

**CLINICAL ISSUES AND MOLECULAR CHARACTERISATION OF
SALMONELLA TYPHI ISOLATES FROM SOUTH EAST ASIA**

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degree of Doctor of Philosophy

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Statement

I, Christiane Dolecek, declare that with the exception of the invaluable help and assistance outlined in the acknowledgements, the work described in this thesis is my own work and has not been submitted for a degree or other qualification to this or another university. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Due to the spread of antimicrobial drug resistance, typhoid fever has become increasingly difficult to treat. In Vietnam, more than 50% of *S. Typhi* isolates are multidrug resistant and 90% are quinolone resistant.

This thesis examines three aspects of typhoid fever; treatment, genotyping of bacteria and the clinical development of an oral vaccine.

We enrolled 358 children and adults with suspected typhoid fever into a randomised controlled trial to compare the efficacy and safety of gatifloxacin (10 mg/kg/day) *versus* azithromycin (20 mg/kg/day) for 7 days. In the blood culture confirmed group, 145 patients received gatifloxacin and 142 patients received azithromycin. Overall treatment failure occurred in 13/145 (9%) patients in the gatifloxacin group and 13/140 (9.3%) patients in the azithromycin group (HR = 0.93; 95% CI 0.43 – 2.0; $p = 0.854$).

We found a statistically significant relationship between drug exposure to gatifloxacin and clinical response. In patients with AUC₀₋₂₄: MIC ratios of greater than 92.7, 93.5% of patients had a favourable response; whilst in patients with AUC₀₋₂₄: MIC ratios equal or less than 92.7, only 75% had a favorable response (OR = 4.81; 95% CI 1.23-18.9; $p = 0.02$).

We investigated the genetic variability and relationship between the *S. Typhi* trial isolates by using a novel SNP genotyping array. The majority of isolates (98%)

belonged to the H58 haplotype, a quinolone resistant haplotype that has expanded globally. Within this group three main subgroups could be distinguished.

We conducted a randomised placebo controlled trial to determine the safety and immunogenicity of a novel oral typhoid vaccine (M01ZH09) with two independently-attenuating deletions (Ty2 aroC- ssaV-) in healthy 5 to 14 year old children in Vietnam. One hundred and fifty-one children were enrolled and followed up for 28 days. Twenty-six percent of M01ZH09 subjects and 22% of placebo subjects experienced an adverse event. There were no serious adverse events and no bacteraemia.

Ninety-seven percent of the subjects showed a positive immune response. M01ZH09 was immunogenic and had an appropriate safety and reactogenicity profile in children in an area with endemic typhoid fever.

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This PhD thesis describes two randomised controlled trials (Chapter 2 and Chapter 5), which inherently require to be conducted by big teams. I am deeply indebted to the doctors who supervised the day to day running of the clinical trial, Dr Tran Thi Phi La, head of the Infectious Ward at An Giang Provincial Hospital and Dr Doan Cong Du, Dr Duong Thanh Long and Dr Nguyen Ngoc Rang; to Dr Le Thi Phuong who recruited patients at Dong Thap Provincial Hospital and to Dr Ha Vinh, head of Ward E at the Hospital for Tropical Diseases. I am grateful to all these doctors for enrolling patients, taking samples and caring for the patients in this trial. The microbiology work included in this thesis was done by Nguyen Van Minh Hoang, Cao Thu Thuy, Pham Van Minh and Tran Thi Thu Nga at the Hospital for Tropical Diseases, Ho Chi Minh City. My special thanks goes to Jim Campbell for supervising the microbiology work, for his patience and for giving me a refresher course in basic microbiology. I am grateful to Dr Nguyen Van Vinh Chau and Dr To Song Diep, both at different times heads of the microbiology department at the

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Abbreviations

FCT	Fever Clearance Time
OR	Odds Ratio
HR	Hazard Ratio
95% CI	95% confidence interval
MDR	multidrug resistant
ITT	intention to treat analysis
PP	per protocol analysis
CLSI	Clinical and Laboratory Standards Institute
CRF	Case Record Form
LPS	Lipopolysaccharide
IgA	Immunoglobuline A
IgG	Immunoglobuline G
ACS	antibody secreting cells
ELISA	Enzyme Linked Immunosorbent Assay
ELISPOT	Enzyme Linked Immunospot Assay
PBMC	peripheral blood mononuclear cells

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Chapter 1

Introduction

1.1 Typhoid and paratyphoid fever

Typhoid and paratyphoid fever are septicaemias caused by the Gram negative bacteria *Salmonella enterica* serovar Typhi (*S. Typhi*) and *Salmonella enterica* serovar Paratyphi (*S. Paratyphi*) A, B and C. Typhoid and paratyphoid fever are summarized as enteric fevers. Whilst *S. Typhi* and *S. Paratyphi* A and B infections are restricted to humans, *S. Paratyphi* C can affect a variety of mammals.

1.2 Epidemiology

Typhoid fever is endemic in Africa, Asia, Central and South America and is also found in parts of the Middle East, southern and eastern Europe (Parry *et al.*, 2002). Improvement of infrastructure and sanitation has virtually eliminated typhoid fever in developed countries and infections seen in Europe, Australia, and North America are usually acquired abroad (mostly from the Indian subcontinent, South East Asia and South America) (Steinberg *et al.*, 2004). Current estimates from the World Health Organization (WHO) suggest that the global burden of typhoid fever is approximately 21 million cases annually with more than 210 000 deaths and that paratyphoid fever causes an additional 5 million cases (Crump *et al.*, 2004). These numbers are based on extrapolating data from 22 studies that used blood culture, the gold standard for the diagnosis of typhoid fever (World Health Organization, 2003). Many institutions in endemic countries lack blood culture facilities, so the true extent of typhoid and paratyphoid fever is difficult to establish. It is likely that the proportion of disease caused by *S. Paratyphi* A particularly in Asia has been underestimated.

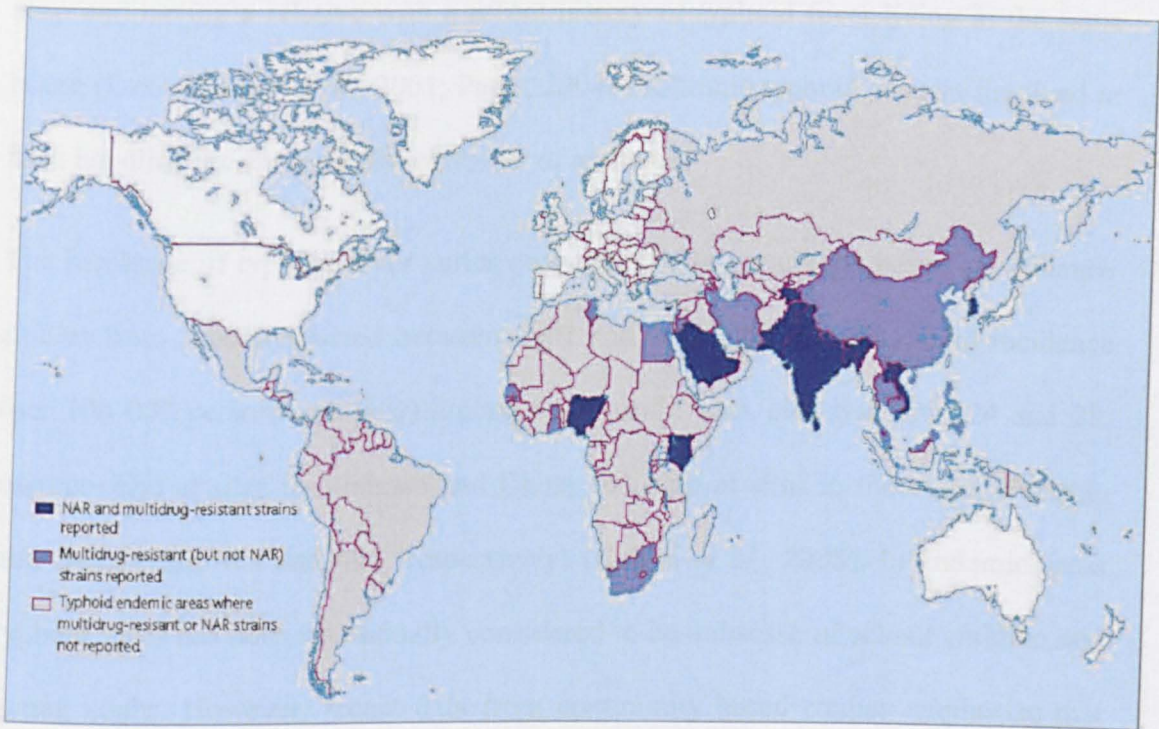


Figure 1.1. Areas of typhoid fever endemicity and distribution of antimicrobial drug resistance to *Salmonella enterica* serotype Typhi, 1990 to 2005.

Modified from (Bhan *et al.*, 2005).

Recent data show that the proportion of diagnosed enteric fevers caused by *S. Paratyphi A* was 64% in China, 24% in India and 33% in Nepal (Maskey *et al.*, 2006; Ochiai *et al.*, 2005).

Transmission of typhoid fever occurs via the faeco-oral route by ingesting contaminated water or food or through direct contact (person to person transmission through poor hygiene). Risk factors for acquiring typhoid fever are eating food prepared outside the home, drinking contaminated water, eating shellfish from polluted water, vegetables that have been fertilised with human waste, the non-use of

soap and having a relative with a recent history of typhoid fever living in the same house (Luxemburger *et al.*, 2001; Parry, 2004b). Chronic typhoid carriers involved in food handling are an important reservoir of infection.

The incidence of typhoid fever varies considerably. In population based surveillance studies from Asia conducted between 2002 and 2004, the annual typhoid incidence (per 100 000 persons per year) among the 5 to 15 year olds was low (24 and 29, respectively) at sites in Vietnam and China and high at sites in Indonesia, Pakistan and India (180, 413 and 493, respectively) (Ochiai *et al.*, 2008). In endemic areas typhoid fever has been traditionally considered to be a disease of school children and young adults. However, recent data from community based studies emphasize that enteric fever is a common cause of morbidity in children between 1 and 5 years and that pre-school children experience similar or greater infection rates than school children (Lin *et al.*, 2000; Ochiai *et al.*, 2008; Sinha *et al.*, 1999).

1.3 The bacterium and identification

The genus *Salmonella* belongs to the family of Enterobacteriaceae and is currently divided into two species, *bongori* and *enterica* (Brenner *et al.*, 2000). The species *Salmonella enterica* is divided into six subspecies (I, *enterica*; II, *salamae*; III, *arizonae*; IV, *diarizonae*; V, *houtenae* and VI, *indica*) and contains more than 2400 serotypes based on the Kaufmann-White scheme (Grimont and Weill, 2007). Most of the *Salmonella* that cause disease in humans (including *S. Typhi*, *S. Paratyphi* and *S. Typhimurium*) are in the sub-species I (*enterica*). In contrast to other *S.* serovars, i.e. *S. enteritidis* and *typhimurium*, which show a wide host range, *S. Typhi* and *S.*

Paratyphi (with the exception of *S. Paratyphi C*) are highly adapted to humans and have no other known natural hosts.

S. Typhi and *S. Paratyphi* are flagellated, non-spore bearing, facultative anaerobic Gram negative bacilli. They are non-lactose fermenters and are identified by a characteristic biochemical pattern on triple sugar iron (TSI) agar slants (acid butt without gas, an alkaline slant and a moderate amount of H₂S production) (World Health Organization, Communicable Disease Surveillance & Response, 2003). The identification is confirmed by serological identification of their somatic lipopolysaccharide (O) and flagellar protein (H) antigens. *S. Typhi* and *S. Paratyphi C* sometimes possess a polysaccharide capsular Vi (virulence) antigen that coats the O antigen and potentially masks it from antibodies.

S. Typhi is O_{9, 12} (group D), Vi and Hd positive and *S. Paratyphi A* is identified as O_{1,2,12}; Ha [1,5] positive. Unique flagella types, Hj and Hz₆₆ are present in *S. Typhi* from Indonesia (Baker *et al.*, 2007; Frankel *et al.*, 1989).

1.4 The genome of *S. Typhi* CT18

The sequenced *S. Typhi* isolate CT18 (Parkhill *et al.*, 2001a) is multidrug resistant (resistant to all first line antibiotics) and was isolated in 1993 from a child with typhoid fever in the Mekong Delta region of Vietnam. The genome of *S. Typhi* CT18 is 4.8 million base pairs in length and encodes approximately 4600 genes (Parkhill *et al.*, 2001a). *S. Typhi* CT18 strain has several large insertions, believed to originate from bacteriophages or plasmids, termed the *Salmonella* pathogenicity islands that

encode genes that are important for survival in the host. *Salmonella enterica* has two type III secretion systems (TTSS), which are important virulence factors of Gram negative bacteria used to translocate proteins into the cytoplasm of eukaryotic host cells and are encoded by *Salmonella* pathogenicity islands SPI-1 and SPI-2. The invasion of epithelial cells is mediated by the SPI-1 TTSS, which is active on contact and injects virulence proteins into the host cell, leading to macropinocytosis of the bacteria and cytoskeletal rearrangement of the host cell to allow translocation. SPI-2 encoded TTSS is expressed after phagocytosis of bacteria by host cells and secretes effectors required for survival and replication in *Salmonella*-containing vesicles (SCV) inside phagocytes and epithelial cells (House *et al.*, 2001; Miao and Miller, 2000).

Another important feature of *S. Typhi* is the presence of more than 200 pseudogenes. The majority of these pseudogenes (124 out of 204) have arisen from introduction of a single frameshift or stop codon, some genes (45) have been inactivated by frameshifts that are due to changes in the length of homopolymeric tracts and some (27) genes are the remnants of insertion sequence (IS) transposases, integrases and genes of bacteriophage origin (Parkhill *et al.*, 2001a). The inactivation of these genes may explain the human host restriction of *S. Typhi*. CT18 harbours two plasmids, one large 220 kbp conjugative multiple drug resistance *incH1* plasmid (pHCM1) which shows a high degree (more than 99%) of sequence identity with R27, an *incH1* plasmid first isolated in the 1960s from *S. enterica* (Sherburne *et al.*, 2000) and a smaller 110 kbp

cryptic plasmid (pHCM2), which shows recent common ancestry with a virulence plasmid of *Yersinia pestis* (Kidgell *et al.*, 2002a; Prentice *et al.*, 2001).

1.5 Pathogenesis and pathology

The best studied model of typhoid fever is the “typhoid-like” illness of mice infected by *S. Typhimurium*, but not all findings from this infection model can be extrapolated to humans (House *et al.*, 2001).

Volunteer studies have shown that the infective dose of *S. Typhi* is between 10^3 to 10^9 bacteria (Hornick *et al.*, 1970). Gastric acidity destroys the organisms but gastric hypoacidity (following gastrectomy, intake of histamine-2 receptor antagonists or proton pump inhibitors) allows a greater number of organisms to enter the small intestine. In a study from India, *Helicobacter pylori* infection has been associated with an increased risk of typhoid fever (Bhan *et al.*, 2002).

After the ingestion in water or food, *S. Typhi* bacteria reach the small intestine where they adhere to the mucosal epithelial cells. They penetrate the mucosal epithelium either via the M (membranous) cells, specialised cells overlying the Peyer’s patches, enterocytes or via a paracellular route. *S. Typhi* bacteria arrive in the lamina propria where they elicit a local influx of macrophages. They are taken up by macrophages and multiply in the mononuclear phagocytic cells of the small intestine, are drained into mesenteric lymph nodes and it is believed that they reach the general circulation (causing an asymptomatic primary bacteraemia) by lymph drainage from the thoracic duct. The incubation period, which depends on the inoculum size and host defence factors, varies between 7 and 14 days. During this phase the bacteria reside and multiply within the organs of the reticuloendothelial system (spleen, liver, bone marrow and lymph nodes and especially the Peyer’s patches of the terminal ileum).

Bacteria are then shed again into the blood stream, marking the onset of fever and symptomatic disease. During the symptomatic stage *S. Typhi* can be cultured from blood, although this may be difficult in low level bacteraemia. In a study that performed quantitative blood cultures in a large number (n = 369) of patients with typhoid fever, the median *S. Typhi* count in blood was 1 colony forming unit per ml (range, <0.3 to 387 CFU/ml), of which a mean of 63% (95% CI, 58 to 67%) were intracellular (Wain *et al.*, 1998). If left untreated, the *S. Typhi* bacteraemia persists for several weeks. In this phase the organism disseminates widely to the organs of the reticuloendothelial system (House *et al.*, 2001; Parry *et al.*, 2002; Wain *et al.*, 2002).

S. Typhi infection produces hyperplasia of the Peyer's patches in the first week, which can resolve or progress to necrosis. Ulcers can lead to perforation and haemorrhage, usually in the third week, although these may occur earlier or later during the disease (Owen, 1994; Parry *et al.*, 2002). It has been hypothesized that the significant inflammation in the ileum causing necrosis and ulceration might be due to the re-exposure of Peyer's patches to *S. Typhi* via the gallbladder, a mechanism similar to the Shwartzman and Koch reactions (Everest *et al.*, 2001). The majority of patients mount local and systemic humoral and cellular immune responses but these do not give complete protection against relapse or re-infection. The mortality of typhoid fever in the pre-antibiotic era used to be about 10 to 20% (Herzog, 1976).

1.6 Clinical presentation

Typhoid fever typically presents with fever, headache, anorexia and abdominal discomfort with either diarrhoea or constipation. This can be accompanied by nausea, vomiting and a dry cough. Diarrhoea is more common in children, whereas constipation is more often found in adults. Profuse diarrhoea has been described in typhoid patients with HIV infection (Parry *et al.*, 2002). On examination, abdominal tenderness, hepatomegaly (in 40 to 70% of patients) and splenomegaly are common. It is rare for patients with typhoid fever to not have any abdominal symptoms and normal bowel movement, however even then typhoid fever cannot be excluded. A small percentage of patients (less than 5%) show rose spots, small blanching erythematous maculopapular lesions of about 2 to 4 mm diameter on the trunk. Haemoglobin levels, white cell counts and platelet counts are normal or reduced. Liver enzymes (AST, ALT) are usually elevated two to three times the upper limit of normal and bilirubin is normal or slightly raised. Abdominal sonography may demonstrate enlargement of liver and spleen and prominent mesenteric lymph nodes.

It has been frequently cited that *S. Paratyphi* causes a milder disease, however recent prospective clinical trials have reported that enteric fever caused by *S. Typhi* and *S. Paratyphi A* are clinically indistinguishable (Maskey *et al.*, 2006; Vollaard *et al.*, 2005). *S. Paratyphi A* has also been described as the cause of severe enteric fever in Indonesia (Hoffman *et al.*, 1984).

1.6.1 Complications and severe typhoid fever

Complications of typhoid fever are more likely to occur in patients who have been sick for longer periods without receiving treatment and in patients infected with non susceptible *S. Typhi* who do not receive appropriate treatment. Complications may develop in up to 10% of hospitalised patients (World Health Organization, 2003). The development of septic shock and acute respiratory distress syndrome are serious complications when treatment is delayed.

Gastrointestinal bleeding (occurs in approximately 10% of patients), intestinal perforation and typhoid encephalopathy are the most common complications. Gastrointestinal bleeding results from a necrotic Peyer's patch eroding the wall of an enteric blood vessel. Often the bleeding is slight and resolves without intervention, however in approximately 2% of cases the bleeding is significant and requires blood transfusion. Gastrointestinal perforation (usually at the terminal ileum) is the most serious complication, and occurs in up to 3% of hospitalised cases (Parry *et al.*, 2002). It manifests as acute abdomen or as worsening of abdominal pain accompanied by shock. Perforation is associated with a high mortality (van Basten and Stockenbrugger, 1994) and needs urgent surgical intervention.

A reduced level of consciousness or encephalopathy, often accompanied by shock, is a serious complication associated with a mortality of up to 50% (Hoffman *et al.*, 1984). Typhoid encephalopathy is a complex neuropsychiatric syndrome with a wide range of symptoms and signs ranging from agitation to delirium and coma. These

symptoms might be worsened by associated liver failure, haematogenic dissemination to the brain or other as yet unknown mechanisms. The incidence of this presentation varies between different countries; ranging from 10 to 40% of hospitalised cases in Indonesia, and Papua New Guinea but less than 2% in Pakistan and Vietnam.

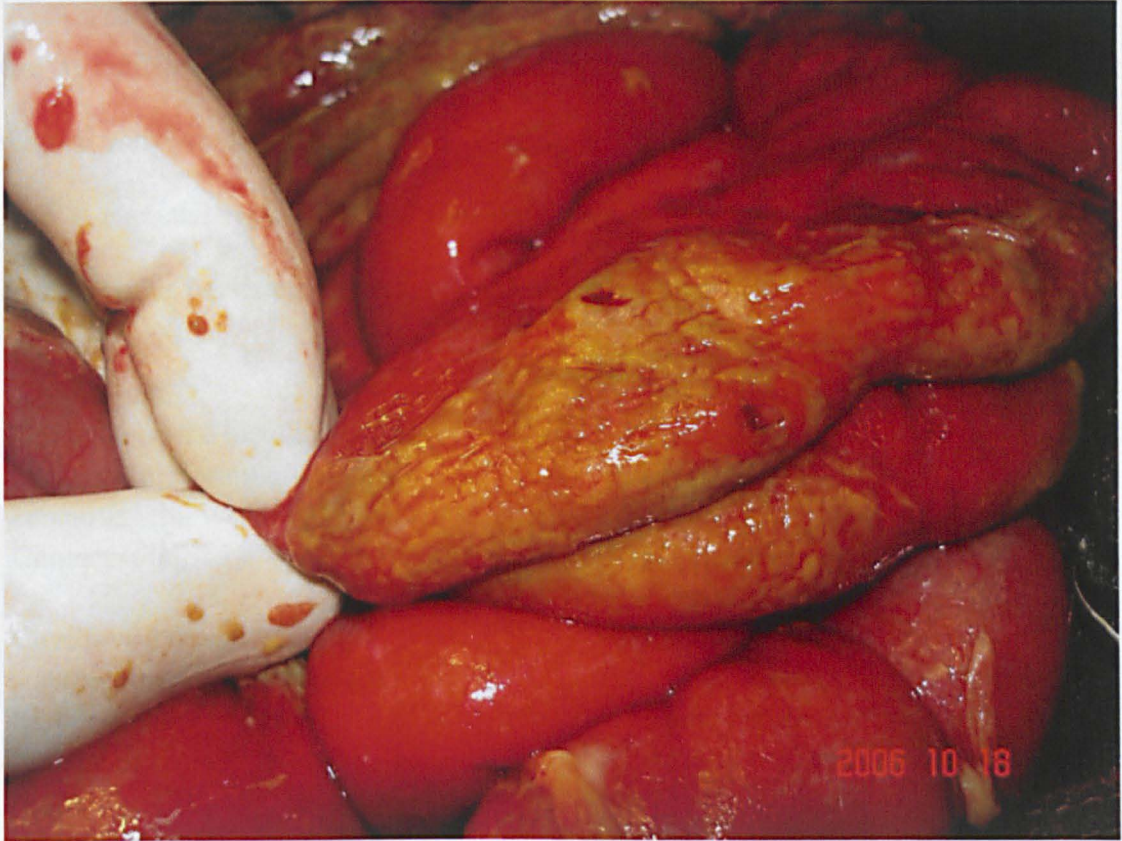


Figure 1.2 Gastrointestinal perforation, usually of the terminal ileum, is one of the most serious complications of typhoid fever.

The picture shows the intraoperative picture of a patient with perforated necrotic ulcer. Photo credit: Pukar Maskey, Patan Hospital, Kathmandu.

This variation is unexplained (Parry *et al.*, 2002). Seizures have been described as frequent complication (up to 10%) in hospitalised children under the age of 10 in Bangladesh (Butler *et al.*, 1991). In patients with encephalopathy, cerebrospinal fluid should be obtained to exclude tuberculous meningitis, other bacterial meningitis and encephalitis. The prognosis of typhoid fever during pregnancy has improved with the use of antibiotics (Seoud *et al.*, 1988), although the optimal treatment for pregnant women remains unclear. Typhoid fever acquired through intrauterine infection can lead to neonatal typhoid fever, a severe sepsis with a mortality of up to 10% or to asymptomatic persistent secretion (Reed and Klugman, 1994).

Abdominal

Gastrointestinal bleeding
 Intestinal perforation and shock
 Hepatitis with/without jaundice
 Cholecystitis

Cardiovascular

Asymptomatic ECG changes
 Myocarditis
 Sudden death

Haematological

Anaemia
 Disseminated Intravascular Coagulation (DIC)

Neuropsychiatric

Encephalopathy- Reduced consciousness levels
 Meningitis
 Seizures

Respiratory

Pneumonia
 Bronchitis

Genitourinary

Retention of urine
 Glomerulonephritis

Focal infections

Abcesses of brain, liver, spleen

Table 1-1. Important complications of typhoid fever.

Modified from (Parry *et al.*, 2002)

1.6.2 Relapse

The re-occurrence of symptoms within one month after the treatment has been completed and symptoms have been resolved is considered a relapse. Depending on the efficacy of the antibacterial treatment to clear the bacteria, relapse rates vary from 0% to 10% in patients (Parry *et al.*, 2002; Parry *et al.*, 2007).

1.6.3 Chronic Carriage

One to five percent of patients with a history of enteric fever become chronic carriers, which means they harbour *S. Typhi* or *S. Paratyphi A* or *B* in their gall bladder and are shedding bacteria intermittently in their stools for at least one year after the illness (World Health Organization, 2003; Ristori *et al.*, 1982). These carriers are an important reservoir of infection and are usually asymptomatic. Up to 25% of chronic carriers do not have a history of typhoid fever. The rate of carriers is higher among females and patients with cholelithiasis. An association between urinary carriage of *S. Typhi* and *S. Paratyphi A* and schistosomiasis has been described, caused by obstructive lesions of the urinary tract (Hathout *et al.*, 1966).

1.6.4 Case Fatality

A WHO report has estimated the case fatality rate in typhoid fever at 1% (Crump *et al.*, 2004). There seems to be considerable geographic variation. In Vietnam and Pakistan the case fatality rate among hospitalised patients is less than 2%, but in patients with severe typhoid fever in Papua New Guinea and Indonesia it can vary

between 30 and 50% (Parry *et al.*, 2002). The most important contributor to a poor outcome is probably a delay in appropriate antibiotic treatment.

1.6.5 Differential diagnosis

Typhoid fever presents with nonspecific symptoms and this makes the diagnosis difficult. Other endemic illnesses, most importantly malaria have to be ruled out. Typhoid can occasionally present as gastroenteritis with vomiting and diarrhoea. The differential diagnosis is broad and varies geographically but includes leptospirosis, rickettsial disease (scrub and murine typhus (rickettsial disease), tuberculosis, brucellosis, other bacterial sepsis, encephalitis, amoebic liver abscesses, visceral leishmaniasis, viral diseases (dengue fever, infectious mononucleosis, hepatitis, influenza), lymphoproliferative disease and autoimmune diseases including systemic lupus erythematosus (SLE).

1.7 Diagnosis

1.7.1 Blood culture and bone marrow culture

The definitive diagnosis of enteric fever requires the isolation of *S. Typhi* or *S. Paratyphi* from blood, bone marrow or an anatomic lesion. Blood culture is the gold standard in the diagnosis of enteric fever. However, the low level of bacteraemia (between 1 and 10 bacteria per ml of blood) is a characteristic feature of typhoid fever and the sensitivity of blood culture is estimated to be only 50 to 60%. Therefore obtaining a large volume of blood is one of the most important factors in the successful isolation of *S. Typhi*. Ideally 10 to 15 ml of blood should be taken from

school children and adults and 2 to 4 ml for toddlers and pre-school children (World Health Organization, 2003; Wain *et al.*, 1998). Bone marrow culture is more sensitive (up to 80%), because of the higher concentration of bacteria found in the bone marrow (Wain *et al.*, 2001).

Antimicrobial susceptibility testing should be performed against a fluoroquinolone, nalidixic acid (the prototype quinolone; strains that are nalidixic acid resistant show reduced susceptibility to fluoroquinolones, even when still within the current breakpoints for fluoroquinolone susceptibility), a third generation cephalosporin, other drugs currently used for treatment and the previous first line antibiotics to which the strains could be resistant (chloramphenicol, ampicillin, trimethoprim–sulfamethoxazole, streptomycin and tetracycline) (World Health Organization, 2003).

1.7.2 Serology

The Widal test was first described more than 100 years ago. It is a tube dilution test based on the presence of agglutinating antibodies to the O and H antigens of *Salmonella*. It is usually performed in settings where culture facilities are not available. In the original format, paired sera (acute and convalescent) were required and a four fold increase in the antibody titre (to O and H antigens) provided support for the diagnosis of typhoid fever. However, the test is usually performed on acute serum only as a screening assessment and there is no consensus regarding the

interpretation of the test, standardisation of the reagents and it lacks sensitivity and specificity.

One of the main problems with the interpretation of the Widal test is the wide variation in the antibody responses of individuals and populations. This is because not everybody mounts a detectable antibody response to *S. Typhi*, healthy populations in endemic areas and patients who received typhoid vaccine show high levels of antibodies and there is cross-reactivity to other *Salmonella* serotypes. For the accurate interpretation of results it is necessary to know the antibody levels of the “background” population in the area where the test is being applied.

Rapid tests have been developed for the serological diagnosis of typhoid fever. The typhidot test (Malaysian Biodiagnostics Research Ltd, Malaysia) is an immunodot ELISA based on the detection of IgG and IgM antibodies specific for the 50 kD outer membrane protein of *S. Typhi*. The Tubex test (IDL Biotech, Sweden) is a semiquantitative colorimetric test which detects anti-*Salmonella* O9 antibodies from a patient's serum by the ability of these antibodies to inhibit the binding between an indicator antibody-bound particle and a magnetic antigen-bound particle. A recent prospective community based study in Kolkata examined more than 6000 blood samples from patients with fever for more than 3 days and compared the performance of the Widal test, Typhidot and Tubex test to the gold standard blood culture (Dutta *et al.*, 2006). An anti-TO titer of 1/80 in the Widal test had a sensitivity, specificity, positive predictive value and negative predictive value of 58%, 85%, 69%, and 77%, respectively for the diagnosis of typhoid fever. The overall performance of sensitivity,

specificity, positive predictive value and negative predictive value of Typhidot and Tubex was found to be 47%, 83%, 85%, and 42% and 56%, 88%, 81%, and 69%, respectively.

1.7.3 Molecular diagnosis

PCR based assays have been developed to diagnose *S. Typhi* infection in blood samples. Most of these assays target the flagellin gene (*fliC*) of *S. Typhi*. Whilst PCR assays which use *Salmonella* isolates show robust results and high sensitivity and specificity (Levy *et al.*, 2008), the results of PCR assays based on patient's blood samples are more variable. In a study by Massi *et al.* real-time PCR based on the amplification of *fliC* was applied to detect *S. Typhi* in blood samples from patients with clinically diagnosed typhoid (Massi *et al.*, 2005). The *TaqMan* assay detected more than 10^3 (range 1.01×10^3 - 4.35×10^4) copies/ml blood of *S. Typhi* in the blood culture positive samples and less than 10^3 copies/ml blood in the blood culture negative samples. Given the low bacterial loads in blood that are characteristic of typhoid fever (Wain *et al.*, 1998), these high copy numbers are surprising. A recent study found that a real-time PCR assay had less than 50% sensitivity when tested on DNA extracted from 2 ml of blood taken from patients with blood culture confirmed typhoid fever (Nga *et al.*, 2010).

1.8 Antimicrobials for the treatment of typhoid and paratyphoid fever

1.8.1 First-line antimicrobial agents for the treatment of enteric fever

1.8.1.1 Chloramphenicol

Chloramphenicol is a broad spectrum antibiotic that was developed in 1947 from the soil bacterium *Streptomyces venezuelae* (Ehrlich *et al.*, 1947). Chloramphenicol is active against many Gram positive and Gram negative organisms (aerobes and anaerobes), rickettsiae, chlamydiae and mycoplasma. It demonstrates mostly bacteriostatic activity and binds to the bacterial 50S ribosomal subunit where it prevents the binding of aminoacyl-tRNA and terminates polypeptide chain synthesis. It was the first antibiotic to be manufactured synthetically on a large scale. It dramatically changed the management of typhoid fever (Woodward *et al.*, 1948).

Chloramphenicol treatment has reduced the typhoid fever mortality from 20% to less than 1%, and the duration of fever from 2 to 4 weeks to four to five days (White, 2002; Woodward *et al.*, 1948; Woodward *et al.*, 1954) and has been the first line drug for the treatment of typhoid fever. However, disadvantages of chloramphenicol are that it does not influence the relapse rate nor the typhoid carrier rate (Herzog, 1976) and the need for prolonged treatment durations of 14 to 21 days (Woodward and Smadel, 1964).

Chloramphenicol is available in three forms, as base, palmitate ester and sodium succinate ester (Neuhauser and Pendland, 2005). Chloramphenicol base is well absorbed and produces serum peak levels within 1 to 2 hours. The palmitate ester is

more soluble and palatable and used as the paediatric formulation, whereas the succinate ester is used for parenteral administration. The esters must be hydrolysed to yield the microbiologically active chloramphenicol base and therefore serum levels achieved with oral base are higher than with the palmitate ester and succinate ester. Chloramphenicol has good penetration into most tissues and body fluids with the exception of bile. It readily crosses the blood-brain barrier and is still used for the treatment of bacterial meningitis in many parts of the world.

The most important adverse effect of chloramphenicol is a dose related, reversible bone marrow depression that results from inhibition of mitochondrial protein synthesis (Yunis, 1989). This is relatively common and results in reversible (when the drug is stopped) reticulocytopenia, anaemia, leukopaenia, or thrombocytopaenia. In contrast, the chloramphenicol associated "idiosyncratic" aplastic anemia is very rare but is not dose related, non reversible and invariably fatal. Aplastic anemia is estimated to occur in 1 in 24500 to 40800 patients who receive the antibiotic, a risk that is about 13 times greater than in the general population (Wallerstein *et al.*, 1969).

Resistance to chloramphenicol was first reported in the 1970s and has been widely spread (Herzog, 1976). The most common mechanism of resistance is the inactivation of the drug by the enzyme chloramphenicol acetyl transferase. The *cat1* gene is usually carried on large incHI1 plasmids (Parkhill *et al.*, 2001a). Despite these limitations chloramphenicol remains an effective first line treatment for typhoid fever in many regions of the world where the bacteria remain sensitive (World Health Organization, 2003; Parry *et al.*, 2002; Thaver *et al.*, 2009)

1.8.1.2 Ampicillin and amoxicillin

The aminopenicillins ampicillin and amoxicillin have been evaluated for the treatment of typhoid fever in several clinical trials and have given similar or slightly inferior results to those obtained with chloramphenicol (Herzog, 1976; White, 2002). Fever clearance times are 1 to 3 days longer than with chloramphenicol and failure rates slightly higher (Herzog, 1976). Relapse and faecal carriage rates are similar to those achieved with chloramphenicol. Ampicillin and amoxicillin have been the treatment of choice in pregnancy and neonates. Resistance is widespread and generally due to the production of the bacterial enzyme β -lactamase that opens the β -lactam ring and causes inactivation of the antibiotic. The gene responsible for resistance in *S. Typhi* has been TEM-1 and is plasmid encoded (White, 2002).

1.8.1.3 Trimethoprim-Sulfamethoxazole (cotrimoxazole)

Trimethoprim-sulfamethoxazole has been widely used for the treatment of typhoid fever and has been shown to be an effective alternative to chloramphenicol (Herzog, 1976). Resistance in *S. Typhi* is mediated by the acquisition of a plasmid encoded sulfonamide resistant dihydropteroate synthase and trimethoprim resistant dihydrofolate reductase (Goldstein and Stein, 2005).

1.8.2 Fluoroquinolones

Nalidixic acid, the prototype 4-quinolone antibiotic was discovered in 1962 (Leshner *et al.*, 1962), it is active against Gram negative bacteria and only achieves modest serum and tissue concentrations.

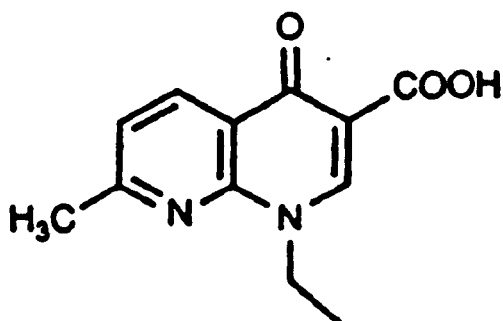


Figure 1.3 Structure of nalidixic acid, the prototype quinolone

Almost 20 years later, the addition of a fluorine molecule at position C6 created the fluoroquinolones. The 6-fluoro substituent confers a greater spectrum of activity against Gram negative and Gram positive pathogens, possibly by improving tissue penetration and binding to the DNA gyrase enzyme.

All quinolone derivatives have a dual ring structure with nitrogen at position 1, a carbonyl group at position 4 and a carboxyl group attached to the carbon at position 3 of the first ring (Hooper, 1995). Several structural modifications have led to compounds with enhanced antibacterial activity. Addition of a piperazinyl group (norfloxacin, enoxacin), methyl-piperazinyl group (pefloxacin, ofloxacin, amifloxacin, lomefloxacin, fleroxacin, temafloxacin), or dimethyl-piperazinyl group (sparfloxacin) at position 7 enhances activity against aerobic Gram negative bacteria.

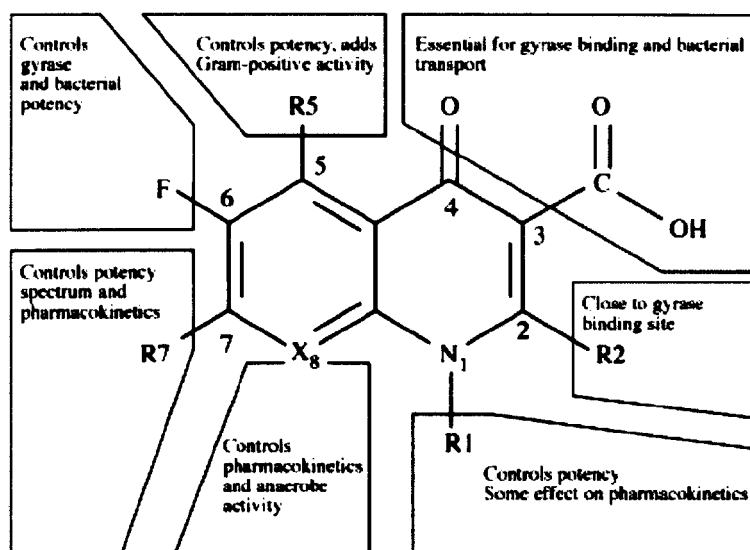


Figure 1.4 Structure activity relationship of the fluoroquinolones.

From (Domagala, 1994)

Ciprofloxacin (a second generation fluoroquinolone) which was discovered in 1981 possesses a cyclopropyl group on position 1 of the quinolone ring which confers excellent activity against Gram negative organisms (*Enterobacteriaceae*, *Haemophilus influenza*, *Neisseria meningitidis* and *Moraxella catarrhalis*) and *Pseudomonas aeruginosa* (Davis *et al.*, 2005).

Ofloxacin (a second generation fluoroquinolone) received FDA approval in 1990 and possesses an additional ring structure bridging positions 1 and 8. Due to its availability and affordability, ofloxacin has been widely used for the treatment of typhoid fever in Vietnam.

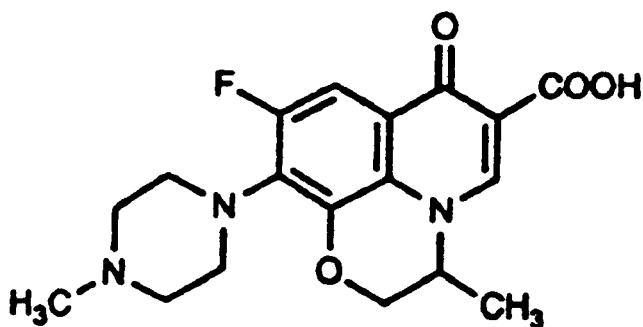


Figure 1.5 Structure of ofloxacin

Gatifloxacin is a broad spectrum 8-methoxy fluoroquinolone with enhanced activity against Gram positive organisms, which has received U.S. Food and Drug Administration (FDA) approval in 1999. It features a cyclopropyl group at position 1 similar to ciprofloxacin. The addition of a methoxy group at position 8 targets both topoisomerase II and IV and probably prevents (or delays) the development of quinolone resistance.

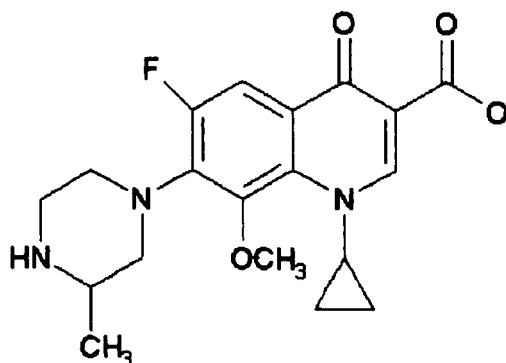


Figure 1.6 Structure of gatifloxacin

1.8.2.1 Mode of action

Fluoroquinolones are considered bactericidal agents and have excellent *in vitro* activity against a wide range of Gram negative and Gram positive organisms. The quinolones rapidly inhibit bacterial DNA synthesis, causing rapid cell death. The targets for the fluoroquinolones are the bacterial topoisomerase enzymes, DNA gyrase (topoisomerase II) and Topoisomerase IV. DNA gyrase supercoils strands of bacterial DNA into the bacterial cell and transiently nicks each chromosomal domain during supercoiling. When supercoiling is complete, DNA gyrase seals the nicked DNA. Topoisomerase IV separates the two linked daughter DNA molecules when replication is complete. These two enzymes are essential in DNA replication and cell partitioning of DNA molecules (Andriole, 2004). DNA gyrase is a tetramer composed of a Gyrase A dimer and Gyrase B dimer which are encoded by *gyrA* and *gyrB* genes, respectively. Topoisomerase IV is a tetramer composed of a ParC and ParE dimer, which are encoded by *parC* and *parE* genes, respectively. For many fluoroquinolones, DNA gyrase is the primary target in Gram negative bacteria and topoisomerase IV may be a secondary target (Piddock, 2002). In Gram positive bacteria, topoisomerase IV is the primary target. Recent microarray data identified an oxidative damage-mediated cell death pathway, which involves reactive oxygen species (ROS) generation and a breakdown in iron regulatory dynamics following norfloxacin-induced DNA damage (Kohanski *et al.*, 2010).

