *Salmonella* Paratyphi A isolates with reduced susceptibility to fluoroquinolones are now common in Asia and are becoming increasingly common in Africa (2, 9), yet complete resistance is rare (4). Here we report an isolate exhibiting extensive resistance against fluoroquinolones, marked by a novel *gyrA* mutation and no additional PMQR sequences. Clearly, additional characterization of this isolate is required to precisely understand the basis of its phenotype, yet its isolation should ring alarm bells within the typhoid community. Indeed, one may speculate that other such strains will emerge rapidly, should the organism have acceptable biological fitness and favorable dissemination conditions. Anecdotally, we find that chloramphenicol is making a comeback in the community in Nepal as a consequence of reducing resistance levels, yet, azithromycin will likely become the preferred drug as ofloxacin and ciprofloxacin become ineffective (3).

Nucleotide sequence accession number. The DNA sequence resulting from the cytosine for thymine substitution at nucleotide 248 has been submitted to EMBL (accession no. HE588040).

ACKNOWLEDGMENTS

We thank the microbiology laboratories at Patan Hospital in Nepal and the Oxford University Clinical Research Unit in Vietnam.

This work was supported by The Wellcome Trust of Great Britain, S.B. is supported by a fellowship from the OAK Foundation through Oxford University.

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The Microbiological and Clinical Characteristics of Invasive *Salmonella* in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal

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Abstract

Gallbladder carriage of invasive *Salmonella* is considered fundamental in sustaining typhoid fever transmission. Bile and tissue was obtained from 1,377 individuals undergoing cholecystectomy in Kathmandu to investigate the prevalence, characteristics and relevance of invasive *Salmonella* in the gallbladder in an endemic area. Twenty percent of bile samples contained a Gram-negative organism, with *Salmonella* Typhi and *Salmonella* Paratyphi A isolated from 24 and 22 individuals, respectively. Gallbladders that contained *Salmonella* were more likely to show evidence of acute inflammation with extensive neutrophil infiltrate than those without *Salmonella*, corresponding with higher neutrophil and lower lymphocyte counts in the blood of *Salmonella* positive individuals. Antimicrobial resistance in the invasive *Salmonella* isolates was limited, indicating that gallbladder colonization is unlikely to be driven by antimicrobial resistance. The overall role of invasive *Salmonella* carriage in the gallbladder is not understood; here we show that 3.5% of individuals undergoing cholecystectomy in this setting have a high concentration of antimicrobial sensitive, invasive *Salmonella* in their bile. We predict that such individuals will become increasingly important if current transmission mechanisms are disturbed; prospectively identifying these individuals is, therefore, paramount for rapid local and regional elimination.

Citation: Dongol S, Thompson CN, Clare S, Nga TVT, Duy PT, et al. (2012) The Microbiological and Clinical Characteristics of Invasive *Salmonella* in Gallbladders from Cholecystectomy Patients in Kathmandu, Nepal. PLoS ONE 7(10): e47342. doi:10.1371/journal.pone.0047342

Editor: Dipshikha Chakravortty, Indian Institute of Science, India

Received July 13, 2012; **Accepted** September 10, 2012; **Published** October 15, 2012

Funding: This work was supported by The Wellcome Trust of Great Britain. SB is funded by an OAK Foundation through Oxford University, UK. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Enteric fever is a systemic infection caused by the invasive bacteria *Salmonella* Typhi (*S.* Typhi) and *Salmonella* Paratyphi A (*S.* Paratyphi A). The disease is contracted by the ingestion of fecal material containing the pathogens [1]. The disease remains common in regions with poor standards of hygiene and sanitation, with global estimates suggesting that 27 million people are affected annually, of which 200,000 people die [2]. With adequate treatment >95% of patients recover completely from typhoid [1]. However, an estimated 2–5% of individuals infected with *S.* Typhi develop a sustained infection of the gallbladder [3]. These individuals are referred to as 'carriers', and like the infamous 'typhoid Mary' [4], are outwardly asymptomatic, continue to intermittently shed organisms for a prolonged period and often have no recollection of an acute episodes of typhoid [1].