1.8.2.2 Mechanism of resistance

The main mechanism of quinolone resistance in *S. Typhi* is the accumulation of amino acid substitutions in the bacterial target enzyme DNA gyrase. The most commonly identified alteration has been a serine to phenylalanine substitution at position 83 of GyrA (Chau *et al.*, 2007; Wain *et al.*, 1997). Amino acid changes at position 87 (aspartate to tyrosine, glycine or asparagine) of GyrA are less frequent. These mutations are focused around a region called the quinolone resistance determining region (QRDR). The QRDR of GyrA is close to tyrosine at position 122, the active site of the enzyme, which is covalently linked to DNA during strand breakage (Drlica, 1999). Single point mutations in *gyrA* of *S. Typhi* lead to nalidixic acid resistance (MIC ≥ 32 $\mu\text{g/ml}$) and reduced susceptibility to the fluoroquinolones (typically ciprofloxacin MICs ranging from 0.125 $\mu\text{g/ml}$ to 1.0 $\mu\text{g/ml}$, compared to the wild type MIC of 0.03 $\mu\text{g/ml}$ or below). Single isolates of fully fluoroquinolone resistant *S. Typhi* and *S. Paratyphi A* have been reported from India (Parry and Threlfall, 2008). The high-level fluoroquinolone resistance seen in these *S. Typhi* (ciprofloxacin MIC ≥ 4 mg/ml) isolates was conferred by dual mutations in *gyrA* and a single mutation in *parC* (Dutta *et al.*, 2008; Gaind *et al.*, 2006).

Alteration in drug transport has been described as an additional mechanism of fluoroquinolone resistance in *E. coli* (Mazzariol *et al.*, 2000) and recently in *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *Streptococcus pneumonia*, *Klebsiella pneumonia* and *S. Typhimurium* (Baucheron *et al.*, 2002; Baucheron *et al.*, 2004; Mazzariol *et al.*, 2002). The expression of the AcrAB-TolC system, the major

multidrug efflux pump, is under the regulation of *ramA*, *marA*, *soxS* or *rob*. Constitutive over expression of AcrAB is present in most ciprofloxacin resistant *E.coli* and also occurs in ciprofloxacin resistant *S. Typhimurium* phage type DT204 (Baucheron *et al.*, 2002). The MarA global activator, which can be derepressed by tetracycline or chloramphenicol, simultaneously up-regulates the AcrAB-TolC transport complex and down-regulates the synthesis of the larger porin OmpF, a mechanism that synergistically blocks the drug penetration and accumulation in the cell (Parry, 2003).

Plasmid mediated quinolone resistance was first described in 1998 in an isolate of *Klebsiella pneumonia* (Martinez-Martinez *et al.*, 1998). Since then five major groups of quinolone resistance determinants, *qnrA*, *qnrS*, *qnrB*, *qnrC* and *qnrD*, have been identified (Strahilevitz *et al.*, 2009). The *qnr* genes encode for a pentapeptide repeat protein that confers reduced susceptibility to the fluoroquinolones (typically with an 8 to 64 fold increased quinolone MIC). Qnr proteins are able to protect DNA gyrase from the quinolones. Aquatic bacteria are thought to be the origin of plasmid encoded quinolone resistance genes. *qnr* genes have been found on plasmids of varying size and incompatibility groups.

Recently additional plasmid mediated quinolone resistance genes have been described. *aac(6')-Ib-cr* encodes a variant aminoglycoside acetyltransferase, which confers resistance to tobramycin, amikacin and kanamycin (Robicsek *et al.*, 2006b). This enzyme reduces the activity of ciprofloxacin by N-acetylation at the amino nitrogen on its piperazinyl substituent. AAC(6')-Ib-cr is selective only for

ciprofloxacin and norfloxacin, which both have an unsubstituted piperazinyl nitrogen. AAC(6')-Ib-cr confers only a modest increase in MIC (3 to 4 fold), but has shown to cause a dramatic increase in the mutant prevention concentration (Robicsek *et al.*, 2006a). The *aac(6')-Ib-cr* gene is located in an integron cassette with an associated *attC* site. It is often found on incF11 plasmids expressing CTX-M-15 that have spread rapidly and become the predominant ESBL in many countries worldwide (Strahilevitz *et al.*, 2009).

So far, *qnr* genes and *aac(6')-Ib-cr* have only been reported in non-Typhi *Salmonella* (Strahilevitz *et al.*, 2009), with the exception of a multiple drug resistant *S. Typhi* strain isolated from an Iraqi traveler that was characterized by the *bla*_{CTX-M15}, *qnrB2* and the GyrA Ser83Phe amino acid substitution (Pfeifer *et al.*, 2009).

1.8.2.3 Adverse events

The frequency of adverse reactions to quinolones is between 6 and 11% with less than 1% of adverse events being recorded as serious (Andriole, 2004). The most frequent adverse effects reported are nausea, upper gastrointestinal discomfort and central nervous system effects such as headache, insomnia and dizziness. The adverse events are typically mild, self limited and mostly resolve when the drug is stopped.

Some adverse effects do not seem to be related to specific modifications, whereas phototoxicity and CNS effects are linked to a specific structure. Each fluoroquinolone tends to produce a characteristic profile of adverse effects.

In their preclinical evaluation, all quinolones studied caused arthropathy in immature animals, especially in young beagle dogs and usually in the major weight bearing joints (Gough *et al.*, 1979; Stahlmann *et al.*, 1990). Histopathological examination showed localized blister formation and erosions in the joint cartilage (Schaad and Wedgwood, 1992). The concern that the fluoroquinolones might also cause cartilage damage in children, have led to cautious use in many countries. However, extensive experience with the fluoroquinolones in children suffering from cystic fibrosis, typhoid fever and bacillary dysentery has provided a body of evidence suggesting that the joint damage seen in young dogs does not occur in children and these antibiotics are safe in children (Bethell *et al.*, 1996; Organization, 2003; Parry *et al.*, 2002; Schaad *et al.*, 1991; Schaad and Wedgwood, 1992).

Fluoroquinolones have been associated with tendinitis and tendon rupture in adults, primarily affecting the Achilles tendon, risk factors were renal dysfunction and concomitant corticosteroid use (Owens and Ambrose, 2005).

Severe neurotoxic reactions are rare. However, hallucinations, depression, and psychotic reaction have been reported. Some fluoroquinolones may inhibit transmission of gamma-aminobutyric acid (GABA) leading to CNS stimulation. These CNS effects may be potentiated by interactions with other medications such as theophyllin (Andriole, 2004; Davis *et al.*, 2005). Thus the quinolones should be used with caution in patients with known CNS disorders (e.g., epilepsy) or conditions predisposing to seizures (Andriole, 2004; Davis *et al.*, 2005).

The most common skin reactions are non specific skin rashes, pruritis and urticaria. Phototoxicity is a rare dermatologic complication of quinolone therapy which is inextricably related to the chemical structure (Owens and Ambrose, 2005).

The “double halogenated” quinolones which possess a halogen moiety (chlorine, fluorine) at position 8 together with the fluorination of position 6 have demonstrated significant phototoxic potential. Agents with these modifications are fleroxacin, lomefloxacin and sparfloxacin. Thrombocytopenia, leucopenia and anaemia are rare (Andriole, 2004). Recently the cardiovascular effects of the quinolones, especially of the newer agents have been closely evaluated. Dose related prolongation of the QTc interval and rare cases of significant arrhythmias including torsades de pointes were reported in patients treated with sparfloxacin and to a lesser extent with grepafloxacin, these two antibiotics were therefore withdrawn.

A study based on post marketing surveillance data reported that the crude incidence rate (95% confidence interval, 95% CI) of cases of Torsades de Pointes (TdP) per 10 million prescriptions in the United States was 0.3 (0.0-1.1) for ciprofloxacin, 2.1 (0.3-7.6) for ofloxacin, 5.4 (2.9-9.3) for levofloxacin and 27 (12-53) for gatifloxacin (Frothingham, 2001). However questions regarding the validity of both the numerators and denominators used in these incidence calculations remain (Owens and Ambrose, 2002). Preclinical and clinical data indicate that levofloxacin, moxifloxacin, and gatifloxacin prolong the QTc interval. The potential for TdP to develop as a result of this is rare and is influenced by many independent variables, especially by concurrent administration of class IA and III antiarrhythmic agents, genetic

susceptibility, underlying cardiac disease, electrolyte imbalance and organ impairment. Therefore gatifloxacin, levofloxacin, moxifloxacin or gemifloxacin should not be used in patients with risk factors predisposing them to TdP (Owens and Ambrose, 2002).

The quinolones as a class have demonstrated the ability to close K^+ -ATP channels in the β cells of the pancreas, resulting in the release of insulin and subsequent hypoglycaemia. However the mechanism for hyperglycaemia remains poorly understood and might be caused by overexposure (failure to adjust the dose in patients with renal failure) (Owens and Ambrose, 2002). Product labels for ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin mention the possibility of hypoglycaemia and hyperglycaemia, whereas the product label for gemifloxacin mentions hyperglycaemia only. Although glucose disturbances appear to be a class effect, the odds of hypo- and hyperglycaemia appear to vary among the agents (Aspinall *et al.*, 2009). A retrospective study in Texas reviewed records of dysglycaemia in hospitalised patients receiving gatifloxacin, levofloxacin, ciprofloxacin or ceftriaxone (Mohr *et al.*, 2005). Dysglycaemic events were more likely to occur in patients receiving gatifloxacin (relative risk, 3.29; 95% CI, 2.33–4.65) or levofloxacin (relative risk, 1.55; 95% CI, 1.29–1.88) versus ceftriaxone.

In another study of elderly inpatients who received gatifloxacin or levofloxacin, gatifloxacin was independently associated with hypoglycaemia (OR, 2.4; 95% CI, 1.1–5.6) and hyperglycaemia (OR, 2.5; 95% CI, 1.6–3.9) versus levofloxacin (Lodise *et al.*, 2007). In diabetic patients treated with gatifloxacin, the overall incidence of

hypoglycaemia was 0.4%, 0.7%, and 1.6% for patients below 65 years, 65 to 69 years and 80 years and above, respectively. The corresponding incidences of hyperglycaemia were 1.0%, 1.6%, and 3.3%, respectively (Owens and Ambrose, 2005).

When exposure to gatifloxacin was simulated in patients with severe hyperglycaemia, AUC values were 2 to 3 times those observed in patients with normal renal function (Ambrose *et al.*, 2003). Therefore the authors suggested to empirically adjust the dose of gatifloxacin to 200 mg daily for patients aged above 65 years with community acquired respiratory tract infections. A detailed discussion of the risk benefit ratio of gatifloxacin for the treatment of enteric fever is presented in Chapter 2.

Only ciprofloxacin, clinafloxacin, enoxacin, grepafloxacin, pefloxacin, and tosufloxacin can inhibit the hepatic cytochrome P₄₅₀ isoform CYP 1A4 isoenzymes. Few drugs are metabolized by these isoenzymes, but important drugs include the methylxanthines (theophylline and caffeine) and warfarin.

1.8.3 Extended spectrum cephalosporins

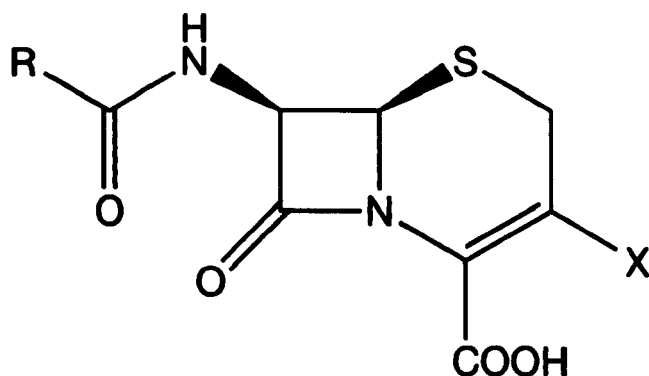


Figure 1.7 Basic structure of cephalosporins

The basic structure of the cephalosporins consists of a four membered β -lactam ring fused with a six-membered dihydrothiazine ring. Cephalosporins exert bactericidal activity by interfering with the later stages of the bacterial cell wall synthesis (Capitano and Kays, 2005). The target site of the β -lactam antibiotics including the cephalosporins are the penicillin-binding proteins (PBPs). During the process of peptidoglycan synthesis, the cephalosporin β -lactam ring becomes covalently bound to the PBPs, acting as a false substrate. This process inhibits the cross linking of the peptidoglycan structure and ultimately leads to bacterial cell lysis.

Production of β -lactamases is the most common mechanism of bacterial resistance. In the late 90s, non-Typhi *Salmonella* producing extended spectrum β -lactamases (ESBL) have been reported in numerous countries. Resistance generally results from the production of Ambler class A or C (also known as AmpC β -lactamases) ESBL.

Class A enzymes such as TEM, SHV, OXA, PER and CTX-M are plasmid mediated and confer resistance against oxyimino- β -lactams such as cefotaxime, ceftazidime and aztreonam, but are not active against cephamycins and can be inactivated by clavulanic acid. Ambler class C enzymes are able to hydrolyse all β -lactams to some extent and are not inhibited by clavulanic acid. Over-expression confers resistance to all β -lactam including the cephamycins, but not to cefpirome, cefipime and the carbapenems (Parry, 2003).

Salmonella producing the β -lactamase CMY-2 have been isolated from food producing animals in Northern America and Europe (Aarestrup *et al.*, 2004; Allen and

Poppe, 2002) and from hospitalised patients in the USA and Taiwan (Parry, 2003). Recent studies in the US, Mexico and South Africa have shown a wider range of ESBL enzymes in non-Typhi *Salmonella* (Parry and Threlfall, 2008). However, prevalence of extended spectrum cephalosporin resistance (ESC) was low in human non-Typhi *Salmonella* isolates collected across 10 European countries in 2000 (Threlfall *et al.*, 2003). Only 0.6% of 27000 *Salmonella* isolates showed resistance to cefotaxime.

Resistance to extended spectrum cephalosporins has been reported in single isolates of *S. Typhi* from Bangladesh and Italy and Paratyphi A from Pakistan and Nepal, however the mechanism was not described in these reports (Parry, 2003; Pokharel *et al.*, 2006). In 2009, a *S. Typhi* isolate with ESBL phenotype caused by *bla*_{CTX-15} was described in a patient returning from Iraq (Pfeifer *et al.*, 2009).

The cephalosporins exhibit time dependent bactericidal activity, therefore the target for antimicrobial therapy is to optimise the duration of exposure (Time above the MIC, T > MIC). Cephalosporins penetrate well into most tissues and body fluids (Capitano and Kays, 2005). High concentrations of ceftriaxone have been demonstrated in human monocytes. Overall, the cephalosporins are a safe class of antibiotics, hypersensitivity reactions are the most common adverse events. Gastrointestinal reactions, including nausea, vomiting and diarrhoea are also reported frequently.

The third generation cephalosporins ceftriaxone and cefixime have been widely used for the treatment of MDR typhoid fever. The average fever clearance times in randomised trials using ceftriaxone have been 7 days and 5 to 10% of patients failed clinically. Relapse rates varied between 4% and 6% (Parry *et al.*, 2002). A study in Pakistan evaluated either 7 or 14 days of ceftriaxone treatment in children with typhoid fever and found a relapse rate of 14% (4 out of 28 patients) in the 7 day treatment group compared to no relapse in the 14 day group (Bhutta *et al.*, 2000). The disadvantage of ceftriaxone is the need for parenteral administration and the high cost, especially for prolonged treatment courses.

Oral cefixime has been a popular choice for the treatment of typhoid fever in children. In randomised controlled trials in children the mean fever clearance times ranged from 5 to 8 days and clinical failure rates were reported to be below 3% (Bhutta *et al.*, 1994; Girgis *et al.*, 1995; Memon *et al.*, 1997). However, a typhoid treatment trial in Vietnam reported much higher failure rates of 23% (10 out of 44 patients) when cefixime was used in children (Cao *et al.*, 1999).

1.8.4 Azithromycin

Azithromycin belongs to the macrolide class of antibiotics and is a derivative of erythromycin. Erythromycin was isolated in 1952 from a strain of *Streptomyces erythreus* obtained from soil from the Philippines. The structure of erythromycin is a 14-membered lactone ring, substituted by two sugars, desosamine and cladinose

(Steigbigel, 1995). Erythromycin is unstable in acidic medium which causes poor and inconsistent bioavailability.

Azithromycin has a 15-membered macrocycle and a methyl-substituted nitrogen, hence the name azalides has been given to this class. Azithromycin exhibits increased activity against Gram negative bacteria and a longer half life.

Macrolides are inhibitors of protein synthesis by impairing the elongation of the peptidyl chain. The main interaction site is the domain V of the 23S rRNA, which lies at the center of the peptidyl transferase site (Mulazimoglu *et al.*, 2005). The macrolide binding site is located at the exit tunnel of the growing peptide chain. The critical interaction occurs between the 2'-OH group of the desosamine and the adenosine residue 2058. The binding site of the macrolides on the 50S subunit of the ribosome overlaps with that of chloramphenicol and the lincosamides, leading to antagonism and cross-resistance between these classes.

Acquired resistance in macrolides can involve three mechanisms; modification of the target, antibiotic inactivation or active efflux (Mulazimoglu *et al.*, 2005). Methylation of the rRNA is currently the most prevalent mechanism of resistance in pathogenic bacteria. This is mediated by the acquisition of *erm* genes which encodes for methyltransferases. Methylation of the N(6) position of adenine 2058 in 23S rRNA results in inhibition of macrolide binding. Monomethylation confers high levels of resistance to lincosamides and streptogramins and low-level resistance to macrolides, whilst dimethylation causes high level resistance to all three drug classes, leading to

the MLS_B phenotype. More than 30 *erm* genes have been described, which are located on conjugative and transferable transposons. The expression of the methylase is either constitutive or inducible. Inducers include the 14-, 15- and 16-membered macrolides, the lincosamides and the streptogramins, but not the ketolides.

Another mechanism of resistance is the substitution of the adenine at position 2058 with guanine, this has been described in *Helicobacter pylori*, *Mycoplasma* and *Mycobacterium spp.* (Mulazimoglu *et al.*, 2005).

Erythromycin-inactivating enzymes have been reported in *Enterobacteriaceae*. These are phosphorylases or esterases (encoded by *ereA* and *ereB*) that confer resistance to all 14-, 15- and 16-membered macrolides.

In Gram positive bacteria, exposure to the antibiotic can induce over-expression of efflux pumps conferring resistance. Examples of inducible efflux pumps are the Msr(A) pump in *Staphylococci* and the Mef efflux system in *Streptococci* (Mulazimoglu *et al.*, 2005).

A *S. Paratyphi A* isolate with an azithromycin MIC (Etest) of 64 mg/L. was recently reported to have caused treatment failure in an enteric fever patient returning from Pakistan, who was treated with oral azithromycin (Molloy *et al.*, 2010).

Azithromycin has a bioavailability of 30% to 50%. The serum peak level is typically reached after 2 hours. Azithromycin has a large volume of distribution which is related to the ability to accumulate inside eukaryotic cells. The ratio of tissue to serum concentration for azithromycin is 50 to 1150 (Mulazimoglu *et al.*, 2005). The half life

is 35 to 40 hours, which allows a single daily dose and shortened treatment regimen (3 to 5 days).

Macrolides are primarily metabolised through cytochrome P450 and eliminated through the bile. Gastrointestinal adverse events are relatively frequent with macrolides. Macrolides have been associated with prolongation of the QT interval and should not be used in patients with concurrent administration of class IA and III anti-arrhythmic agents and underlying cardiac disease.

Azithromycin has become a popular treatment choice for the treatment of MDR typhoid fever. The MICs for *S. Typhi* to azithromycin range from 4 to 16 µg/ml (Metchock, 1990). The peak serum level after a single dose of 500 mg of azithromycin is 0.4 mg/L (Mulazimoglu *et al.*, 2005). However, as azithromycin is concentrated more than 100-fold inside polymorphonuclear cells and macrophages (Panteix *et al.*, 1993) and *S. Typhi* is primarily an intracellular pathogen (Wain *et al.*, 1998), effective drug concentrations are considerably above the MIC.

In randomized clinical trials, azithromycin has been used for the treatment of MDR typhoid fever in children and adults in Egypt, India and Vietnam (Butler *et al.*, 1999; Chinh *et al.*, 2000; Frenck *et al.*, 2000; Frenck *et al.*, 2004; Girgis *et al.*, 1999). Cure rates were excellent and outcomes in patients infected with nalidixic acid resistant *S. Typhi* were satisfactory (Chinh *et al.*, 2000).

1.9 Antimicrobial drug resistance of *S. Typhi* and *S. Paratyphi A*

In 1948 the introduction of chloramphenicol revolutionised the management of typhoid fever (Woodward *et al.*, 1948). Chloramphenicol was effective for more than 20 years, but in the 1970s outbreaks of chloramphenicol resistant typhoid fever took place in Mexico, India and Vietnam. In the late 1980s and early 1990s outbreaks of typhoid fever occurred that were resistant against all ‘first line’ antimicrobials (multidrug resistance (MDR) defined as resistance to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole) (Parry *et al.*, 2002). These MDR *S. Typhi* isolates have been responsible for numerous outbreaks in countries in the Indian subcontinent, South East Asia and Africa (Bhan *et al.*, 2005). All MDR strains so far examined, have been harbouring plasmids of the *incHI1* incompatibility group.

Consequently, the fluoroquinolones have become the treatment of choice for typhoid fever. The fluoroquinolones show excellent tissue penetration, accumulation in monocytes and macrophages and high drug levels in the gall bladder. However, there have been reports from Vietnam, India and Tajikistan of the emergence of *S. Typhi* isolates that respond less well to the fluoroquinolones (Bhan *et al.*, 2005; Parry *et al.*, 2002). In 1997, a typhoid epidemics in Tajikistan caused by such isolates caused more than 10000 illnesses and 108 deaths (Mermin *et al.*, 1999). Technically these isolates remain within the breakpoints set for fluoroquinolone susceptibility by the Clinical Laboratory Standard Institute (CLSI) (Clinical Laboratory Standard Institute, 2007), but they are resistant to nalidixic acid (the prototype quinolone) and show higher

MICs to the fluoroquinolones. Patients infected with these isolates show a poor clinical response when treated with ciprofloxacin or ofloxacin.

Nalidixic acid resistance is usually caused by single point mutations in the bacterial target enzyme GyrA, either at codon 83 or 87 (Chau *et al.*, 2007). High-level ciprofloxacin resistant *S. Typhi* (Dutta *et al.*, 2008; Gaind *et al.*, 2006) and *S. Paratyphi A* (Gaind *et al.*, 2006) with double mutations in *gyrA* and single mutation in *parC* have been described in the Indian subcontinent. Recently there have been reports of *S. Typhi* isolates with reduced susceptibility to the fluoroquinolones that test nalidixic acid sensitive (Cooke *et al.*, 2007), suggesting another mechanism of resistance. There have been sporadic reports of ceftriaxone resistant *S. Typhi*, but these isolates still seem to be rare (Parry and Threlfall, 2008). A decreasing trend of chloramphenicol resistant *S. Typhi* has been reported in recent years from the Indian subcontinent (Dutta *et al.*, 2005; Mohanty *et al.*, 2006). MDR as well as nalidixic acid resistant *S. Paratyphi A* is an emerging problem in the Indian subcontinent (Chandel *et al.*, 2000; Maskey *et al.*, 2006).

1.10 Treatment of typhoid and paratyphoid fever

Enteric fevers are systemic infections and appropriate antimicrobial treatment should be initiated early. It is important to provide supportive measures, such as oral and intravenous fluids, appropriate nutrition and antipyretics. More than 90% of patients are managed as outpatients, with oral antibiotics, reliable care and close medical follow up for complications or failure to respond to therapy. Patients with persistent

vomiting, severe diarrhoea and abdominal distension need admission to hospital (World Health Organization, 2003).

The management of patients with enteric fever should include blood cultures and stool cultures after completion of treatment to check for failure to clear the bacteria in the blood and stools (convalescent stool carriage), as well as follow up for at least 6 months to identify chronic faecal carriage. However in most endemic areas such follow up is not possible and therefore the management of the acute illness with effective drugs, that also prevent chronic carriage and relapse, are essential.

1.10.1 Current recommendations of the World Health Organization for the treatment of typhoid fever

The choice of treatment depends on the antimicrobial susceptibility of the isolates, but also on the cost, which is an important factor especially in endemic regions. Table 1-2 shows the current recommendations of the World Health Organization (WHO) for the treatment of typhoid fever (World Health Organization, 2003).

The fluoroquinolones are the most effective treatment for typhoid fever. In patients infected with nalidixic acid susceptible isolates, fever usually resolves within 4 days, cure is achieved in 96% of patients and rates for faecal carriage and relapse are below 2% (Parry *et al.*, 2002).

The fluoroquinolones are also recommended for the treatment of children with typhoid fever. Extensive experience with the fluoroquinolones in children suffering from cystic fibrosis, typhoid fever and bacillary dysentery has provided a body of

evidence suggesting that the cartilage damage seen in young animals does not occur in children (Bethell *et al.*, 1996; World Health Organization, 2003; Parry *et al.*, 2002). Alternatives to the fluoroquinolones are third generation cephalosporins (cefixime or ceftriaxone) and azithromycin. For infections with nalidixic acid (quinolone) resistant *S. Typhi*, azithromycin, prolonged courses of high dose fluoroquinolones (e.g. ofloxacin at 20 mg/kg/day) and third generation cephalosporins (ceftriaxone) are recommended. Ceftriaxone should be used for 10 to 14 days. Ceftriaxone therapy for 7 days was associated with a relapse rate of 14% (Bhutta *et al.*, 2000; Frenck *et al.*, 2000).

Susceptibility	OPTIMAL THERAPY			ALTERNATIVE EFFECTIVE DRUGS		
	Antibiotic	Daily dose (mg/kg)	Days	Antibiotic	Daily dose (mg/kg)	Days
Fully sensitive	Fluoroquinolone e.g. ofloxacin or ciprofloxacin ^a	15	5-7 ^a	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				Trimethoprim- sulfamethoxazole	8-40	14
Multidrug resistance	Fluoroquinolone or cefixime	15	5-7	Azithromycin	8-10	7
		15-20	7-14	Cefixime	15-20	7-14
Quinolone (nalidixic acid) resistance ^b	Azithromycin or ceftriaxone	8-10 75	7 10-14	Cefixime	20	7-14

^a Three day courses are also effective and are particularly so in epidemic containment.

^b The optimum treatment for quinolone resistant typhoid fever has not been determined. Azithromycin, the third generation cephalosporins, or a 10-14 day course of high-dose fluoroquinolones is effective. Combinations of these are now being evaluated.

Table 1-2. World Health Organization treatment recommendations for uncomplicated typhoid fever.

From (World Health Organization, 2003)

There are few data on the treatment of typhoid fever in pregnancy (Seoud *et al.*, 1988). Ampicillin for fully susceptible isolates and ceftriaxone are considered safe for this indication (World Health Organization, 2003; Parry *et al.*, 2002).

1.10.2 Management of severe typhoid fever

Both, in- and out-patients should be closely monitored for the development of complications. The parenteral fluoroquinolones are probably the first choice for the treatment of severe typhoid fever (Dutta *et al.*, 1993; World Health Organization, 2003) but there have been no randomised trials to date. In severe typhoid fever, the fluoroquinolones should be given for a minimum of 10 days. For patients infected with nalidixic acid resistant isolates, ceftriaxone is effective (World Health Organization, 2003).

A trial conducted in the 1980s showed a dramatic beneficial effect of high dose steroid treatment (dexamethasone at 3 mg/kg for the first dose given over 30 minutes and 1 mg/kg every 6 hours for 48 hours) in severely sick typhoid fever patients with encephalopathy and shock, given in addition to chloramphenicol. Dexamethasone adjunctive treatment reduced the mortality from 56% to 10% when compared to placebo (Hoffman *et al.*, 1984). Hydrocortisone at a lower dose was not effective (World Health Organization, 2003).

Intestinal perforation is a surgical emergency. Early intervention is crucial, and mortality rates increase when surgery cannot be performed immediately and vary between 10% and 32% (van Basten and Stockenbrugger, 1994). Metronidazole should

be added to the antibiotic regimen to treat leakage of intestinal bacteria into the abdominal cavity. Patients with intestinal haemorrhage need intensive care, monitoring and blood transfusion. Intervention is needed if there is significant blood loss, then crossmatched blood should be ready and the operating theatre should be prepared (Parry *et al.*, 2002).

In patients presenting with a relapse, cultures should be obtained and patients should be treated according to the susceptibility pattern of the infecting isolate.

1.10.3 Typhoid carriers

Chronic carriers play an important role in the transmission of typhoid and paratyphoid fever. *S. Typhi* and *S. Paratyphi* are only shed intermittently in the faeces. Therefore, stool samples need be obtained repeatedly to detect typhoid carriers. Long antimicrobial treatment courses of up to six weeks should be given according to the susceptibility of the isolates to eradicate carriage (World Health Organization, 2003).

In susceptible isolates, clearance was achieved in up to 80% with the administration of 750 mg ciprofloxacin or 400 mg norfloxacin twice daily for 28 days (Ferrecchio *et al.*, 1988; Gotuzzo *et al.*, 1988). If cholelithiasis is present the patient may require antibiotic therapy as well as cholecystectomy. In patients with chronic urinary carriage resulting from infection with *Schistosoma haematobium*, antiparasitic medication in addition to antibiotics is needed to achieve bacteriological cure (World Health Organization, 2003).

1.11 Control and prevention of typhoid fever

Typhoid fever can be prevented by the provision of safe drinking water, good food hygiene and safe sewage disposal. Chronic carriers pose a special risk to the community, therefore programmes to detect and treat chronic carriers should be in place.

There are two licensed, safe typhoid vaccines available, the parenteral (intramuscular injectable) Vi polysaccharide and the oral live attenuated Ty21 vaccine, both vaccines are based on the pathogenic *S. Typhi* strain Ty2 (Felix and Pitt, 1951). The oral Ty21 vaccine (Vivotif, Berna) needs to be administered in three doses (day 1, 3, 5) and is licensed for adults and children above 6 years. Field studies with the oral Ty21 vaccine in the 1980s have shown a protective efficacy after 3 years of 96% in Egypt (Wahdan *et al.*, 1982) and up to 77% in Chile when using the liquid formulation (Levine *et al.*, 1990). The parenteral Vi vaccine is licensed for adults and children above 2 years. A single intramuscular injection confers a protective efficacy of 77% after 21 months in South Africa (Klugman *et al.*, 1987). The WHO recommends that the immunization of school-age children should be undertaken wherever the control of the disease is a priority (World Health Organization, 2003). Unfortunately, these two vaccines are both underutilised in developing countries.

1.12 Vietnam

1.12.1 Introduction

The Socialist Republic of Vietnam is the second largest country in South East Asia. Vietnam borders to China, Laos and Cambodia and stretches 1650 km north to south. Vietnam's coastline along the South China Sea extends 3400 km. The country is divided in the Red River Delta in the north, the Central Highlands and the Mekong Delta in the south. Vietnam has an estimated population of 87 million with a median age of 27 years (Central Intelligence Agency, 2009). The capital is Hanoi. There are four other municipalities (Ho Chi Minh City, Danang, Can Tho and Hai Phong) and 58 provinces.

Economically, Vietnam is classified as a developing low income country by the World Bank. In 2008, the gross domestic product (GDP) was US\$ 90 billion and the GNI (gross national income) per capita (Atlas method) was US\$ 890 (World Bank, 2009). Twelve % of the population are estimated to be below the poverty line (i.e. living on less than US\$ 1 per day) (Central Intelligence Agency, 2009). The total expenditure on health care is 7.1% of the GDP or US\$ 58 per capita per annum. The government expenditure on healthcare is US\$ 23 per capita or 39% of the total expenditure (World Health Organization, 2010). There are six doctors, eight nurses and three pharmaceutical personnel and 28 hospital beds per 10,000 population. The maternal mortality rate is 150 per 100 000 live births and the mortality rate in children under 5 years was 14 per 1000 live births in 2007. Life expectancy at birth is currently

74 years and the adult literacy rate is 90% (Central Intelligence Agency, 2009). Adult HIV prevalence is estimated at 0.5%.



Figure 1.8. Map of Vietnam, showing the major cities.

From (Central Intelligence Agency, 2009)

1.12.2 The Hospital for Tropical Diseases, Ho Chi Minh City

The Hospital for Tropical Diseases was initially built in 1865 and is a 500 bed infectious diseases hospital in Ho Chi Minh City, Vietnam and was formerly known

as the Centre for Tropical Diseases and before that as Cho Quan Hospital. It serves the local community in Ho Chi Minh City and is a tertiary referral centre for infectious diseases for the southern provinces of the country. Randomised controlled trials for the treatment of typhoid fever have been conducted on the adult and paediatric wards since 1992. The Wellcome Trust Major Overseas Programme Vietnam, Oxford University Clinical Research Unit, Ho Chi Minh City is based within the Hospital for Tropical Diseases, in a state of the art modern research facility, allowing close collaborations between clinicians and researchers. The research undertaken in this PhD was based at the Oxford University Clinical Research Unit, The Hospital for Tropical Diseases, Ho Chi Minh City.

1.12.3 Typhoid fever and antimicrobial drug resistance in Vietnam

Typhoid fever has been endemic in Vietnam, especially in the densely populated Mekong Delta area.

Chloramphenicol resistant typhoid fever was first described at the Hospital for Tropical Diseases in 1973 (Butler *et al.*, 1973). In 1993, during the initial outbreak of MDR *S. Typhi* in Kien Giang province in the south of Vietnam, the fluoroquinolone antibiotics were introduced as the first choice for the treatment of typhoid fever (Nguyen *et al.*, 1993). Since 1993, the proportion of MDR *S. Typhi* plateaued at high levels in southern Vietnam and there has been a dramatic increase in nalidixic acid resistance. In 2004, 50% (101/202) of the *S. Typhi* isolates from southern Vietnam were MDR. In 1998, five years after ofloxacin and ciprofloxacin become widely used

to treat typhoid fever but also a broad range of other febrile illnesses, often purchased without prescription in an uncontrolled market, 87% of the *S. Typhi* isolates were resistant to nalidixic acid (Chau *et al.*, 2007).

In 2004, 97% (196/201) of the *S. Typhi* isolates were resistant to nalidixic acid and showed reduced susceptibility to the older fluoroquinolones, such as ofloxacin. This combination of MDR and nalidixic acid resistance is a particular problem in Vietnam, leading to poor clinical response and high failure rates (up to 36% clinical failure rates) in typhoid fever patients treated with ofloxacin (Chinh *et al.*, 2000; Parry *et al.*, 2006), and severely restricts the therapeutic options in patients with typhoid fever. Data from a cross-sectional study from eight Asian countries (Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan and Vietnam) underline that antimicrobial drug resistance in *S. Typhi* is a problem in Asia (Chau *et al.*, 2007). In 2003 and 2004, the prevalence of chloramphenicol resistant *S. Typhi* remained high with 18% (9/50) of isolates in Laos, 19% (28/149) in Nepal, 26% (6/23 and 9/34 isolates, respectively) in India and Pakistan and 40% (16/40) in Bangladesh. All countries in the region, with the exception of China and Laos, faced an additional problem of widespread nalidixic acid resistance. Again southern Vietnam was a particular hot spot with more than 90% of all *S. Typhi* resistant to nalidixic acid. Roumagnac *et al* recently suggested that fluoroquinolone use has stimulated the clonal expansion of a nalidixic acid resistant *S. Typhi* haplotype H58 in southeast Asia (Roumagnac *et al.*, 2006).

Provinces	2004		2005		2006	
	Cases	Deaths	Cases	Deaths	Cases	Deaths
Ho Chi Minh City	106	0	75	0	42	0
An Giang	483	0	724	0	545	0
Dong Thap	700	1	557	0	413	0
Southern Provinces	2707	1	3088	0	2173	0
Whole country	4323	1	5030	5	3018	0

Table 1-3. Reported cases of syndromic typhoid fever in Vietnam, 2004 to 2006.

Table 1-3 summarises the reported cases of syndromic typhoid fever in Vietnam (blood culture confirmed and unconfirmed) between 2004 and 2006 shown for three selected provinces (Ho Chi Minh City, An Giang and Dong Thap province), the southern provinces and the whole country. Source: Statistics on Infectious Diseases, National Institute of Health and Epidemiology (NIHE), Ministry of Health, Vietnam

Compared to the 1990s, the number of reported cases of typhoid fever has declined in Vietnam. In 1995, more than 700 *S. Typhi* strains were isolated from blood cultures from patients admitted at the Hospital for Tropical Diseases in Ho Chi Minh City (Parry, 2004a). In 2009, there were less than 50 blood culture confirmed cases of typhoid fever at this hospital. The reduction in the incidence of typhoid fever in Vietnam has been attributed to improvements in infrastructure that have accompanied the economic development and to better treatment. There have been limited targeted

vaccination campaigns, using the Vi vaccine (Lin *et al.*, 2001), however the extent to which vaccination contributed to the reduction in incidence is not clear.

1.13 Nepal

Nepal is a landlocked country in Southern Asia that shares borders with the Republic of India in the south, west and east and with the Peoples Republic of China in the north. The total land area is about 140 000 km², of which only 16% are arable land. Nepal is a very mountainous country with eight of the world's ten highest peaks on its territory. Mount Everest, the world's tallest mountain with a height of 8844 m is located on the Nepalese-Tibetian border. Kanchenjunga, the world's third tallest mountain (8586 m) is located on the Nepalese-Indian border.

The Tarai plain, the northern rim of the Ganges plain, is situated at 300 m above sea level in the south of Nepal. Due to the diverse geography and enormous range of altitudes, the weather in Nepal varies from cool summers and extreme alpine winters in the north to sub tropic summers and mild winters in the south.

Nepal has an estimated population of 29 million and is among the poorest and least developed countries in the world. Approximately 31% of its population are living below the poverty line (less than 1 US\$ per day). Agriculture provides an income for 75% of the population and accounts for approximately one third of the GDP.



Figure 1.9. Map of Nepal showing the major cities.

Source: Central Intelligence Agency, 2009

After ten years of civil war (from 1996 to 2006) which was led by Maoist insurgents, nationwide elections took place in 2008 and the newly elected Constituent Assembly declared Nepal a Federal Democratic Republic and abolished the 240 year old monarchy.

The gross national income (GNI) per capita is 400 US\$ (Central Intelligence Agency, 2009). The adult literacy rate (above 15 years) is 57% and the life expectancy is 67 years. The maternal mortality rate (adjusted) is one of the world's highest with 830 death per 100 000 live births and the infant mortality rate is 41 deaths per 1000 live births (World Health Organization, 2010). The mortality rate in children under 5 years is 51 per 1000 live births. Access to trained health care workers is a major problem in

this mountainous country, where travel is often by foot. There is a huge disparity in the quality of health care offered in urban and rural areas. In Kathmandu 10 doctors serve for every 10 000 people, whereas in rural Nepal 0.3 doctors serve 10 000 inhabitants. Only 19% of pregnant women have access to a skilled medical professional (doctor, nurse or midwife) at delivery, in some rural areas this percentage tends to be as low as 3% (Unicef, 2009).

1.13.1 Patan Hospital, Kathmandu

Kathmandu, Nepal's capital, has approximately 1.5 million inhabitants and is situated at an elevation of 1300 m. Kathmandu has four seasons, cold winters (December to February), spring (March to May), hot monsoon summers (June to August) and autumn (September to November). Patan Hospital is one of the three general hospitals serving the Kathmandu metropolitan area. The hospital has 450 beds and provides inpatient and outpatient medical, surgical, paediatric, gynaecology and obstetric services for approximately 300 000 outpatients and 16 000 inpatients per year. It serves as a primary and secondary health care facility.

Part of the research presented in Chapter 3 of this thesis was conducted at Patan Hospital, Kathmandu.

1.13.2 Enteric fever in Nepal

Enteric fever is a major public health problem in Nepal. Data regarding the burden of disease are difficult to obtain, this is partially due to self medication which is purchased without prescription from local pharmacies and the absence of diagnostic

facilities in rural areas. A recent study reported that *S. Typhi* and *S. Paratyphi A* accounted for 75% (9124 out of 12252) of all positive blood cultures that were performed at Patan Hospital from 1993 till 2003, during an 11 year period (Maskey *et al.*, 2008). The proportion of enteric fever caused by *S. Paratyphi A* increased from 18% in 1993 to 34% in 2003.

Multidrug resistant (MDR) *S. Typhi* was first reported in Nepal in 1991 and ciprofloxacin replaced chloramphenicol as first line treatment in 1994, but increasing rates of treatment failure were noted since 2000 (Maskey *et al.*, 2008). Faecal contamination of water sources is significant. In 2002, an outbreak of enteric fever in 5936 people in Bharatpur was reported (Lewis *et al.*, 2005). The source of the outbreak was the municipal water supply.

Kathmandu is served by an antiquated water supply. The water frequently contains coliform bacteria due to possible cross connections between water supply and sewage system. Chlorination is often inadequate. Often inhabitants do not have access to piped water in their homes and collect their water from local water spouts (Figure 1.9) (Karkey *et al.*, 2008).



Figure 1.10. Community water spout in Kathmandu, Nepal.

Villagers, mostly women and children are seen collecting their daily water provision from the community's stone spouts. This water is usually not boiled.

1.14 Aims of the thesis

The work described in this thesis includes two clinical trials (Chapters 2 and 5) and two laboratory studies (Chapters 3 and 4) that examine different aspects of typhoid fever and aim to improve our understanding of this disease. The work for this thesis was undertaken at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam and partly (Chapter 3) at Patan Hospital, Kathmandu, Nepal, in collaboration with the

Wellcome Trust Sanger Institute, UK and the Institute for Clinical Pharmacodynamics, US.

Chapter 2 describes a randomised controlled trial conducted in the Mekong Delta region of Vietnam with a high proportion of multidrug and nalidixic acid resistant *S. Typhi* strains. This chapter examines whether the newer fluoroquinolone gatifloxacin or azithromycin is the better treatment for uncomplicated typhoid fever in Vietnam. In the discussion the results of this trial are compared with two recent enteric fever trials using gatifloxacin, performed in Kathmandu, Nepal.

Chapter 3 uses existing data (patients' weight, height, serum creatinine and drug doses) from the gatifloxacin treatment trial (Chapter 2) to model the patients' drug exposure and put this into context with their clinical response to gatifloxacin treatment. It also identifies evidence based *S. Typhi* gatifloxacin MIC breakpoints to predict treatment success or failure.

The second section of Chapter 3 evaluates the pharmacokinetics of gatifloxacin in patients with typhoid fever. Blood samples were taken during a randomised trial of gatifloxacin *versus* chloramphenicol in Nepal and gatifloxacin plasma concentrations were measured. The pharmacokinetic profile of the Nepalese enteric fever patients is compared to North American adult subjects and paediatric patients with otitis media.

Chapter 4 explores the question of why the Mekong Delta has continued to be a hot spot for typhoid fever infections, particularly multidrug and nalidixic acid resistant typhoid fever, whilst the incidence of typhoid fever has declined in the rest of

Vietnam. This chapter looks at the population structure of the *S. Typhi* population isolated in the Mekong delta region during the clinical trial described in Chapter 2 and attempts to determine whether typhoid infections in this area are due to a certain genotype of *S. Typhi*.

Chapter 5 highlights possible mechanisms of prevention and describes a randomised controlled Phase II clinical trial of a novel oral typhoid fever vaccine in healthy Vietnamese children. The M01ZH09 (*S. Typhi* (Ty2 *aroC ssaV*) ZH9) vaccine has a well-defined dual mechanism of attenuation and is given in a single oral dose. This chapter describes the safety and immunogenicity of this vaccine in healthy children in an endemic area.

In the conclusion the key findings of this thesis are summarised and other research, current developments and possible future directions are discussed.

Chapter 2

A Multi-Center Randomised Controlled Trial of Gatifloxacin *versus* Azithromycin for the Treatment of Uncomplicated Typhoid Fever in Children and Adults in Vietnam

2.1 Introduction

The emergence of antimicrobial drug resistance in *S. Typhi* is a major problem particularly in South East Asia and the Indian sub-continent (Bhan *et al.*, 2005; Bhutta, 2006; Parry *et al.*, 2002). There is a need for an efficacious, safe and affordable oral treatment, particularly in regions with a high proportion of both multidrug and nalidixic acid resistant *S. Typhi*.

The World Health Organization (see Chapter 1) recommends the fluoroquinolones or cefixime for the treatment of MDR typhoid fever and azithromycin, the third-generation cephalosporins, or a 10-14 day course of high-dose older generation fluoroquinolones (e.g. ofloxacin or ciprofloxacin) for the treatment of nalidixic acid resistant typhoid fever (World Health Organization, 2003).

A trial from southern Vietnam used ofloxacin at the maximum recommended dose of 20 mg/kg/day for 7 days for the treatment of MDR and nalidixic acid resistant typhoid fever and showed high clinical failure rates (36%), high immediate post-treatment faecal carriage (19%), which may lead to transmission in the community after discharge from hospital, and prolonged mean fever clearance times of 8.2 days (95% CI, 7.2-9.2 days) (Parry *et al.*, 2007). These results underline that the older generation fluoroquinolones are clearly failing in the treatment of nalidixic acid resistant typhoid fever.

Of the newer fluoroquinolones, gatifloxacin, a broad spectrum synthetic 8-methoxyfluoroquinolone is available and affordable in South and South East Asia

including Vietnam. Of all the fluoroquinolones, gatifloxacin showed the lowest minimum inhibitory concentrations (MICs) for nalidixic acid resistant *S. Typhi* from Nepal (Maskey *et al.*, 2006) and Vietnam and a rapid bactericidal effect in time-kill experiments involving *S. Typhi* isolates with single and double mutations in *gyrA* of *S. Typhi* (Chau *et al.*, 2007). Gatifloxacin can be administered as a once daily oral dose.

Gatifloxacin was shown to be safe and efficacious in a pilot study in patients with a median fever clearance time (FCT) of 76 hours (unpublished data Dr. Nguyen Chinh).

Azithromycin, an azalid antibiotic, has achieved excellent clinical results in the treatment of MDR and nalidixic acid resistant typhoid fever (Chinh *et al.*, 2000; Parry *et al.*, 2007). However azithromycin is expensive and often not available in developing countries.

We conducted a randomised controlled trial comparing the efficacy of gatifloxacin to azithromycin in southern Vietnam, an area characterised by a very high proportion of MDR (88%) and nalidixic acid resistant (93%) *S. Typhi* isolates (Parry *et al.*, 2007).

2.2 Methods

2.2.1 Study design and objectives

The study was designed as a multicenter, open-label randomised controlled trial to compare the efficacy and safety of gatifloxacin versus azithromycin for the treatment of uncomplicated typhoid fever in children and adult in-patients in southern Vietnam.

The overall objective of the trial was to identify an efficacious, safe, available and affordable oral treatment for MDR and nalidixic acid resistant typhoid fever.

2.2.2 Participants

Patients were eligible to be included in the study if they had clinically suspected or culture confirmed uncomplicated typhoid fever and if fully informed written consent had been obtained. For children, consent was obtained from the parent. Exclusion criteria were pregnancy, age under 6 months, history of hypersensitivity to either of the trial drugs, any signs of severe typhoid fever (shock, deep jaundice, encephalopathy, convulsions, bleeding, suspicion or evidence of gut perforation), or previous reported treatment with a fluoroquinolone antibiotics, a third generation cephalosporin or macrolide antibiotics within one week prior to hospital admission.

2.2.3 The study sites and ethical approval

The study was conducted at three hospitals in the south of Vietnam (Figure 2.1.). Adult and paediatric patients were recruited at the Hospital for Tropical Diseases in Ho Chi Minh City, at the Dong Thap Provincial Hospital in Cao Lanh, Dong Thap province and at the An Giang Provincial Hospital in Long Xuyen in An Giang province.



Figure 2.1. Map of southern Vietnam.

The trial was conducted as a multicenter trial in three hospitals in southern Vietnam.

The study was approved by the Ethical and Scientific Committee of the Hospital for Tropical Diseases in Ho Chi Minh City and the Oxford University Tropical Research Ethics Committee (OXTREC), UK for all three study sites. The clinical and microbiological data from the first 40 patients recruited to each arm of the study were sent to the independent Data Safety and Monitoring Committee for their advice regarding the continuation of the study. The study was not stopped. The trial was registered at Current Controlled Trials (ISRCTN67946944).

2.2.4 Intervention

According to their randomisation number patients were assigned to oral treatment with either 20 mg/kg azithromycin (Zithromax® suspension, Pfizer, USA; 200 mg/5 mL or Zithromax® tablets, Pfizer, USA; 500 mg/tablet) or 10 mg/kg gatifloxacin (Tequin®, Bristol-Myers Squibb, USA; 400 mg/tablet) once daily for 7 days. Tablets

were cut to obtain the appropriate study dosage and administered with water. Inevitably, the dose administered was an estimate of 10 mg/kg/day of gatifloxacin or 20 mg/kg/day of azithromycin (number of tablets or proportions of tablets were documented in the CRFs). Gatifloxacin was only available as tablets, which were cut to obtain the appropriate dosage and crushed if necessary for children.

The maximum dose of azithromycin was 1 g per day. All drugs were purchased commercially.

2.2.5 Procedures

2.2.5.1 In-patient procedures

On admission to the hospital the patient's full history was taken, a standard clinical examination was performed and axillary temperature, weight and height were measured. Before treatment, full blood counts including white blood differential counts, serum aspartate transaminase (AST), serum alanine transaminase (ALT) and bilirubin were checked and blood cultures were obtained. For adult patients, creatinine, blood urea nitrogen (BUN) and glucose levels were additionally measured. In some patients bone marrow cultures were obtained. Urines were checked with dipstick and pre-treatment stool cultures were obtained. Chest X-ray and abdominal ultrasound were performed and repeated as clinically indicated. Randomisation and initiation of therapy took place either immediately on admission to hospital or patients were observed until results of blood tests including blood cultures were available and then randomised. Vital signs including measurement of axillary temperatures were measured and recorded every 6 hours (at 6, 12, 18 and 24 hours) until discharge.

Patients were examined daily until discharge from hospital, with particular reference to clinical symptoms, FCT, side effects of the drug and any complication of the disease. Additionally laboratory tests were scheduled if clinically indicated. All adverse events were recorded. On day 7 to 9 after the start of treatment full blood counts, liver function tests, blood and stool cultures were checked. In case of insufficient response to therapy, development of complications or drug-associated adverse events, the initial treatment was suspended and parenteral ceftriaxone (2 g per day) in two divided doses was used as rescue treatment for 10 days.

2.2.5.2 Follow-up procedures

Out-patient follow-up appointments were scheduled at 1 month, 3 months and 6 months after discharge from hospital to seek evidence for relapse (1 month visit) and check for chronic typhoid carriage (all visits). At these appointments a full history was taken, relevant examinations performed and stool cultures obtained. Blood or bone marrow cultures were only obtained if clinical symptoms were indicative of acute infection. If patients did not attend their follow up appointment, they were reminded by letter or a member of the study team visited their home. If stool samples were not available, a rectal swab was obtained.

Patients with convalescent stool carriage of *S. Typhi* or *S. Paratyphi A* were retreated according to the sensitivity of the isolate and were further followed up. Ultrasound was performed to exclude biliary or kidney stones if carriage was persistent.

2.2.6 Microbiology

Five to 8 mL of blood was collected from adults and inoculated into Bactec Plus Aerobic Blood bottles, and 3 to 5 mL of blood from children was inoculated into Bactec Peds Plus culture bottles (Becton Dickinson, New Jersey, USA). The bottles were incubated at 37°C in the BACTEC 9050 automated analyser for 7 days and sub-cultured according to standard methods when the machine indicated a positive signal, or incubated at 37°C in a standard laboratory incubator (An Giang hospital) and examined daily.

Stool samples or rectal swabs were inoculated onto MacConkey agar and Xylose Lysine Decarboxylase (XLD) agar plates, and in 10 mL of selenite F broth. Plates and broth were incubated at 37°C overnight and the broth was sub-cultured on MacConkey and XLD agar plates the next morning.

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 antiserum (Murex, Dartford, UK).

Antimicrobial susceptibility testing was performed by disc diffusion according to Clinical Laboratory Standards Institute (CLSI) guidelines (CLSI, 2006), using CLSI breakpoints (CLSI, 2007). Antimicrobial agents tested were: ampicillin, chloramphenicol, trimethoprim-sulfamethoxazol, nalidixic acid, ofloxacin, ciprofloxacin and ceftriaxone (Oxoid, Basingstoke, UK). MICs for amoxicillin, chloramphenicol, nalidixic acid, ofloxacin, ciprofloxacin, gatifloxacin, ceftriaxone

and azithromycin were determined by E-test (AB Biodisk, Solna, Sweden). Multidrug resistance (MDR) of isolates was defined as resistance to chloramphenicol (MIC \geq 32 $\mu\text{g}/\text{mL}$), ampicillin (MIC \geq 32 $\mu\text{g}/\text{mL}$) and trimethoprim-sulfamethoxazole (MIC \geq 8/152 $\mu\text{g}/\text{mL}$). Nalidixic acid resistance was defined as an MIC \geq 32 $\mu\text{g}/\text{mL}$. The CLSI breakpoints for ofloxacin and gatifloxacin were \leq 2 $\mu\text{g}/\text{mL}$ susceptible and \geq 8 $\mu\text{g}/\text{mL}$ resistant, for ciprofloxacin \leq 1 $\mu\text{g}/\text{mL}$ susceptible and \geq 4 $\mu\text{g}/\text{mL}$ resistant and for ceftriaxone \leq 8 $\mu\text{g}/\text{mL}$ susceptible and \geq 64 $\mu\text{g}/\text{mL}$ resistant. There were no CLSI MIC breakpoints for azithromycin (CLSI, 2007). The control strains used for all susceptibility tests were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213.

All cultures, identification of *S. Typhi* and *S. Paratyphi A* and disc diffusion were performed at the three study sites. All isolates were sent to the Hospital for Tropical Diseases, Ho Chi Minh City, for confirmation of identity, susceptibility testing and MIC testing.

2.2.7 Outcomes of the study

The primary endpoint of the study was the resolution of fever (fever clearance time, FCT), which was defined as the time from the start of the antibiotic treatment to when the axillary temperature first fell \leq 37.5°C and remained there for at least 48 hours. Secondary endpoints were the overall failure to treatment, which was defined a priori as any of the following: clinical failure (persistence of fever and symptoms two days after the end of treatment, i.e. on day 10) or need for re-treatment due to insufficient

treatment response as judged by the treating physician; microbiological failure (positive blood culture on day 7 to 9 after the start of treatment); the development of typhoid fever-related complications during hospital-stay; the occurrence of relapse (symptoms and signs suggestive of typhoid fever) within 1 month after completion of treatment or the detection of faecal carriage of *S. Typhi* at the follow-up visits at 1, 3 and 6 months (to exclude faecal carriage a minimum of two consecutive follow-up visits had to be attended).

2.2.8 Sample Size

The primary outcome measure for the study was the fever clearance time (FCT).

Previous studies that used azithromycin to treat typhoid fever patients, reported a mean fever clearance time of 130 hours (Chinh *et al.*, 2000) and 139 hours (Parry *et al.*, 2007). For gatifloxacin, clinical observations from a small number of typhoid fever patients were available and indicated a mean FCT of 76 hours. We calculated that 139 patients with culture confirmed typhoid fever would be needed in each treatment arm to detect a Hazard Ratio of 1.40 with two-sided alpha of 0.05 and power of 0.80 (Machin *et al.*, 1997). Therefore, assuming a median fever clearance time of 130 hours for azithromycin, the sample size of 140 patients with culture-confirmed typhoid fever in each arm would give power of at least 0.80 to detect a difference between treatments if the fever clearance time in the gatifloxacin group was 92 hours or less.

2.2.9 Randomisation

An administrator independent from the study generated the random number sequence in Excel using RAND function. These randomised codes were blocked in a size of 50. Treatment assignments were folded and kept in opaque, sealed, sequentially numbered envelopes at all three study sites. Due to logistic reasons randomisation was not stratified by centre.

After all inclusion and exclusion criteria were checked, and informed consent given, the study doctor opened the envelope to determine which treatment the subject would receive. The sealed envelopes were opened in strict numeric sequence. This study was conducted as an open study.

2.2.10 Statistical methods

Binary outcomes (clinical failure, microbiological failure, typhoid fever-related complications) were compared between the two treatment groups using Fisher's exact test, assuming the worst case scenario (all lost to follow up treated as failures). The un-adjusted Odds Ratio (OR) and Cornfield's 95% Confidence Interval (Breslow and Day, 1980) were calculated to show the relative risk of developing individual secondary outcomes (clinical, microbiological failure, typhoid fever-related complications) in the gatifloxacin group compared to the azithromycin group.

Fever clearance time, time to relapse and time to overall failure were analysed using survival methods. The time to overall failure equaled the earliest time individual failure was recorded. Kaplan-Meier estimates of probabilities of each event were

calculated at any time-point, and they were compared between the two treatment groups using the log-rank test. Data of patients who were lost to follow-up were censored at the time of the last recorded outcome. The Hazard Ratio was derived from Cox proportional hazard model (Collett, 2003).

All patients with positive blood or bone marrow culture for *S. Typhi* and *S. Paratyphi A* (per protocol analysis) and separately all randomised patients (intention to treat analysis) were analysed.

All data were recorded prospectively into individual Case Record Forms (CRF) and entered into an electronic database (Epi Info 2003, CDC, Atlanta, USA) and double-checked.

Analysis was performed using STATA version 8.0 (Stata Corporation, Texas, USA) statistical software program.

2.3 Results

2.3.1 Participant flow and recruitment

During the study period, 460 patients were assessed for eligibility (Figure 2.2). One hundred and two patients were ineligible; the main reason was the reported previous use of fluoroquinolone, macrolide or third generation cephalosporin antibiotics (41 patients) in the week before hospitalisation.

Between April 2004 and August 2005, 358 patients with suspected typhoid fever were randomised to receive either gatifloxacin or azithromycin. Two hundred eighty-eight

of these patients had blood or bone marrow confirmed typhoid fever and 70 patients were culture negative for *S. Typhi*. One culture positive patient was excluded from the per protocol analysis (PP), because he had received ciprofloxacin before entry to the trial. The PP group consisted of 287 patients, 145 in the gatifloxacin group and 142 in the azithromycin group. All PP patients, except two in the azithromycin group, finished the full course of treatment.

The total number of patients visiting the follow-up at 1 month was 275 out of 287 (96%), at 3 months 268 out of 287 (93%), at 6 months 128 out of 287 (44%) patients.

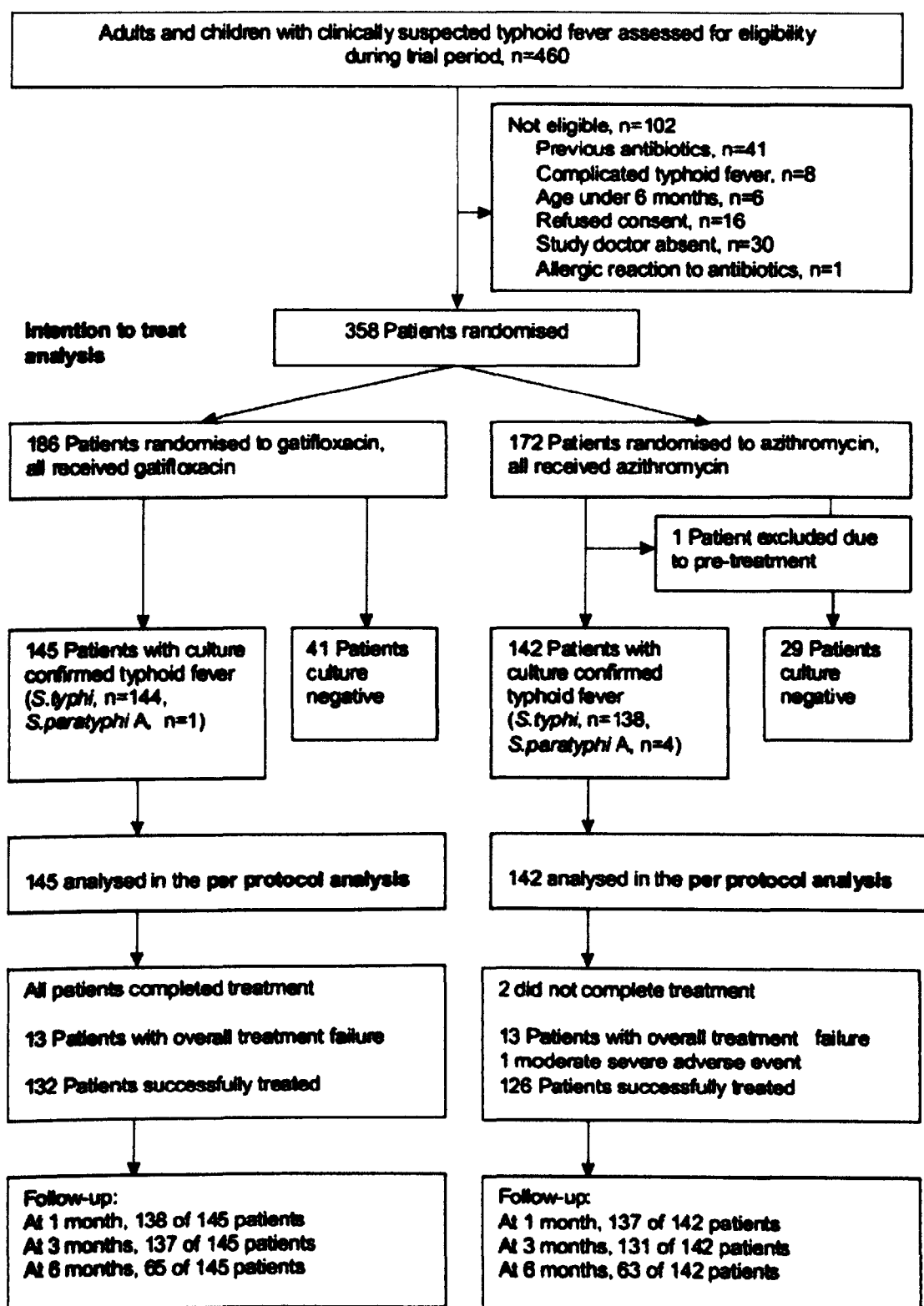


Figure 2.2. Profile of the trial

All 358 randomised patients were analysed in the intention to treat (ITT) analysis. Two hundred and eighty-seven patients with culture confirmed typhoid fever, 145 treated with gatifloxacin and 142 with azithromycin, were analysed in the pre-specified PP analysis.

2.3.2 Baseline Data

The median age of patients recruited in this trial was 11 years (range 1-41) in the PP group.

The baseline characteristics of the patients were similar in the two treatment groups and in the culture negative patients (Table 2-1)

Patients with suspected and blood culture confirmed typhoid fever were eligible for this trial. In the PP group, the median delay in time between hospital admission and randomisation was 3 days (interquartile range 1-4) in the gatifloxacin group and 3 days (interquartile range 2-4) in the azithromycin group. In the ITT group, the median delay in time between hospital admission and randomisation was 2 days (interquartile range 0-4) in the gatifloxacin group and 3 days (interquartile range 1-4) in the azithromycin group.

Characteristics	Culture confirmed patients treated with		Blood culture negative patients, n=70
	Gatifloxacin, n=145	Azithromycin, n=142	
Median age in years (range)	11 (2-30)	11 (1-41)	9 (2-42)
Number of children defined as age under 15 (%)	109 (75.2)	101 (71.1)	56 (80)
Number of males (%)	71 (49)	76 (53.5)	29 (41)
Median weight in kilograms (range)	25 (8.5-55)	24.5 (9.5-57)	19.5 (10.5-53)
Median duration of fever before admission in days (range)	7 (2-30)	7 (2-30)	7 (3-30)
Number of patients who received pretreatment (%)	21 (14.5)	18 (12.7)	16 (22.9)
Median temperature at admission in °C (range)	39 (37-40.5)	39 (37.3-41)	38.75 (37-40)
Hepatomegaly, number (%)	69 (47.6)	63 (44.4)	36 (51.4)
Splenomegaly, number (%)	17 (11.7)	14 (9.8)	2 (2.9)
Abdominal pain, number (%)	82 (56.5)	76 (53.5)	43 (61.4)
Weight loss, numbers (%)	69 (47.6)	71 (50)	21 (30)
Vomiting, number (%)	47 (32.4)	54 (38)	19 (27.1)
Diarrhoea, number (%)	95 (65.5)	82 (57.7)	49 (70)
Mild jaundice, number (%)	12 (8.3)	20 (14.1)	1 (1.4)
Median haematocrit in % (range)	34.3 (19.2-54.3)	34.6 (20.7-60.5)	34.2 (24.6-46.7)
Median white cell count, 10 ⁹ /L (range)	6.9 (2-17.2)	7.05 (2.4-16.8)	7.25 (2.8-11.7)
Median platelet count, 10 ⁹ /L (range)	172 (34-500)	172.5 (45-578)	208 (51-496)
Median AST, U/L(range)	85 (16.9-773)	72 (17.6-1190)	50.1 (11-533)
Median ALT, U/L (range)	67.4 (10.3-276)	59.4 (10.2-734)	44.1 (10-375)
Numbers of <i>S.Typhi</i> / <i>S.Paratyphi A</i> isolated from culture	144/1	138/4	0
Positive pretreatment faecal cultures, numbers (%)	11/124 (8.9)	6/118 (5.1)	0

AST, Serum Aspartate Aminotransferase AST (normal range, 12-30 U/L)
ALT, Serum Alanine Aminotransferase ALT (normal range, 13-40 U/L)

Table 2-1. Baseline characteristics of culture confirmed (PP analysis) and culture negative typhoid fever patients

2.3.3 Protocol deviations

At one study site, the An Giang Provincial Hospital, the follow-up visit at 6 months was not possible due to logistic reasons. It was therefore agreed to carry out two follow-up visits at 1 and 3 months and to schedule additional (cross-sectional) follow-up dates to invite as many patients as possible to a third follow-up visit. From the PP population, 22 out of 91 patients in the gatifloxacin arm and 17 out of 87 patients in the azithromycin arm attended the third visit.

2.3.4 Primary outcomes

There was no significant difference in the resolution of fever (FCT) between the two treatment groups (Table 2-2).

Outcome Type	Outcomes	Sub-Categories	Treatment group (n=287)		p-Value
			Gatifloxacin n=145	Azithromycin n=142	
Primary	Fever Clearance Time in hours (95% CI)		106 (94-118)	106 (88-112)	0.984 [^]
Secondary	Overall Treatment Failure, numbers of patients (%)		13/145 (9)	13/140 (9.3)*	0.854 [^]
		Did not complete full treatment course, n (%)	0	2	
		Clinical failure, n (%)	6/145 (4.3)	6/140 (4.2)	1.000 [#]
		Microbiological failure, n (%)	2/145 (1.4)	3/140 (2.2)	0.680 [#]
		Typhoid-fever related complications, n (%)	0/145 (0)	8/140 (5.7)	0.003 [#]
		Gastrointestinal bleeding	0	4	
		Pneumonia	0	2	
		Liver dysfunction	0	2	
		Relapse after discharge from hospital, n (%)	4/137 (2.9)	0/127 (0)	0.052 [^]
		Number of patients with faecal carriage ^{&} (%)	1/137 (0.7)	0/131 (0)	

Patients can fail in more than one subcategory. *In the worst case scenario: 15/142 (10.6%) showed overall treatment failure in the azithromycin group, p = 0.570. [^]The p value is based on the logrank test. [#]The p value is based on Fisher's exact test. [&]Evaluated in patients who attended at least two follow up visits.

Table 2-2. Primary and secondary outcomes of culture confirmed patients (PP analysis)

By PP analysis, the median FCT was 106 hours in both treatment arms (95% Confidence Interval [CI]; 94 to 118 hours for gatifloxacin *versus* 88 to 112 hours for azithromycin), (logrank test $p = 0.984$, HR = 1.0; 95% CI 0.80 to 1.26). The Kaplan-Meier survival curve for the fever clearance time is shown in Figure 2.3. At day 7, fever clearance rate was 82.8% (95% CI; 76.2% to 88.4%) in the gatifloxacin group and 80.5% (95% CI; 73.6% to 86.6%) in the azithromycin group.

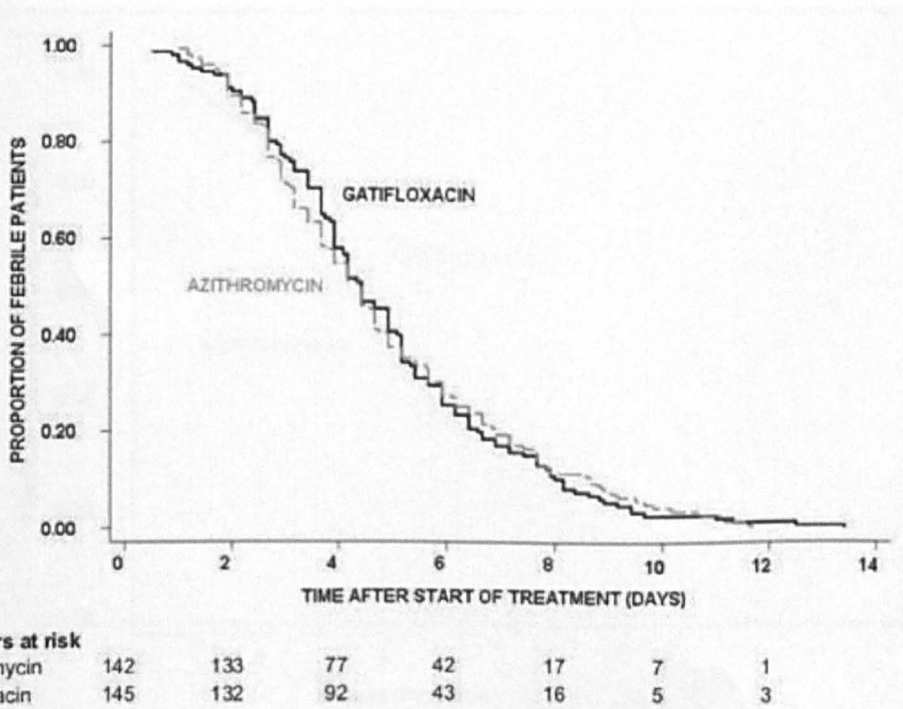


Figure 2.3 Proportion of culture confirmed patients still febrile.

Kaplan- Meier survival curve showing the proportion of culture confirmed patients (PP analysis) still febrile through time by treatment group.

In the ITT population, the median FCT was 100 hours in both treatment arms (95% CI; 92 to 106 hours for gatifloxacin versus 88 to 112 hours for azithromycin), (logrank test $p = 0.914$, HR = 1.01; 95% CI 0.82 to 1.25). At day 7, fever clearance rate was 84.2% (95% CI; 78.5% to 89%) in the gatifloxacin group and 82.6% (95% CI; 76.5% to 87.9%) in the azithromycin group (Figure 2.4).

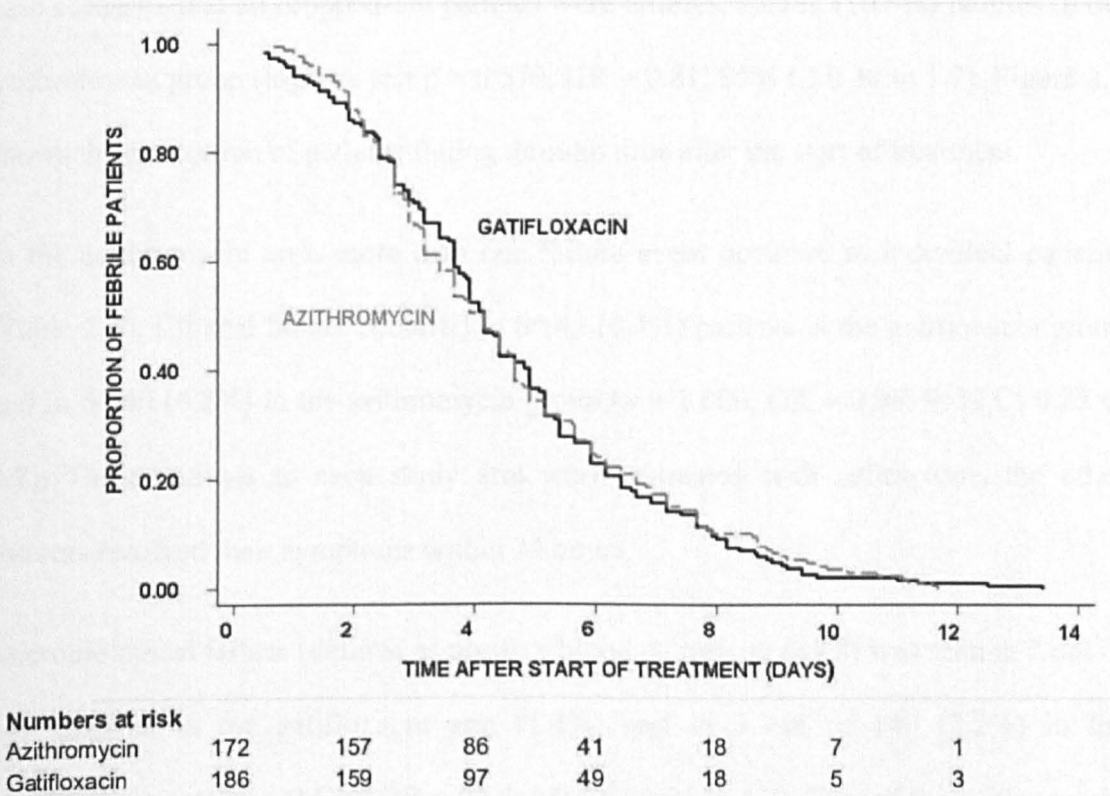


Figure 2.4. Proportion of all randomised patients still febrile.

Kaplan-Meier survival curve showing the proportion of all randomised patients (ITT analysis) still febrile through time by treatment group.

2.4 Secondary outcomes

There was no death in the study. There was no significant difference in overall failure to treatment between the two groups (Table 2-2).

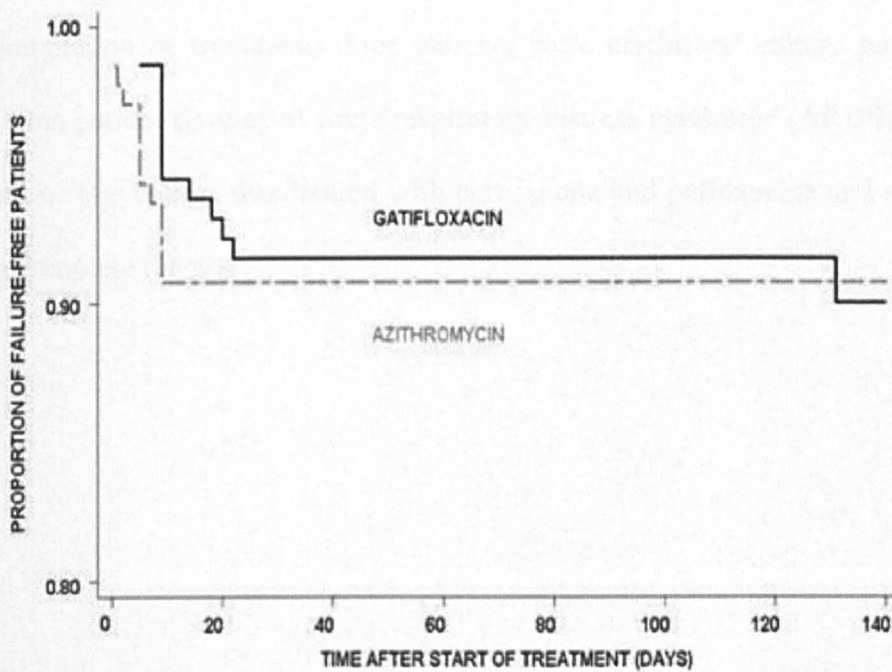
By PP analysis, the number of patients that showed overall failure to treatment was 13/145 (9%) in the gatifloxacin group and 13/140 (9.3%) in the azithromycin group (logrank test $p = 0.854$, HR = 0.93; 95% CI 0.43 to 2.0), or when assuming the worst case scenario, that all dropped-out patients were failures, 15/142 (10.6%) failures in the azithromycin group (logrank test $p = 0.570$, HR = 0.81; 95% CI 0.38 to 1.7). Figure 2.5 shows the proportion of patients failing through time after the start of treatment.

In the azithromycin arm, more than one failure event occurred in individual patients (Table 2-2). Clinical failure occurred in 6/145 (4.3%) patients in the gatifloxacin group and in 6/140 (4.2%) in the azithromycin group ($p = 1.000$, OR = 0.96; 95% CI 0.25 to 3.7). Three patients in each study arm were re-treated with ceftriaxone, the other patients resolved their symptoms within 24 hours.

Microbiological failure (defined as positive blood culture on day 8) was seen in 2 out of 145 patients in the gatifloxacin arm (1.4%) and in 3 out of 140 (2.2%) in the azithromycin arm ($p = 0.680$, OR = 0.64; 95% CI 0.05 to 5.7). Two of the azithromycin recipients showed additionally signs of clinical failure.

There were no typhoid fever-related complications in the 145 gatifloxacin patients compared to 8 out of 140 (5.7%) patients in the azithromycin arm ($p = 0.003$, OR = 0; 95% CI 0 to 0.4). Two azithromycin recipients developed signs of liver dysfunction

(elevated AST and ALT, deepening of jaundice) in addition to signs of clinical failure. Study treatment was continued and symptoms resolved by the time of discharge. Four patients, three children and one adult, suffered from gastrointestinal bleeding on day 3, day 5 (2 cases) and day 7 of treatment respectively, three patients received blood transfusions. One of these patients developed shock but responded to intravenous fluids and supportive treatment. Treatment was discontinued immediately in all the patients and re-treatment with ceftriaxone was initiated. Two adult patients developed pneumonia during treatment.



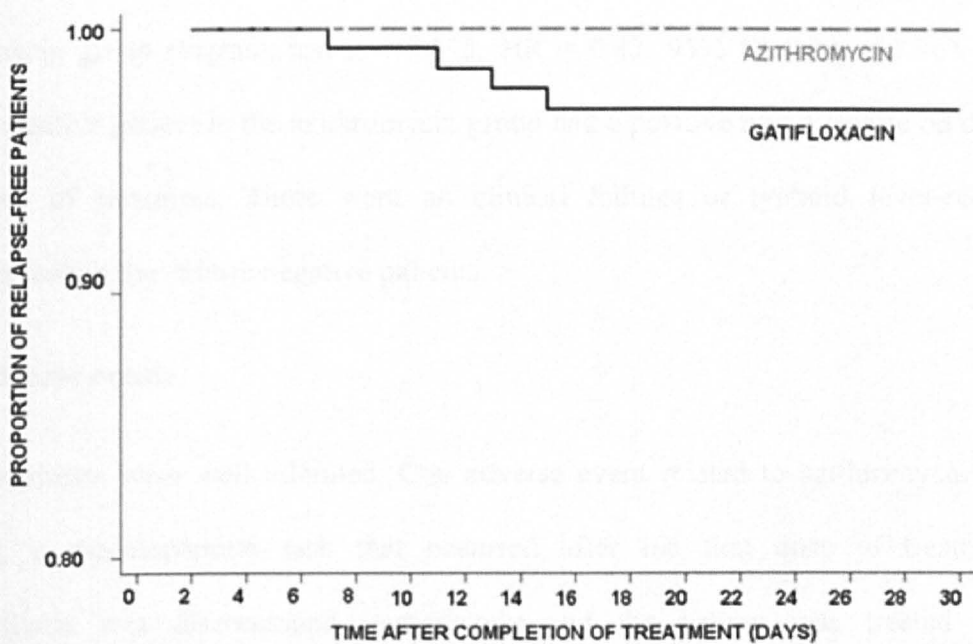
Numbers at risk								
Azithromycin	142	124	121	119	118	51	49	47
Gatifloxacin	145	130	128	128	127	60	58	52

Figure 2.5 Proportion of patients with overall failure in the culture confirmed population.

Kaplan-Meier survival curve showing the proportion of patients with overall failure in the culture confirmed population (PP analysis) by treatment group.

Relapse was evaluated only in patients that were initially categorised as successfully treated, patients with clinical failure, microbiological failure or complications were not evaluated. Four patients out of 137 (2.9%) relapsed in the gatifloxacin group compared to none out of 127 in the azithromycin group (logrank test $p = 0.052$, HR = not

estimable due to zero observations in one group), (Figure 2.6). These relapses with symptoms suggestive of typhoid fever occurred on day 7, 11, 13 and 15 respectively, after completion of treatment, three patients were confirmed culture positive for *S. Typhi*. One patient developed acute respiratory distress syndrome (ARDS) and needed ventilation. The patient was treated with ceftriaxone and pefloxacin and subsequently made a complete recovery.



Numbers at risk

Azithromycin	127	125	123	122	122	121	110	52
Gatifloxacin	137	134	129	128	126	126	115	57

Figure 2.6 Proportion of patients with relapse in the culture confirmed population.

Kaplan-Meier survival curve showing the proportion of patients with relapse in the culture confirmed population (PP analysis) by treatment group.

Chronic faecal carriage was evaluated in patients who attended at least two follow-up appointments, 137 in the gatifloxacin group and 131 in the azithromycin group. Only one patient with chronic faecal carriage was detected after 6 months (An Giang study site), the patient had received gatifloxacin.

In the ITT analysis (all 358 randomised patients), overall treatment failure was reported in 13 out of 185 (7%) in the gatifloxacin group compared to 14 out of 168 (8.4%) in the azithromycin group (logrank test $p = 0.615$, HR = 0.82; 95% CI 0.39 to 1.76). One culture negative patient in the azithromycin group had a positive blood culture on day 7 after start of treatment. There were no clinical failures or typhoid fever-related complications in the culture negative patients.

2.4.1 Adverse events

Both treatments were well tolerated. One adverse event related to azithromycin was reported, a maculopapular rash that occurred after the first dose of treatment. Azithromycin was discontinued immediately and the patient was treated with ceftriaxone.

Gastrointestinal side effects (change in consistency and frequency of stools) that were probably typhoid fever related were relatively frequent in both treatment arms at the start of treatment. In the gatifloxacin group, one patient experienced vomiting on day 2 and day 3 and one patient experienced diarrhoea (4 episodes/ day) on day 4 and day 5 of treatment. These episodes were self-limiting and did not require the interruption of therapy.

The median levels of serum AST and ALT fell in both groups after 7 days of therapy. In the PP group, the median post-treatment AST was 46.4 U/L (range 12.8 – 217.5) in the gatifloxacin arm and 45 U/L (range 5 – 358) in the azithromycin arm. The median post-treatment ALT fell to 46.8 U/L (range 7.4 – 278) and 49.9 (1.1 – 494),

respectively. In the culture-negative patients, the median post-treatment AST was 44.8 U/L (range 12 – 654) and ALT was 40 U/L (range 10 – 424.4).

2.4.2 Antimicrobial susceptibilities of *S. Typhi* and *S. Paratyphi A* isolates

From the PP population, 282 (98%) *S. Typhi* and 5 (2%) *S. Paratyphi A* strains were isolated. Two hundred and sixty three *S. Typhi* and five *S. Paratyphi A* were received at the Hospital for Tropical Diseases for antimicrobial susceptibility testing.

Fifty-eight percent of the *S. Typhi* isolates were MDR and 96% were nalidixic acid resistant and showed reduced susceptibility to the older generation fluoroquinolones (Table 2-3). However technically, using current CLSI breakpoints, all isolates remained susceptible in vitro to ciprofloxacin and ofloxacin. The MIC₉₀ of gatifloxacin was the lowest of all the fluoroquinolones tested at 0.19 µg/mL (range 0.004 - 0.5). All isolates were susceptible to ceftriaxone.

The 5 *S. Paratyphi A* strains were fully susceptible to all the antimicrobials tested.