During acute typhoid, invasive *Salmonella* cross the intestinal epithelial barrier, invade and survive within macrophages, eventually reaching the bone marrow, liver, pancreas and spleen [5]. Invasion of the gallbladder occurs either directly from the blood or by retrograde spread from the bile [1]. In a subset of individuals infected with *S.* Typhi, the organisms chronically colonize the gallbladder and carriers shed these organisms intermittently into the intestinal lumen and thus in the feces. It is gallbladder colonization and fecal shedding that form a central dogma for the transmission and persistence of typhoid fever. As a consequence of the internal localization of organisms, this dogma is difficult to challenge in humans and the host-restricted nature of the relevant pathogens make carriage difficult to replicate precisely in non-mutant mouse models [6]. As a result, data regarding the prevalence, bacteriology and mechanisms of carriage are sparse. The only population-based study estimating chronic *Salmonella* carriage in an endemic setting is from Chile where investigators gathered data from autopsies, calculating a carriage rate of 694 per 100,000 [3].

Investigations of *Salmonella* carriage suggest that the propensity to become a chronic carrier follow the typical epidemiology of gallbladder disease. Thus, the likelihood of carriage increases with age and is more common in females [7]. Existing data also imply that individuals with gallstones or other gallbladder abnormalities are at increased risk of carriage [8]. These epidemiological theories

are supported by laboratory-based investigations, which have shown that *Salmonella* can form biofilms and survive for prolonged period on gallstones [9,10].

There remains a significant burden of typhoid fever across Asia, yet the understanding of *Salmonella* carriage in these populations is limited. We have found previously that *S.* Paratyphi A can be isolated from the gallbladders of patients undergoing cholecystectomy and we suggested that carriage of invasive *Salmonella* play a pivotal role in the persistence of these pathogens in Kathmandu, Nepal [11]. We aimed to define the microbiology and epidemiology of invasive *Salmonella* carriage in Kathmandu. We demonstrate that *S.* Typhi and *S.* Paratyphi A are present in the gallbladder in a high concentration, are less common than other Gram-negative organisms, are not associated with lymphocytic infiltration in the gallbladder tissue, and do not exhibit resistance to multiple antimicrobials.

Results

Microbiological Examination of Bile from Cholecystectomy Patients

From June 2007 until October 2010, a total of 1,496 patients underwent cholecystectomy for acute or chronic cholecystitis at Patan hospital in Kathmandu. From the 1,496 patients, bile samples from 1,377 individuals were obtained and subjected to microbiological examination; 119 (8%) patients either denied consent or were unavailable for recruitment. A Gram-negative organism was isolated from 20% (274/1,377) of the bile samples. *E. coli*, *Salmonella spp.* and *Klebsiella spp.* were the most commonly isolated organisms, found in 78 (5.7%), 48 (3.5%) and 41 (3.0%) of the bile samples, respectively (Table 1). The remainder of the culture positive bile samples contained a range of organisms including *Psuedomonas spp.*, *Acinetobacter spp.*, *Enterobacter spp.*, *Citrobacter freundii*, *Vibrio spp.* and *Serratia marcescens* (Table 1). Of the 48 *Salmonellae* isolated, 24 (1.7%) were *S.* Typhi, 22 (1.6%) were *S.* Paratyphi A and two (0.1%) were *S. enterica* group C.

Forty-six *Salmonella* isolates were available for antimicrobial susceptibility testing by disc diffusion. Fifty-nine percent (27/46) of the *Salmonella* isolates were resistant to nalidixic acid, and a single *S.* Paratyphi A isolate was resistant to both nalidixic acid and ciprofloxacin. All *S.* Typhi and *S.* Paratyphi A strains were susceptible to ceftriaxone, chloramphenicol, gatifloxacin and ofloxacin and we identified no multi-drug resistant (MDR) (resistant to chloramphenicol, ampicillin and co-trimoxazole) isolates. One *S. enterica* group C isolate demonstrated resistance to nalidixic acid, ceftriaxone, gatifloxacin and chloramphenicol (Table 1).