		Treatment with		
		All isolates n = 263	Gatifloxacin n = 137	Azithromycin n = 126
Multidrug resistant, numbers (%)		153 (57)	87 (63)	66 (50.8)
Nalidixic acid resistant, numbers (%)		254 (94.8)	132 (95.6)	121 (93)
Amoxicillin (µg/ml)	MIC 50	>256	>256	>256
	MIC 90	>256	>256	>256
	range	0.125 to >256	0.5 to >256	0.125 to >256
Chloramphenicol (µg/ml)	MIC 50	>256	>256	>256
	MIC 90	>256	>256	>256
	range	0.38 to >256	2 to >256	0.38 to >256
Nalidixic acid (µg/ml)	MIC 50	>256	>256	>256
	MIC 90	>256	>256	>256
	range	1.5 to >256	1.5 to >256	1.5 to >256
Ofloxacin (µg/ml)	MIC 50	0.75	0,75	1
	MIC 90	1,5	1,5	1,5
	range	0.23-2	0.32-2	0.23-2
Ciprofloxacin (µg/ml)	MIC 50	0.38	0.38	0.38
	MIC 90	0.5	0.5	0.5
	range	0.004-0.75	0.006-0.75	0.004-0.38
Gatifloxacin (µg/ml)	MIC 50	0.125	0.125	0.125
	MIC 90	0.19	0.19	0.19
	range	0.004-0.5	0.006-0.25	0.004-0.5
Ceftriaxone (µg/ml)	MIC 50	0.125	0.125	0.125
	MIC 90	0.125	0.125	0.19
	range	0.064-0.25	0.064-0.19	0.064-0.25
Azithromycin (µg/ml)	MIC 50	8	8	8
	MIC 90	12	12	12
	range	1.5-16	1.5-16	4-16

Table 2-3 Antimicrobial susceptibilities and MICs of 263 *S. Typhi* isolates.

MIC_{50/90}, at which 50% and 90% of the organisms are inhibited respectively. MDR is defined as resistance to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole. CLSI MIC breakpoints are as follows: for chloramphenicol, ampicillin and nalidixic acid resistance $\geq 32 \mu\text{g/mL}$; ofloxacin and gatifloxacin $\leq 2 \mu\text{g/mL}$ susceptible and $\geq 8 \mu\text{g/mL}$ resistant; ciprofloxacin $\leq 1 \mu\text{g/mL}$ susceptible and \geq

4 µg/mL resistant; ceftriaxone ≤ 8 µg/mL susceptible and ≥ 64 µg/mL resistant; there are none for azithromycin.

2.5 Discussion

The results of this trial show that both antibiotics worked well for the treatment of MDR and nalidixic acid resistant typhoid fever in Vietnam. A seven day oral course of gatifloxacin had similar efficacy and safety as a seven day course of azithromycin, which is recommended for the treatment of MDR and nalidixic acid resistant typhoid fever (Chinh *et al.*, 2000; World Health Organization, 2003).

However, azithromycin is not available throughout much of the developing world and it is expensive. The costs of a 7-day treatment course of gatifloxacin (at 10 mg/kg per day) for an adult patient in Vietnam were approximately 25 US\$, the costs of azithromycin (at 20 mg/kg per day) were more than 90 US\$.

The results for gatifloxacin in this trial are comparable to the excellent clinical outcomes achieved with ofloxacin in Vietnam in the early 1990s, when *S. Typhi* isolates were still nalidixic acid susceptible (Cao *et al.*, 1999; Tran *et al.*, 1995; Vinh *et al.*, 1996).

Gatifloxacin has a higher affinity to GyrA and is less inhibited by the common mutations in the *gyrA* gene (Lu *et al.*, 1999). The gatifloxacin MIC₅₀ of the study isolates was 0.125 µg/mL compared to the ofloxacin MIC₅₀ of 0.75 µg/mL. We would not recommend the continued use of the older generation fluoroquinolones (ofloxacin and ciprofloxacin) in regions with high rates of nalidixic acid resistant typhoid fever for

fear of selecting further mutations in *gyrA* (Tam *et al.*, 2007). This could put at risk the potential clinical benefit of the newer fluoroquinolones, including gatifloxacin.

There have been several case reports of gatifloxacin-associated dysglycaemia in patients with type II diabetes mellitus, overweight or with other comorbidity (Ambrose *et al.*, 2003; Baker and Hangii, 2002; Frothingham, 2005). There have been concerns about the use of gatifloxacin, after a retrospective case-control study in 1.4 million individuals over the age of 66 years (mean age 77 years) in Canada was published ((Park-Wyllie *et al.*, 2006). Subsequently, the US FDA required a 'Black Box' warning to be added to the package insert information. Later, "Tequin" (gatifloxacin) was voluntarily withdrawn in the US, Canada and Japan.

However, gatifloxacin remains approved in many countries where enteric fever is endemic and drug resistant strains are present, including India, Vietnam, Nepal, Bangladesh and China. It is also still available as an ophthalmic solution for eye infections in Canada and the US (Olliaro P., 2011).

As our trial was completed before publication of this report (Park-Wyllie *et al.*, 2006), we did not systematically monitor for hypo- and hyperglycaemia. Blood glucose levels taken as part of the routine care were normal. All patients were managed as in-patients and potential symptoms of hypo- and hyperglycaemia would have been noted by the study physicians. No dysglycaemia events were reported during the in-patient period or during the follow up period of 3 to 6 months.

The patients in our trial were healthy, young and non-obese individuals. In our setting and in our patient population gatifloxacin was highly effective despite very high rates of drug resistance and was well tolerated.

A trial in 867 children with otitis media with glucose monitoring and a one year follow-up (Pichichero *et al.*, 2005), as well as two recent enteric (typhoid and paratyphoid) fever trial in Nepal used gatifloxacin and did not report any clinically relevant dysglycaemias (Arjyal *et al.*, 2011; Pandit *et al.*, 2007). Other newer generation fluoroquinolones, i.e. gemifloxacin and moxifloxacin have shown low MICs for nalidixic acid resistant *S. Typhi* and *S. Paratyphi A* (Maskey *et al.*, 2006), unfortunately these drugs are not available in Vietnam and they are considerably more expensive. The *in vitro* results seen with these other newer generation fluoroquinolones should be evaluated in clinical trials.

The emergence of nalidixic acid resistant *S. Typhi* and *S. Paratyphi A* with reduced susceptibility to the fluoroquinolones is a widespread problem throughout Asia and therefore our study is relevant to the whole region (Bhan *et al.*, 2005; Chau *et al.*, 2007). Many case reports and some randomised controlled trials have described the worsening clinical response to ciprofloxacin and ofloxacin (Aarestrup *et al.*, 2003; Kadiravan *et al.*, 2005; Parry *et al.*, 2007).

The search for effective antibiotics to treat typhoid fever is imperative.

Typically trials in typhoid fever are limited by small sample sizes. A recent Cochrane review stressed the need for large well-designed trials in enteric fever (Thaver *et al.*,

2005). The evidence from our trial is strengthened by a sample size of 287 patients with culture confirmed typhoid fever (358 patients randomised). Both antibiotics also worked well for the patients with negative blood cultures. This is an important finding because the sensitivity of blood culture for the diagnosis of typhoid fever is only approximately 50 to 80% (World Health Organization, 2003).

One possible limitation of our trial was the low rate of stool cultures positive for *S. Typhi*. Faecal carriage is usually characterised by intermittent shedding and the stool culture for *S. Typhi* is not very sensitive. When comparing our data with other studies that demonstrate that azithromycin is highly efficacious for the treatment of typhoid fever, we find similar low rates of faecal carriage at follow-up (Chinh *et al.*, 2000; Frenck *et al.*, 2004). It could be hypothesized that antibiotics that show high intracellular concentrations and good tissue penetration like azithromycin and the fluoroquinolones, achieve rapid bacterial killing and elimination throughout the body, which reduces faecal carriage.

The dose of gatifloxacin and azithromycin tablets was prepared by careful cutting of the tablets (proportions of the tablets administered were recorded in the CRFs). Inevitably, it was therefore an estimation of the exact dose. Hence we cannot guarantee that each patient received exactly 10 mg/kg/day of gatifloxacin or 20 mg/kg/day of azithromycin.

A MEDLINE search for “azithromycin, clinical trial, typhoid/enteric fever” and the recent enteric fever Cochrane review (Thaver *et al.*, 2008) identified 6 clinical trials in the literature. In total, 251 typhoid fever patients were treated with azithromycin.

Four trials, three from Egypt and one from India, used azithromycin to treat MDR typhoid fever (Butler *et al.*, 1999; Frenck *et al.*, 2000; Frenck *et al.*, 2004; Girgis *et al.*, 1999). Azithromycin achieved cure rates between 88% and 100%, the mean FCT ranged from 3.8 to 4.5 days. Two trials performed in Vietnam used azithromycin at 20 mg/kg/day (Chinh *et al.*, 2000) and at 10 mg/kg/day (Parry *et al.*, 2007) for the treatment of MDR and nalidixic acid resistant typhoid fever. In total, 107 patients with culture confirmed typhoid fever were enrolled. The cure rate was 93% and 82% and the FCT was 5.6 and 5.8 days, respectively. Our results concur with these excellent data.

Two recently completed trials conducted in Kathmandu, Nepal also used gatifloxacin at the same dose and duration for the treatment of nalidixic acid resistant typhoid and paratyphoid fever (Arjyal *et al.*, 2011; Pandit *et al.*, 2007). Both trials reported excellent results for patients treated with gatifloxacin. The first trial compared the efficacy of gatifloxacin *versus* cefixime (20 mg/kg per day given in two divided doses for 7 days) (Pandit *et al.*, 2007). Successful treatment in the gatifloxacin group was achieved in 96.5% (85 out of 88) patients and the median FCT (95% CI) was 92 hours (84-114 hours). This trial was stopped early by the independent Data and Safety Monitoring Committee as a result of the poor clinical response in the patients randomised to cefixime. In the cefixime group the overall failure rate was 37.6 % (26 out of 70 patients) and the median FCT was 138 hours.

The second trial compared the efficacy of gatifloxacin *versus* chloramphenicol (75 mg/kg per day in four divided doses for 14 days) (Arjyal *et al.*, 2011). Treatment failure occurred in 12 out of 177 patients (6.8%) in the gatifloxacin arm compared to 14 out of

175 (8%) in the chloramphenicol arm (HR 0·86, 95% CI 0·40 to 1·86; $p = 0·70$). The median FCT was 3·9 days in both treatment groups.

I believe on the basis of this and other recently published trials, that gatifloxacin or azithromycin are now the treatments of choice for enteric fever in areas of MDR and nalidixic acid resistance (Arjyal *et al.*, 2011; Chinh *et al.*, 2000; Pandit *et al.*, 2007; Parry *et al.*, 2007). However it is important to use these antimicrobial agents sparingly (i.e. at the recommended dose and duration) because indiscriminate use would inevitably encourage further drug resistance.

Chapter 3

Population Pharmacodynamics and Population Pharmacokinetics of Gatifloxacin in Patients with Typhoid Fever

3.1 Introduction

3.1.1 Pharmacokinetics

Pharmacokinetics (PK) describes the changes of drug concentrations over time in the body. The concentration *versus* time is a result of the processes of adsorption, distribution, metabolism and elimination.

The appropriate use of antimicrobial agents requires an understanding of the characteristics of the drug, host factors and the pathogen. Pharmacokinetic studies describe parameters such as the peak serum concentration C_{\max} , the serum half life $t_{1/2}$ and the cumulative exposure to an agent by the area under the concentration time curve (AUC) for a 24 hour period (McKinnon and Davis, 2004). Severe disease and sepsis can significantly alter the pharmacokinetics of drugs, especially distribution and elimination.

3.1.2 Pharmacodynamics

Pharmacodynamics (PD) describes the relationship between the drug exposure in serum, tissues and body fluids and the pharmacological and toxicological effects of the drugs (Craig, 2007). For antimicrobials, the success of a given drug dose depends on a measure of drug exposure (such as the area under the concentration-time curve, the serum peak concentration and the duration of time the serum concentrations exceed a certain level) and a measure of the potency of the drug against the infecting organisms, e.g. the minimum inhibitory concentration (MIC) or the minimum bactericidal concentration (Craig, 2001). The relationship between drug exposure and MIC of the

pathogen has been shown to be predictive of microbiological eradication, this is often summarised as exposure-response relationship (Craig, 2007).

PK/PD indices that are used as surrogate markers for clinical and antimicrobial efficacy are the ratio of peak plasma concentration (C_{max}) of the antimicrobial to MIC of the pathogen (C_{max}/MIC), the ratio of the area under the concentration/time curve 0 to 24 hours to the MIC ($AUC_{0-24}:MIC$) and the time above MIC ($T>MIC$).

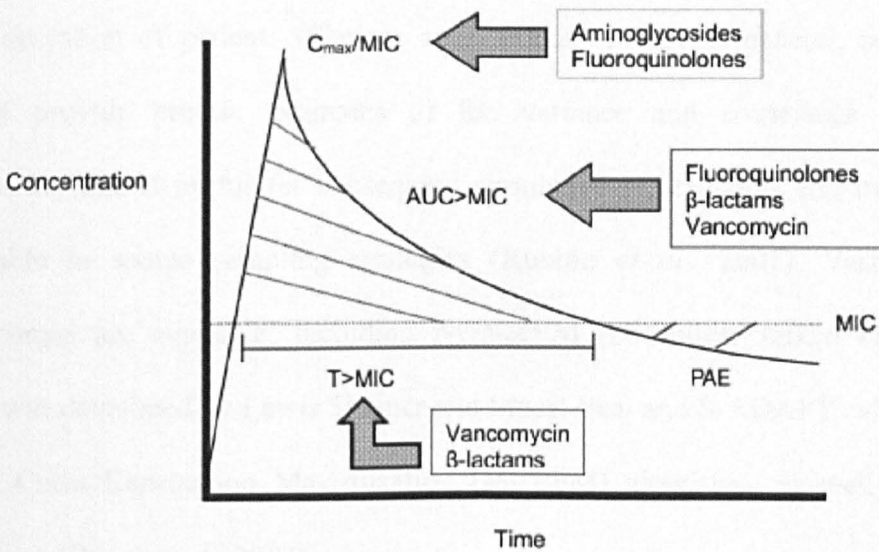


Figure 3.1 Concentration versus time with MIC superimposed and pharmacokinetic and pharmacodynamic markers.

From (McKinnon and Davis, 2004).

There is renewed interest in pharmacodynamic concepts as clinicians and scientists realise that our antimicrobial armamentarium is limited (Becker *et al.*, 2006) and only few new antibiotics are likely to be developed.

PD concepts can help to determine how to best use current available antibiotics and how to prevent antibiotic resistance. Mathematical modelling and pharmacodynamics have demonstrated that it is possible to delineate a drug exposure that would prevent the emergence of mutant subpopulations (Jumbe *et al.*, 2003).

3.1.3 Population pharmacokinetics and pharmacodynamics

Population pharmacokinetics and pharmacodynamics has been developed in the late 1980s and aims to quantify determinants of drug concentration (PK) or response (PD) in a population of patients (Sheiner and Ludden, 1992). In general, population PK models provide precise estimates of the variance and covariance between PK parameters, this is useful for subsequent simulation experiments and the models are applicable to sparse sampling strategies (Rubino *et al.*, 2007). Various software programmes are available, including NONMEM (non-linear mixed effects model) which was developed by Lewis Sheiner and Stuart Beal and S-ADAPT, which performs Monte Carlo Expectation Maximization (MCP-EM) algorithm, as well as Bayesian estimation (Bauer *et al.*, 2007).

3.1.4 Patterns of antimicrobial killing

Antimicrobials exhibit two primary patterns of microbial killing (Graig, 2007). The first pattern is characterised by concentration dependent killing which occurs over a wide range of concentrations and also shows moderate to prolonged persistent effects. Higher drug concentrations result in a greater rate and extent of microbial killing. This pattern

is observed with the fluoroquinolones, aminoglycosides, daptomycin, ketolides, metronidazole and amphotericin B.

The second pattern is the time dependent (or minimal concentration dependent) killing. The extent of the microbial killing is primarily dependent on the duration of the exposure. The killing rate is saturated at low multiples of the MIC, usually around four or five times the MIC. Higher drug concentrations do not kill microbes faster or more extensively. Time dependent killing with minimal to none persistent effects are observed with β -lactam antibiotics and flucytosine. Macrolides, clindamycin, glycopeptides, tetracyclines and linezolid exhibit a time dependent pattern with moderate to prolonged persistent effects that can prevent re-growth of bacteria during the dosing interval (Graig, 2007).

3.1.5 The pharmacokinetics and pharmacodynamics of gatifloxacin

Gatifloxacin (see Chapter 1) is a broad spectrum 8-methoxy fluoroquinolone with enhanced activity against Gram positive and Gram negative organisms, anaerobes and mycobacteria, which received U.S. Food and Drug Administration (FDA) approval in 1999. In clinical trials, gatifloxacin was shown to be effective in the treatment of acute respiratory infections, including community-acquired pneumonia, acute exacerbation of chronic bronchitis, sinusitis and urinary tract infections in adults and otitis media in children (Capparelli *et al.*, 2005).

Gatifloxacin is readily absorbed from the gastrointestinal tract, with an absolute bioavailability of approximately 96% (LaCreta *et al.*, 2000). Gatifloxacin has a large

volume of distribution (approximately 1.8 L/kg), low protein binding (approximately 20%), broad tissue distribution and is primarily (more than 80%) excreted unchanged in the urine (Grasela, 2000b; Nakashima *et al.*, 1995). It has an elimination half life of 8 to 10 hours, independently of the dose (Grasela, 2000b; Nakashima *et al.*, 1995).

In a single dose study, gatifloxacin concentrations in serum reached a peak between 1 and 2 hours and the peak concentrations were 0.87, 1.71, 3.35, and 5.41 µg/ml at the doses of 100, 200, 400, and 600 mg, respectively (Nakashima *et al.*, 1995).

The PK/PD profile of the fluoroquinolones has been well characterized (Ambrose *et al.*, 2007). For fluoroquinolones, the area under the concentration-time curve at 24 hours to MIC ratio ($AUC_{0-24}: MIC$) has correlated most strongly with efficacy in animal and *in vitro* models and in patients with a variety of diseases (Ambrose *et al.*, 2007). Studies by Forrest A. *et al.* evaluated intravenous ciprofloxacin for the treatment of pneumonia caused predominantly by Gram negative organisms and *Pseudomonas aeruginosa* in seriously ill patients (Forrest *et al.*, 1993). At an $AUC_{0-24}: MIC$ above 125 the probability of therapeutic response, defined as clinical and microbiological cure was 80%.

In patients with hospital acquired pneumonia treated with levofloxacin, patients in whom $AUC_{0-24}: MIC$ ratios ≥ 87 were attained had a 90% probability of a positive microbiological response, while those with lesser exposures had only a 43% favourable response to therapy ($p = 0.01$) (Drusano *et al.*, 2004).

In vitro gatifloxacin PK/PD data against *S. Typhi* suggest that similar AUC: MIC ratios (approximately 105) correlate with bacterial eradication (Booker *et al.*, 2005).

However, no clinical PK/PD data exist for infections involving *S. Typhi*. This chapter describes the pharmacokinetics and pharmacodynamics of gatifloxacin and is divided into two sections. The first section uses data from a randomised controlled trial of gatifloxacin *versus* azithromycin (described in Chapter 2) for the treatment of typhoid fever in Vietnam, to model the relationship between drug exposure (AUC₀₋₂₄: MIC) and therapeutic response in patients treated with gatifloxacin and to identify appropriate susceptibility breakpoints for gatifloxacin. The current CLSI breakpoints for Enterobacteriaceae (including *Salmonella*) for ofloxacin and gatifloxacin are ≤ 2 mg/L susceptible and ≥ 8 mg/L resistant, for ciprofloxacin ≤ 1 mg/L susceptible and ≥ 4 mg/L resistant (CLSI, 2007). The breakpoints for nalidixic acid are ≥ 32 mg/L (resistant) and ≤ 16 mg/L (susceptible). There has been discussion whether the current fluoroquinolone breakpoints are not too generous and therefore misleading clinicians (Aarestrup *et al.*, 2003; Crump *et al.*, 2003). The CLSI guidelines (2007) on the “Performance Standards for Antimicrobial Susceptibility testing” have responded to this criticism and recommend that extraintestinal *Salmonella* isolates should be screened for nalidixic acid resistance, and that in case of nalidixic acid resistance the physician should be informed that the isolate may not be eradicated by fluoroquinolone treatment (Institute, 2007).

However some of the newer fluoroquinolones do remain effective against nalidixic acid resistant *S. Typhi* infection and therefore this recommendation is leading clinicians

towards less effective treatments. Therefore a review of the breakpoints for *Salmonella* is necessary but prospective clinical and pharmacological data are lacking. We also determined the positive and negative predictive value of the nalidixic acid screening test for the clinical efficacy of gatifloxacin treatment in the same patient population.

The second section describes the pharmacokinetics of gatifloxacin in adult and paediatric patients with enteric fever in Nepal. Blood samples to measure gatifloxacin concentrations in Nepalese adults and children with enteric fever were obtained during a randomised trial of gatifloxacin *versus* chloramphenicol, described in the discussion of Chapter 2. The pharmacokinetics (PK) of gatifloxacin has been reported in healthy volunteers and in selected North American patient populations, who included adult patients with community-acquired respiratory tract infections and paediatric patients with acute or recurrent otitis media (Grasela, 2000a; Grasela *et al.*, 1998; Rubino *et al.*, 2007). However, the PK of gatifloxacin in adult and paediatric patients with enteric fever is unknown. Such data will be useful to construct exposure-response relationships for the efficacy of gatifloxacin in this patient population.

3.2 Materials and Methods

3.2.1 Section 1. Pharmacodynamics of gatifloxacin in adult and paediatric typhoid fever patients in Vietnam

3.2.1.1 Patients and procedures

Typhoid fever patients who were treated with an oral dose of 10 mg/kg/day of gatifloxacin (Tequin®, Bristol-Myers Squibb, USA; 400 mg/tablet) once daily for 7

days in a randomised clinical trial conducted in Vietnam were analysed (Trial registration: <http://controlled-trials.com/ISRCTN67946944>).

Patients, inclusion and exclusion criteria, procedures and outcomes have been described in detail in Chapter 2 of this thesis. In brief, favourable clinical response was defined as the resolution of fever and symptoms within 48 hours of the end of therapy (i. e. on day 10). Fever was evaluated every 6 hours. Relapse was defined as the recurrence of fever and symptoms and/or the isolation of *S. Typhi* from blood within 1 month after the completion of therapy. Patients were followed up at 1, 3 and 6 months, at these visits the recent history was obtained, physical examination carried out and stool cultures were performed to check for chronic faecal carriage of *S. Typhi*.

3.2.1.2 Antimicrobial susceptibility testing of S. Typhi

The antimicrobial susceptibility testing of *S. Typhi* has been described in chapter 2. Multidrug resistance was defined as resistance to ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole.

3.2.1.3 Drug exposure determination

For children under 16 years we used a previously validated population pharmacokinetic model to determine the drug exposure as expressed by the area under the concentration time curve at 24 hours (AUC_{0-24}) (Rubino *et al.*, 2007). The PK model used is a one-compartment model with first order absorption and elimination, and linear relationships between body surface area (BSA) and apparent oral clearance (CL/F ; where F is the oral bioavailability), and weight and apparent volume of distribution (V/F), respectively. We used patients' specific demographic and baseline information (age,

sex, weight, height and serum creatinine) to estimate individual patient drug exposures. Body surface area (BSA) was calculated according to the method of Gehan and George (Gehan and George, 1970).

$$\text{EQUATION 1: } \text{BSA (m}^2\text{)} = 0.0235 \times \text{Height (cm)}^{0.42246} \times \text{Weight (kg)}^{0.51456}$$

For patients below 16 years, clearance was estimated according to Rubino et al. (Rubino *et al.*, 2007).

$$\text{EQUATION 2: } \text{Clearance}_{(<16 \text{ years})} = 8.46 \cdot \text{body surface area}$$

For patients above 16 years of age, creatinine clearance (CL_{cr}) was estimated according to the Cockcroft-Gault formula (Cockcroft and Gault, 1976), which estimates the glomerular filtration rate:

$$\text{EQUATION 3: } \text{estCL}_{\text{cr}} = (140 - \text{age}) \times \text{weight (kg)} / 72 \times \text{serum creatinine (mg/dL)}^*$$

*Multiply with factor 0.85 if female

Clearance was estimated for patients above the age of 16 years according to Ambrose *et al.* (Ambrose *et al.*, 2001).

$$\text{EQUATION 4: } \text{Clearance}_{(>16 \text{ years})} = 8.11 + 0.0629 (\text{creatinine clearance} - 75.0)$$

The clearance estimates were then used in conjunction with dose to estimate gatifloxacin exposure AUC_{0-24} for each patient:

$$\text{EQUATION 5: } \text{AUC}_{0-24} = \text{dose/clearance}$$

The ratio of drug exposure to MIC (AUC₀₋₂₄: MIC ratio) was calculated by dividing the AUC₀₋₂₄ by the MIC of the patient's infecting *S. Typhi* isolate:

$$\text{EQUATION 6: AUC}_{0-24} : \text{MIC ratio} = \text{AUC}_{0-24} / \text{MIC}$$

The analysis included all patients with sufficient data to estimate the AUC₀₋₂₄, who were blood culture positive and for whom the gatifloxacin MICs of the infecting *S. Typhi* isolate was available.

3.2.1.4 Pharmacodynamic analysis

All statistical analyses were implemented in the statistical program SYSTAT 11, Richmond, CA. Univariate and multivariate logistic regression were used to evaluate the probability of clinical response. Multivariate logistic regression modelling was carried out using likelihood ratio testing and backwards stepping. Categorical breakpoint values for continuous variables, such as AUC₀₋₂₄: MIC ratio, were determined by classification and regression tree (CART) analysis. Variables evaluated included age, sex, weight, multidrug resistance, AUC₀₋₂₄, MIC, and AUC₀₋₂₄: MIC ratio.

Stratified Kaplan-Meier and Cox proportional hazards regression analyses were used to examine the relationship between independent variables of interest and the time to fever resolution. The time until fever resolution was defined as the first time the patient's axillary temperature was less than 37.5 °C and remained so for a minimum of 48 hours.

3.2.2 Section 2. Pharmacokinetics of gatifloxacin in paediatric and adult enteric fever patients in Nepal

3.2.2.1 Patient population, ethical approval, inclusion and exclusion criteria

Blood samples were collected during a randomised controlled trial comparing the efficacy and safety of an oral dose of 10 mg/kg/day of gatifloxacin (Broadband, Acme Formulation Private Limited, Solan, India; marketed by Novartis AG, Basel, Switzerland; 400 mg/tablet) once daily for 7 days *versus* an oral dose of 75 mg/kg/day of chloramphenicol (Chloro, National Healthcare, Nepal) in four divided doses for 14 days for the treatment of enteric fever.

The trial was conducted at Patan Hospital, Kathmandu, Nepal. Patients aged 2 years to 60 years with suspected enteric fever (more than 3 days of fever and clinically diagnosed typhoid fever) were invited to participate. The trial was approved by the Oxford University Tropical Research Ethics Committee and the Nepal Health Research Council. The trial registration number was ISRCTN53258327 (<http://www.controlled-trials.com/ISRCTN53258327>).

Patients who lived in a pre-designated area of approximately 20 square kilometres 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 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, known previous treatment with chloramphenicol, quinolone antibiotic or third generation cephalosporin or macrolide within one week of hospital admission. Patients who had received

amoxicillin or cotrimoxazole were included as long as they did not show evidence of clinical response.

3.2.2.2 Procedures

Patients were enrolled into the study by dedicated physicians at Patan hospital, who took a full history and carried out the clinical examination. Once the study physicians had enrolled the patient, they were managed as outpatients. Trained community medical auxiliaries (CMAs) visited each patient's house every 12 hours until the patient was cured, as described previously (Pandit *et al.*, 2007). At each visit the CMAs recorded oral temperature, inquired about symptoms and directly observed each patient ingesting the single dose of gatifloxacin and the two doses of chloramphenicol coinciding with their daily visits.

Patients were re-examined by the study physicians at Patan Hospital on day 8 and blood and stool cultures were collected if the initial blood culture was positive. Patients also visited the study physicians on day 15, and at one, three and six months. At these visits stool of blood culture positive patients was checked for faecal carriage. Also febrile illnesses in household members were inquired. Random plasma glucose was measured on day 1, days 2 to 7 (using finger prick testing using OneTouch SureStep, Johnson & Johnson, USA during the CMAs' evening visits), on day 8, day 15 and one month. Haemoglobin A1C was measured at three months. Full blood counts, liver enzymes and creatinine were studied on days 1, 8, and 15.

3.2.2.3 Outcomes

In brief, favourable clinical response was defined as the resolution of fever and symptoms within 48 hours of the end of therapy (i. e. on day 10). Fever was evaluated every 12 hours. Relapse was defined as the recurrence of fever and symptoms and/or the isolation of *S. Typhi* or *S. Paratyphi A* from blood within 1 month after the completion of therapy. At the 1, 3 and 6 months follow up visits, stool cultures were performed to check for chronic faecal carriage of *S. Typhi* or *S. Paratyphi A*.

3.2.2.4 Blood samples and sparse plasma sampling schedule

Blood sampling was performed in steady state concentrations. Steady state concentrations describe equilibrium between administration and elimination, which is usually reached after 5 drug half lives. For gatifloxacin with a half life of approximately 8 hours, steady state is reached after administration of the second dose.

Two ml of blood were collected into lithium heparin tubes using a sparse sampling scheme. The first of two samples was taken 3 to 6 hours after administration of the third or a later dose and the second sample was taken 12 to 24 hours after the third or a later dose. Blood tubes were inverted several times and immediately placed on ice. Samples were processed as soon as possible after collection. The samples were centrifuged for 10 minutes at 100 x g in a centrifuge which cups have been chilled. The plasma layer was transferred to polypropylene screw-capped tubes and labelled with the study number, study day, date and the actual sampling time. Plasma and cell pellets were immediately stored at -80°C. Information about each blood sample (date, time), the

exact time and doses (in mg; all tablets were weighed before administration) of all previous gatifloxacin administrations were recorded in a spreadsheet.

For children, one sample was taken 6 to 24 hours after the third dose. Sampling times were spread out to allow for the best possible validation using a single sample.

3.2.2.5 Gatifloxacin assay

Human plasma samples (50 μ l) were deproteinated with 250 μ l acetonitrile. The samples were centrifuged and an aliquot of the supernatant (50 μ l) was transferred into an autosampler vial containing 1 ml of HPLC water.

Samples were analyzed by high pressure liquid chromatography tandem mass spectrometry (LC/MS/MS). The LC/MS/MS system consists of a Shimadzu Prominence HPLC system and an Applied Biosystems/MDS Sciex API5000 LC/MS/MS.

Chromatographic separation was performed using a Phenomenex Luna Phenyl-Hexyl column, 5 μ m, 150 x 3.0 mm column and a mobile phase consisting of 85% 0.1% formic acid in water and 15% 0.1% formic acid in acetonitrile, at a flow rate of 0.75 ml/min. Gatifloxacin concentrations were obtained using LC/MS/MS monitoring the MS/MS transition m/z 376 \rightarrow m/z 332. Analysis run time was 4.0 minutes.

The assay was linear over a range of 0.050 to 10.0 μ g/ml ($r^2 > 0.996$). The inter-day precision (%CV) ranged from 2.61 to 9.69%, with an accuracy ranging from 106% to 111% on quality control samples at three levels (0.10 μ g/ml, 1.00 μ g/ml, and 8.00 μ g/ml) in replicates of three at each level on each analysis day.

3.2.2.6 Development of a population pharmacokinetic model

Population PK datasets were constructed using the exact dose and sampling times. The data were vetted for the presence of errors in dose and/or sampling times. Potential outlier concentrations were explored using accepted methodology.

Separate population pharmacokinetic candidate models for adults (>16 years of age) and paediatric patients (≤ 16 years of age) were fit to the data using Monte-Carlo parametric expectation maximization (MCPM) as implemented in the open-source software program S-ADAPT, which was written by Robert J. Bauer and is available at <http://bmsr.usc.edu/Software/ADAPT/SADAPTsoftware.html> (Bauer and Guzy, 2004). The program performs parametric population analysis, including maximum likelihood estimation, via the Expectation Maximization (EM) algorithm with sampling as implemented in the Monte Carlo Expectation Maximization (MCPM) algorithm, as well as Bayesian estimation (University of South Carolina, 2009).

Due to the sparse nature of the PK sampling scheme, the structure and covariate relationships from previous gatifloxacin adult and paediatric population PK models derived from North American patients were retained, but were revised to fit the data from this population (Grasela, 2000a; Grasela *et al.*, 1998; Rubino *et al.*, 2007).

These PK models had the structure of one compartment models with first-order absorption and elimination.

The equations and covariate relationships are provided below.

For the adult patients (>16 years) the following equations were used to estimate total clearance (CL_t) and central volume of distribution (V_c). Creatinine clearance (CL_{cr}) was calculated using the method of Cockcroft and Gault (Cockcroft and Gault, 1976).

$$CL_t \text{ (L/hr)} = CL_t\text{-intercept} + CL_t\text{-slope} \times CL_{cr} \text{ (ml/min/1.73m}^2\text{)}$$

$$V_c \text{ (L)} = V_c\text{-slope} \times \text{Body Weight (kg)}$$

The following equations were used for the paediatric patients (≤ 16 years) to estimate total clearance (CL_t) and central volume of distribution (V_c). Body surface area (BSA) was estimated according to the Gehan and George method (Gehan and George, 1970).

$$CL_t \text{ (L/hr)} = CL_t\text{-slope} \times BSA \text{ (m}^2\text{)}$$

$$V_c \text{ (L)} = V_c\text{-slope} \times \text{Body Weight (kg)}$$

3.3 Results

3.3.1 Section 1. Pharmacodynamics of gatifloxacin in adult and paediatric typhoid fever patients in Vietnam

3.3.1.1 Patient population and clinical data

Of the 186 patients randomised to receive gatifloxacin therapy in this trial, 185 could be evaluated for this analysis. Of these, 144 patients were blood culture positive for *S. Typhi* and 124 patients had sufficient data to estimate gatifloxacin exposure.

These 124 patients represent the population used in all pharmacodynamic analyses. Sixty three patients were male and 61 were female; their mean (standard deviation, SD)

age was 11.3 (5.48) years; their mean (SD) weight was 27.8 (12.4) kg; and their mean (SD) body surface area was 1.02 (0.299) m². Nine of the 124 (7%) patients had positive bone marrow cultures for *S. Typhi* in addition to their positive blood cultures. Five (6/124) percent of patients were infected with nalidixic acid susceptible (MIC ≤ 16 mg/L) and 95% (118/124) with nalidixic acid resistant (MIC ≥32 mg/L) *S. Typhi* strains. Sixty one (76/124) percent of patients were infected with multidrug resistant *S. Typhi* strains.

The median (range) gatifloxacin MIC of *S. Typhi* isolates was 0.018 (0.006-0.09) mg/L for nalidixic acid susceptible isolates and 0.12 (0.06-0.25) mg/L for nalidixic acid resistant isolates. The median (range) ciprofloxacin MIC value for nalidixic acid susceptible *S. Typhi* isolates was 0.014 (0.006-0.12) mg/L and for nalidixic acid resistant isolates was 0.38 (0.12-0.75) mg/L.

3.3.1.2 Pharmacodynamic analysis

Clearance estimates from previously validated adult and paediatric population pharmacokinetic models were used in conjunction with the dose to obtain an estimate for AUC (Ambrose *et al.*, 2001; Rubino *et al.*, 2007).

In these typhoid fever patients, there was a statistically significant relationship between the intensity of the drug exposure to gatifloxacin and clinical response.

Risk Factor	<i>p</i> value	Risk Factor	<i>p</i> value
Age	0.206	MDR	0.867
Sex	0.795	AUC ¹	0.519
Weight	0.136	AUC ²	0.0833
<i>S. Typhi</i> in bone marrow	0.165	MIC ¹	0.0256
		MIC ²	0.0716
		AUC:MIC ¹	0.0849
		AUC:MIC ²	0.0243

¹Treated as a continuous variable

² Treated as a categorical variable

Table 3-1 Univariate logistic regression analysis results.

Risk factors were tested for association with clinical response in typhoid fever patients (n =124) using univariate logistic regression analysis.

We could identify a significant dichotomous categorical breakpoint for AUC₀₋₂₄: MIC ratio which was predictive of clinical success. Patients in whom an AUC₀₋₂₄: MIC ratio of greater than 92.7 was obtained, had a favourable response to treatment in 93.5%, whilst in patients with AUC₀₋₂₄: MIC ratios ≤ 92.7 only 75% had a favourable response (Odds Ratio = 4.81, 95% CI 1.23-18.9; *p* = 0.02) (Figure 3.2).

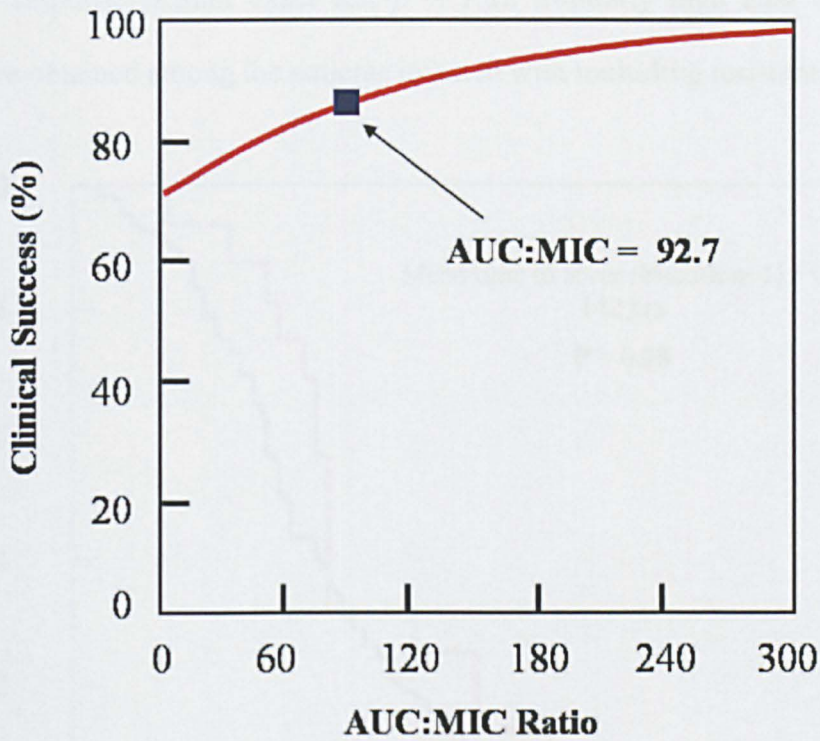


Figure 3.2 Relationship between gatifloxacin exposure and clinical response in typhoid fever patients.

Univariate logistic regression analysis with AUC: MIC as a continuous variable, for AUC: MIC breakpoint (92.7); $p = 0.02$.

The mean time to fever resolution was 113 hours in patients in whom $AUC_{0-24}: MIC$ ratios greater than 92.7 were attained, compared to 142 hours in those patients with lesser exposures ($p = 0.08$) (Figure 3.3). The proportion of patients who had a positive clinical response was 91% (113/124) for all patients. All six patients infected with nalidixic acid susceptible strains had successful clinical response to therapy; while 91% (107/118) of those patients infected with nalidixic acid resistant strains had a positive

therapeutic response (Fisher exact test $p = 1.0$). Similarly high cure rates of 91% (69/76) were obtained among the patients infected with multidrug resistant *S. Typhi*.

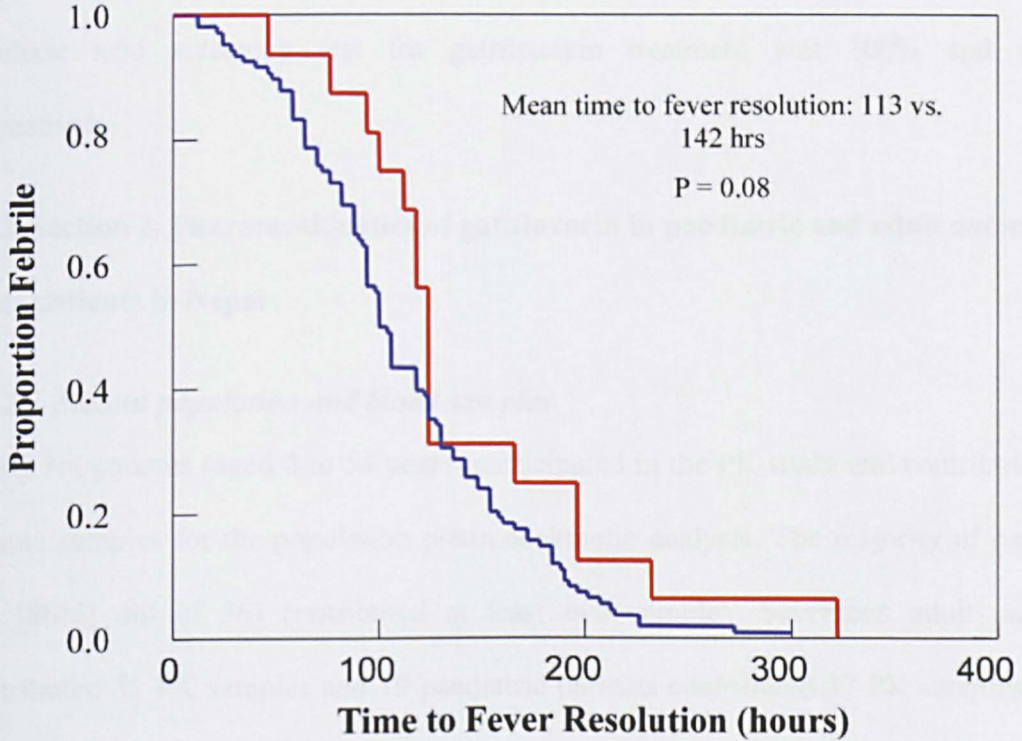


Figure 3.3 Kaplan-Meier survival curve.

The Kaplan-Meier survival curve shows the proportion of culture confirmed patients still febrile through time stratified by $AUC_{0-24}:MIC$ ratio breakpoint. The mean time to fever resolution tended to be shorter in patients with $AUC_{0-24}:MIC > 92.7$ (blue line) than those patients with $AUC_{0-24}:MIC \leq 92.7$ (red line).

We could identify a borderline significant dichotomous categorical gatifloxacin MIC breakpoint for *S. Typhi*, which was predictive for clinical response. *S. Typhi* gatifloxacin MIC values ≥ 0.19 mg/L were associated with 83.8% of patients having a

positive clinical response, while patients with gatifloxacin MIC values less than 0.19 mg/L had in 94.3% positive response ($p = 0.0716$; odds ratio 3.17, 95% CI 0.93-11.2). The positive (predicts success) and negative (predicts failure) predictive value of the nalidixic acid screening test for gatifloxacin treatment was 100% and 9.3%, respectively.

3.3.2 Section 2. Pharmacokinetics of gatifloxacin in paediatric and adult enteric fever patients in Nepal

3.3.2.1 Patient population and blood samples

Thirty six patients (aged 3 to 54 years) participated in the PK study and contributed 68 plasma samples for the population pharmacokinetic analysis. The majority of patients (31 [86%] out of 36) contributed at least two samples. Seventeen adult patients contributed 31 PK samples and 19 paediatric patients contributed 37 PK samples. The median age of the 19 children was 10 years (range 5 to 16) and that of the 17 adult patients was 23 years (range 17 to 54 years), respectively. The following co-variables were analysed: age, sex, weight, height, body surface area (BSA), and serum creatinine concentrations. The demographic characteristics of the 36 patients are shown in Table 3-2.

Variable	Population	N	Mean (SD)	Median	Range
Age (years)	Adults	17	27.4 (10.6)	23.0	17.0 - 54.0
	Children	19	10.5 (3.01)	10.0	5.0 - 16.0
Weight (kg)	Adults	17	56.6 (9.28)	53.0	44.0 - 72.0
	Children	19	26.9 (8.87)	25.0	15.0 - 50.0
BSA (m ²)	Adults	17	1.61 (0.152)	1.58	1.36 - 1.87
	Children	19	0.999 (0.212)	0.955	0.665 - 1.53
CL _{cr} (mL/min/1.73m ²)	Adults ^a	17	67.1 (12.2)	69.4	46.2 - 93.0
	Children ^b	19	79.6 (11.8)	79.8	56.7 - 104

n number of patients

^acalculated using the method of Cockcroft and Gault (Cockcroft and Gault, 1976)

^bcalculated using the Schwartz equation (Schwartz *et al.*, 1976)

Table 3-2 Demographic characteristics of the adult and paediatric pharmacokinetic analysis population

3.3.2.2 Pharmacokinetic analysis and population pharmacokinetic model

Gatifloxacin pharmacokinetics was best fit by a linear one compartment model with first order absorption and elimination. Fits of data were excellent ($R^2 > 0.9$ for paediatric and adult data); the inter-individual variability in pharmacokinetics was modest. None of the gatifloxacin concentrations were deemed to be significant outliers.

Standard diagnostic goodness-of-fit and weighted residual plots were employed to establish whether the pharmacokinetic model was adequate for describing the data.

Figure 3.4 shows the goodness-of-fit plots for the observed versus the individual fitted

gatifloxacin concentrations for the final adult (Panel A) and paediatric population (Panel B) PK model, respectively. Panels C and D are the plots of individual weighted residuals versus individual fitted gatifloxacin concentrations for the final adult and paediatric population PK models, respectively.

Figure 3.5 shows a scatter plot of gatifloxacin concentrations ($\mu\text{g/ml}$) measured in blood versus the time since the previous dose was administered for adult and paediatric patients, respectively. Gatifloxacin steady state concentrations measured at 3 hours after the previous dose ranged from 4.5 $\mu\text{g/ml}$ to 8.9 $\mu\text{g/ml}$ in children and from 5 $\mu\text{g/ml}$ to 9.8 $\mu\text{g/ml}$ in adults, respectively.

Table 3-3 presents the final population pharmacokinetic parameter estimates for the adult and paediatric data. Given the relatively small size of the datasets and the sparseness of the sampling scheme, the precision of parameter estimates was acceptable.

We compared the gatifloxacin PK parameter estimates between the previous PK models, developed from North American patients/subjects and those resulting from the application of this model to Nepalese patients with enteric fever (Table 3-4). Compared to the North American children with otitis media (Rubino *et al.*, 2007), the Nepalese paediatric enteric fever patients had approximately 50% slower clearance (Table 3-4). The distributions of age and body surface area (BSA) were similar between the North American and Nepalese paediatric populations, but the Nepalese population had higher serum creatinine values, suggesting comparatively lower renal function (Figure 3.6).

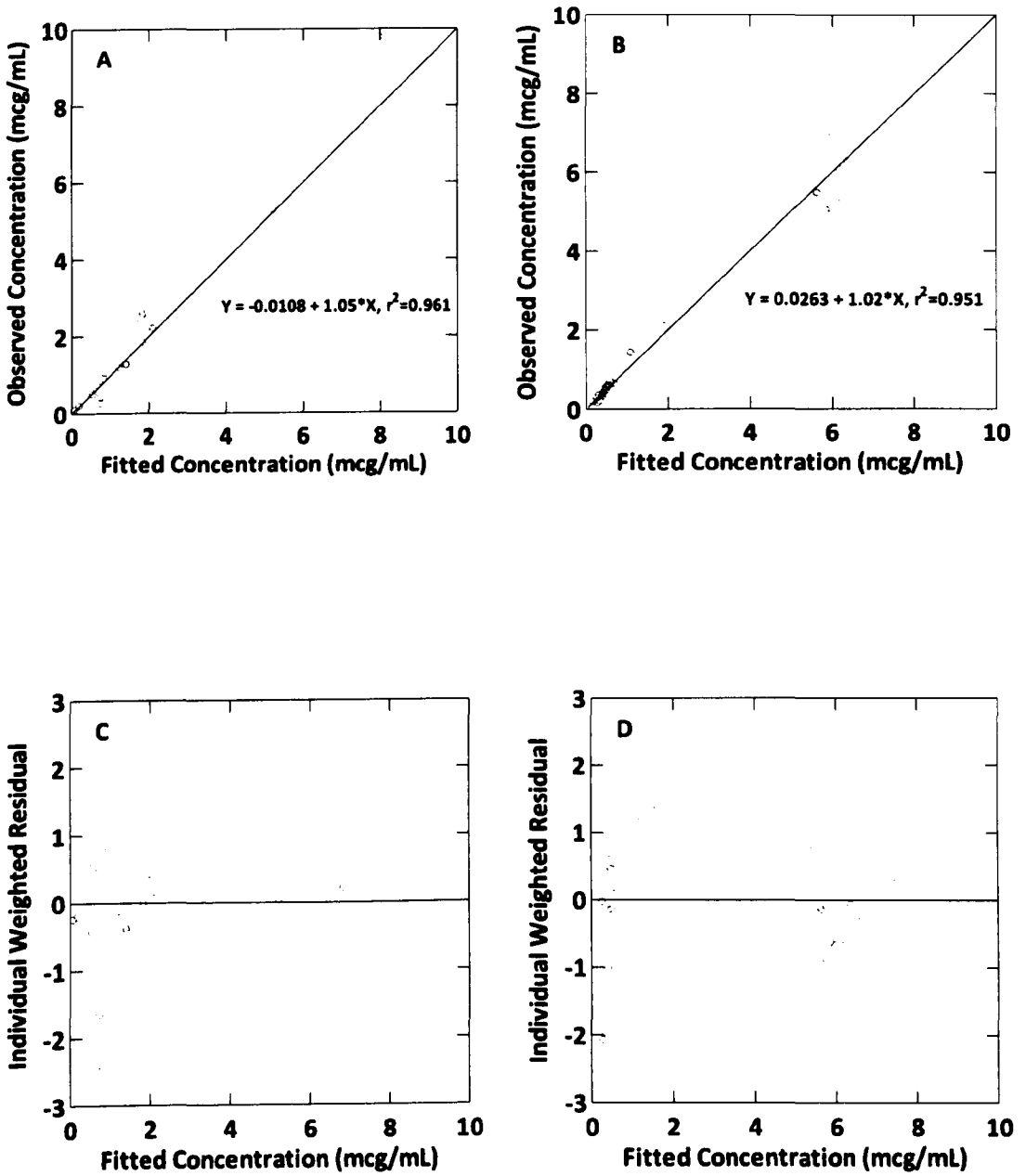


Figure 3.4 Goodness-of-fit plots.

Goodness-of-fit plots for the observed versus the individual fitted gatifloxacin concentrations for the final adult (Panel A) and paediatric population (Panel B) PK model, respectively. Panels C and D are the plots of individual weighted residuals

versus individual fitted gatifloxacin concentrations for the final adult and paediatric population PK models, respectively.

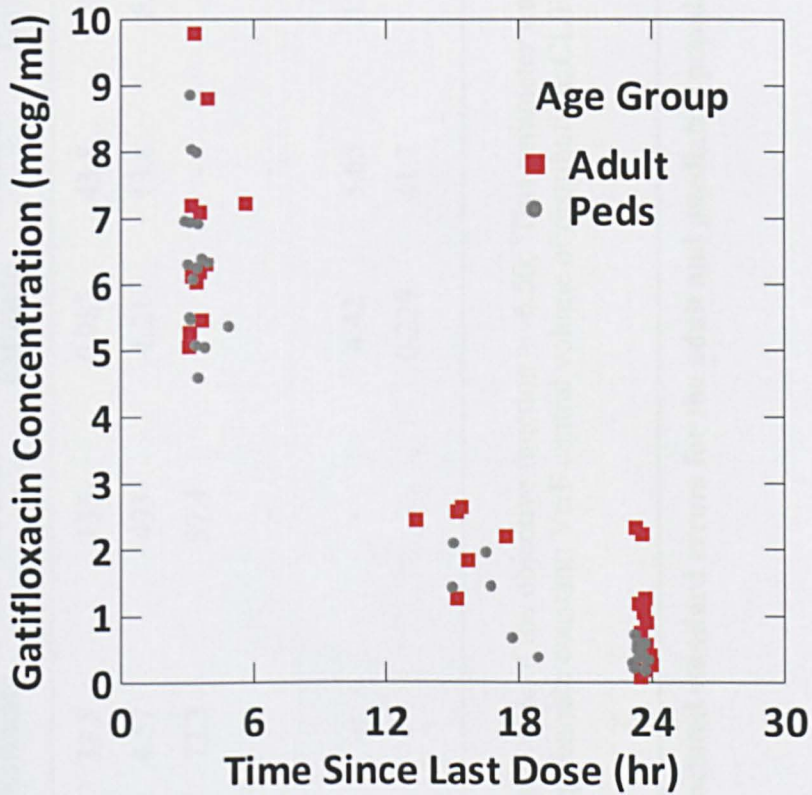


Figure 3.5 Scatter plot of gatifloxacin concentrations versus time since the last dose, stratified by population.

Adult patients (red squares) are defined as age above 16 years,
paediatric patients (grey dots) as age \leq 16 years.

Parameter	Adult Population PK Model ^a				Paediatric Population PK Model ^b			
	Population Mean		Magnitude of Interindividual Variability (%CV)		Population Mean		Magnitude of Interindividual Variability (%CV)	
	Final Estimate	%SEM	Final Estimate	%SEM	Final Estimate	%SEM	Final Estimate	%SEM
Ka (1/hr)	3.34	116	38.5	135	0.982	43.8	28.4	485
Vc/F (L/kg)	1.28	17.7	4.57	493	1.21	13.8	6.33	119
CL/F, non-renal (L/hr)	2.91	21.9	72.3	57.4	-	-	-	-
CL/F, renal slope ^c (L/hr/mL/min)	0.0629	-	-	-	-	-	-	-
CL/F(L/hr/m ²)	-	-	-	-	4.42	5.65	15.0	101
SD _{sl}	0.250	17.2	-	-	0.229	41.7	-	-

^aMinimum value of the objective function = 6.34; ^bMinimum value of the objective function = -6.50; ^cThis parameter was not fit due to the narrow range of renal function in this population; Ka absorption rate constant; Vc/F central volume of distribution; CL/F apparent oral clearance; SD_{sl} standard deviation of the slope

Table 3-3 Final PK parameter estimates and associated standard errors for the adult and paediatric population PK models

Patient population	PK parameter	Mean (%SEM) parameter estimates	
		Previous models	Current data
Paediatric	CL/F (L/h/m ²)	8.46 (3.50)	4.41 (5.65)
	V _c (L/kg)	2.15 (3.30)	1.21(13.8)
	CL/F, nonrenal (L/h)	8.11(35.3)	2.91 (21.9)
Adult	CL/F, renal-slope (L/h/mL/min)	0.0629 (37.8)	0.0629 (---)
	V _c (L/kg)	1.45 (7.9)	1.28 (17.7)

V_c central volume of distribution; CL/F apparent oral clearance

Table 3-4 Comparison of gatifloxacin PK parameters.

Comparison of gatifloxacin PK parameters between previous PK models (developed from North American patients/subjects) and the application of this model in the analysis of Nepalese patients with enteric fever (current data).

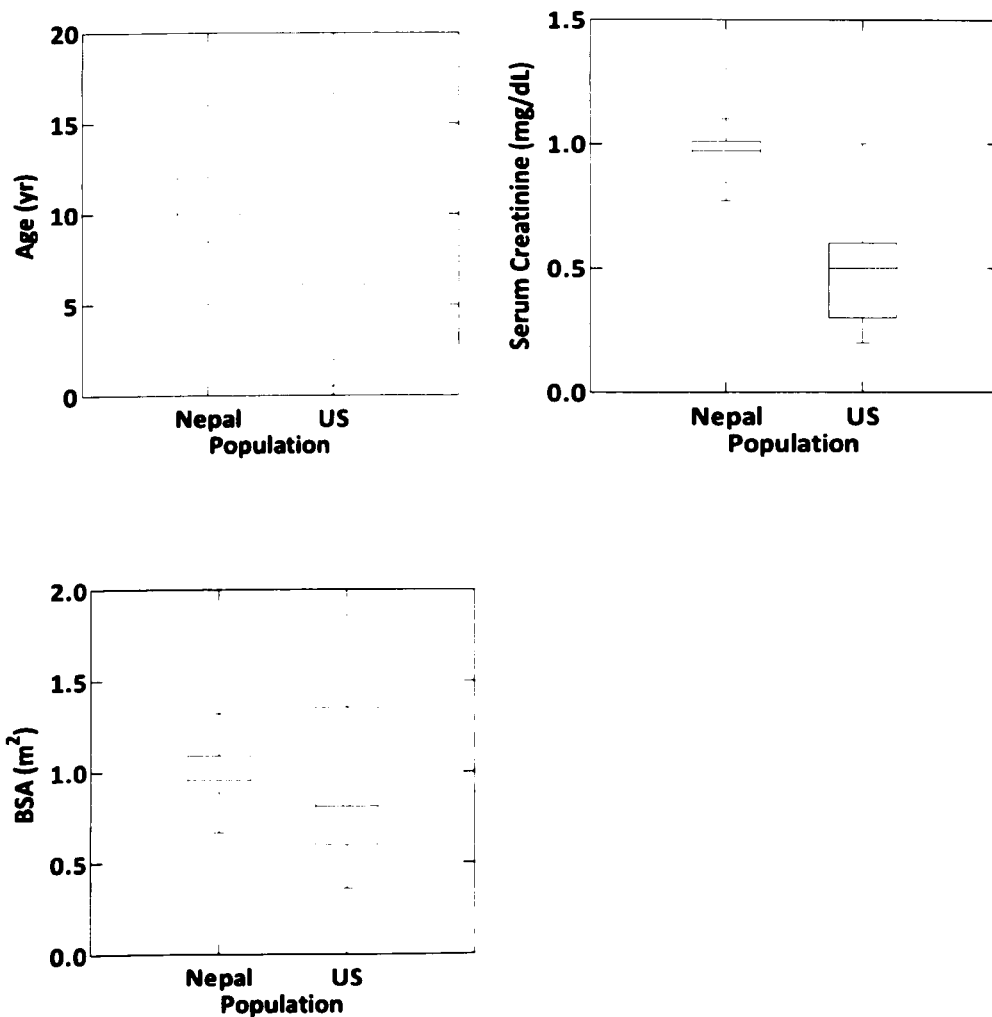


Figure 3.6 Box plots of age, serum creatinine (in mg/dl) and body surface area (BSA) in paediatric patients.

The data are stratified by population (Nepal = current analysis, US = model development population). The line inside the box represents the median, the upper and lower limits of the box are the 75th and 25th percentiles, respectively, and the upper and lower error bars indicate the values that are 1.5 times the inter-quartile range.

Individual points outside the error bars represent values that are >1.5 times the inter-quartile range (i.e., outliers).

3.4 Discussion

This chapter describes two studies that analyse the pharmacodynamic (section 1 of this chapter) and the pharmacokinetic profile (section 2) of gatifloxacin in patients infected with *S. Typhi* and *S. Paratyphi A*.

The first study had two objectives, to examine the relationship between gatifloxacin exposure and clinical efficacy and to determine the positive and negative predictive value of the nalidixic acid screening test for the clinical efficacy of gatifloxacin in the same patient population. A relationship between exposure to gatifloxacin and clinical response was identified. In patients in whom an $AUC_{0-24}: MIC$ ratio of greater than 92.7 was attained, 93.5% were classified as clinically cured, while in patients in whom $AUC_{0-24}: MIC$ ratios of less than or equal to 92.7 were achieved only 75% of patients had a favourable response (Odds Ratio = 4.81, 95% CI 1.23 - 18.9; $p = 0.02$). These findings are supported by clinical (Drusano *et al.*, 2004; Forrest *et al.*, 1993) and non-clinical studies (Booker *et al.*, 2005). A study in critically ill patients infected with Gram negative enteric pathogens and *Pseudomonas aeruginosa* (Forrest *et al.*, 1993) treated with intravenous ciprofloxacin, concluded that achieving an $AUC_{0-24}: MIC$ ratio of greater than 125 was associated with improved clinical outcomes. Drusano *et al.* showed that an $AUC_{0-24}: MIC$ ratio of greater than 100 also prevented the emergence of bacterial resistance, particularly in the critically ill (Drusano *et al.*, 2004).

In a one compartment in vitro pharmacodynamic model of gatifloxacin and *S. Typhi*, a $AUC_{0-24} : MIC$ ratio of 105 correlated with 90% bacterial eradication (Booker *et al.*, 2005). Importantly, the relationship between exposure and response based on this model did not differ for a strain that was gatifloxacin susceptible (MIC 0.5 mg/L) and one that was resistant (MIC 4.0 mg/L), despite the latter having two GyrA (Ser83→Tyr; Asp87→Gly) and two ParC (Thr 57→Ser; Ser80→Ile) mutations. This observation is supported by a neutropenic murine thigh infection model involving *Salmonella* and levofloxacin, where the $AUC_{0-24} : MIC$ ratios necessary for net bacterial stasis or a 90% reduction in bacterial burden did not differ by nalidixic acid susceptibility status (Craig *et al.*, 2006).

Patients in whom $AUC_{0-24} : MIC$ ratios greater than 92.7 were attained also tended towards a more rapid time (29 hours) to fever resolution ($p = 0.08$). Administering drugs in a manner that leads to clinical improvements, such as fever and symptom resolution or the more rapid eradication of faecal pathogens, may have profound public health implications. As large epidemics are often related to faecal contamination of food and water supplies, decreasing the duration of *S. Typhi* shedding may limit secondary infections ((Luxemburger *et al.*, 2001). Perhaps more importantly, it has been demonstrated that for fluoroquinolones and other Enterobacteriaceae and *P. aeruginosa*, it is possible to suppress the emergence of resistant subpopulations by administering high intensity (e.g. large $AUC_{0-24} : MIC$ ratio) regimens for shorter durations (Jumbe *et al.*, 2003).

The predictive value of the nalidixic acid screening test for gatifloxacin in patients with typhoid fever was poor ($p = 1.0$). While all patients infected with nalidixic acid susceptible strains were treated successfully, so were 90.7% of patients infected with nalidixic acid resistant strains.

This is not true for the older generation fluoroquinolones such as ofloxacin and ciprofloxacin for which the nalidixic acid screening test was highly predictive of clinical success in the treatment of typhoid fever (Parry, 2004a). This observation has important clinical implications, as current CLSI guidelines state that “Fluoroquinolone-susceptible strains of *Salmonella* that test resistant to nalidixic acid may be associated with clinical failure or delayed response in fluoroquinolone-treated patients with extra-intestinal Salmonellosis. Extra-intestinal isolates of *Salmonella* should also be tested for resistance to nalidixic acid. For isolates that test susceptible to fluoroquinolones and resistant to nalidixic acid, the physician should be informed that the isolate may not be eradicated by fluoroquinolone treatment” (CLSI, 2007). As fluoroquinolones are associated with higher cure rates and more rapid fever resolution than other agents (Parry *et al.*, 2002), these CLSI guidelines may direct clinicians to use other suboptimal antimicrobial therapies.

The explanation for the failure of the nalidixic acid screening test to predict gatifloxacin efficacy likely relates to the methoxy group at the 8-position of the quinolone bicyclic aromatic core of gatifloxacin. The C-8 methoxy group allows for dual enzymatic targeting of DNA gyrase and DNA topoisomerase IV in *Escherichia coli*, which results in a modest MIC increase in DNA gyrase mutants (Lu *et al.*, 1999). This is exemplified

by the ratio of median MIC of nalidixic acid susceptible strains to the median MIC of resistant strains which was 7-fold for gatifloxacin compared to 27-fold for ciprofloxacin.

This data represent, to our knowledge, the first clinical validation of the importance of this structure-activity relationship for Enterobacteriaceae and highlights that not all fluoroquinolones are the same. The poorer clinical responses to the older fluoroquinolones now reported for enteric fever should not be extrapolated to the latest generation of drugs from this class.

Finally, these clinical data suggest gatifloxacin, and perhaps other 8-methoxy fluoroquinolone, specific susceptibility breakpoints. Gatifloxacin MIC values ≥ 0.19 mg/L were associated with 83.8% of patients having a positive clinical response, while patients with MIC values less than 0.19 mg/L had a 94.3% positive response ($p = 0.0716$). This MIC breakpoint is similar to that derived from the aforementioned in vitro PK/PD *S. Typhi* infection model and Monte Carlo simulation (Booker *et al.*, 2005). Simulation results suggested susceptible breakpoint of 0.12 mg/L for gatifloxacin. Moreover, these data indicate that the nalidixic acid screening test does not predict gatifloxacin efficacy. Gatifloxacin attained high favourable clinical response rates regardless of nalidixic acid susceptibility or multidrug resistance status. Continued use of this screening test to predict gatifloxacin efficacy, and perhaps that of other 8-methoxy fluoroquinolones, may drive clinical use away from effective treatment regimens to less effective regimens. One possible remedy is the use of *S. Typhi* specific susceptibility breakpoints, which to date have not been determined by the CLSI.

As drug clearance was markedly lower in Nepalese enteric fever patients compared to North American infected patients, these data demonstrate the importance of evaluating pharmacokinetics in different patient populations. The PK models described in this chapter will be used in future pharmacokinetics-pharmacodynamics analyses of efficacy in Asian populations with enteric fever.

Chapter 4

High throughput genotyping of *S. Typhi* strains isolated in the Mekong

Delta region of Vietnam

4.1 Introduction

S. Typhi is a recently evolved and genetically monomorphic organism. *S. Typhi* is exceptional amongst the 2400 serovars of *S. enterica*, as it doesn't have a promiscuous life style infecting a wide range of animals and has an ecologically adapted niche as a human invasive pathogen. This host restriction has been attributed to the acquisition of several *Salmonella* pathogenicity islands (SPIs) and the accumulation of pseudogenes (Achtman, 2008; Parkhill et al., 2001a).

The *S. Typhi* genome contains several large insertions, which encode virulence-associated genes, the *Salmonella* pathogenicity islands (SPI) (Parkhill *et al.*, 2001a). Bacterial pathogenicity islands often exhibit features of mobile genetic elements (Hou, 1999) and are believed to be acquired through recent horizontal acquisition from other bacteria. Features of SPIs include a GC content that differs from that of the rest of the bacterial genomes, the presence of flanking direct repeats and insertion elements that are reminiscent of those of phages and plasmids and the ability to encode 'mobility genes', such as integrases, transposases and origins of plasmid replication (Hou, 1999). Often these elements overlap with tRNA genes suggesting that the 3' sites of transfer RNAs may serve as recombination site for pathogenicity islands (Hou, 1999).

Two hundred and four pseudogenes have been described in the genome of *S. Typhi* strain CT 18 (Parkhill *et al.*, 2001a). Most of these pseudogenes have been introduced by a single frameshift or stop codon and many of these pseudogenes are found in housekeeping genes and genes that are potentially involved in virulence (Parkhill *et al.*, 2001a).

As a young and monophyletic organism, *S. Typhi* shows little DNA sequence variation. The analysis of a global collection of 334 *S. Typhi* isolates by multilocus enzyme electrophoresis (MLEE), which tested 24 conserved neutral cytoplasmic enzymes for variation in charge identified only two multilocus enzyme genotypes (Selander *et al.*, 1990). Multi Locus Sequence Typing (MLST) of seven *S. Typhi* housekeeping genes also demonstrated that genetic polymorphisms were rare in *S. Typhi* (Kidgell *et al.*, 2002b). Only three polymorphic sites were found when seven genes from a diverse collection of 26 *S. Typhi* isolates were sequenced. It was estimated that the last common ancestor of *S. Typhi* is approximately 50,000 years (Kidgell *et al.*, 2002b).

For epidemiological purposes to identify genetic relationship of isolates, especially in outbreak situations and to trace sources of infection, different typing schemes for *S. Typhi* have been deployed. These include Pulsed Field Gel Electrophoresis (PFGE) (Thong *et al.*, 1994; Thong *et al.*, 1995), Variable Number of Tandem Repeat (VNTR) analysis (Liu *et al.*, 2003), IS200 typing (Threlfall *et al.*, 1994) and ribotyping ((Navarro *et al.*, 1996). These typing schemes can identify multiple variants and can capture strain variation and properties, especially in outbreak situations. It has been shown that differences in PFGE and ribotype patterns of *S. Typhi* reflect the genomic rearrangement of the chromosome through recombination between rRNA loci (Ng *et al.*, 1999). Also the modular nature and distribution of prophages can contribute significantly to strain variation (Hou, 1999). However, whilst these techniques capture variation, there are some disadvantages, as they are difficult to standardise within and in

between laboratories and they do not readily provide phylogenetic relationships (Achtman, 2008).

Recently, Roumagnac *et al.* (Roumagnac *et al.*, 2006) have developed a typing scheme based on Single Nucleotide Polymorphisms (SNP) for *S. Typhi*. Denatured High Performance Liquid Chromatography (dHPLC) was performed to analyse the diversity of 199 gene fragments of a global collection of 105 *S. Typhi* isolates. Fifty-five polymorphic gene fragments were analysed in an additional 350 isolates, which were isolated mostly in Southern Asia. Mutation discovery detected 97 bi-allelic Polymorphisms (BiPs). *S. Typhi* was resolved into a fully parsimonious phylogenetic tree defining 85 distinct haplotypes (H1-H85), which developed from a common root node, *S. Typhi* H45 (Figure 4.1) (Achtman, 2008; Roumagnac *et al.*, 2006).

This study revealed that *S. Typhi* has a neutral population structure, which is defined by the absence of strong selection in housekeeping genes and pathogenicity genes. However, on the backbone of this neutral population structure the clonal expansion of nalidixic acid (quinolone) resistant *S. Typhi* haplotype H58 emerged recently in South East Asia, presumably due to high antibiotic selective pressure. This clone has become the predominant *S. Typhi* haplotype in Vietnam. *S. Typhi* haplotype H58 is more closely related to Ty2 than to CT 18 (Figure 4.1) (Roumagnac *et al.*, 2006).

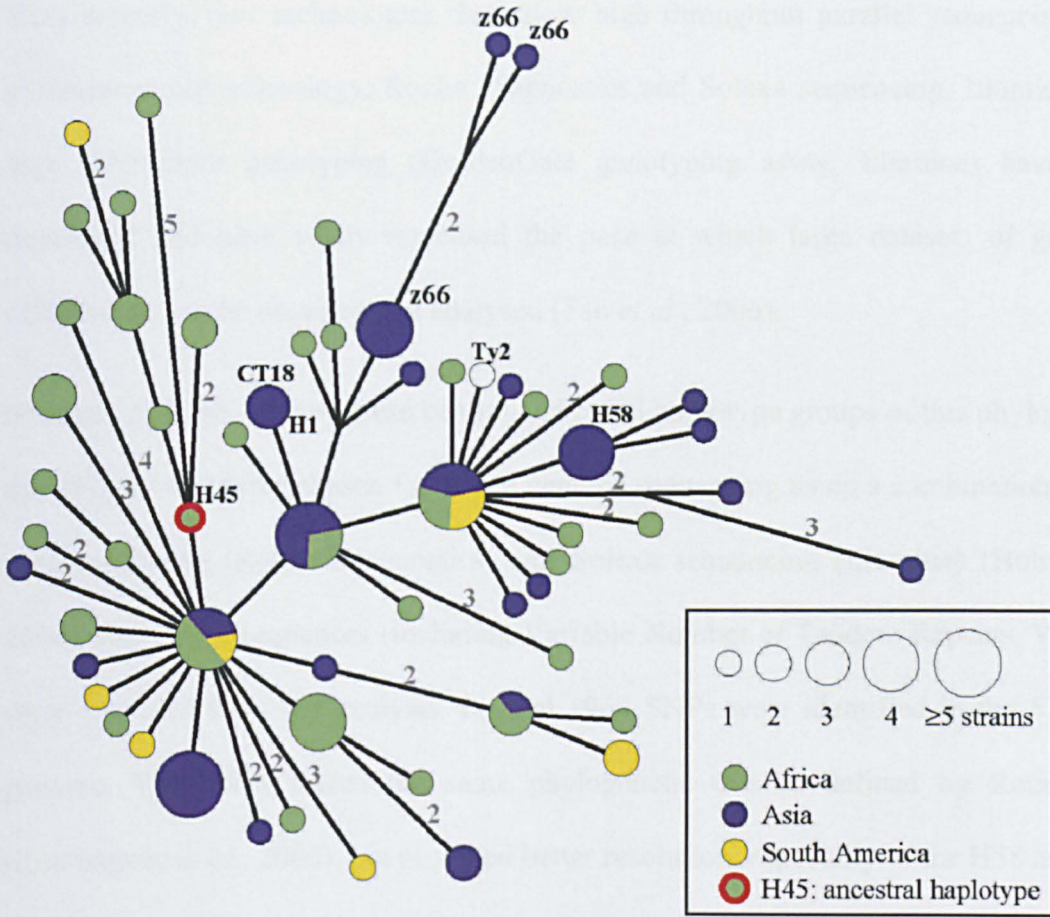


Figure 4.1 Minimal spanning tree of 105 global *S. Typhi* isolates.

Minimal spanning tree of 105 global *S. Typhi* isolates based on sequence polymorphisms in 199 gene fragments (88,739 base pairs). From (Roumagnac *et al.*, 2006). The tree shows 59 haplotypes (nodes) based on 88 BiPs, the colour coding of the pie charts indicates the continental source of the *S. Typhi* strains. The numbers along some edges indicate the number of BiP) that separate the nodes that they connect; unlabeled edges reflect single BiPs. The genomes of the CT18 and Ty2 strains have been fully sequenced. z66 refers to a flagellar variant that is common in Indonesia.

Very recently, new technologies that allow high throughput parallel sequencing (454 pyrosequencing technology, Roche Diagnostics and Solexa sequencing, Illumina) and high throughput genotyping (GoldenGate genotyping assay, Illumina) have been developed and have vastly increased the pace at which large datasets of genomic information can be obtained and analysed (Fan *et al.*, 2006).

Nineteen *S. Typhi* isolates from central and radial haplotype groups of this phylogenetic tree (Figure 4.1) were chosen for whole genome sequencing using a combination of 454 pyrosequencing (Roche Diagnostics) and Solexa sequencing (Illumina) (Holt *et al.*, 2008). Repetitive sequences (including Variable Number of Tandem Repeats, VNTRs) were excluded from the analysis. In total 1964 SNPs were identified in the *S. Typhi* genome. The SNPs traced the same phylogenetic tree as defined by Roumagnac (Roumagnac *et al.*, 2006), but provided better resolution, especially of the H58 and H59 haplotypes (Holt *et al.*, 2008) (Figure 4.2). The study revealed that pseudogene formation has occurred independently in different lineages of *S. Typhi* and is ongoing. This supports the hypothesis that evolution in this human restricted pathogen is dominated by loss of gene function (Holt *et al.*, 2008). Reduction in genome size and accumulation of large numbers of pseudogenes have been described as mechanisms of host restriction in other pathogens, including *Yersinia pestis* (Parkhill *et al.*, 2001b) and *Mycobacterium leprae* (Vissa and Brennan, 2001).

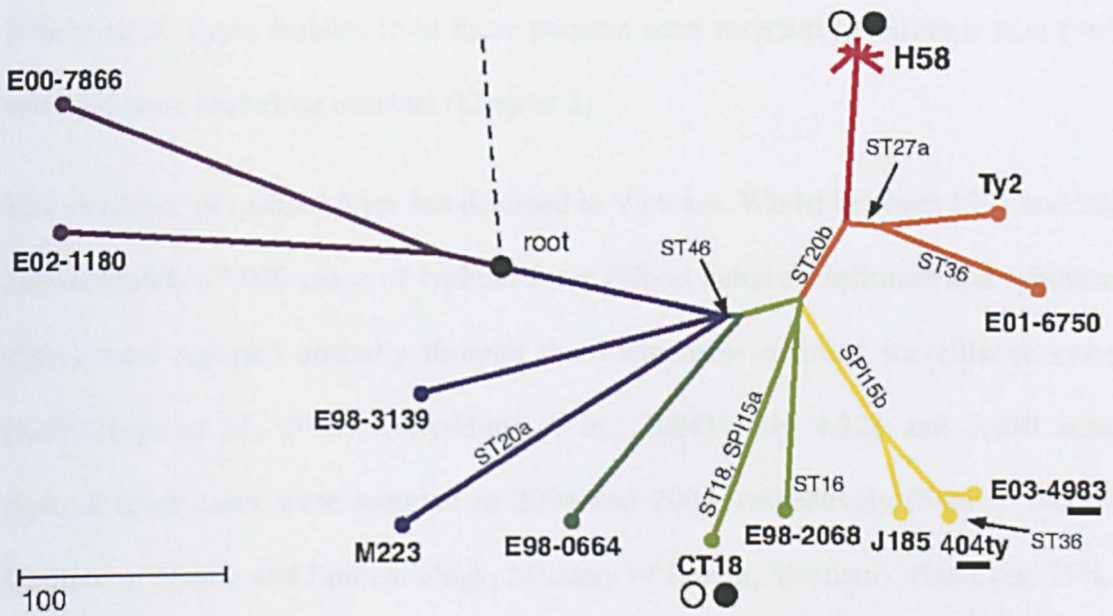


Figure 4.2 Phylogenetic tree of *S. Typhi* based on SNP data from whole-genome sequencing of 19 *S. Typhi* isolates.

From (Holt *et al.*, 2008). Branch colours indicate different lineages of *S. Typhi*; branch lengths are measured in number of SNPs (scale as indicated). Central, small black circle indicates the ancestral root, dashed line represent the *Salmonella* lineage; phage (ST) and SPI15 insertion events are shown along branches; plasmids detected in each isolate are indicated by filled circles (IncHII multidrug resistance plasmids), open circles (cryptic plasmid) and filled lines (linear plasmids carrying z66 flagella variant).

In this chapter we used a GoldenGate (Illumina) assay to type 1500 single nucleotide polymorphisms (SNPs) and analyse the genetic variation of *S. Typhi* isolated from 264 typhoid fever patients during a randomised multicenter treatment trial (reported in Chapter 2) conducted in the Mekong delta region of Vietnam between 2004 and 2005.

Nearly all *S. Typhi* isolates from these patients were resistant to nalidixic acid (96%) and 58% were multidrug resistant (Chapter 2).

The incidence of typhoid fever has declined in Vietnam. Whilst between 1991 and 2001 approximately 17,000 cases of typhoid fever (blood culture confirmed and syndromic cases) were reported annually through the Vietnamese national surveillance system (Kelly-Hope *et al.*, 2007; Kelly-Hope *et al.*, 2008), only 4,323 and 5,030 annual typhoid fever cases were reported in 2004 and 2005, respectively (Source: National Institute of Health and Epidemiology, Ministry of Health, Vietnam). However, 75% of these cases occurred in the Mekong delta (Kelly-Hope *et al.*, 2007; Kelly-Hope *et al.*, 2008).

4.2 Methods

4.2.1 *S. Typhi* isolates

S. Typhi isolates were collected during a multicenter clinical trial (described in Chapter 2), conducted at the (a) the Hospital for Tropical Diseases in Ho Chi Minh City (n=10), (b) Dong Thap Provincial Hospital, Cao Lanh, Dong Thap province (n=25) and (c) An Giang Provincial Hospital, Long Xuyen, An Giang province (n=232). Children (above 6 months) and adults were eligible to be included in the study if they had clinically suspected or culture confirmed uncomplicated typhoid fever and if fully informed written consent had been obtained.

4.2.2 Bacterial isolation and DNA preparation

After initial isolation, *S. Typhi* isolates were stored at -70°C in a 20% glycerol solution until required for further analysis and DNA extraction. To revive frozen organisms, MacConkey and Xylose Lysine Decarboxylase (XLD) agar plates were inoculated from the glycerol solution and incubated at 37°C overnight. To ensure correct identification, colonies were checked using slide agglutination with serotype specific antiserum (Vi, O9) and an irrelevant antiserum as a negative control (O4) (Murex, Dartford, United Kingdom). Two mL of nutrient broth were inoculated with single *S. Typhi* colonies and incubated overnight. Overnight cultures were centrifuged and *S. Typhi* DNA was extracted using Wizard Genomic DNA Purification kit (Promega, USA) as recommended by the manufacturer's guidelines. DNA was stored at -20°C and was quantified using the Quant-IT PicoGreen dsDNA Reagent and Kit (Invitrogen, UK). *S. Typhi* DNA concentrations were adjusted to 50 ng/mL and 250 ng of DNA were pipetted into 96-well plates. Each 96-well plate contained two isolates in duplicate and the sequenced *S. Typhi* isolate CT18 as control for assay reproducibility.

4.2.3 Selection of *S. Typhi* SNPs

S. Typhi SNPs identified by dHPLC (Roumagnac *et al.*, 2006) and by whole genome sequencing of 19 *S. Typhi* isolates (Holt *et al.*, 2008), were used to design a custom GoldenGate assay (Figure 4.3). In total, 1485 chromosomal SNPs, (listed in (Holt *et al.*; Kariuki *et al.*) and 172 IncH11 plasmid haplotype SNPs (Kariuki *et al.*; Phan *et al.*,

2009) including antibiotic resistance gene sequences were probed in sample *S. Typhi* using the GoldenGate assay.

4.2.4 High throughput SNP genotyping

The GoldenGate assay (Illumina) provides high throughput SNP genotyping platform. The assay was originally developed for high throughput SNP calling in complex human genetic linkage studies and is modified in this study for SNP identification in bacteria. The assay generates SNP specific PCR products that are subsequently hybridised to beads either on a solid matrix or in solution (Figure 4.3). Three oligonucleotides are synthesized for each SNP, two allele specific oligonucleotides that distinguish the SNP, and a locus specific sequence just downstream of the SNP. The allele specific and locus specific oligonucleotide sequences also contain target sequences for a set of universal primers (universal PCR sequences 1-3). Each locus specific oligonucleotide also contains a unique sequence (address or “illumicode”) which is complementary to sequences attached to the 3 μ M beads. The pooled oligonucleotides are hybridized simultaneously to genomic DNA in a single DNA sample per well reaction. Highly multiplexed allele specific oligonucleotide hybridization, ligation and extension assays are followed by universal PCR amplification, which reduces amplification bias to a minimum. The address sequences of the PCR amplification products hybridize with its cognate sequences on the 3 μ M beads in the 96-sample Sentrix Array Matrices (SAMs). The fluorescence on each bead is quantified. Each address sequence translates to a particular SNP locus and the presence of Cy3 and Cy5 indicates the allele. For the

GoldenGate array designed in this study it facilitated the interrogation of 1536 custom designed SNPs per sample.

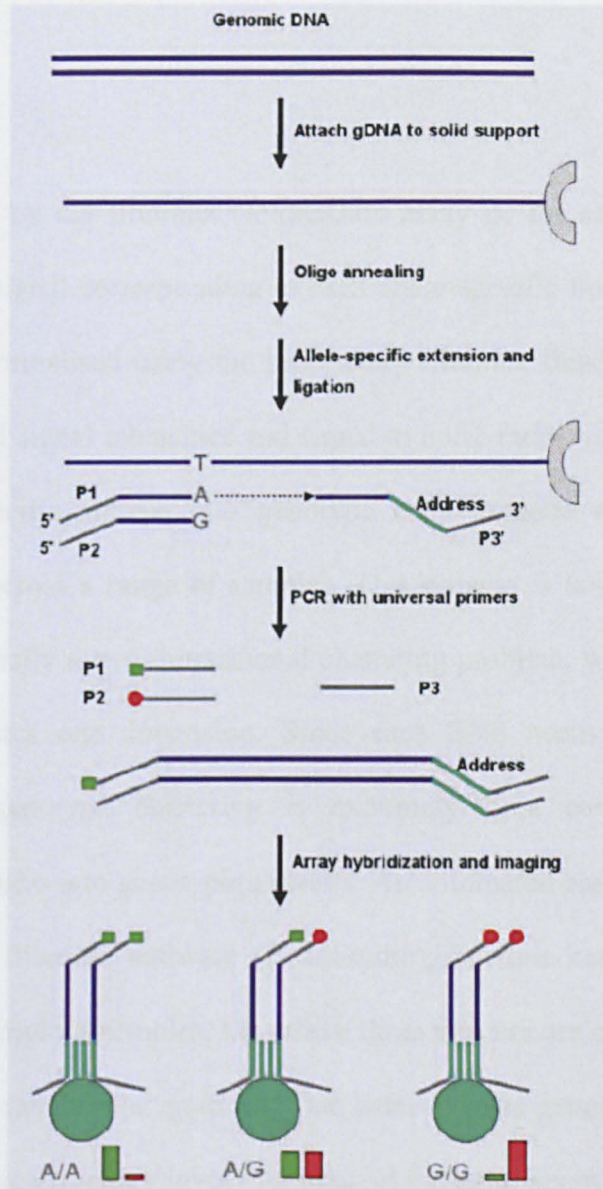


Figure 4.3 Schematic representation of GoldenGate assay workflow (GoldenGate, Illumina).

The GoldenGate assay uses allele-specific extension and ligation for genotype calling, enabled by an enzymatic discrimination step. Modified from www.illumina.com.