Baseline data, stratified by microbiological culture result are shown in Table 2. Notably, fitting with the typical epidemiological characteristics of cholelithiasis, 77% (1,066/1,377) of the patients were female and the median age was 39 years (range: 16 to 76 years). The median age of those with *Salmonella* in their bile was 35 years (range: 18 to 67 years) and 73% were female. It is noteworthy that none of the pre-surgical stool cultures from any patients were *Salmonella* positive and, when questioned, only 15% (7/48) of the *Salmonella* bile-positive patients had a memorable history of typhoid, none of which had been confirmed by microbiological culture. From available records, 16% (7/43) of *Salmonella* bile-positive patients reported >5 days of fever on entry, 7% (3/46) were admitted with jaundice, 5% (2/41) had a palpable gallbladder and 4% (2/45) were admitted with pancreatitis.

Table 1. Antimicrobial resistance patterns of Gram-negative organisms from the bile of patients undergoing cholecystectomy.

Organism	Patients	Antimicrobial resistance n (%)								
	n (%)	Amoxicillin	Cefotaxime	Ciprofloxacin	Ofloxacin	Chloramphenicol	Cotrimoxazole	Gentamycin	Amikacin	Nalidixic Acid
Typhi	24 (1.7)	0/23 (0)	0/24 (0)	0/24 (0)	0/24 (0)	0/22 (0)	4/23 (17.4)	0/11 (0)	0/0 (0)	11/22 (50.0)
Paratyphi A	22 (1.6)	0/22 (0)	0/22 (0)	1/22 (4.5)	1/22 (4.5)	2/22 (9.1)	0/22 (0)	0/16 (0)	0/1 (0)	16/19 (84.2)
Other	2 (0.1)	1/2 (50.0)	0/2 (0)	0/2 (0)	0/2 (0)	1/2 (50)	0/2 (0)	0/1 (0)	0/0 (0)	1/2 (50.0)
Total *Salmonella*	**48 (3.5)**	**1/47 (2.1)**	**0/48 (0)**	**1/48 (2.1)**	**1/48 (2.1)**	**3/46 (6.5)**	**4/47 (8.5)**	**0/28 (0)**	**0/1 (0)**	**28/43 (65.1)**
Escherichia coli	78 (5.7)	32/76 (42.1)	12/76 (15.8)	23/77 (29.9)	21/77 (27.3)	14/69 (20.3)	22/76 (28.9)	4/40 (10.0)	0/39 (0)	20/42 (47.6)
Klebsiella spp	41 (3.0)	36/39 (92.3)	9/39 (23.1)	6/39 (15.4)	6/38 (15.8)	12/38 (31.6)	11/39 (28.2)	4/24 (16.7)	1/25 (4.0)	9/18 (50.0)
Pseudomonas spp	33 (2.4)	23/31 (74.2)	4/31 (12.9)	4/31 (12.9)	4/31 (12.9)	21/28 (75.0)	21/30 (70.0)	2/20 (10.0)	1/18 (5.6)	9/13 (69.2)
Acinetobacter spp	19 (1.4)	14/19 (73.7)	7/19 (36.8)	6/19 (31.6)	4/19 (21.1)	11/19 (57.9)	9/19 (47.4)	5/9 (55.6)	4/10 (40.0)	6/11 (54.5)
Enterobacter spp	21 (1.5)	16/19 (84.2)	6/20 (30.0)	2/20 (10.0)	1/20 (5.0)	3/19 (15.8)	3/20 (15.0)	1/8 (12.5)	0/5 (0)	2/14 (14.3)
Other	34 (2.5)	14/18 (77.8)	5/20 (25.0)	3/20 (15.0)	2/17 (11.8)	5/18 (27.8)	3/18 (16.7)	1/4 (25.0)	1/3 (33.3)	5/19 (26.3)
Total Non-*Salmonella*	**226 (16.4)**	**135/202 (66.8)**	**43/205 (21.0)**	**44/206 (21.4)**	**38/202 (18.9)**	**66/191 (34.6)**	**69/202 (34.2)**	**17/105 (16.2)**	**7/100 (7.0)**	**51/117 (43.6)**

doi:10.1371/journal.pone.0047342.t001

Table 2. The baseline characteristics of the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups.

	Women			Men		Typhoid fever history		Surgery	
Culture group	**Patients**	**Age**	**Patients**	**Age**	**Patients**	**Clinical diagnosis**	**Culture diagnosis**	**Elective**	**Acute**
	n (%)	**Median (range)**	**n (%)**	**Median (range)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**	**n (%)**
Salmonella positive	48 (3.5)	34.5 (20–67)	35 (72.9)	38 (18–57)	13 (27.1)	7 (14.6)	0 (0)	33 (68.8)	10 (20.8)
Culture positive for non-*Salmonella*	226 (16.4)	39 (16–76)	167 (73.9)	46 (11–80)	50 (22.1)	35 (15.5)	12 (5.3)	200 (88.5)	10 (4.4)
Culture negative	1103 (80.1)	38 (16–76)	864 (78.3)	44 (11–75)	214 (19.4)	176 (16.0)	29 (2.6)	983 (89.1)	61 (5.5)
Total	1377 (100)	39 (16–76)	1066 (77.4)	45 (11–80)	277 (20.1)	218 (15.8)	41 (3.0)	1216 (88.3)	81 (5.9)

doi:10.1371/journal.pone.0047342.t002

Bacterial Load of Salmonella in Bile

To quantify the bacterial load in the bile, real-time PCR was performed on total nucleic extracted from the bile of six *S.* Paratyphi A positive individuals and 12 *S.* Typhi positive individuals. All qualitative serovar specific PCR data corresponded precisely with the culture data. The median target copy numbers/bacterial loads were 9.3×10^4 (IQR 5×10^4–2.3×10^5) CFU/ml^{-1} for *S.* Paratyphi A and 5.2×10^4 (IQR 2×10^4–7.28×10^5) CFU/ml^{-1} for *S.* Typhi. The difference in bacterial load between the two organisms was non-significant ($p=0.93$; Mann-Whitney U test), yet, were approximately two and three orders of magnitude greater than those previously reported in bone marrow and blood, respectively [12,13].

Hematological and Biochemical Characteristics

The 1,377 individuals undergoing cholecystectomy were divided into three groups on the basis of their bile culture results: *Salmonella* positive, culture negative, and culture positive for non-*Salmonella*. Individuals that were *Salmonella* positive were more likely to have experienced continuous right upper-quadrant pain (10%, 5/48) compared to those that were culture negative (3%, 37/1,151) ($p=0.008$, chi squared test) and those that were culture positive for non-*Salmonella* (2%, 5/214) ($p=0.008$, chi squared test). Hematology and biochemistry data from the patients were compared using the Mann-Whitney U test (Table 3). There was no significant difference in liver enzyme or bilirubin levels between the *Salmonella* positive group and the other two groups. Yet, the *Salmonella* positive group had a higher median neutrophil count and a lower median lymphocyte count than the culture negative group and the culture positive non-*Salmonella* group (Table 3).

Surgical and Histopathological Characteristics

The major surgical and post-surgical characteristics of the gallbladders from the three groups were compared using Fisher's exact test (Table 4). The majority of *Salmonella* positive individuals had gallstones (96%, 46/48); yet, there was no significant difference in the proportion of individuals with gallstones between the three groups. We did, however, identify several gallbladder characteristics that were associated the presence of *Salmonella*. Namely, gallbladder distension and inflammation was more frequently observed in the *Salmonella* positive group than the culture negative group and the non-*Salmonella* culture positive group (Table 3). Furthermore, the presence of an empyema (pus within the gallbladder cavity) was also more common in the *Salmonella* positive group than the other two groups. Inflammation was more likely to be due to polymorphonuclear infiltration than lymphocytic infiltration in the *Salmonella* infected gallbladder tissue, with 13% (6/48) of the *Salmonella* positive gallbladder specimens having massive neutrophil infiltrate near the lumen, compared to 4% (51/1,151) and 5% (10/214) of the culture negatives and the non-*Salmonella* culture positives, respectively. Furthermore, an additional 15% (7/48) of the *Salmonella* positive gallbladder specimens had acute-on-chronic cholecystitis (neutrophil infiltrate near the lumen with lymphocyte infiltrate and dysplasia in the mucosa) compared to 5% (10/214) and 7% (14/214) of the culture negatives and the non-*Salmonella* culture positives, respectively (Table 3.). Correspondingly, chronic inflammation without large neutrophil infiltrate was not observed in gallbladder tissue from the *Salmonella* positive group.