4.2.5 Genotype calling

The raw data provided by the Illumina GoldenGate assay is, for each SNP in each sample, a fluorescence signal corresponding to each allele-specific fluorescent-labelled probe. Raw data was normalised using the proprietary Illumina BeadStudio software, but the mean normalised signal intensities and signal to noise ratios vary among SNPs. Thus turning allele-specific signals into genotype calls requires each SNP to be analysed individually, across a range of samples. This process is known as genotype clustering, and is essentially a two-dimensional clustering problem, where each allele-specific probe contributes one dimension. Since each SNP needs to be clustered individually, manual genotype clustering is extremely time consuming and is considered to introduce bias into genotyping results. An automated clustering algorithm is implemented in the Illumina software (BeadStudio), but has been optimised for clustering genotypes in diploid samples, i.e. where three clusters are expected for each locus, corresponding to two homozygous and one heterozygous genotype (AA, BB or AB). However for the present study involving haploid bacterial genotyping, we expect no heterozygous genotype clusters but some 'no signal' genotype clusters. Illumina BeadStudio is proprietary software and unable to be modified, but third-party opensource genotype clustering algorithms are available. Among the best performing for the Illumina genotyping platform is Illuminus (Teo *et al.*, 2007), whose author Y. Teo modified to search for a third 'no signal' cluster centred at the origin rather than a heterozygous cluster. This version, referred to hereafter as Illuminus-P, was applied to genotype clustering of all GoldenGate data presented in this thesis.

4.2.6 Phylogenetic analysis

Alleles determined by genotype clustering with Illuminus-P as described above were analysed as follows. A Perl script was written to extract allele data from subsets of high-quality SNPs. The script outputs alignments of concatenated strings of SNP alleles in phylip format, suitable for analysis using phylogenetic methods. Chromosomal alleles from a global collection of 180 *S. Typhi* isolates plus 19 sequenced isolates were analysed in ModelTest (Posada and Crandall, 1998), a tool to select the best-fit model of nucleotide substitution (implemented in FindModel at <http://www.hiv.lanl.gov/content/sequence/findmodel/findmodel.html>). The analysis suggested a general time reversible (GTR) model provided the most appropriate phylogenetic model for these data. Phylogenetic analysis was performed using maximum likelihood approaches to fit a GTR model, implemented in the software program RAxML (Stamatakis *et al.*, 2005). SNP genotyping with the Illumina GoldenGate only provides genetic information at the specific assayed loci; in the case of chromosomal SNPs, these are mostly loci determined by whole genome comparison of 19 *S. Typhi* strains (Holt *et al.*, 2008). Thus the genotyping analysis essentially places each *S. Typhi* isolate at the appropriate position along branches defining the phylogenetic tree of these 19 strains, and branch lengths reflect genetic divergence only at the assayed loci. Short branches separating very closely related clusters (e.g. within the H58 group) were verified by manually inspecting cluster plots for SNPs that differentiated within those clusters.

4.2.7 Statistical analysis

Clinical data were deposited into an electronic database (Epi Info 2003, CDC, Atlanta, USA). For comparison of patient characteristics according to infecting *S. Typhi* haplotypes, Kruskal-Wallis tests were used for analysis of continuous variables (age, length of stay in hospital, fever clearance time) and χ^2 tests were used for categorical variables (presence of symptoms). Two-tailed p-values are reported; statistical analysis was performed using the R package (<http://www.r-project.org/>).

4.2.8 PCR amplification and sequencing of *gyrA* gene in *S. Typhi*

Oligonucleotide primers for the amplification of the quinolone resistance determining regions in *gyrA* gene in *S. Typhi* were as follows (Chau *et al.*, 2007): GYRA/P1 5' TGTCCGAG ATGCCTGAAGC and GYRA/P2 5' TACCGTCATAAGTTATCCACG. Predicted PCR amplicon size was 347 bp. PCR was performed under the following conditions; 30 cycles of: 92°C for 45 seconds, 45 - 62°C for 45 seconds and extension at 74°C for 1 minute, followed by a final extension step at 74°C for 2 minutes. PCR products were purified and directly sequenced using the CEQ DTCS - Quick Start Kit (Beckman Coulter, USA) and the CEQ 8000 capillary sequencer. The resulting DNA sequence was analyzed using CEQuence Investigator CEQ2000XL (Beckman Coulter, USA). All sequences were verified, aligned and manipulated using Bioedit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared to other *gyrA* sequences by BLASTn at NCBI.

4.2.9 Spatial data collection and analysis

Patient addresses were recorded at the time of hospital admission. The latitude and longitude of the residences of typhoid fever patients (to the hamlet/village level) was assigned from the collected address data using 1/50,000 scale maps (Source: Cartographic Publishing House and VinaREN, Ministry of Natural Resources and Environment, Vietnam) and cross-checked using the websites <http://www.basao.com.vn> and <http://ciren.gov.vn>.

Location data was analysed using Quantum GIS version 1.4.0 (<http://www.qgis.org/>). Locations were colour coded according to *S. Typhi* haplotype and clustering of specific haplotypes was calculated using the nearest neighbour analysis function. Nearest neighbour analysis examines the distances between each point and the closest point to it, and then compares these to expected values for a random sample of points from a CSR (complete spatial randomness) pattern. Significant clustering was inferred by Z-score value (standard normal variate) of less than 0; a positive score was interpreted as dispersion of locations.

4.3 Results

4.3.1 *S. Typhi* population structure

A recently developed typing system, which is based on the simultaneous interrogation of 1,485 single nucleotide polymorphisms (SNPs) distributed in the *S. Typhi* chromosome using a custom Illumina GoldenGate array, was used to analyse each of the *S. Typhi* isolates. This approach facilitates the unequivocal assignment of isolates to

haplotypes, allowing closely related strains to be distinguished phylogenetically based on single nucleotide changes (Holt *et al.*, 2010; Kariuki *et al.*, 2010). From the 287 patients with culture confirmed typhoid fever recruited between January 2004 and December 2005, 267 *S. Typhi* were available for SNP typing. This included 264 *S. Typhi* isolated from blood culture at admission (see clinical trial described in Chapter 2), one relapse *S. Typhi* isolate and two *S. Typhi* faecal carriage isolates. A total of 261 isolates (98%) were of the common H58 haplogroup. The remaining isolates were of haplotypes H1 (n=3), H45, H50 and H52 (see Figure 4.4 and Table 4-1).

The H58 *S. Typhi* isolates displayed variation at 10 SNP loci (detailed in (Holt *et al.*, 2010)), which differentiated seven distinct sub-H58 haplotypes, shown in Figure 4.4. However, 242 (93%) of these isolates belonged to just three closely related H58 haplotypes, designated C, E1 and E2 in Figure 4.4. The genome of *S. Typhi* strain AG3, isolated during the study (March 2004) from a typhoid fever patient living in An Giang province, was sequenced previously (Holt *et al.*, 2008). AG3 belongs to the H58-E2 haplotype, and the SNPs separating E2 from haplotypes E1 and C were originally identified by analysis of the AG3 genome. Therefore, the ability to differentiate within the cluster of 242 *S. Typhi* isolates was dependant on the inclusion of strain AG3 in the initial SNP detection study (Holt *et al.*, 2008).

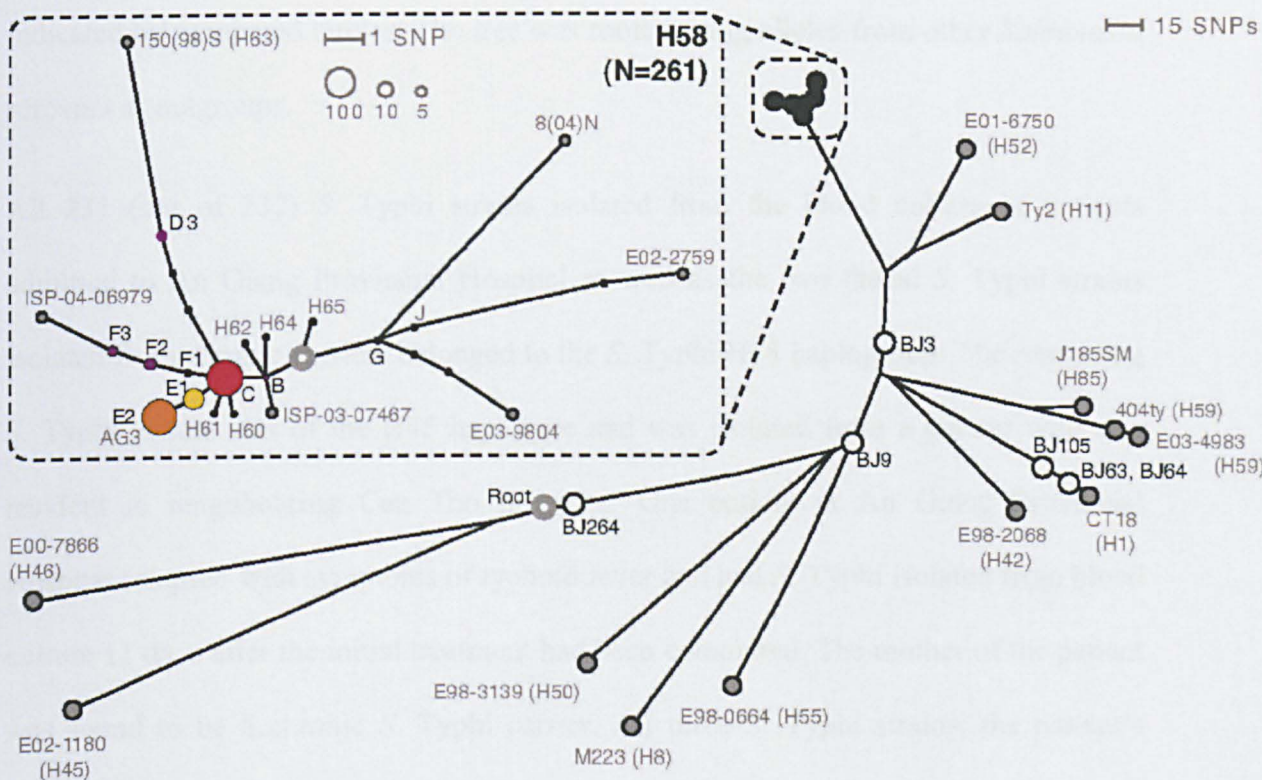


Figure 4.4 Phylogenetic distribution of *S. Typhi* isolates from the Mekong delta region of Vietnam.

From (Holt *et al.*, 2011).

Grey nodes represent control isolates (labelled by isolate code and haplotype group), unfilled grey circle indicates tree root (H45 (Roumagnac *et al.*, 2006)), white nodes correspond to non-H58 *S. Typhi* isolated in this study (labelled with isolate code), black nodes show H58 isolates. Inset: Enlargement of the H58 haplogroup; grey nodes represent control isolates (labelled by isolate code or haplotype code), unfilled grey circle indicates tree root; coloured circles indicate nodes corresponding to H58 *S. Typhi* isolated in this study, node labels are as in the text, node colours indicates the haplotype of the *S. Typhi* isolate, node sizes indicate the number of isolates on the scale as

indicated by numbered circles. The tree was rooted using alleles from other *Salmonella* serovars as outgroups.

All 231 (out of 232) *S. Typhi* strains isolated from the blood culture of patients admitted to An Giang Provincial Hospital as well as the two faecal *S. Typhi* strains isolated from chronic carriers belonged to the *S. Typhi* H58 haplogroup. The remaining *S. Typhi* isolate was of the H45 haplotype and was isolated from a patient who was resident in neighbouring Can Tho province. One patient at An Giang Provincial Hospital relapsed with symptoms of typhoid fever and had *S. Typhi* isolated from blood culture 11 days after the initial treatment had been completed. The mother of the patient was found to be a chronic *S. Typhi* carrier. All three *S. Typhi* strains, the patient's admission and relapse blood culture isolates and the mother's faecal isolate belonged to the *S. H58-E2* subtype. The patients' isolates were both MDR and carried the IncHI1 ST6 plasmid (see below), whereas the mother's *S. Typhi* isolate was susceptible to all first line antimicrobials at the time of isolation. Patients were followed up at 1, 3 and 6 months to obtain stool samples. Chronic faecal carriage of *S. Typhi* was detected in only one trial patient. This was a MDR H58-C strain isolated from stool after 6 months of follow-up, which was indistinguishable in all SNP loci from the patient's original blood culture isolate.

At Dong Thap Provincial Hospital, only 3 of the 25 *S. Typhi* isolates did not belong to the H58 haplogroup. Two H1 isolates (BJ63 and BJ64) were identical at all assayed SNP loci (Figure 4.4) and were isolated on consecutive days from two patients in Dong Thap. A third H1 strain (BJ105; Figure 4.4) differed from BJ63 and BJ64 at 16 SNP

loci and was isolated in Dong Thap 14 months after these isolates. Two siblings (BJ75 and BJ76) from Dong Thap province were admitted on two consecutive days in 2004 and were both infected with MDR *S. Typhi* of the haplotype H58-C.

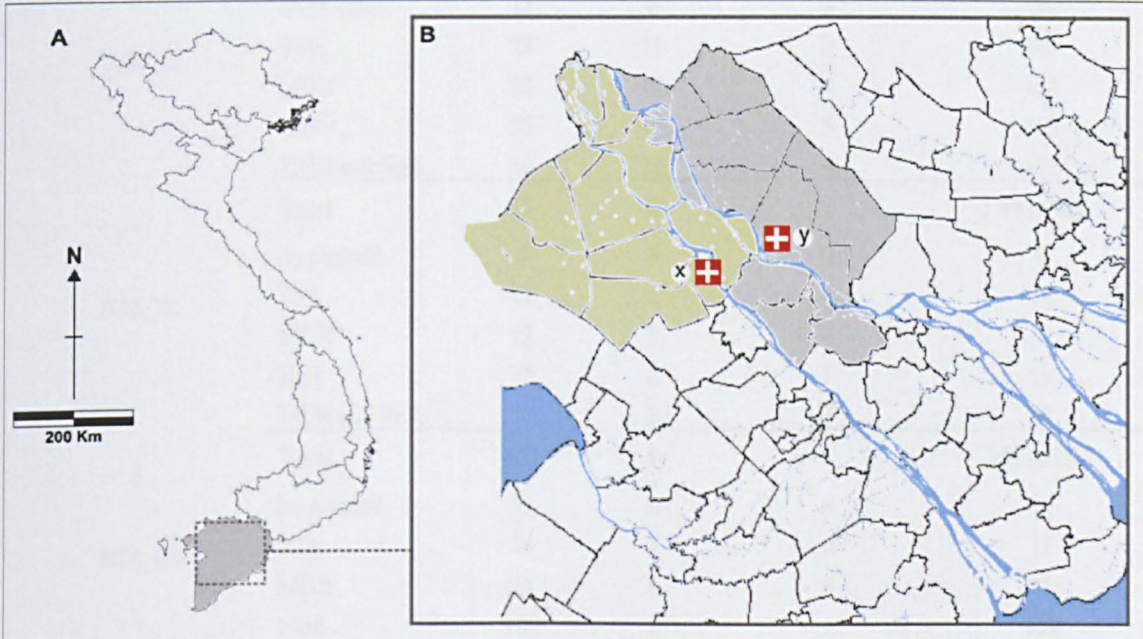


Figure 4.5 Location of the hospitals in the Mekong delta of Vietnam.

The map shows the 8 Vietnamese regions. Highlighted in grey is Mekong delta region in the south, which covers 40,000 km². The dotted box corresponds to the area magnified in Figure 1b. Highlighted in Figure 1 B are An Giang province (green) and Dong Thap province (grey) and the provincial hospitals of An Giang province (x) and Dong Thap (y). The direct distance between the two hospitals is 22.5 km.

S. Typhi isolates	Province	Province			Total study n=267
		An Giang n=232	Dong Thap n=25	Ho Chi Minh City n=10	
Characteristics		n	n	n	n (% of all isolates)
H58 haplotypes	Total	231	22	8	261 (98%)
	Total	95	17	6	118 (44%)
	no plasmid	17	6	3	26
H58_C	ST6	78	11	3	92
	MDR	86	12	4	102
	NaR	95	17	5	117
	MDR and NaR	86	12	4	102
	Total	12	2	1	15 (6%)
	no plasmid	0	0	0	0
H58_E1	ST6	12	2	1	15
	MDR	12	2	1	15
	NaR	12	2	1	15
	MDR and NaR	12	2	1	15
	Total	107	1	1	109 (41%)
	no plasmid	91	0	1	92
H58_E2	ST6	16	1	0	17
	MDR	20	1	0	21
	NaR	107	1	1	109
	MDR and NaR	20	1	0	21
	Total	17	2	0	19 (7%)
	no plasmid	3	0	0	3
Other H58 subtypes	ST6	14	2	0	16
	MDR	14	2	0	16
	NaR	14	2	0	16
	MDR and NaR	14	2	0	16
Non H58 haplotypes	Total	1*	3^a	2^a	6 (2%)
	no plasmid	1	3	2	6
	ST6	0	0	0	0
	MDR	0	0	0	0
	NaR	0	0	0	0
	MDR and NaR	0	0	0	0

Table 4-1 *S. Typhi* haplotypes according to region.

S. Typhi haplotypes are listed according to the hospital (An Giang Provincial Hospital, An Giang; Dong Thap Provincial Hospital, Dong Thap and the Hospital for Tropical Diseases, Ho Chi Minh City) where they were isolated.

*One *S. Typhi* H45 haplotype was isolated. &Three *S. Typhi* H1 haplotypes were collected. ^One *S. Typhi* isolate of the H50 and one of the H52 haplotype were isolated. NaR nalidixic acid resistance.

Of the ten *S. Typhi* strains isolated at the Hospital for Tropical Diseases in Ho Chi Minh City, eight were members of the H58 haplogroup, with patients reporting to be residents in Ho Chi Minh City (n=5), Long An (n=1), Kien Giang (n=2) and An Giang (n=1) provinces, reflecting the larger catchment area of the hospital. The remaining two *S. Typhi* were of haplotypes H52 (BJ3) and H50 (BJ9) and were isolated from patients living in Binh Hoa province (north of Ho Chi Minh City) and Ho Chi Minh City, respectively. There was no obvious association between *S. Typhi* haplotype and patient age, length of stay in hospital, fever clearance time or relapse (Table 4-2). However, upon admission, patients infected with *S. Typhi* haplotype H58-E2 tended to report lower frequencies of diarrhoea and headache and higher frequencies of constipation compared to patients infected with other haplotypes, including H58-C (see Table 4-2).

	<i>S. Typhi</i> H58-E2 n=107	Non-H58- E2 <i>S. Typhi</i> n=157	<i>S. Typhi</i> H58-C n=117	H58-E2 vs all other <i>S. Typhi</i> [95% CI]	p-value
Age (yrs)	11.9	12.2	12.7	Diff. -0.8 (-2.0,1.0)	0.83
Time in hospital (days)	13.9	13.7	13.8	Diff. 0.2 (-1.0,1.0)	0.74
Fever clearance (hrs)	116	115	121	Diff. 1 (-12,18)	0.70
Constipation	13.6%	5.8%	6.0%	OR 2.6 (1.1,6.1)	0.03
Headache	55.7%	70.1%	70.1%	OR 0.54 (0.32,0.90)	0.02
Diarrhoea	55.1%	72.6%	73.5%	OR 0.46 (0.28,0.78)	0.004

Table 4-2 Selected characteristics of 264 typhoid fever patients, based on baseline presentation, history and outcomes.

For continuous variables age, time in hospital and fever clearance, values shown are means and test statistic given is the difference in means (Diff.); other variables indicate frequency of constipation, headache or diarrhoea self-reported at time of admission, test statistic is odds ratio (OR); 95% confidence intervals are given in brackets. All comparisons shown are for patients infected with H58-E2 *S. Typhi* versus those infected with other *S. Typhi* haplotypes (including H58-C and others).

4.3.2 Plasmids and antimicrobial resistance

The GoldenGate SNP assay incorporated probes targeting IncHI1 plasmid sequences, allowing for detection of the presence of IncHI1 plasmid within the *S. Typhi* genomic DNA. The SNP assay indicated that a total of 139 of the *S. Typhi* isolates harboured an IncHI1 plasmid. All plasmids were of the IncHI1 ST6 sequence type (Phan *et al.*, 2009) and all plasmid-bearing isolates belonged to the *S. Typhi* H58 haplogroup (see Table 4-1). The MDR IncHI1 plasmid was more common among H58-C isolates than H58-E2 isolates (86% vs 19%, see Table 4-1). Of the 139 *S. Typhi* isolates giving positive signals for IncHI1 SNP loci, 137 (99%) were classified as MDR by antimicrobial susceptibility testing conducted at the time of isolation (see Chapter 2). One other IncHI1-positive isolate tested positive by GoldenGate SNP typing for the genes *sul1*, *sul2*, *dfrA7*, *tetACDR*, *strAB*, *bla* and *cat*, like the MDR isolates, yet had low MICs for chloramphenicol, ampicillin and co-trimoxazole. An additional *S. Typhi* isolate, BJ5, was resistant to ampicillin and co-trimoxazole but sensitive to chloramphenicol. This was consistent with GoldenGate results, which gave positive signals for the *repC* replication initiation gene of IncHI1, resistance genes *strAB*, *bla*, *sul1*, *sul2*, *dfrA7*, but no signal for sequences from the *cat* gene which encodes chloramphenicol resistance. A further 17 *S. Typhi* isolates were recorded as MDR according to their antimicrobial susceptibility pattern at the time of isolation, but did not test positive for IncHI1 plasmid SNPs. This likely reflects loss of the IncHI1 plasmid during culture or in storage between the time of isolation and DNA extraction.

A total of 257 *S. Typhi* isolates were resistant to nalidixic acid. All of these isolates belonged to the H58 haplogroup and all were susceptible to gatifloxacin, ciprofloxacin and ofloxacin according to current CLSI guidelines (Clinical and Laboratory Standards Institute, 2007). *S. Typhi* haplotypes H58-C, H58-E1 and H58-E2 were uniformly resistant to nalidixic acid with the exception of a single H58-C isolate which had an intermediate MIC of 28 µg/mL (resistance defined as MIC ≥ 32 µg/mL). The sequenced H58-E2 isolate AG3 harbours a mutation changing serine (TCC) to phenylalanine (TTC) at codon 83 in the *gyrA* gene (Holt *et al.*, 2008), which is known to confer resistance to nalidixic acid (Hopkins *et al.*, 2005). In the present study we sequenced the *gyrA* gene in 223 of the nalidixic acid resistant isolates (88%) and found the GyrA Ser83Phe amino acid substitution in all isolates tested.

4.3.3 Spatial and temporal distribution

Figure 4.6 shows the spatial distribution of the residences of 160 of the 267 typhoid patients (this information was not available for the remaining patients). At the time of the study prospective GPS mapping of patients' residences was not possible, however we used patients address data to allocate longitude and latitude. Of the patients admitted at An Giang Provincial Hospital and Dong Thap Provincial hospital, sufficient address detail to allow for assignment of latitude and longitude was provided in 61% and 73% of cases, respectively. In An Giang, the patients' homes clustered around the An Giang Provincial Hospital, but also around the Sông Hậu branch of the Mekong river (arrow in Figure 4.6). Mainly *S. Typhi* of the H58-E2 haplotype were isolated from patients living near this point in An Giang province and this group demonstrated significant

clustering using nearest neighbour analysis (orange in Figure 4.6; $n = 57$, $Z\text{-score} = -14.145$). In contrast, *S. Typhi* of the H58-C haplotype were isolated relatively frequently in neighbouring provinces and had a more sporadic, yet significant, clustering pattern (red in Figure 4.6; $n = 31$, $Z\text{-score} = -5.747$).

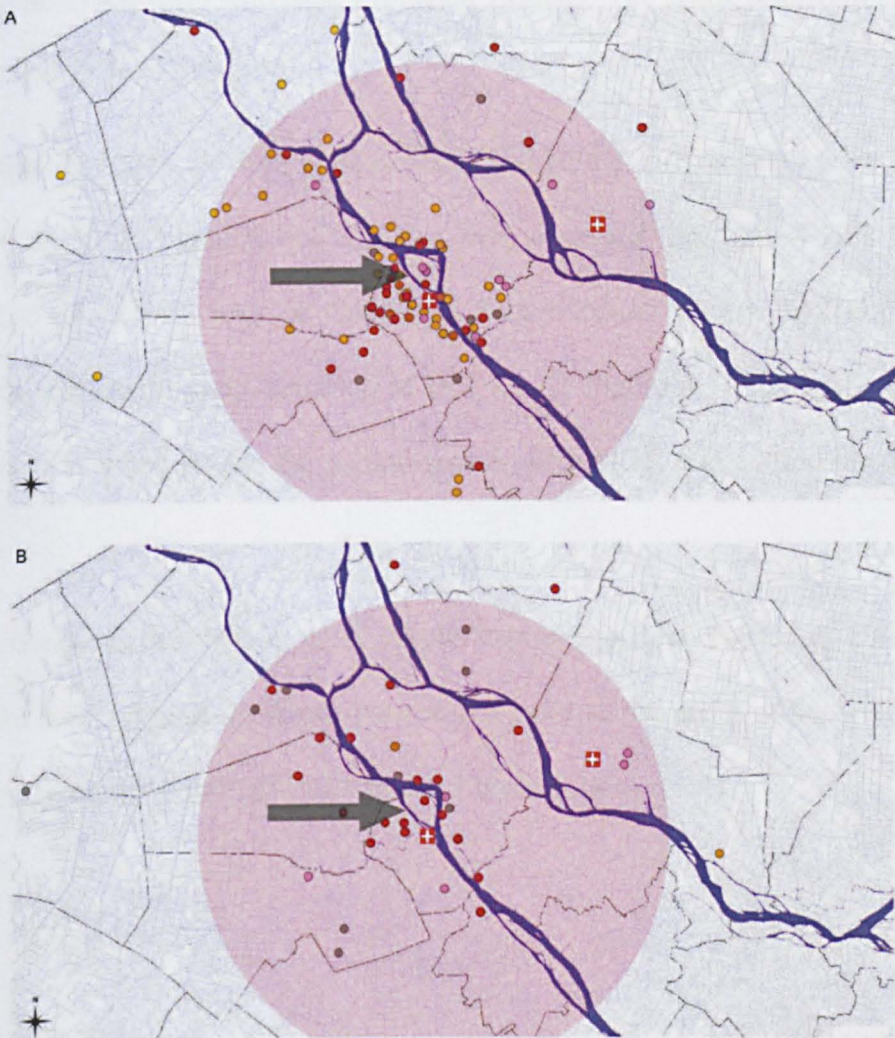


Figure 4.6 The spatial distribution of *S. Typhi* haplotypes surrounding An Giang provincial hospital in 2004 (a) and 2005 (b).

Each point corresponds to the residential location of a typhoid fever patient and the colour represents the haplotype of the *S. Typhi* isolate (with or without plasmid): dark orange, H58-E2 with MDR plasmid; light orange, H58-E2 without MDR plasmid; dark red, H58-C with MDR plasmid; pink, H58-C with MDR plasmid; grey, other *S. Typhi* haplotypes. Locations of the hospitals are shown by a white cross on a red background. The pink circle corresponds to a range of 15 km from An Giang Provincial Hospital. The arrow distinguishes the Sông Hậu branch of the Mekong river.

In 2004, *S. Typhi* H58-E2 (103 isolates) and H58-C (62 isolates) were both prevalent. In 2005, 55 *S. Typhi* H58-C isolates were observed, but only 4 H58-E2 Typhi. The decline of H58-E2 may be associated with selection for the IncHI1 MDR plasmid, which was much more common in H58-C. As Figure 4.7 highlights, the majority of isolates collected during the second season were MDR and carried the IncHI1 plasmid ST6.

The temporal distribution of *S. Typhi* haplotypes over 2004 and 2005 is shown in Figure 4.7. Typhoid incidence peaked just prior to the onset of the wet season in each year (see monthly rainfall, solid line in Figure 4.7).

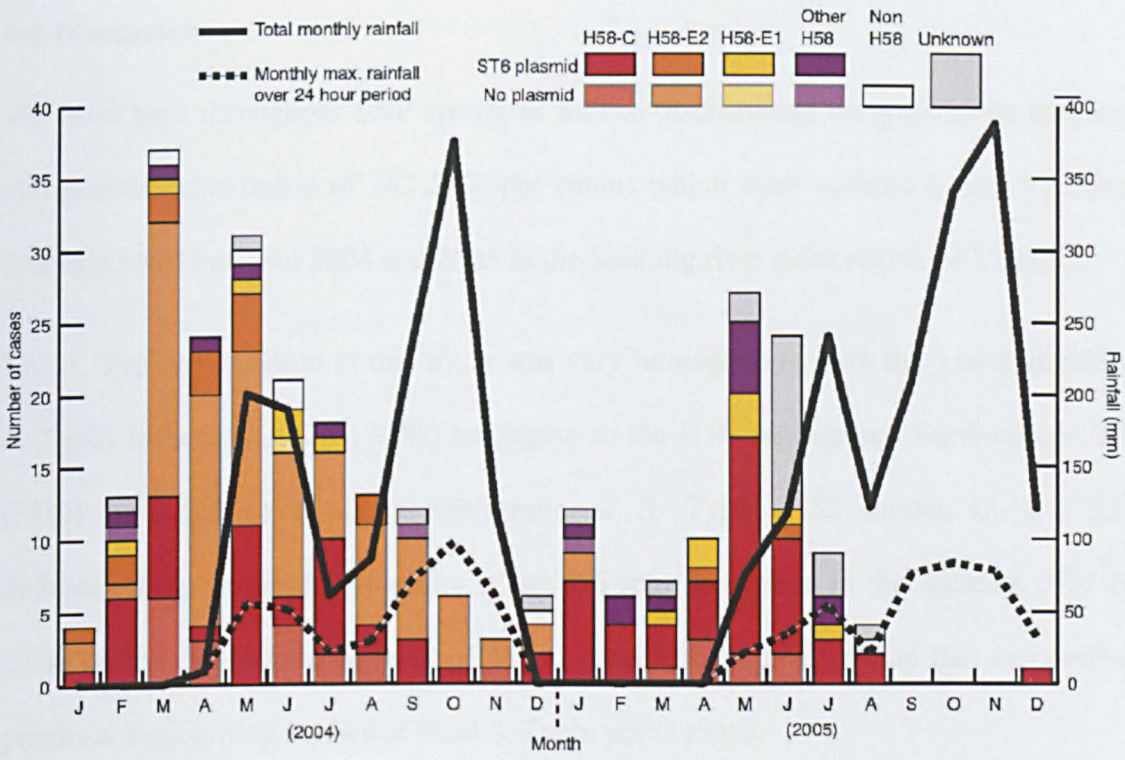


Figure 4.7 Monthly incidence of typhoid fever cases by haplotype in An Giang province

Bar heights indicate the total number of *S. Typhi* isolated each month during the study, according to the scale given on the left-hand y-axis; colours indicate *S. Typhi* haplotype and presence of IncHI1 plasmid as given in the legend. Solid black line = total rainfall each month recorded in An Giang, dashed line = maximum rainfall occurring in a 24 h period during each month in An Giang; scale given on right-hand y-axis.

4.4 Discussion

We used high throughput SNP typing as tool to discriminate the population structure and genetic relationship of 267 *S. Typhi* strains which were isolated during a clinical treatment trial between 2004 and 2005 in the Mekong river delta region of Vietnam.

The *S. Typhi* population in this study was very homogenous, with the vast majority of *S. Typhi* isolates (n = 261, 98%) belonging to the H58 haplogroup. Furthermore, 242 (91%) belonged to a single sublineage of *S. Typhi* H58 (nodes C, E1, E2), demonstrating remarkable clonality in the *S. Typhi* population in this location over the study period. The observed level of clonal dominance is greater than that observed in previous haplotyping studies of local *S. Typhi* populations.

Among 54 *S. Typhi* isolates from Jakarta, Indonesia, a total of nine haplotypes were detected, with the dominant H59 haplotype accounting for 53% of isolates; the next most frequent haplotype was genetically distant from H59 and comprised 24% of isolates (Baker *et al.*, 2008). In Kathmandu, Nepal, a collection of *S. Typhi* isolated from children hospitalised with typhoid fever was dominated by the H58-G haplotype (66%) but the distant H42 haplotype was also present at a significant frequency (19%) (Holt *et al.*). Among *S. Typhi* isolated between 2001 and 2008 in Nairobi, Kenya, 87% were H58, although two distinct lineages of this haplogroup (nodes B and J) were co-circulating at high frequencies (>40% each) over the period between 2004 to 2008 (Kariuki *et al.*). *S. Typhi* H58 nodes B and J (concomitant in Nairobi) represent distinct lineages, each acquiring unique SNPs since the last common ancestor of H58 (Figure 4.2). H58 nodes C, E1 and E2, which account for 91% of isolates in this study in

Vietnam, are closely related and formed a tight clonal complex differentiated by just two SNPs (Figure 4.4). The overall level of clonality of the *S. Typhi* population analysed in this study was unexpectedly high. The clonal complex comprising H58-C, H58-E1 and -E2 was not detected in study populations in Nepal and Kenya where the same SNP typing method was used (Holt *et al.*, 2010; Kariuki *et al.*, 2010), suggesting it may have arisen locally in Vietnam.

Despite the genetic homogeneity we observed, the availability of whole-genome sequence data for *S. Typhi* isolate AG3 (Holt *et al.*, 2008), collected during the study, allowed us to differentiate closely related organisms within the H58 group. Just two SNPs identified in strain AG3 (haplotype H58-E2) subdivided the homogeneous group into three nodes (Figure 4.2), of which two, subgroup C and E2 were dominant (> 40% of isolates each). Isolates belonging to the H58-C node were present at a constant rate during the two years of the study (62 isolates in 2004 and 55 in 2005). However, isolates belonging to the H58-E2 node were common during 2004 (103 isolates), yet were virtually undetected in 2005 (3 isolates). This change in both haplotype distribution and total number of typhoid cases from 2004 to 2005 is striking, and suggests an outbreak caused by *S. Typhi* H58-E2 during 2004. This decline is also reflected in hospital data from the An Giang Provincial Hospital, where in 2004 193 patients were admitted with blood culture confirmed typhoid fever, whereas in 2005 only 91 patients had *S. Typhi* isolated from blood culture.

It was additionally found that H58-C strains had a much stronger association with the ST6 IncHI1 MDR plasmid than H58-E2 (Table 4-2). It is possible to speculate that the

persistence of H58-C strains and the corresponding disappearance of H58-E2 may be associated with a competitive phenotypic advantage conferred by the IncHI1 MDR plasmid. However, it is important to remember that node C is a precursor of node E2 and we can only differentiate E2 from C because we had whole genome sequence data for an H58-E2 strain from which to identify SNPs (Holt *et al.*, 2008). Thus the population of *S. Typhi* isolates assigned to node C by our SNP typing assay may be more diverse than that assigned to H58-E2.

We identified two cases of chronic faecal carriage of *S. Typhi* during the course of the study, one in a patient's relative and one in a patient after 6 months of follow-up. This underlines the importance of screening procedures to identify carriers and effective treatment to eliminate carriage and reduce transmission. The faecal *S. Typhi* isolates were of the dominant H58-E2 and H58-C haplotypes, respectively. In a previous case-control study performed in the Mekong delta, close contact with a patient with typhoid fever was significantly associated with developing the disease compared to hospital controls (adjusted odds ratio (OR) = 5.2; 95% CI 1.7-15.9) or community controls (adjusted OR = 11.9; 95% CI 2.3-60.7) (Luxemburger *et al.*, 2001). This suggests that close contacts and faecal carriers play an important role in the transmission and persistence of these haplotypes in the community.

We were able to collect residential location data from 160 typhoid patients (61%); these provided roughly equal representation of patients infected with *S. Typhi* H58-C (65%) and H58-E2 (62%). Spatial clustering of *S. Typhi* H58-E2 was evident particularly around the Sông Hậu branch of the Mekong river, while other *S. Typhi* haplotypes were

more broadly distributed. The spatial clustering of H58-E2 *S. Typhi* further supports an outbreak in 2004 caused by these isolates. In contrast, the broader spatial and temporal distribution of *S. Typhi* H58-C during the study suggests it may be well established in the community and can persist over longer distances and time periods.

We also observed that some symptoms of patients infected with H58-E2 *S. Typhi* differed from those infected with other *S. Typhi* haplotypes (Table 4.2). Infections associated with H58-E2 *S. Typhi* were less likely to be associated with diarrhoea and headache compared with other *S. Typhi* haplotypes (Odds Ratio 0.46; 95% CI 0.28-0.78 and OR 0.54; 95% CI 0.32-0.9, respectively), but were more commonly associated with constipation (OR 2.6; 95% CI 1.1 – 6.1). This suggests there may be some differences between H58-E2 and other *S. Typhi* with respect to the disease phenotype, however these were post-hoc analyses, no adjustments were made for multiple comparisons and hence these associations should be interpreted with caution. However, if confirmed in subsequent prospective studies, it would be of interest to know whether these phenotypic characteristics were associated with specific mutations in the H58-E2 clone. The two SNPs differentiating the E2 node from E1 and C are both synonymous mutations (C->T in *mela* (nt 315); G->A in *rbsA* (nt 576)) and the phage insertions and large deletions detected in our earlier analysis of the AG3 strain sequence data were also detected in other sequenced H58 isolates (Holt *et al.*, 2008). However, we were unable to verify if other single-base insertions or deletions were present, which may result in gene inactivation with corresponding phenotypic effects.

Patterns of antimicrobial resistance of *S. Typhi* tend to vary markedly between different typhoid endemic regions. In this present work, as in the recent study of Kenyan isolates (Kariuki *et al.*, 2010), there were high rates of MDR associated with IncHI1 ST6 plasmids among strains of the *S. Typhi* H58 haplogroup. This suggests that the presence of the plasmid may contribute to the success of the dominant *S. Typhi* haplotypes, and the results of our study corroborate this hypothesis. The *S. Typhi* H58-E2 subtype (which was generally not associated with a plasmid) was only transient, while the H58-C subtype (which was more commonly associated with the IncHI1 MDR plasmid) was present in 2004 and 2005 in southern Vietnam. In a Kenyan study, almost all isolates of the dominant haplotypes carried the MDR plasmid, while the plasmid-free H58-G subtype was only detected twice (Kariuki *et al.*, 2010). All the H58 isolates analysed in the present study were resistant to nalidixic acid, conferred by an identical mutation in *gyrA*. This is consistent with previous studies reporting strong associations between the nalidixic acid resistance phenotype and the H58 haplogroup of *S. Typhi* (Holt *et al.*, 2010; Kariuki *et al.*, 2010; Roumagnac *et al.*, 2006). The presence of the same mutation conferring nalidixic acid resistance in all isolates of H58-C, -E1 and -E2 suggests that this mutation may have arisen in the common ancestor of this clonal complex, perhaps *in situ* in the Mekong delta region, and its continued presence is likely maintained by selective pressure exerted by the administration of fluoroquinolones.

Chapter 5

Safety and Immunogenicity of the Novel Single Oral Dose Live Typhoid Vaccine M01ZH09 in Healthy Vietnamese Children

5.1 Introduction

The previous chapters of this thesis have highlighted the problem of antimicrobial drug resistance of *S. Typhi*. In industrialised countries typhoid fever has been essentially eliminated. The most potent measures to eliminate typhoid fever are improvements in infrastructure and sanitation.

Typhoid vaccines are another effective strategy to prevent typhoid fever. Whole-cell typhoid fever vaccines have been introduced by Almroth E. Wright in England and by Richard Pfeiffer in Germany at the end of the 19th century. These vaccines were given to soldiers in developing and developed countries and were applied in mass vaccination campaigns in several countries in the 1960s (DeRoeck *et al.*, 2008; Engels *et al.*, 1998; Tarr *et al.*, 1999). The three year cumulative efficacy of the whole-cell typhoid vaccine was 73% for two doses (based on seven trials), however fever after vaccination occurred in 15.7% of recipients (Engels *et al.*, 1998). The high rates of adverse events necessitated the introduction of new generation vaccines.

There are currently two licensed, safe typhoid vaccines available, the live oral Ty21a vaccine and the parenteral (intramuscular injectable) Vi polysaccharide. The World Health Organization recommends that countries should consider the programmatic use of these typhoid vaccines for controlling endemic diseases and that the immunization of school age and/or pre-school age children should be undertaken particularly in areas where antibiotic resistant *Salmonella enterica* serovar Typhi (*S. Typhi*) is prevalent (World Health Organization, 2008).

Ty21a typhoid vaccine was developed in the 1970s by random chemical mutagenesis of the pathogenic *S. Typhi* strain Ty2. The mutations responsible for its attenuation include an inactivation of *galE* (which encodes UDP-galactose-4-epimerase, involved in synthesis of lipopolysaccharide), an inability to express Vi polysaccharide presumably through mutation and other mutations that are collectively responsible for the stable, highly attenuated phenotype (Germanier and Fuer, 1975; Kopecko *et al.*, 2009).

Ty21a vaccine is available in enteric-coated capsules or liquid formulation. It is administered in three doses (four doses in the US and Canada) two days apart and is licensed for adults and children above 6 years. Field studies with the oral Ty21a vaccine in the 1980s have shown a protective efficacy between of 96% after 3 years in Egypt (Wahdan *et al.*, 1982), 77% in Chile after 3 years when using the liquid formulation (Levine *et al.*, 1999) and up to 53% in Indonesia after 2.5 years {Simanjuntak, 1991 #920. Ty21a cannot be used in immunocomprised patients and pregnant women, and antibiotics should be avoided seven days before and after immunisation. Revaccination is recommended every 5 years.

The parenteral capsular polysaccharide Vi vaccine is given in a single dose (25 µg) and is licensed for individuals above 2 years. A single intramuscular injection conferred a protective efficacy of 77% after 21 months in South Africa {Klugman, 1987 #616}, 72% after 17 months in Nepal (Acharya *et al.*, 1987) and 61% after 2 years in India (Sur *et al.*, 2009). Revaccination is recommended every three years.

Similar to other T-cell-independent polysaccharide vaccines, Vi vaccine is not immunogenic in children under 2 years of age and does not induce immunological memory. To overcome this limitation, a conjugate Vi vaccine bound to recombinant protein *Pseudomonas aeruginosa* exotoxin A (rEPA) was developed (Szu *et al.*, 1994). Two doses of the conjugate Vi-rEPA vaccine administered 6 weeks apart had a protective efficacy of 91.5% in children aged two to five years in Vietnam after 27 months (Lin *et al.*, 2001), but this vaccine is not yet commercially available.

The major limitation of the Ty21a vaccine is the need to administer at least 3 doses. From a public health perspective, a single dose oral typhoid vaccine would have major advantages (Fraser *et al.*, 2007; Levine, 2003). Modern genetic techniques allow the construction of oral one dose typhoid vaccines with defined attenuating mutations. These next generation typhoid vaccines include *S. Typhi* strain Ty800 (attenuated by a double deletion in the genes *phoP* and *phoQ*) (Hohmann *et al.*, 1996), CVD908-*htrA* (attenuated by deletion mutations in *aroC*, *aroD* and *htrA* genes) (Tacket *et al.*, 2000) and M01ZH09.