Discussion

The mechanism of gallbladder infection/colonization remains contentious, and it is unknown if *Salmonella* promote gallbladder

Table 3. The haematological and the biochemical characteristics of the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups.

Hematology	Culture negative			Culture positive non-*Salmonella*			*Salmonella* positive			p1*	p2*
	n	median	IQR	n	median	IQR	n	median	IQR		
Total cell ($\times10^3/\mu L$)	953	7.9	6.6–9.5	188	7.85	6.45–10.15	42	9.45	6.4–14	**0.025**	0.058
Neutrophil ($\times10^3/\mu L$)	917	66	58–74	177	65	58–75	41	72	60–82	**0.012**	**0.042**
Lymphocyte ($\times10^3/\mu L$)	914	31	24–38	175	31	23–38	40	24.5	17–36	**0.007**	**0.040**
Monocyte ($\times10^3/\mu L$)	270	1	1–2	70	1.5	1–2	9	1	1–2	0.676	0.848
Eosinophil ($\times10^3/\mu L$)	641	2	1–4	131	2	1–4	22	2	2–4	0.999	0.515
Basophil ($\times10^3/\mu L$)	54	0	0–1	14	0	0–0	1	6	–	0.058	**0.020**
Total bilirubin (mg/mL)	965	0.8	0.68–1	190	0.8	0.7–1	42	0.86	0.7–1.1	0.119	0.280
Conjugated bilirubin (mg/mL)	950	0.2	0.19–0.26	186	0.2	0.18–0.24	41	0.2	0.2–0.28	0.414	0.419
AST (u/L)	961	30	23–41	190	29.5	23–40	42	28	24–38.9	0.878	0.986
ALT (u/L)	960	30	21.9–43	188	29.5	21–43.5	42	30.5	24–41	0.898	0.953
ALP (u/L)	941	122	82–209	188	150.5	94.5–232.5	42	124.5	96–191	0.622	0.276
Amylase (u/L)	136	61.5	41.5–252.5	20	57.5	38–86.5	9	87	34–190	0.867	0.437

*Mann-Whitney U test, boldface indicates $p \leq 0.05$.
p1: Comparing culture-negative to *Salmonella*-positive patients.
p2: Comparing culture positive for non-*Salmonella* to *Salmonella*-positive patients.
IQR: Interquartile range.
doi:10.1371/journal.pone.0047342.t003

damage during chronic infection or if the organisms exploit existing gallbladder damage to stimulate colonization [14]. Bile is typically sterile, and consists of organic and inorganic compounds, bile acids, cholesterol, phospholipids and the pigment biliverdin. Sterility is partially maintained by the secretion of IgA and mucus, preventing bacterial survival and adhesion to the surface of the lumen and the major bile duct, respectively [15]. Here, we found a wide array of organisms in the bile of individuals undergoing cholecystectomy, some of which have been previously isolated from the gallbladder [16–18]. Again, whether these organisms

Table 4. The gallbladder characteristics within the *Salmonella* positive, culture negative and the culture positive for non-*Salmonella* bile culture groups.