M01ZH09 (*S. Typhi* (Ty2 *aroC ssaV*) ZH9) is a promising defined attenuated typhoid vaccine candidate, it has a well-defined dual mechanism of attenuation (Hindle *et al.*, 2002) and has been safe and immunogenic in a single dose in Western (Hindle *et al.*, 2002; Kirkpatrick *et al.*, 2005b; Kirkpatrick *et al.*, 2006) and Vietnamese adult volunteers (Hien TT, unpublished).

Historically, oral live vaccines often showed reduced immunogenicity in developing country populations compared to Western populations (Levine, 2006), therefore M01ZH09 was evaluated at an early stage of its development in children in an endemic country.

This chapter describes the results of a randomised placebo controlled trial that evaluated the safety and immunogenicity of M01ZH09 in 151 healthy Vietnamese children aged between 5 and 14 years.

5.2 Methods

5.2.1 Study design and objectives

The study was designed as a randomised placebo controlled single blind trial to evaluate the safety and immunogenicity of the novel oral single dose live typhoid vaccine M01ZH09 in Vietnamese children aged 5 to 14 years (inclusive).

5.2.2 The study site and ethical approval

The trial was conducted at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam. Ethical approval for the trial and all trial related documents was obtained from the Oxford Tropical Research Ethics Committee (OXTREC) and the Institutional Review Board of the Ministry of Health, Hanoi, Vietnam. The trial was conducted in accordance with the Declaration of Helsinki and its amendments and according to Good Clinical Practice guidelines and was monitored by Matrix Contract Research Ltd., UK

(now Novella Clinical). The trial was also conducted under an US Investigational New Drug (IND) license. Trial registration: www.controlled-trials.com/ISRCTN91111837.

5.2.3 Investigator and sponsor roles

The trial was sponsored by Emergent Product Development UK Ltd., UK and funded by a Strategic Translational Award from the Wellcome Trust, UK (grant code: B9RKYTO).

The data were subject to a confidentiality agreement between the sponsor and investigator, which established full access to the study data. Prior to publication the manuscript was reviewed by the sponsor but there was no obligation on behalf of the academic authors to modify the publication. The data entry and analysis of data were managed by Statwood (now Quanticate), UK, an independent Clinical Research Organisation.

5.2.4 Participants

Healthy Vietnamese children aged 5 to 14 years (inclusive) were invited to participate in the trial. Recruitment was carried out by word of mouth and flyers. Families who were interested in the trial were invited to attend one of several information evenings at the Hospital for Tropical Diseases. At these meetings the study was presented by the principal investigator (TTH) and all questions could be discussed and answered. Families who remained interested in the trial were invited to attend the screening visit. Children were eligible if they were available during the trial period and at least one of their parents gave written informed consent for their child to participate after the trial

procedures and potential risks were carefully explained by the study investigators. All children were invited to give their assent to the study and written informed assent was obtained from subjects starting at the age of 6 years. After informed consent was obtained, screening tests were performed. Children were screened by history, physical examination (including height, weight and vital signs), blood tests (biochemistry, haematology and HIV test), urine dipsticks and pregnancy tests (for female subjects of 11 years and above). Stool cultures were performed to check for the presence of *Salmonella species*.

Subjects with a history of typhoid fever, Ty21a vaccination in the last 10 years or any other typhoid vaccine in the last 5 years, any clinically significant illness, abnormal blood test results, immune suppression, positive HIV or pregnancy test were excluded. Also excluded were subjects whose body weight was under 17 kg in the 5 to 10 year old group or under 27 kg in the 11 to 14 year old group and subjects who suffered from an acute febrile illness at the time of dosing. Only one child per family was allowed to participate in the trial.

The results of the screening tests were reviewed and subjects who continued to meet the inclusion criteria were invited to continue in the trial.

5.2.5 The M01ZH09 vaccine and dose

S. Typhi (Ty2 *aroC ssaV*) ZH9 was constructed using a rational genetic attenuation strategy. Two defined independently attenuating deletion mutations were introduced into *S. Typhi* Ty2. Deletion of *aroC*, encoding chorismate synthase, prevents the

biosynthesis of aromatic amino acids and deprives the live vaccine bacterium of essential nutrients. Deletion of *ssaV*, encoding a structural component of the *Salmonella* pathogenicity island-2 (SPI-2) type III secretion system, which prevents systemic spread of *S. Typhi* (Hindle *et al.*, 2002). The vaccine was manufactured according to Good Manufacturing Practice protocols by Eurogentec S.A. and SynCo Bio Partners B.V; batch number M-STZH9-F16 was shipped to Vietnam. The vaccine kits were stored at 2-8°C.

Previous studies in adult volunteers demonstrated that a nominal dose of 5×10^9 CFU of the vaccine strain was immunogenic and safe (Hindle *et al.*, 2002; Kirkpatrick *et al.*, 2005b; Kirkpatrick *et al.*, 2006). The Ty21a oral typhoid vaccine capsules are licensed for adults and children above 6 years using the same dose and immunization schedule and large Ty21a field trials in children used the same dose and regimen as in adults (Levine *et al.*, 1987). It was therefore determined that the appropriate dose for the children's study was a nominal dose of 5×10^9 CFU of *S. Typhi* (Ty2 *aroC ssaV*) ZH9.

The vaccine (containing 5×10^9 CFU of vaccine strain plus excipients) and the placebo (vaccine excipients only) were supplied as freeze-dried formulations in single dose vials, which were labeled identically, containing "M01ZH09 oral typhoid vaccine or placebo" but with a unique subject number corresponding to the randomisation list. The bicarbonate solution was prepared by dissolving one effervescent bicarbonate tablet (provided in the vaccine kit and containing 2.6 g sodium bicarbonate, 1.65 g ascorbic acid and 30 mg aspartame) in 150 ml of bottled drinking water (final concentration: 1.75% wt/vol sodium bicarbonate, 1.1% wt/vol ascorbic acid, and 0.02% wt/vol

aspartame). The lyophilised vaccine or placebo was reconstituted in either 150 ml (for children above 10 years) or in 75 ml of the bicarbonate solution (the other 75 ml were discarded) for children below 10 years and was administered immediately.

The study used two age group specific randomisation lists, one for the 11 to 14 year old and one for the 5 to 10 year old children to ensure at least 70% children were between 5 to 10 years old.

5.2.6 Intervention

On the day of vaccination (day 0) which took place within 28 days of the screening, inclusion and exclusion (including history of antibiotic medication in the last 2 weeks) criteria were reviewed. Pregnancy tests (female subjects of 11 years and above only), urine dipstick test and stool cultures were performed. Blood samples for haematology, biochemistry, ELISA and ELISPOT assays (only in children 11 years and above) were obtained. After the subjects had fasted for at least 2 hours (with the exception of drinking water), the candidate typhoid vaccine or placebo was administered.

Subjects were allocated the next age-group specific subject number and the medication pack bearing the same number was prepared and issued by the pharmacist, who was otherwise not involved in the trial. The subjects were randomly assigned to receive either M01ZH09, consisting of 5×10^9 CFU of *S. Typhi* (Ty2 *aroC ssaV*) ZH9 or the placebo reconstituted in bicarbonate solution as described above.

Volunteers were observed for at least 90 minutes at the hospital. During this time pulse and blood pressure were recorded periodically and only drinking water was provided.

Diary cards were issued for all the volunteers and all subjects received a basic hygiene kit containing soap, gloves and spatulas for the collection of stool samples. The subjects and their parents were instructed to measure and record the oral temperature of the children twice daily (morning and evening) and to record any adverse events (including headache, fever, nausea, vomiting, abdominal pain, frequency and consistency of stools and any other symptoms) for 14 days.

5.2.7 Follow up procedures and monitoring of adverse events

Children were followed up daily from days 1 to 14 and again on day 28 after dosing. At these appointments diary cards were checked and adverse events and concomitant medication reviewed. A history of the last 24 hours with special emphasis on temperatures of 38.5°C and above and adverse events (diarrhoea, loss of appetite, vomiting, headache and chills) was obtained. Oral temperatures and vital signs were recorded and children were examined for signs of splenomegaly. Stool cultures were performed daily from day 1 to day 14. Blood samples for biochemistry and haematology were obtained on days 7, 14 and 28; for the LPS specific serum IgA ELISA on days 7 and 14; for the IgG ELISA on days 14 and 28 and for the LPS specific IgA antibody secreting cell (ASC) ELISPOT assay (only in subjects aged 11 years and above) on day 7. The total amount of blood taken during this study was approximately 28 ml from the 5 to 10 years old and 44 ml from the 11 to 14 years old children.

5.2.8 Unscheduled visits

Subjects and parents were instructed to make additional visits to the clinic, if the child felt unwell and/or had a fever of $\geq 38.5^{\circ}\text{C}$. At these visits the subject was assessed and samples taken for culture as clinically indicated. Blood cultures to investigate for the presence of *S. Typhi* in blood would be obtained if a fever of $\geq 39.0^{\circ}\text{C}$ was recorded twice over a 48 hours period, or a severe fever of $\geq 39.5^{\circ}\text{C}$ was recorded once.

5.2.9 Definition and reporting of serious adverse events

There was no Data Safety and Monitoring Committee for this trial. Data from all children were reviewed daily and there were *a priori* defined stopping rules which would trigger a suspension of the trial and a safety review. Serious adverse events were reported to AKOS Ltd (Hitchin, UK), a pharmacovigilance company within 24 hours.

5.2.10 Detection of *Salmonella* in stool samples at the screening visit and day 0

The detection of *Salmonella* species at the screening visit and on day 0 was performed according to microbiological standard procedures. In brief, stool samples were inoculated onto MacConkey agar and xylose lysine deoxycholate (XLD) agar plates, and in 10 ml of selenite F broth. Plates and broth were incubated at 37°C overnight and the broth was sub-cultured on MacConkey and XLD agar plates the next morning. Isolates were screened using standard biochemical tests and *Salmonella* were identified by slide agglutination with specific antisera (Oxoid Ltd., UK) and API20E profiling (bioMérieux, UK).

5.2.11 Detection of *S. Typhi* in stool samples

Stool samples were collected daily between days 1 and 14. Stool samples were cultured directly on deoxycholate citrate agar (DCA) Hynes plates (direct method) and in selenite F broth (enriched method). Both media were supplemented with aromatic compounds (DCA-aro and selenite F-aro, respectively) to detect *S. Typhi*, including the auxotrophic vaccine strain, in stools. Following overnight incubation at 37°C, an aliquot of the inoculated selenite F-aro broth was sub-cultured on DCA-aro Hynes plates. Suspected *S. Typhi* colonies were inoculated on brain heart infusion agar plates supplemented with aromatic compounds (BHI-aro). Oxidase negative colonies were evaluated by agglutination with Hd, Vi and O9 anti-sera (Oxoid Ltd., UK) and API20E profiling (bioMérieux, UK). Stool samples containing isolates that were positive in at least 2 out of 3 agglutinations and identified as *S. Typhi* by API20E profiling were considered to be positive for *S. Typhi*. All isolates were stored in 10% (v/v) glycerol at -80°C.

5.2.12 Detection of *S. Typhi* in blood samples

Blood samples were collected into either Bactec Peds Plus/F culture bottles (1-3 ml blood; BD, USA) or Bactec Plus Aerobic/F culture bottles (4-10 ml blood; BD, USA) and supplemented with aromatic compounds. Blood cultures were incubated at 35°C in the Bactec detection system and monitored for up to 5 days. Gram stain was performed on all bottles triggering a positive reaction. Positive cultures and all cultures that were negative after 5 days of incubation were sub-cultured on XLD agar plates. Suspected *S.*

Typhi colonies were sub-cultured onto BHI-aro agar plates. Oxidase negative isolates were evaluated by agglutination and API20E profiling (bioMérieux, UK) as above.

5.2.13 PCR identification of *S. Typhi* isolates

Genomic DNA was isolated from glycerol stocks of *S. Typhi* isolates using a DNeasy blood and tissue kit (Qiagen, UK). Multiplex PCRs were performed using a *Taq* PCR core kit (Qiagen, UK). Each reaction mixture contained 200 μ M dNTPs, 0.4 μ M *ssaV4* (5' ATCCCCACGACTTCAGCAAG 3') and *ssaV7* (5' CTTTCTGGCTCATCATGAGG 3'), and 0.1 μ M *aroC.Z1* (5' GACAACTCTTTCGCGTAACC 3') and *aroC.Z3* (5' TTACATCCGCATTCTGTGCC 3'), 10 ng genomic DNA and 1.25 u *Taq* DNA polymerase in a total volume of 50 μ l reaction buffer. PCRs were performed for 25 cycles as follows: 94°C for 30 sec, 57°C for 30 sec and 72°C for 2.5 min. The PCR products were visualised by ethidium bromide staining and UV transillumination after electrophoresis on a 0.8% (w/v) TAE agarose gel. The expected sizes of the PCR products were 1.04 kb (*aroC*) and 2.59 kb (*ssaV*) for *S. Typhi* wild-type strains and 0.45 kb (*aroC*) and 0.70 kb (*ssaV*) for *S. Typhi* (Ty2 *aroC ssaV*) ZH9.

5.2.14 Detection of antibody secreting cells producing *S. Typhi* LPS specific IgA antibodies by ELISPOT assay

ELISPOT assays to detect antibody secreting cells (ACS) producing *S. Typhi* LPS specific IgA antibodies were performed on days 0 and 7 as described previously (Kirkpatrick *et al.*, 2005a; Kirkpatrick *et al.*, 2006). In brief, whole blood was collected in

heparinised cell preparation tubes (Vacutainer CPT; BD, UK) and centrifuged. Peripheral blood mononuclear cells (PBMCs) were washed, resuspended in culture medium and adjusted to three cell concentrations ($1 \times 10^7/\text{ml}$, $5 \times 10^6/\text{ml}$ and $2.5 \times 10^6/\text{ml}$). One hundred microlitres of each concentration were added to LPS coated and uncoated wells (for subtraction of non-specific results) of nitrocellulose microtiter plates (Millipore, USA) and incubated overnight at 37°C in a 5% CO_2 incubator. PBMC collected from a healthy volunteer who had received three doses of Ty21a (Vivotif, Berna, Switzerland) were included as positive control and PBMC from a non-vaccinated person as negative control. Plates were washed and an alkaline phosphatase-conjugated anti-human IgA antibody (Immune Systems Ltd., UK) was added and incubated for one hour. Plates were washed and spots were visualised by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. Antibody secreting cell (ASC) spots were counted manually using an inverted microscope. If more than 100 spots per well were present, the result was described as “too many spots to be counted”. A positive response in the ELISPOT assay on day 7 was defined as ≥ 4 IgA ASC specific for LPS per 10^6 PBMCs and a negative response as < 4 IgA ASC specific for LPS per 10^6 PBMCs. Subjects with a day 0 result of ≥ 4 ASC per 10^6 PBMC were excluded from the ELISPOT analysis. ELISPOT assays were performed at the Hospital for Tropical Diseases.

5.2.15 Analysis of *S. Typhi* LPS specific serum IgG and IgA by ELISA and definition of a positive immune response

Quantitative ELISA methods for measuring *S. Typhi* LPS specific serum IgG and IgA were developed and qualified by Emergent Product Development UK Ltd using serum samples from recipients of M01ZH09 who participated in prior clinical trials and who had given informed consent for retention and usage of their samples. For the IgG ELISA, serum which demonstrated more than 4-fold increase from pre-dose in a previously described end point titre assay (Kirkpatrick *et al.*, 2005b) were pooled and used as reference standard. The LPS specific IgG concentration in the standard serum was set arbitrarily at 30,000 units/ml. For the IgA ELISA, serum from past recipients of M01ZH09 who demonstrated positive response in IgA ELISPOT were pooled and used as reference standard. The LPS specific IgA concentration in the standard serum was set arbitrarily at 100 units/ml.

The standard error of measured sample means and the least significant difference (LSD) between two samples at the 1% significance level were calculated using PRISM (PRISM Training & Consultancy Ltd, UK). This was used as the cut-off value for a positive result in the respective assay. A positive serum IgG response was defined as a 70% increase (fold change of 1.7) as compared to the corresponding baseline sample, whereas a positive serum IgA response was defined as a 50% increase (fold change of 1.5) as compared to baseline.

Serum samples for measurement were frozen at -20°C and shipped to Emergent Product Development UK Ltd for the ELISA analyses. For the IgG ELISA, microtiter plates were coated with *S. Typhi* LPS, washed and then blocked. Washing occurred between each step. Calibration standards and diluted test samples were added, and the plates were incubated. Bound IgG was detected using an anti-human IgG antibody conjugated to horseradish peroxidase (HRP) (Dako, Denmark) followed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. The plates were read at 450 nm within 30 minutes of stopping the reactions with 0.3 mol/L sulphuric acid. The standard curve was constructed by plotting the optical densities (ODs) of standards against concentrations and fitted by a 4-parameter logistic equation (SoftMax Pro 4.6, Molecular Devices, USA). The concentration of LPS specific IgG in each sample was determined from the standard curve. The IgA quantitative ELISA was performed in a similar manner, except using a double detection system of biotinylated anti-human IgA antibody (Southern Biotech, USA) followed by streptavidin-HRP conjugate (Dako, Denmark).

5.2.16 Outcomes of the study

5.2.16.1 Safety Outcomes

The primary safety endpoint was the proportion of subjects with any adverse events attributed to M01ZH09. The secondary safety endpoint was the proportion of subjects with any serious related adverse events; any related or unrelated adverse events; persisting faecal shedding of *S. Typhi* (Ty2 aroC⁻ ssaV⁻) ZH9 after day 7; and/or had a fever of 38.5°C or greater in the 14 days post vaccination, withdrew from the trial due

to adverse events, including bacteraemia, and/or had clinically significant changes in laboratory parameters related to the candidate vaccine.

All subjects who received a dose of the vaccine or placebo were analysed in the safety population. Post-vaccination adverse events were categorised according to body system and preferred term using the Medical Dictionary for Regulatory Activities (MedDRA, Version 9.1), allocated before unblinding. Adverse events were graded by severity (mild, moderate, severe) and judged for the relatedness to the study vaccine (unlikely, possibly, probably) by the investigator. Only possibly and probably related adverse events were attributed to the vaccine. Moderate fever was defined as an oral temperature of $\geq 38.5^{\circ}\text{C}$ and severe fever as an oral temperature of $\geq 39.5^{\circ}\text{C}$. Moderate diarrhoea was defined as more than 4 unformed stools and severe diarrhoea as more than 6 unformed stools in a 24 hour period or evidence of significant dehydration. All adverse events were recorded in the CRFs and monitored until return to normal.

The numbers and proportion of subjects reporting adverse events were listed by body system. A per subject analysis of adverse events was performed e.g., if a subject reported the same adverse event on three occasions that adverse event was only counted once. Subjects reporting more than one adverse event per body system were counted only once in that body system total.

5.2.16.2 Immunogenicity Outcomes

The primary immunogenicity endpoint was the proportion of subjects who developed a positive immune response to *S. Typhi* LPS defined by an increase of 70% (1.7 fold

change) in LPS specific serum IgG on day 14 or 28 and/or an increase of 50% (1.5 fold change) in LPS specific serum IgA on day 7 or 14 compared to baseline.

The secondary immunogenicity endpoints were defined as the proportion of subjects who developed a positive immune response in each of the following assessments: *S. Typhi* LPS specific IgA ELISA assay on days 7 or 14, *S. Typhi* LPS specific IgG ELISA assay on days 14 or 28 and *S. Typhi* LPS specific IgA ELISPOT on day 7. A positive ELISPOT was defined as ≥ 4 IgA antibody secreting cells specific for *S. Typhi* LPS per 10^6 PBMCs.

5.2.17 Sample Size

The planned sample size was 150 subjects, of whom at least 70% should be aged 10 years or younger, as this was the target age of the vaccine, randomised to M01ZH09 or placebo in a 2:1 ratio.

No formal sample size calculation was considered appropriate; it was aimed to include a sufficient sample size to assess safety and immunogenicity based on previous observations in adult studies and immunogenicity rates of licensed typhoid vaccines in children.

5.2.18 Randomisation procedures

Randomisation codes were computer generated in blocks of 9 by Statwood Ltd, UK. The vaccine and the placebo were labeled identically but with a unique subject number corresponding to the randomisation list. The study used two age group specific

randomisation lists, one for the 11 to 14 year old and one for the 5 to 10 year old children to ensure at least 70% children were between 5 to 10 years old.

Subjects were allocated the next age-group specific subject number in strict numerical sequence from this list and the medication pack bearing the same number was prepared by the pharmacist.

5.2.19 Blinding

This study was formally a single blind study due to slight differences in taste and aroma between the treatment preparations but it was conducted under the principles of a double blind study. M01ZH09 and placebo were packaged and labeled identically but with a unique sequential number. Possible sources of unblinding could have been the preparation of the vaccine. Therefore the study pharmacist was otherwise not involved in the trial. The subjects were asked to not report the taste of the vaccine. Microbiology results were not reviewed by the investigators for at least 14 days after vaccination to avoid potential unblinding through shedding in stools. Immunology results were not reviewed by the investigators. The study site received code break envelopes in case an emergency made unblinding for a single subject necessary. No codes were broken during this study. The unblinding of treatment allocations took place after the trial had been completed and the whole database had been entered and locked.

5.2.20 Data collection, data entry and statistical methods

All data were recorded in Case Record Forms (CRFs). CRFs were reviewed and collected by the study monitor. Data entry, data management and statistical analysis

were conducted by Statwood, UK using SAS software (version 9.1). Data were double entered and analysed according to an *a priori* defined statistical analysis plan which included the definition of all subject populations and the trial endpoints. The safety population included all subjects who received the study medication. The intention to treat (ITT) population comprised all dosed subjects who had any post-dose immunogenicity data available. The per protocol (PP) population excluded major protocol violators (failure to meet the inclusion/exclusion criteria, to comply with the study medication or use of other vaccinations or antibiotics two weeks before until 2 weeks after vaccination, or use of antacids or proton-pump inhibitors prior to vaccination and/or did not provide samples for the ELISAs). The protocol stated that a confirmatory analysis of the primary immunogenicity endpoint in the PP population was planned if more than 5% of subjects were excluded.

The proportion of subjects who experienced post-dose adverse events was presented together with their two-sided 95% confidence intervals (95% CI). Post dose adverse events, adverse events considered to be related to the vaccine and adverse events that occurred in more than 10% of the trial population were tested using a two-sided Fisher's exact test to compare between the two groups.

The proportion of subjects who developed a positive immune response was presented together with their two-sided 95% CI calculated by using an exact binomial distribution. The treatment difference and associated 95% CI were presented as above. All available data from withdrawn subjects was included in the analysis.

5.3 Results

5.3.1 Participant flow and recruitment

The trial was conducted between April and July 2007. In total, 205 healthy Vietnamese children between 5 and 14 years (inclusive) were screened for eligibility. Fifty-four children were not eligible (Figure 5.1), the most common reasons were unavailability for the whole study period ($n = 10$) and a positive stool culture for *Salmonella* species at screening (non-typhoid *Salmonella*, $n = 22$). No *S. Typhi* or *S. Paratyphi A* were detected in stools at the screening visits.

One hundred and fifty-one children were randomised, 101 children received the candidate typhoid vaccine M01ZH09 and 50 children received placebo. All subjects fulfilled the inclusion and exclusion criteria at screening and dosing, however two subjects (both in the M01ZH09 group) had clinically significant elevated white blood counts (16.3 and $18.2 \times 10^9/L$ respectively) on day 0, these results were only available after dosing.

One subject in the M01ZH09 group vomited after taking approximately 50% of the required vaccine dose. The subject agreed to take another dose, but failed to retain it. Three subjects withdrew from the study, one placebo recipient withdrew due to non-compliance (refused to provide stool samples) after day 2 and two vaccine recipients left the study after day 6 (one wished to withdraw, the second subject went on holiday). All three subjects attended the day 28 visit. The remaining subjects attended all study visits. Two subjects had unscheduled visits. One subject in the vaccine group attended

the clinic on day 16 because of fever of 38.1°C and one subject in the placebo group returned on day 26 with a temperature of 38.0°C. Blood cultures were obtained from both subjects and both cultures were negative. One M01ZH09 recipient had unscheduled tests performed. The subject presented on day 10 with a temperature of 38.6°C and reported diarrhoea, vomiting and fever on the previous day. The white blood count was elevated with $14.2 \times 10^9/L$ and the blood culture result was negative.

5.3.2 Numbers analysed

All 151 children who were randomised and received either M01ZH09 (n=101) or placebo (n=50) constituted the intention to treat (ITT) population. Seven subjects, 4 in the vaccine group and 3 in the placebo group were protocol violators (see Figure 5.1) and were excluded from the per protocol (PP) population. The analysis of the primary endpoints in the PP population was planned if more than 5% of subjects were excluded from the ITT. The PP population comprised 95% (144/151) of subjects and therefore no per protocol analysis was conducted.

All outcomes were evaluated for the ITT population.

5.3.3 Baseline data

The two groups did not differ significantly at enrolment in terms of sex, age and laboratory parameters (Table 5-1). One hundred and seven (71%) children were aged 10 years or younger.

Two subjects in the M01ZH09 group had clinically significant elevated white blood counts on day 0 (see above). Twelve subjects in total, five in the placebo group and seven in the M01ZH09 group (this included the subject who vomited on the day of dosing) had a positive stool culture for non-typhoid *Salmonella* on day 0, these results were only available after dosing.

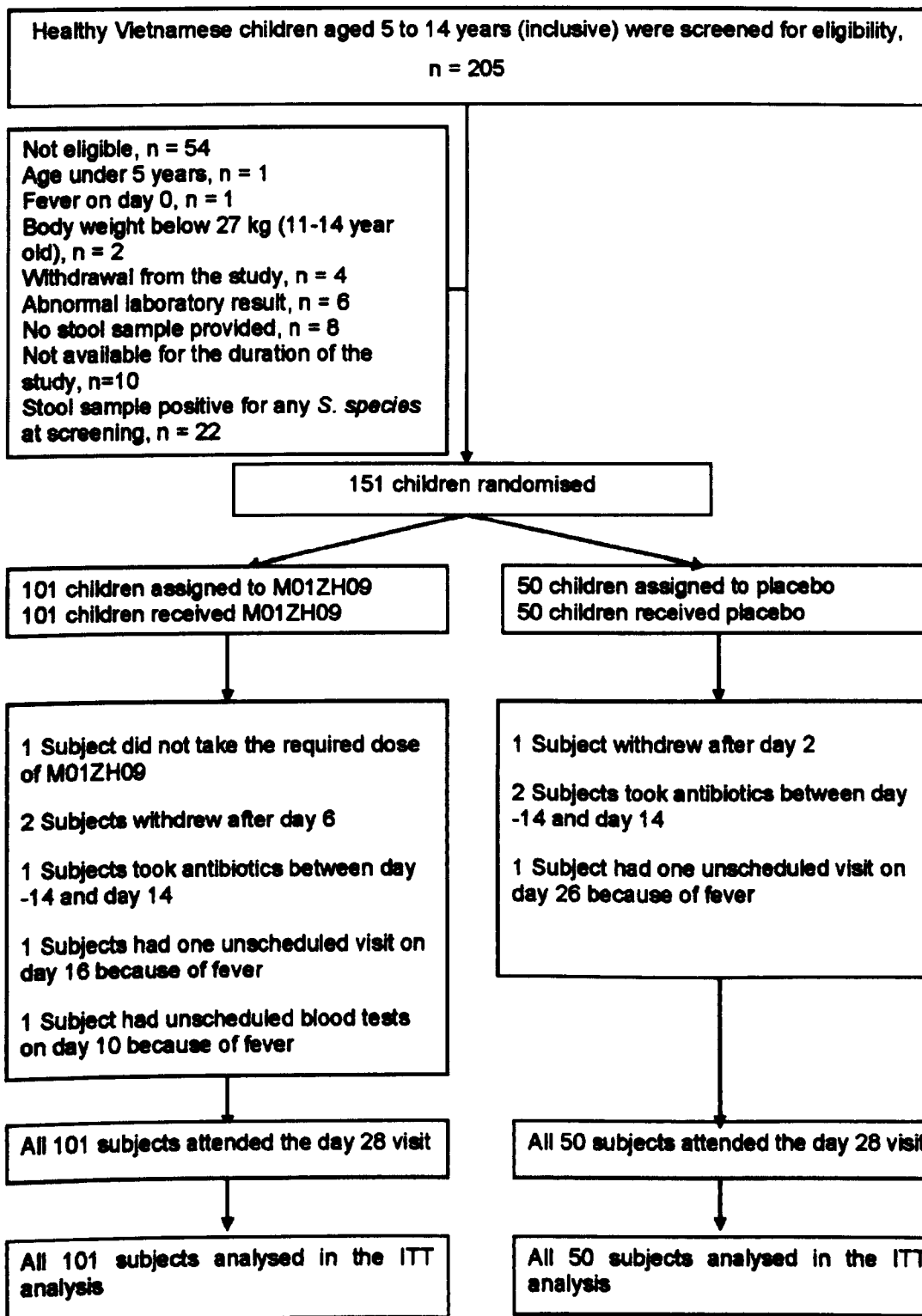


Figure 5.1 Flow of patients

Characteristics	M01ZH09 group (n = 101)	Placebo group (n = 50)	Overall (n = 151)
Age in years	9 (5-14)	9 (5-14)	9 (5-14)
Number of children under 10 years (%)	109 (75.2)	101 (71.1)	56 (80)
Number of males (%)	54 (53)	27 (54)	81 (54)
Weight in kilograms	28 (17-53)	26.5 (17-66)	27 (17-66)
Height in cm	132 (97-165)	130.50 (100-165)	132 (97-165)
Oral temperature in °C	36.80 (36.1-37.5)	36.75 (35.1-37.4)	36.80 (35.1-37.5)
Haemoglobin, g/dl*	13.3 (10.5-15.3)	13.4 (10.9-15.5)	13.3 (10.5-15.5)
White cell count, 10 ⁹ /L ^	7.2 (5-18.2)	8.0 (4.9-10.7)	7.4 (4.9-18.2)
Lymphocytes, %^	39.6 (14.9-55.5)	39.9 (21.4-67.4)	39.7 (14.9-67.4)
Neutrophils, %^	48.6 (29.2-70)	50.5 (19.5-73.8)	49.9 (19.5-73.8)
Monocytes, %^	5.3 (2.3-12.3)	5.0 (1.4-13.2)	5.2 (1.4-13.2)
Basophiles, %^	0.3 (0-0.7)	0.3 (0-0.7)	0.3 (0-0.7)
Eosinophiles, %^	4.0 (0.1-18.4)	3.0 (0.4-15.2)	3.8 (0.1-18.4)
Platelet count, 10 ⁹ /L &	295 (190-503)	311 (190-467)	300 (190-503)
AST, U/L	27 (14-52)	26 (14-51)	27 (14-52)
ALT, U/L	16 (6-58)	17 (5-43)	16 (5-58)
Creatinine, mM/L	0.47 (0.33-0.70)	0.47 (0.27-0.73)	0.47 (0.27-0.73)
Stool culture positive for <i>Salmonella sp.</i>	7	5	12

**Table 5-1 Baseline characteristics of the subjects on day 0
(Intention to Treat population).**

All data are presented as median (range) unless otherwise specified. Data from one*, two^ and seven& subjects not available.

5.3.4 Protocol deviations

The protocol stated that the primary safety endpoint would be the proportion of subjects reporting serious adverse events attributed to M01ZH09. Due to concerns that these numbers might be small and would not be sufficient to detect a difference in safety between the two groups, the primary endpoint was changed to the proportion of subjects with any related adverse event. This change was made after the completion of the clinical phase but prior to database lock and unblinding.

5.3.5 Outcomes and estimation

5.3.5.1 Safety Outcomes

There were no serious adverse events, no bacteraemia and none of the subjects withdrew due to adverse events in this trial.

Similar proportion of subjects reported adverse events in both treatment groups during the 28 days of follow up, but the total number of events was higher in the M01ZH09 group. In the vaccine group, 26 (26%; 95% CI 18-35%) of 101 subjects reported 64 adverse events compared to 11 (22%; 95% CI 12-36%) of 50 subjects in the placebo group who reported 17 adverse events (odds ratio (OR) = 1.23, 95% CI 0.550-2.747; $p = 0.691$) (Table 5-2). Repeated occurrences of a particular adverse event in the same subject were included in the total number of 64 and 17 adverse events, respectively. Of the 64 adverse events reported by M01ZH09 recipients, 55 were mild (8 of these were considered to be related to the candidate vaccine), 8 moderate (one related) and one was

severe and related to M01ZH09 (Table 5-2). Of the 17 adverse events in the placebo group, 12 were mild, 5 moderate and none was related.

Four (4%) M01ZH09 recipients experienced 10 adverse events that were related to the candidate vaccine compared to none in the placebo group ($p = 0.302$). Of these, 8 were mild, one moderate (diarrhoea) and one event of pyrexia was severe. The moderate and the severe related adverse events occurred in the same subject. This subject had a normal temperature on day 0, but the pre-dose blood test showed an elevated white blood cell count ($16.3 \times 10^9/L$). The subject experienced five post vaccination adverse events occurring on day 0, including fever of 38.5 and 39.0°C, diarrhoea, headache, abdominal pain and anorexia. The subject received paracetamol and recovered.

Similar proportions of subjects experienced fever post vaccination (Table 5-2), only one subject reported fever related to M01ZH09 (see above).

Adverse events classified as gastrointestinal disorders, nervous system disorders and investigations were experienced by a higher proportion of M01ZH09 recipients (Table 5-2). Twelve (12%) vaccine recipients experienced gastrointestinal disorders compared to 1 (2%) placebo recipient ($p = 0.061$). Nervous system disorders (headache) occurred in 9 (9%) vaccine recipients compared to 1 (2%) placebo recipient ($p = 0.166$) and investigations were reported by 4 (4%) vaccine recipients compared to none of the placebo recipients ($p = 0.302$). Cough was the most frequently reported adverse event, occurring in 6 (6%) M01ZH09 recipients versus 7 (14%) placebo recipients ($p = 0.124$).

On day 1 after vaccination, faecal shedding of *S. Typhi* occurred in 47 (49%) of 95 vaccine recipients; shedding was detected by the direct method in 11 (12%) subjects and by the enriched method of culturing stools in 36 (38%) subjects. On day 2 after vaccination, faecal shedding was detected in 12 (12%) of 97 subjects (in 1 (1%) subject by direct and in 11 (11%) subjects by enriched method). Only one (1%) of 98 subjects experienced shedding on Day 3 (detected by enrichment method). In total, 51 (51%; 95% CI 41-61%) of 100 M01ZH09 subjects experienced shedding on either days 1, 2 or 3 and no subjects experienced shedding on day 4 after vaccination or later.

The presence of *S. Typhi* was detected in the stools of 1 (2%; 95% CI 0-11%) of 50 subjects in the placebo group. This occurred on day 2 and was detected using the enriched method. No fever or adverse events were recorded for this subject. The finding of a positive stool culture for *S. Typhi* in a placebo subject was only available after unblinding of the trial. All previous and all sequential stool cultures up to day 14 of this subject were negative. This isolate was identified as the vaccine strain *S. Typhi* (Ty2 *aroC ssaV*) ZH9 by subsequent PCR analysis.

Seven (7%) of 101 M01ZH09 recipients and 3 (6%) of 50 placebo recipients were detected to have a positive stool culture for non-typhoid *Salmonella* between day 1 and 14 after vaccination.

Event	M01ZH09 (n=101)		Placebo (n=50)	
	Number of subjects	Percent (95% CI)	Number of subjects	Percent (95% CI)
Any adverse event	26	26 (18-35)	11	22 (12-36)
Gastrointestinal disorders (%)	12	12 (6-20)	1	2 (0-11)
<i>related to vaccine</i>	3	3	0	0
Abdominal pain (%)	8	8	0	0
<i>related to vaccine</i>	2	2	0	0
Constipation (%)	2	2	0	0
Diarrhoea	5	5	1	2
<i>related to vaccine</i>	3	3	0	0
Nausea	3	3	0	0
<i>related to vaccine</i>	1	1	0	0
Vomiting	3	3	0	0
General disorders and administration site conditions	9	9 (4-16)	5	10 (3-22)
Chills	1	1	1	2
Fatigue	0	0	2	4
Pyrexia	8	8	3	6
<i>related to vaccine and severe*</i>	1	1	0	0
Infections and infestations	1	1 (0-5)	0	0 (0-7)
Viral infection	1	1	0	0
Investigations	4	4 (1-10)	0	0 (0-7)
Urine colour abnormal	2	2	0	0
White blood cell count increased	2	2	0	0
Metabolism and nutrition disorders	3	3 (1-8)	0	0 (0-7)
Anorexia	1	1	0	0
<i>related to vaccine</i>	1	1	0	0
Decreased appetite	2	2	0	0
Nervous system disorders	9	9 (4-16)	1	2 (0-11)
Headache	9	9	1	2
<i>related to vaccine</i>	2	2	0	0
Respiratory, thoracic and mediastinal disorders	6	6 (2-12)	7	14 (6-27)
Cough	6	6	7	14
Rhinorrhoea	1	1	0	0
Skin and subcutaneous tissue disorders	1	1 (0-5)	1	2 (0-11)
Rash	1	1	1	2
Vascular disorders	1	1 (0-5)	0	0 (0-7)
Hypertension	1	1	0	0

**Table 5-2 Incidence of adverse events after vaccination during 28 days of follow up
(Intention to Treat population)**

Per-subject analysis of adverse events (unlikely, possibly and probably related to the vaccine) reported during 28 days of follow up. Subjects could experience more than one adverse event. Each adverse event was only counted once for each subject and system class. There were 56 adverse events in the M01ZH09 group and 16 in the placebo group, when repeated occurrences of a particular event in the same patient were only counted once. Adverse events that were possibly or probably related to the vaccine are presented in *italic*. * One severe adverse event was reported.

5.3.5.2 Immunogenicity Outcomes

Ninety-eight (97%; 95% CI, 92-99%) of 101 subjects in the M01ZH09 group and 8 (16.0%; 95% CI, 7-29%) of 50 subjects in the placebo group developed a positive immune response in either the *S. Typhi* LPS specific serum IgG or IgA ELISA, defined as the primary endpoint (Table 3). The difference in proportions of responders between the vaccine group and the placebo group was 81.0% (95% CI; 68-89%), the lower limit of the 95% CI of this difference was greater than 50% and fulfilled the a priori defined criterion for an acceptable immune response.

Median baseline LPS specific antibody levels were comparable in both groups (Figure 5.2). In the M01ZH09 group, median IgA antibody levels increased from 3 (IQR; 3-7.2) units/ml at baseline to 94 (IQR; 19.8-231.5) units/ml and 103 (IQR; 23.9-253.5) units/ml on days 7 and 14 respectively. On day 7, the 88 immune responders in the vaccine group (Table 5-3) displayed a median 16.4 (IQR 3.75-60.25) fold rise in serum IgA antibodies relative to baseline (Table 5-4).

In the vaccine group, median LPS specific IgG antibody levels were 66650 (IQR; 31075-123900) units/ml and 55700 (IQR; 25450-106800) units/ml on day 14 and 28, respectively, compared to median baseline levels of 6300 (IQR 3620-16560) units/ml. On day 14, the 91 immune responders in the M01ZH09 group (Table 5-3) showed a median 8.18 (IQR; 3.57-20.68) fold increase in serum IgG antibodies relative to baseline.

Forty-two out of 44 eligible subjects provided samples for the ELISPOT assay on day 7. All baseline ELISPOT samples were negative (defined as < 4 ASC per 10^6 PBMC). On day 7, 28 (100%) of 28 M01ZH09 subjects who provided samples showed a positive ELISPOT response compared to none (0%) of the 14 evaluable subjects in the placebo group. Sixteen (57%) of 28 M01ZH09 recipients displayed results of >100 spots per 10^6 PBMC and among the remaining 12 vaccine subjects numbers of spots ranged from 8 to 128 per 10^6 PBMC. The median number of spots in the M01ZH09 recipients was > 100 (IQR 46.5- >100) spots per 10^6 PBMC, as counting stopped above 100 spots, this was recorded as "too many spots to be counted". All 14 placebo recipients showed < 4 spots per 10^6 PBMC, this was recorded as "too few spots to be counted."

There was strong correlation between the results of the IgA ELISA and the IgA ELISPOT assays on day 7. Twenty-eight (100%) of 28 M01ZH09 recipients showed a positive immune response and 14 (100%) of 14 placebo recipients showed a negative response in both assays.

	M01ZH09 group n=101			Placebo group n=50		
	Positive Immune reponse			Positive Immune reponse		
	No.	No. (%)	95% CI	No.	No. (%)	95% CI
Detected in IgA ELISA assay						
Day 7	99	88 (89)	81-94	49	1 (2)	0-11
Day 14	99	92 (93)	86-97	49	1 (2)	0-11
Day 7 or day 14	99	94 (95)	89-98	49	2 (4)	1-14
Detected in IgG ELISA assay						
Day 14	99	91 (92)	85-97	49	6 (12)	5-25
Day 28	101	90 (89)	81-94	50	6 (12)	5-24
Day 14 or 28	101	93 (92)	85-97	50	8 (16)	7-29
Detected in either IgA or IgG ELISA assay						
Day 7, 14 or 28	101	98 (97)	92-99	50	8 (16)	7-29
Detected in IgA ELISPOT						
Day 7	28	28 (100)	88-100	14	0 (0)	0-23

Table 5-3 Proportions of responders to the candidate typhoid vaccine M01ZH09

(Intention to Treat population).

No., number of subjects who provided samples.

A positive immune response in the ELISA assay was defined by an increase of 50% (1.5 fold change) in LPS specific serum IgA and/or an increase of 70% (1.7 fold change) in LPS specific serum IgG compared to baseline.

*44 subjects aged 11 years and above (29 subjects in the M01ZH09 group and 15 subjects in the placebo group) were eligible for the ELISPOT.

A positive ELISPOT result was defined as ≥ 4 IgA antibody secreting cells specific for *S. Typhi* LPS per 10^6 PBMCs. None of the subjects had a positive day 0 ELISPOT result.