Characteristic	Culture negative	Culture positive non-*Salmonella*	*Salmonella* positive	*p1**	*p2**
	n = 1,103	n = 214	n = 48		
Gallbladder tissue thickness					
Thick (>4 mm)	173 (15.7)	36 (16.8)	10 (20.8)	0.350	0.602
Normal (4 mm)	493 (44.7)	86 (40.0)	21 (43.8)		
Thin (<4 mm)	74 (6.7)	12 (5.6)	1 (2.1)		
Gallbladder size					
Contracted	108 (9.8)	25 (11.7)	1 (2.1)	**0.026**	**0.035**
Distended	221 (20.0)	52 (24.3)	15 (31.3)		
Gall stones					
None	19 (1.7)	6 (2.8)	3 (6.3)	0.101	0.481
Single	344 (31.2)	62 (29.0)	14 (29.2)		
Multiple	684 (62.0)	133 (62.1)	28 (58.3)		
Pathology					
Inflammation	93 (8.4)	17 (7.9)	8 (16.7)	**0.046**	0.060
Empyema	90 (8.2)	21 (9.8)	10 (20.8)	**0.003**	**0.033**
Sludge	57 (5.2)	8 (3.7)	1 (2.1)	0.338	0.581
Mucocele	50 (4.5)	7 (3.3)	1 (2.1)	0.427	0.664

*Fisher's exact test, boldface indicates $p \leq 0.05$.
p1: Comparing culture-negative to *Salmonella*-positive patients.
p2: Comparing culture-positive, *Salmonella*-negative to *Salmonella*-positive patients.
doi:10.1371/journal.pone.0047342.t004

functionally stimulate cholecystitis or cholelithiasis, or merely have the ability to colonize damaged gallbladders, remains unclear. Our data confirm that non-*Salmonellae* organisms, with a spectrum of pathogenic potential, are as equally adept at colonizing the gallbladder and surviving within the bile as typhoidal *Salmonella*. Yet, non-*Salmonellae* appear not to stimulate the same pathology as *Salmonella*; *Salmonella* infected tissue was more commonly associated with systemic and local acute inflammatory responses. Mouse experiments, utilizing *Salmonella* Typhimurium, have shown that *Salmonella* can replicate within the epithelial cells of the gallbladder [19], and that colonized gallbladders displayed evidence of the epithelial destruction and local neutrophil infiltrate. Here, we also find extensive neutrophil infiltrate, yet are unable to confirm if the bacteria are damaging the tissue or colonizing previously damaged tissue. However, as shown by an increased prevalence of gallbladder distention, right upper quadrant pain, empyema and a raised systemic neutrophil count, there is an evident association of invasive *Salmonella* in the gallbladder with an acute inflammatory response.

We have previously noted the presence of individuals in Kathmandu with *Salmonella* in their gallbladder, highlighting the presence of *S.* Paratyphi A [11]. The role of chronic carriage of *S.* Paratyphi A has received much less attention than that of *S.* Typhi and it is unknown as to what extent chronic gallbladder carriage is contributing to the increasing burden of *S.* Paratyphi A across many parts of Asia [20]. Enteric fever caused by *S.* Paratyphi A increased from 17.5% (155/885) in 1993 to 34% (926/2,718) in 2003 in the location of this study [21].

Furthermore, we found an almost equal ratio of *S.* Typhi and *S.* Paratyphi A (1:0.9) isolated from bile, yet the isolates from blood from acutely infected patients over the same period is lower (1:0.4) [22]. This disparity may result from a multitude of factors, but may predict that *S.* Paratyphi A is more adept at inducing carriage in this population, or, once in the gallbladder, may be more likely to induce an acute inflammatory response, requiring a surgical intervention, than S. Typhi.

We found that 3.5% of the individuals undergoing gallbladder surgery had invasive *Salmonella* in their bile in this area with a high incidence of enteric fever [23]. A report from a similar patient demographic in India suggest an equivalent rate of $<5\%$, and in Chile, 7.3% of bile cultures were found to be positive for *Salmonella* [7]. The long-term carriage of invasive *Salmonella* in the gallbladder is thought to be central to the maintenance and transmission of these human-restricted pathogens [14]. However, data from our previous work in Kathmandu suggests that direct transmission plays a negligible role in acute infections, and we have hypothesized that carriers merely act as a reservoir for maintaining local strain diversity in areas of high endemicity [22]. Here, we found antimicrobial resistance to only nalidixic acid in the *Salmonella* from the gallbladder. Although nalidixic acid resistance often precedes resistance to other fluoroquinolones, these isolates were susceptible to gatifloxacin and ofloxacin. Firstly, these data show that infection with an antimicrobial resistant organism is not likely to be associated with *Salmonella* carriage. Secondly, if one considers nalidixic acid resistance as a proxy marker of contemporary strains, the organisms in the gallbladder have probably been there for some time (i.e. from a period when nalidixic acid resistance was less prevalent) [21,24]. Nalidixic acid resistance is a growing problem in Kathmandu. From an ongoing clinical trial enrolling enteric fever patients over the last two years at Patan Hospital, 80% (171/214) of invasive *Salmonella* isolates demonstrated resistance to nalidixic acid, which is greater than the proportion (59%) found from bile isolates in the current study (unpublished data). This evidence supports our current hypothesis of gallbladder carriage playing a limited role in the acute transmission of typhoid in Kathmandu.

Whilst we argue that in locations such as Kathmandu, the role of carriers in typhoid fever transmission may be negligible, it is reasonable to suggest that those shedding invasive *Salmonella* play a vital important role in low transmission setting. In the USA, up to 30% of typhoid fever infections are anticipated to result from contact with a chronic carrier [25]. Therefore, these individuals will become increasingly important as indirect transmission this area begins to subside after the introduction of an effective intervention strategy. However, currently there is no appropriate diagnostic test for the detection of long-term carriers [26]. Bile cultures from string devices are considered effective [27], but are impractical for screening large cohorts [28]. The presence of gallbladder disease is, perhaps, currently the best clinical predictor of carriage of invasive *Salmonella* [7]. However, it remains unclear as to why some patients progress to become chronic shedders and others do not. The development of a rapid diagnostic for the detection of invasive *Salmonella* carriage should accelerate regional elimination of typhoid and add insight into the epidemiological role of these individuals.

One of the major caveats of our study that limits the generalizability of our findings is the fact that our passively acquired patient population may not accurately reflect the general population of Kathmandu. Additionally, all patients in the study had some form of gallbladder abnormality, although it is unclear whether such abnormalities had been induced by the infecting organisms. Nevertheless, in the absence of an alternative methodology, our study represents a reasonable estimation of the burden and mechanism of invasive *Salmonella* carriage.

In conclusion, we have calculated a prevalence of 3.5% of invasive *Salmonella* in bile from patients undergoing cholecystectomy in Kathmandu, Nepal. We demonstrate that *S.* Paratyphi A is almost as prevalent as *S.* Typhi in the gallbladder in this population and that carriage is not driven by antimicrobial resistance. The overall role of invasive *Salmonella* carriage in settings such as Kathmandu is not understood, and we suggest that organisms in the gallbladder may not play a dominant role in acute typhoid fever in this location. We predict, however, that carriers will become more important if current transmission mechanisms are disturbed; prospectively identifying these individuals is paramount for rapid local and regional elimination.

Methods

Ethics Statement

This study was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the institutional ethical review boards of Patan Hospital, The Nepal Health Research Council and The Oxford University Tropical Research Ethics Committee (OXTREC, Reference number: 2108). All enrollees were required to provide written informed consent for the collection and storage of all samples and subsequent data analysis. In the case of those under 18 years of age, a parent or guardian was asked to provide written informed consent.

Setting and Study Population

The study was conducted at Patan Hospital, a 318-bed government hospital located in the Lalitpur Sub-Metropolitan City in the Kathmandu valley, Nepal. Patan Hospital provides both emergency and elective inpatient services. Typhoid fever is a common complaint at Patan Hospital and *S.* Typhi and *S.* Paratyphi A are the most common bacteria cultured from blood

of febrile patients in this location. Antimicrobials are available without prescription in the community in a variety of public and private outlets and there are numerous private physician clinics where patients may seek advice and clinical diagnosis for febrile disease. There has been no widespread implementation of a typhoid vaccine in this area, yet a generic typhoid Vi vaccine is available for purchase in some health care settings. However, at the time of this investigation there was limited community uptake of the vaccine.

The surgical department performs approximately 400 cholecystectomies annually. For the purposes of this study, consecutive patients admitted to the surgical ward from June 2007 to October 2010 for either open cholecystectomy or laparotomy surgery for symptomatic cholelithiasis between 8 am and 4 pm were approached for participation. All patients who gave written informed consent were eligible for the study; there were no exclusion criteria. All enrollees were also required to provide written informed consent for the collection, use and storage of the tissue removed during surgery samples. A questionnaire related to the patient's health and demographics was administered prior to surgery along with a stool sample for microbiological culture. Surgeons collected bile samples and gallbladder tissue during the procedure.

Gallbladder Morphology and Histopathology

Patients were routinely examined by ultrasonography before surgery to assess the presence of gallstones and to detect inflammation. The surgeon performing the procedure curated a report, assessing the thickness of the gallbladder wall (stratified into three categories, thick: >4 mm, normal: 4 mm and thin: <4 mm), the presence and the number of gallstones, the presence and characteristics of fluid (pus: empyema, mucoid/clear/watery: mucocele and sludge) and overall morphology (contracted or distended). Hematocrit, total leukocytes with differential count, total bilirubin, conjugated bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST), and amylase were measured prior to surgical intervention. All extracted tissue was subjected to a histopathological examination to assess/confirm the extent of the inflammation; all histopathology was performed by the same skilled technician who was blinded to presence or absence of bacteria within the bile. All sections we examined by light microscopy after staining with heamatoxylin and eosin. Inflammation was identified by tissue morphology and the presence of neutrophils (acute) and lymphocytes (chronic).

Microbiological Culture, Antimicrobial Susceptibility Testing and Real-time PCR

Bile and stool were collected for culture from all patients undergoing cholecystectomy. Bile was inoculated into equal volumes of Selenite F broth and Peptone broth and incubated at 37°C overnight. Broths were sub-cultured onto MacConkey agar and Xylene Lysine Deoxycholate (XLD) agar. After overnight incubation at 37°C the plates were examined for the growth of Gram-negative bacteria and colonies were identified by standard microbiological methods and identified by API20E manufactured by bioMerieux, Inc. *S.* Typhi and *S.* Paratyphi A isolates were confirmed by slide agglutination by specific antisera (Murex Biotech, Biotech, England). The antimicrobial sensitivity profile was performed by Kirby Bauer disc diffusion method using standard BSAC and CLSI guidelines [29]. The antimicrobials tested were amoxicillin, chloramphenicol, co-trimoxazole, nalidixic acid, ciprofloxacin, ofloxacin, ceftriaxone and gatifloxacin. The minimum inhibitory concentrations (MICs) were performed for nalidixic acid, ciprofloxacin, ofloxacin and azithromycin by E-test (AB Biodisk, Sweden). Susceptibility to ciprofloxacin and ofloxacin were evaluated using newly suggested susceptibly breakpoints for these antimicrobials; $\geq$0.125 μg/ml and $\geq$0.25 μg/ml for ciprofloxacin and ofloxacin respectively [30]. Real-time PCR was performed using a standard curve for quantitation as previously described [12], using DNA extracted from 200μl bile samples as template.

Data Analysis

Data were entered into a database using Excel 2007 (Microsoft) and analyzed using Stata/IC version 9.2 (StataCorp, TX, USA). Chi-square and Fisher's exact tests were used to compare proportions between groups and Mann-Whitney U tests were used for continuous non-parametric data. P-values $\leq$0.05 were considered to be statistically significant.

Acknowledgments

We wish to acknowledge the ongoing efforts of the microbiology laboratory and the surgical support staff as well as the histopathology department, who were essential for the completion of this study.

Author Contributions

Conceived and designed the experiments: SD SB. Performed the experiments: SC TVTN PTD PM SP VKJ SV. Analyzed the data: SD CT. Contributed reagents/materials/analysis tools: AK AA SK NSK GD JF CD BB. Wrote the paper: SB SD CT.

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