Day	M01ZH09 group		Placebo group	
	Median units/ml	IQR	Median units/ml	IQR
	n=101		n=50	
Serum IgA antibody levels specific for LPS				
Day 0	3	3-7.2	4	3-7.9
Day 7	94 [^]	19.8-231.5	3.1*	3-7.4
Day 14	103 [^]	23.9-253.5	3.4*	3-7.4
Serum IgG antibody levels specific for LPS				
Day 0	6300	3620-16560	6925	3950-10762.5
Day 14	66650 [^]	31075-123900	7680*	4170-11500
Day 28	55700	25450-106800	8175	4402.5-12437.5

Data from one* and two[^] subjects missing.

IQR interquartile range

Table 5-4 Serum IgA and IgG antibody levels specific for *S. Typhi* LPS (Intention to Treat population)

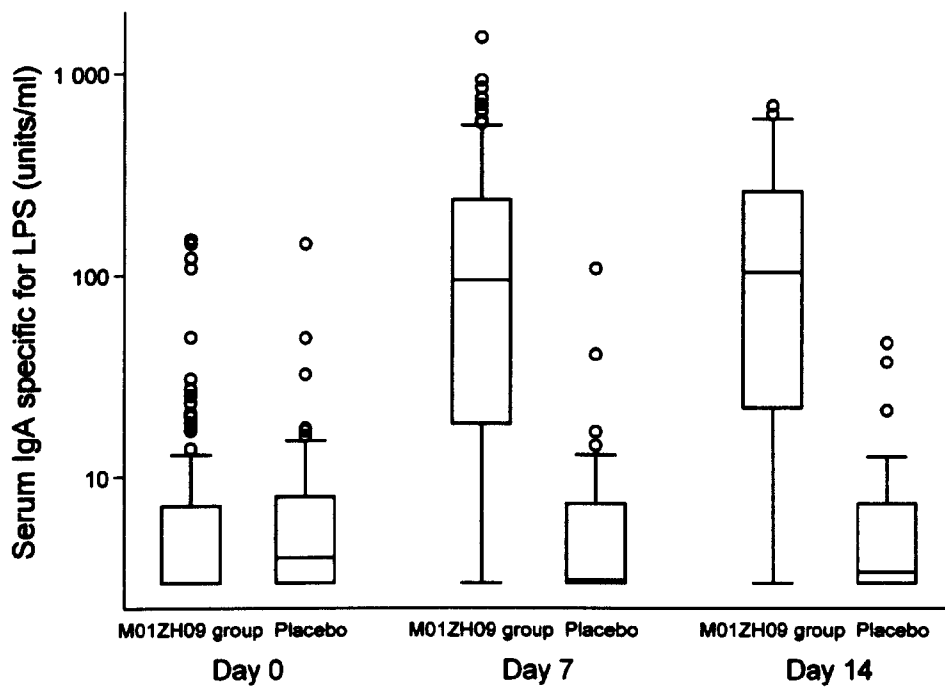
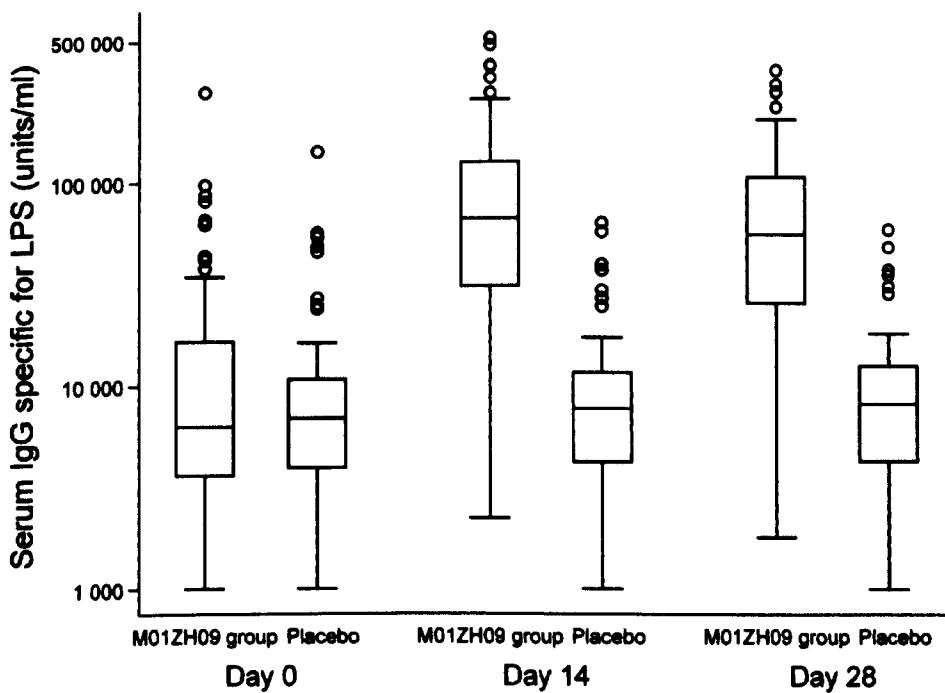
A**B**

Figure 5.2 Time course of LPS specific serum IgA and IgG antibody levels.

Time course of LPS specific serum IgA (A) and IgG (B) antibody levels, according to vaccination groups (Intention to Treat population).

Box and whisker plots showing the distribution of antibodies according to time point and vaccination groups. The horizontal line within each box represents the median, the top and bottom of each box represents the 75th and 25th percentiles, respectively, and the I bar represents the highest and lowest values within 1.5 times the interquartile range. Circles show outliers.

5.4 Discussion

This is the first evaluation of a novel oral typhoid vaccine in school children in an endemic country. *S. Typhi* (Ty2 *aroC ssaV*) ZH9 (contained in M01ZH09) is characterised by two well defined deletion mutations, one in an aromatic amino acid biosynthesis pathway gene and the other in a functional gene of the SPI-2 type III secretion system (Hindle *et al.*, 2002). A single dose of 5×10^9 CFU of the vaccine strain was well tolerated and had an acceptable safety profile. There were no serious adverse events, no withdrawals due to adverse events and none of the subjects experienced bacteraemia.

In general, adverse events were mild. Similar proportions of subjects, 26% (26 of 101) in the candidate vaccine group and 22% (11 of 50) in the placebo group reported adverse events during the 28 day follow up period ($p = 0.691$). The overall number of adverse events tended to be higher in the M01ZH09 group, especially those classified as gastrointestinal disorders, nervous system disorders and investigations.

There was one severe related adverse event in this trial, a high fever of 39.0°C which occurred on day 0 after vaccination in a subject who had a pre-dose elevated white blood count ($18.2 \times 10^9/L$) and might have suffered from an underlying infection. One other subject vomited after drinking approximately half of the vaccine dose, this subject was found to have a positive stool culture for non-typhoid *Salmonella* on day 0.

S. Typhi was isolated from the stools of one placebo recipient on day 2 after vaccination which was later identified as *S. Typhi* (Ty2 *aroC ssaV*) ZH9 by PCR

analysis. The previous stool cultures and all following stool cultures of this subject up to day 14 were negative. After a thorough check, which included the randomisation codes and vaccination paperwork, the possibility that the subject received M01ZH09 by error was excluded. The subject also did not display any positive results in the immunogenicity assays. It was concluded that the most likely cause for isolating *S. Typhi* (Ty2 *aroC ssaV*) ZH9 in the stools of a placebo recipient was the mislabelling or mismatch of stool samples.

The candidate vaccine elicited a positive immune response in 97% (98/101) of the M01ZH09 recipients by ELISA and in 100% (28/28) of M01ZH09 recipients who were evaluable by ELISPOT assay. In conclusion M01ZH09 was safe and immunogenic in Vietnamese children.

The observed safety and immunogenicity profile of the candidate typhoid vaccine in children compares favourably to that seen in Western adult volunteers. M01ZH09 has been tested so far up to a nominal dose level of 5×10^9 CFU in nine UK volunteers (Hindle *et al.*, 2002) and 80 US volunteers (Kirkpatrick *et al.*, 2005b; Kirkpatrick *et al.*, 2006). Immunogenicity results from previously published M01ZH09 trials used a 4 fold or higher increase in LPS specific IgG antibody levels as definition of a positive immune response in the endpoint titre ELISA and seroconversion rates were 50% (8/16 subjects) (Kirkpatrick *et al.*, 2006) and 77.4% (24/31 subjects) (Kirkpatrick *et al.*, 2005b). In this study, allowing for these different cut-offs, the magnitude of the immune response seen in the children was approximately 30 fold and 10 fold increase of median levels of LPS specific IgA and IgG antibodies, respectively. Furthermore. the

median number of ASCs producing LPS specific IgA antibodies, a measure for priming of the mucosal immune system, was greater than 100 per 10^6 PBMC in this trial, this compares favourably to an arithmetic mean of 118 ASC/ 10^6 PBMC seen in a previous M01ZH09 trial in adults (Kirkpatrick *et al.*, 2006) and a geometric mean of 119 ASC/ 10^6 PBMC (producing IgA and IgG) seen in American volunteers who received 4 doses of the licensed Ty21a typhoid vaccine at a dose of $2-6 \times 10^9$ CFU (Kantele *et al.*, 1998).

This is encouraging as one major concern for the development of many oral vaccines has been their reduced immunogenicity when tested in developing country populations compared to Western volunteers (Dougan *et al.*, 2002; Levine, 2006). For oral vaccines a brisk colonisation of the intestine is necessary to become immunogenic, it might be possible that drug resistant commensals, bacterial overgrowth, enteric viruses or helminths interfere with the colonisation of the new vaccine (Levine and Campbell, 2004). In this study, 51% (51/100) of vaccine recipients shed *S. Typhi* (Ty2 *aroC ssaV*) ZH9 in stools after vaccination, one subject excreted the vaccine strain on day 3, but no shedding was observed on day 4 and beyond. In Western adult volunteers shedding of *S. Typhi* in stools was reported for slightly longer durations and ranged from 1 to 6 days and 1 to 7 days in a small number of volunteers, respectively (Hindle *et al.*, 2002; Kirkpatrick *et al.*, 2005b).

Typhoid fever is still a major health problem in developing countries, with high incidence (Crump *et al.*, 2004; Ochiai *et al.*, 2008) and high rates of antimicrobial drug resistance, especially in Asia (Chau *et al.*, 2007; Ochiai *et al.*, 2008). The WHO

recommends the immunisations of school and preschool children in endemic areas, especially where drug resistant typhoid fever is prevalent as well as in epidemic situations (World Health Organization, 2006, 2008). M01ZH09 is a promising novel oral one dose typhoid vaccine and large trials are necessary to evaluate vaccine efficacy. If protection from typhoid fever is demonstrated, M01ZH09 may facilitate large vaccination campaigns due to its simpler logistic and broader acceptance from children.

Chapter 6
Conclusions

This thesis aims to evaluate different aspects of typhoid fever and to provide better understanding of this disease. This chapter will summarise key findings from the thesis and point at future avenues of research.

Chapter 2 described a randomised clinical trial conducted in the Mekong Delta in Vietnam that compared gatifloxacin to azithromycin for the treatment of multidrug and nalidixic acid resistant typhoid fever. Two hundred and eighty-seven patients, both adults and children, with blood culture confirmed typhoid fever were enrolled, 145 were treated with gatifloxacin and 142 patients with azithromycin. Both antibiotics showed an excellent efficacy and safety profile. The median FCT was 106 hours in both arms and overall treatment failure (defined as any of the following: persistence of fever till day 10, need for re-treatment, blood culture positive on day 7, development of complications, relapse or faecal carriage) occurred in approximately 9% of patients in both arms (13/145 patients in the gatifloxacin group and 13/140 patients in the azithromycin group, respectively). Ninety-six percent (254/263) of the *S. Typhi* isolates were resistant to nalidixic acid and 58% (153/263) were multidrug resistant.

This trial was part of a series of clinical trials to evaluate the antibiotic regimen recommended for the treatment of typhoid fever by the WHO (World Health Organization, 2003). Particular attention was paid to the design of these trials, to respond to criticism made by the authors of a recent Cochrane review (Thaver *et al.*, 2008). Typically clinical trials in typhoid fever have been small, outcomes have been defined at the discretion of the investigators, length of follow up varied, and they have often not included children, although this is the age group predominantly affected by

the disease (Thaver *et al.*, 2008). The trial presented in this thesis and subsequent work (not included here) aimed to address these reasonable criticisms by designing large clinical trials using a standardised trial design with adequate sample sizes that include all age groups to facilitate subsequent meta-analyses.

Blood culture, the gold standard for the diagnosis of typhoid fever is estimated to have only 40% to 60% sensitivity (World Health Organization, 2003; Parry *et al.*, 2002). Therefore, a large proportion of patients in typhoid trials is blood culture negative for *S. Typhi* and only diagnosed clinically (World Health Organization, 2003), until now these patients have been excluded from the analysis. The trial described in Chapter 2 reported an *a priori* defined “intention to treat” analysis including blood culture negative patients, as well as the “per protocol” analysis of those patients with blood culture confirmed typhoid fever. In my view this has addressed some of the criticisms from the Cochrane review (Thaver *et al.*, 2008) and has helped establishing standard methods for the design of clinical trials in typhoid fever.

These excellent results for gatifloxacin seen in this trial are supported by two subsequent trials conducted in Kathmandu, Nepal (Arjyal *et al.*, 2011; Pandit *et al.*, 2007). In total, these two trials enrolled 265 patients with culture confirmed typhoid fever who were treated with gatifloxacin (Arjyal *et al.*, 2011; Pandit *et al.*, 2007), both trials also monitored daily blood glucose levels. There were no clinically relevant dysglycaemias in these young patients.

Gatifloxacin has also been used for the treatment of pulmonary tuberculosis. A randomised controlled trial evaluating gatifloxacin-containing short-course (4 months) regimen against standard regimen for the treatment of pulmonary tuberculosis has been conducted in 5 African countries (Benin, Guinea, Kenya, Senegal and South Africa) between 2005 and 2011 (Olliaro P., 2011). A total of 1836 patients have been recruited in this trial, 917 were randomised to the gatifloxacin arm. The incidence rates of dysglycaemic events were similar in the gatifloxacin and control arms (Olliaro P., 2011).

As outlined in the discussion of Chapter 2, gatifloxacin was withdrawn from the US and Canadian market following a report of increased risk of dysglycaemia in elderly Canadians (Park-Wyllie *et al.*, 2006). A group of investigators including myself has recently submitted an application to WHO to include gatifloxacin in the Essential Medicines List (EML) for the indications typhoid fever and tuberculosis (Olliaro P., 2011). Unfortunately, this has been rejected by the WHO committee in April 2011 on the basis, that there were alternative therapeutic options available. We are in the process of challenging the decision from the WHO committee and discussing options to try and ensure gatifloxacin remains available for specific populations and indications.

There is clearly a different risk/benefit ratio of gatifloxacin in different populations. Whilst there have been side effects of hypo- and hyperglycaemia in multimorbid elderly patients, possibly because of age-related decreases in renal function (Ambrose *et al.*, 2003), these side effects have not been seen in young and otherwise healthy patients. We have few drugs available for the treatment of multidrug and nalidixic acid resistant

typhoid fever. The third generation cephalosporins are no real alternatives, as intravenous ceftriaxone shows slow fever clearance and clinical response (average FCT of one week) (Parry *et al.*, 2002), and a clinical trial using oral cefixime had to be stopped early by the Data and Safety Monitoring Board due to poor performance (Pandit *et al.*, 2007).

From the evidence of these studies in predominantly young and otherwise healthy patients gatifloxacin is effective, safe and affordable. It would be a shame if gatifloxacin were not available for the treatment of typhoid fever and tuberculosis in young people due to adverse events in a different patient population, i.e. elderly multi-morbid Canadians. We are running out of antibiotics, we should be careful before we discard effective and safe drugs.

A lot of thought and effort has been put into the development of protocols and the design of case record forms (CRFs) during these studies. These resources will be made available for other researchers on www.enterics.org, which will be linked with the e-research hub, accessible via <http://ght.globalhealthhub.org/>.

The WHO guidelines (World Health Organization, 2003) which still recommend cefixime for the treatment of multidrug and nalidixic acid resistant *S. Typhi*, urgently need to be updated on the basis of clinical evidence. The Oxford University Clinical Research Unit Vietnam is involved in producing up-to-date guidelines in collaboration with the Coalition Against Typhoid.

Chapter 3 described the pharmacodynamic analysis of the gatifloxacin arm of the trial presented in Chapter 2. A dichotomous categorical breakpoint for AUC₀₋₂₄: MIC ratio was identified. Patients in whom an AUC₀₋₂₄: MIC ratio of greater than 92.7 was obtained, had a favourable response to treatment in 93.5%, whilst in patients with AUC₀₋₂₄: MIC ratios \leq 92.7 only 75% had a favourable response. A borderline significant dichotomous categorical gatifloxacin MIC breakpoint for *S. Typhi*, predictive for clinical response, could be identified. *S. Typhi* gatifloxacin MIC values \geq 0.19 mg/L were associated with 83.8% of patients having a positive clinical response, while patients with gatifloxacin MIC values less than 0.19 mg/L had in 94.3% positive response. In gatifloxacin treated patients infected with nalidixic acid susceptible (n=6) and nalidixic acid resistant (n=118) organisms, clinical success rates were 100% and 91%, respectively. Thus, based on the nalidixic acid screening test, nalidixic acid susceptibility predicts success 100% of the time (n=6/6), whereas nalidixic acid resistance predicts failure only 9.3% of the time (n=11/118). Therefore the nalidixic acid screening test is not suited to predict gatifloxacin failure.

There has been criticism that the CLSI fluoroquinolone breakpoints for *Salmonella* were too generous. The MIC breakpoints for ciprofloxacin against extraintestinal *Salmonella* have been recently revised by CLSI and these new breakpoints will be published in 2012. In June 2011 the gatifloxacin breakpoints will be reviewed. The data presented in Chapter 3 has been made available to CLSI.

Chapter 4 described the population structure of the *S. Typhi* strains isolated during the clinical trial. The *S. Typhi* isolates were very homogenous, the vast majority of *S. Typhi*

(n = 261, 98%) in the Mekong delta region belonged to the H58 haplogroup, which expanded and persisted under antibiotic pressure.

Chapter 5 looked at novel and improved typhoid vaccines and described the safety and immunogenicity trial of a novel oral one dose typhoid vaccine in healthy Vietnamese children.

The adverse events were generally mild and the vaccine showed acceptable immunogenicity in these children. The next step would require an efficacy trial in an endemic region, however these trials are prohibitively expensive, at an estimated cost of 1 Million US per 1000 subjects. Therefore the next phase for the M01ZH09 vaccine is a typhoid challenge study that is currently being performed at Oxford University, UK (Principal Investigator Professor Andrew Pollard).

This thesis has addressed clinical issues, in particular aiming to establish a standard design for randomised clinical trials in typhoid fever and the use of pharmacokinetic and pharmacodynamic parameters, a better understanding of the population structure of *S. Typhi* in a region of high transmission and the assessment of a one dose oral typhoid vaccine.

In 2011, it is the tenth anniversary of the publication of the genome sequence of *S. Typhi* CT18, isolated in Vietnam (Parkhill *et al.*, 2001a). A lot has been accomplished in the last decade since this seminal publication, but much still remains to be done.

We need to define the best treatment strategies and ensure we design trials in a way that permits more informative meta-analyses. There has been renewed interest in the

development of new vaccines for typhoid and paratyphoid fever and also for non-Typhi *Salmonella*. Although there are great challenges to the development of these vaccines, it should be possible. Perhaps a greater question is whether these vaccines can be fully developed and made available to low income countries.

It is possible to consider the elimination or even eradication of typhoid fever. To do this a multidisciplinary approach would be required, including appropriate treatment of patients with effective antibiotics that cure the patient and also prevent secondary transmission and chronic carriage, the use of appropriate vaccines and the improvement of the infrastructure in low-income countries.

Publications arising from this thesis

1. Gatifloxacin versus chloramphenicol for uncomplicated enteric fever: an open-label, randomised, controlled trial. Arjyal A, Basnyat B, Koirala S, Karkey A, Dongol S, Agrawaal KK, Shakya N, Shrestha K, Sharma M, Lama S, Shrestha K, Khatri NS, Shrestha U, Campbell JI, Baker S, Farrar J, Wolbers M, Dolecek C. *Lancet Infect Dis*. 2011 Jun;11(6):445-54. Epub 2011 Apr 29.
2. Gatifloxacin for treating enteric fever. Submission to the 18th Expert Committee on the Selection and Use of Essential Medicines (2010). P. L. Olliaro, C. Dolecek, J. Farrar, B. Basnyat, T. T. Hien, J. Horton, P. Garner, P. G. Ambrose, N. J. White, K. Fielding, C. Merle, C. Sismanidis. http://www.who.int/selection_medicines/committees/expert/18/applications/Cochrane_Gatifloxacin.pdf
3. Temporal fluctuation of multidrug resistant salmonella typhi haplotypes in the mekong river delta region of Vietnam. Holt KE[^], Dolecek C[^], Chau TT, Duy PT, La TT, Hoang NV, Nga TV, Campbell JI, Manh BH, Vinh Chau NV, Hien TT, Farrar J, Dougan G, Baker S. *PLoS Negl Trop Dis*. 2011 Jan 4;5(1):e929. [^]These authors contributed equally.
4. The burden and characteristics of enteric fever at a healthcare facility in a densely populated area of Kathmandu. Karkey A, Arjyal A, Anders KL, Boni MF, Dongol S, Koirala S, My PV, Nga TV, Clements AC, Holt KE, Duy PT, Day JN, Campbell JI, Dougan G, Dolecek C, Farrar J, Basnyat B, Baker S. *PLoS One*. 2010 Nov 15;5(11):e13988.
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Relationship thesis-publications

Chapter 1 of this thesis is in part based on the Review “Typhoid fever and other enteric fevers” written for “Infectious diseases. J. Cohen, W.G. Powderley (Eds.)”. Parts of Chapter 1 have been used for the gatifloxacin EML application to WHO (http://www.who.int/selection_medicines/committees/expert/18/applications/Cochrane_Gatifloxacin.pdf). The results of the clinical trial reported in Chapter 2 have been published in PLoS One (Dolecek *et al.*, 2008). The discussion has been updated to include more recent trials. Part of the results described in Chapter 3 of this thesis, have been reported at the Interscience Conference on Antimicrobial Agents and Chemotherapy (ICAAC) 2008, Washington D. C. Chapter 4 is based on a publication in PLoS Negl Trop Dis. 2011 (Holt *et al.*). The results described in Chapter 5 have been published in PLoS ONE 2010 (Tran *et al.*).

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Appendix

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.

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Introduction

Enteric fever is a disease that predominantly affects children and is caused by the faecal–oral transmission¹ 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^{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.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.⁷ Additionally, no trials have been done to investigate the efficacy of chloramphenicol versus a newer fluoroquinolone, such as gatifloxacin, in the

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treatment of enteric fever in children.¹³ Recent reports suggest a general decline in the prevalence of MDR typhoid fever in Asia,⁹⁻¹¹ 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*.²⁰

Gatifloxacin was effective in the treatment of nalidixic-acid-resistant enteric fever in two previous randomised trials done in Nepal¹⁶ and Vietnam.²⁰ The drug targets both DNA gyrase and topoisomerase IV,^{21,22} 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

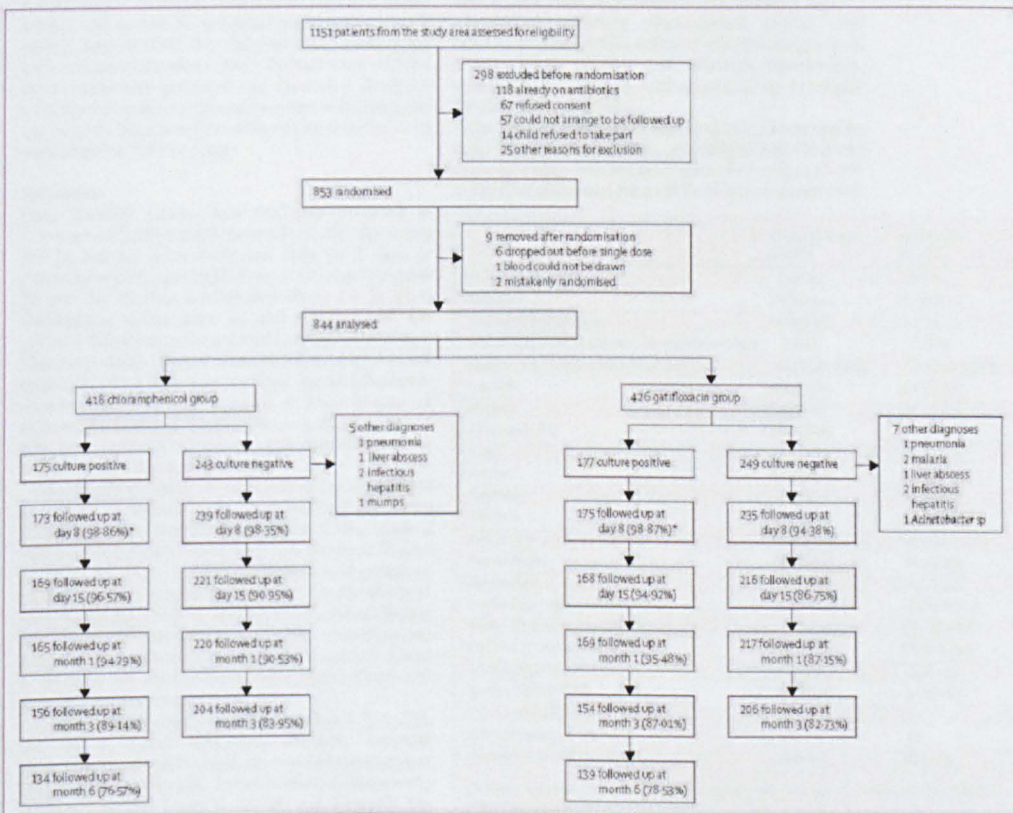


Figure 1: Trial profile
*Two culture-positive patients in both the chloramphenicol and gatifloxacin groups were lost to follow up before day 8.

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 cotrimoxazole 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 previously¹⁶ 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 (BioMérieux, Paris, France) and slide agglutination with specific antisera (Murex Biotech, 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 (43.3%)	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 ($\times 10^9/L$)	6.4 (5.0-8.1)	6.2 (5.1-8.1)
Median (IQR) platelet count ($\times 10^9/L$)	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)
<i>Salmonella typhi</i> isolated	125	124
<i>Salmonella paratyphi</i> A isolated	50	55
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 -0.02 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 estimate); *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 $<37.5^{\circ}\text{C}$ 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.

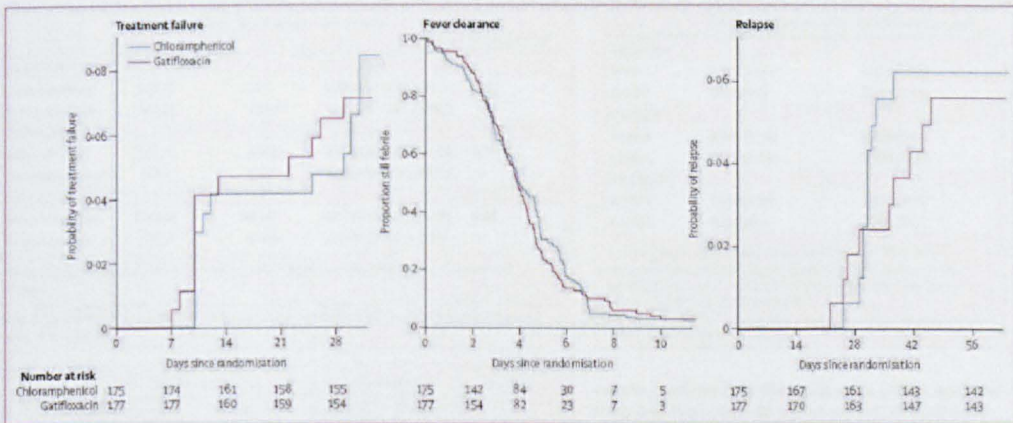


Figure 2: Kaplan-Meier estimates for time to treatment failure, fever clearance, and relapse for culture-positive patients

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 2.9.1.²³

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 ^a	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 0.93 (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	-
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	-
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). ^aPatients 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 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				
<i>Salmonella typhi</i>	11/125	8/124	0.73 (0.29-1.82, p=0.50)	0.51
<i>Salmonella paratyphi A</i>	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

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

	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 2000-2500-10⁹/L. Grade 2: WBC count 1500-1999-10⁹/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

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%)	0.50
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 $\times 10^6$ 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, $p=0.21$).

	<i>Salmonella paratyphi</i> A (n=103)	<i>Salmonella typhi</i> (n=249)	p value
Chloramphenicol			
MIC 50 (μ g/ml)	8.00	4.00	–
MIC 90 (μ g/ml)	12.00	8.00	<0.0001
Range	2.00–64.00	1.50 to >256.00	–
Amoxicillin			
MIC 50 (μ g/ml)	1.00	0.50	–
MIC 90 (μ g/ml)	2.00	1.00	<0.0001
Range	0.50–3.00	0.04 to >256.00	–
Cotrimoxazole			
MIC 50 (μ g/ml)	0.12	0.05	–
MIC 90 (μ g/ml)	0.19	0.06	<0.0001
Range	0.02–0.38	0.01 to >32.00	–
Tetracycline			
MIC 50 (μ g/ml)	1.50	1.00	–
MIC 90 (μ g/ml)	2.90	2.00	<0.0001
Range	0.50–8.00	0.38 to >256.00	–
Ceftriaxone			
MIC 50 (μ g/ml)	0.19	0.12	–
MIC 90 (μ g/ml)	0.25	0.19	<0.0001
Range	0.12–0.38	0.05–0.25	–
Azithromycin			
MIC 50 (μ g/ml)	12.00	6.00	–
MIC 90 (μ g/ml)	16.00	12.00	<0.0001
Range	1.00–48.00	0.38–24.00	–
Nalidixic acid			
MIC 50 (μ g/ml)	>256.00	>256.00	–
MIC 90 (μ g/ml)	>256.00	>256.00	<0.0001
Range	1.50 to >256.00	0.38 to >256.00	–
Ciprofloxacin			
MIC 50 (μ g/ml)	0.50	0.25	–
MIC 90 (μ g/ml)	0.75	0.38	<0.0001
Range	0.02–1.50	0.00–1.00	–
Ofloxacin			
MIC 50 (μ g/ml)	1.50	0.38	–
MIC 90 (μ g/ml)	2.00	0.50	<0.0001
Range	0.06–6.00	0.02–4.00	–
Gatifloxacin			
MIC 50 (μ g/ml)	0.50	0.12	–
MIC 90 (μ g/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 cotrimoxazole. 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%.²¹ 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.²² 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.¹⁴ Finally, in another study comparing gatifloxacin with ofloxacin for the

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,²³ and a recent meta-analysis of fluoroquinolones versus other antibiotics in the treatment of typhoid fever.²⁴ There were ten trials^{25–34} 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 trial²⁷ 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.^{15,21}

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.

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.¹³ Our trial was completed

in an outpatient setting with the help of CMAs, as described in our earlier trial.¹⁴ 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 precisely 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).¹⁷

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.

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Gatifloxacin for treating enteric fever

**Submission to the 18th Expert Committee on the
Selection and Use of Essential Medicines**

Gatifloxacin for enteric fever

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Gatifloxacin for enteric fever

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1 Summary statement of the proposal for inclusion

Enteric fever (*Salmonella typhi* and *S. paratyphi*) affects 26 million mostly young people in resource limited setting annually (conservative estimates). Resistance has developed and spread widely against all the traditional treatments and there are few therapeutic options that treat the patient effectively and prevent long term carriage. No antibiotics have ever been developed specifically for the treatment of enteric fever. Very few countries use typhoid vaccines and there is no vaccine for paratyphoid.

Multidrug resistance (MDR = resistance to chloramphenicol, ampicillin and trimethoprim/sulfamethoxazole) and nalidixic acid resistance (reducing the sensitivity to the classical fluoroquinolones ofloxacin and ciprofloxacin) is widespread. Resistance causes higher failure rates and prolonged carriage, increasing the risk of complications in an individual and increasing the potential for continued transmission to the community.

There is good evidence from a series of randomised controlled trials that gatifloxacin can be applied universally in all endemic areas, irrespective of *Salmonella* susceptibility profiles. There is also pre-clinical and clinical pharmacokinetic/pharmacodynamic (PK/PD) information to support the proposed gatifloxacin treatment.

A once-a-day gatifloxacin 7-day regimen is effective and safe against both sensitive, MDR and nalidixic acid resistant strains of *Salmonella typhi* and *S. paratyphi*. No susceptibility screening is required. It is the least expensive treatment currently available.

1.1 Rationale for this submission

The claim is supported by

- In-vitro, clinical (randomised controlled trials, RDTs and meta-analysis) and pharmacological (PK/PD) evidence that gatifloxacin is effective for the treatment of enteric fever, including multi-drug resistant and nalidixic acid resistant strains.
- Safety information based on RDTs of enteric fever and longer exposure for the treatment of tuberculosis.
- Cost and cost-effectiveness data - gatifloxacin is the least expensive option for treating enteric fever.

The product is widely available across disease-endemic countries as a generic product; while approved as a general antibiotic it is not specifically indicated at present for the treatment of enteric fever. However, gatifloxacin has been approved for treating urinary tract infections involving non-*Salmonella* Enterobacteriaceae, such as *Escherichia coli*, which is genetically closely related to *Salmonella*.

2 Focal point in WHO submitting the application

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Leader - Drug development and evaluation for helminths and other neglected tropical diseases

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4 International Nonproprietary Name (INN, generic name) of the medicine

INN: Gatifloxacin

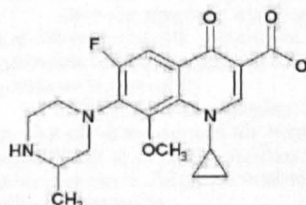
Chemical name: (±)-1-Cyclopropyl-6-fluoro-1,4-dihydro-8-methoxy-7-(3-methyl-1-piperazinyl)-4-oxo-3-quinolinecarboxylic acid sesquihydrate

Molecular formula: $C_{29}H_{37}FN_5O_4 \cdot 1.5H_2O = 402.4$

CAS: 160738-57-8 (anhydrous gatifloxacin); 180200-66-2 (gatifloxacin sesquihydrate)

ATC code: J01MA16; S01AX21

Chemical structure:



5 Formulation proposed for inclusion

Solid oral forms (200mg and 400mg tablets and capsules) are available.

No specific paediatric formulation currently exists.

5.1 Prospective formulation improvements

Ways of stimulating manufacturers to optimize gatifloxacin formulation will be sought. The enteric fever patient population is generally young and small and dosing is based on body weight. While tablet crushing is customary, and a practical dosing schedule is proposed here, smaller (lower strength) tablets, scored tablets or a suspension will improve dosing accuracy.

An oral suspension was developed and used in Phase 3 clinical trials as part of the Bristol-Myers Squibb Company gatifloxacin paediatric New Drug Application. Additionally, a 50 mg paediatric tablet was also studied. Formulation details will be sought from the Bristol-Myers Squibb Company (Princeton, New Jersey, USA) for possible technology transfer.

Scored 200mg and 400mg tablets could be developed easily.

6 International availability

Several generic products are on the market.

6.1 Patent status

The patent situation for gatifloxacin is publicly available in "Drugs in Focus January '10" (1), (details in Appendix 1). In addition WHO/TDR commissioned a search to Withers & Rogers in 2009.

Of the four patent families reported by the Key Patent Indicator (KPI), only the first family (claims to its chemical formula) is relevant to the use of gatifloxacin products for enteric fever. All expired in 2010 or earlier, except: (i) 2012 in Germany and Austria (through extension of patent) and (ii) 2011 in Germany, France and UK (data exclusivity expiry) but (a) extension in Australia and Germany were granted for brand name Tequin® which is discontinued and (b) no marketing authorization exists for gatifloxacin in Australia, France or UK. The latest patent to expire is in Canada although the product was voluntarily discontinued. There is no patent in the disease-endemic countries.

6.2 Production

Gatifloxacin is currently manufactured and sold by generic companies in India and China and freely available for export.

In India, the principal manufacturer of Approved Pharmaceutical Ingredient (API) is CIPLA Pharmaceuticals of Mumbai, who manufacture gatifloxacin sequihydrate as bulk material for export (2) and use by other companies in India (3).

CIPLA also manufacture gatifloxacin as tablets under the trade name Gatiquin as 200 and 400 mg tablets (4). There are at least 80 generic manufacturers currently supplying gatifloxacin formulated material in India. The individual presentations of generic gatifloxacin in India are in Appendix 2.

In China, there are a number of producers of API recorded, several of which produce to GMP standards, although the status of formulated gatifloxacin in the China market is more difficult to determine. Gatifloxacin is also available in Nepal, Vietnam, Pakistan and other countries in the region. Availability in other countries with endemic enteric fever is difficult to determine.

7 Listing is requested as an individual medicine

Individual medicine - gatifloxacin

Reasons are: resistance to first-generation fluoroquinolones; specific efficacy, safety data and supportive pharmacokinetic/pharmacodynamic and in vitro data; specific cost of product.

8 Information supporting the public health relevance

Enteric fever is widespread; conservative estimates have 26 million cases per year between *S. typhi* and *S. paratyphi*.
Multidrug resistance (MDR = resistance to chloramphenicol, ampicillin and trimethoprim/sulfamethoxazole) and nalidixic acid resistance (NAR = reducing the sensitivity to the classical fluoroquinolones ofloxacin and ciprofloxacin) is widespread.
Where MDR and NAR are common azithromycin and gatifloxacin are now the best options for treatment and can additionally treat other pathogens which may cause a clinical syndrome similar to enteric fever.

8.1 Epidemiology

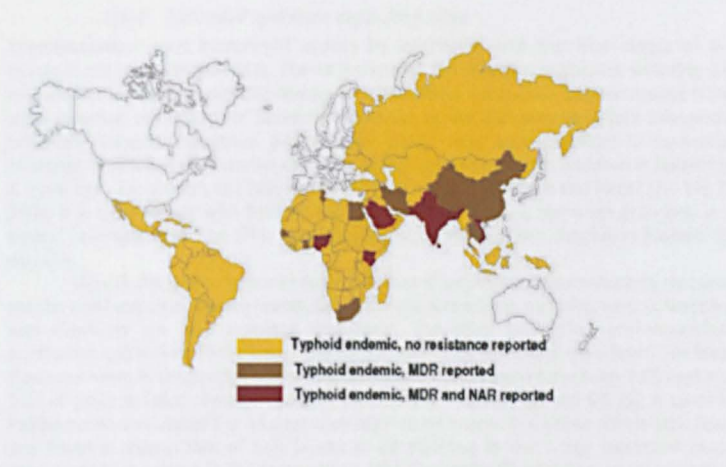
Typhoid fever and paratyphoid fever are septicaemias caused by the Gram negative bacteria *Salmonella enterica* serovar Typhi (*S. typhi*) and *Salmonella enterica* serovar Paratyphi (*S. paratyphi*) A, B and C. Typhoid and paratyphoid fever are summarized as enteric fevers. Whilst *S. typhi* and *S. paratyphi* A and B infections are restricted to humans, *S. paratyphi* C can affect a variety of animals.

Enteric fever is endemic in Africa, Asia, Central and South America and found in parts of the Middle East, southern and eastern Europe (5). Improvement of infrastructure and sanitation has virtually eliminated typhoid fever in developed countries and infections seen in Europe, Australia, and North America are usually acquired abroad (mostly from the Indian Subcontinent, South East Asia and South America) (6). Current estimates from the World Health Organization (WHO) suggest that the global burden of typhoid fever is approximately 21 million cases annually with more than 210 000 deaths and that paratyphoid fever causes an additional 5 million cases (7). These numbers are based on extrapolating data from 22 studies that used blood culture, the gold standard for the diagnosis of typhoid fever. Many institutions in endemic countries lack blood culture facilities and the sensitivity of blood culture is less than 50% and so the true magnitude of the problem is undoubtedly greater. Transmission of typhoid fever occurs via the faeco-oral route by ingesting contaminated water or food or through direct contact. Chronic typhoid carriers involved in food handling are an important reservoir of infection. In endemic areas enteric fever is a disease of young school children through to young adults.

A WHO report has estimated the case fatality rate in enteric fever at 1% (7). The most important contributor to a poor outcome is a delay in appropriate antibiotic treatment made more likely by the presence of drug resistant strains in the community.

The geographical distribution of *S. typhi* and areas of multi-drug and nalidixic acid resistance are in Figure 1.

Figure 1 Areas of typhoid fever endemicity and distribution of antimicrobial drug resistance to *Salmonella enterica* serotype Typhi, 1990 to 2005. Modified from (8). MRD = Multidrug Resistance; NAR = Nalidixic Acid Resistance.



8.2 Current treatment options and antibiotic resistance

8.2.1 Chloramphenicol

Chloramphenicol is a broad spectrum antibiotic with bacteriostatic activity. It was developed in 1947. Chloramphenicol was the first antibiotic to be used in the treatment of typhoid fever (9).

Chloramphenicol treatment reduced typhoid fever mortality from 20% to approximately 1%, and the duration of fever from 2-4 weeks to 4-5 days (9-11). The most important adverse effect of chloramphenicol is a dose related, reversible bone marrow depression that results from inhibition of mitochondrial protein synthesis. This is relatively common and is reversible when the drug is stopped. In contrast, the chloramphenicol associated "idiosyncratic" aplastic anemia is very rare but is not dose related, non reversible and invariably fatal. Aplastic anemia is estimated to occur in 1 in 24,500 to 40,800 exposed (12). Resistance to chloramphenicol was first reported in the 1970s and has spread widely (13). Chloramphenicol remains of use for enteric fever in regions of the world where the bacteria are fully sensitive (5, 14, 15). However, disadvantages of chloramphenicol include the need for knowledge of the local sensitivity pattern, higher relapse and typhoid carrier rates (13) plus the need for treatment four times a day for 14-21 days (16) which reduces adherence.

8.2.2 Ampicillin and amoxicillin

The aminopenicillins ampicillin and amoxicillin have been evaluated for the treatment of typhoid fever in several clinical trials proved inferior to chloramphenicol (10, 13). Resistance is widespread and generally due to the production of the bacterial enzyme β -lactamase.

8.2.3 Trimethoprim-Sulfamethoxazole (cotrimoxazole)

Trimethoprim-sulfamethoxazole was widely used for the treatment of typhoid fever but with widespread resistance and an inferior efficacy it is rarely used today (13).

8.2.4 Extended spectrum cephalosporins

Cephalosporines exert bactericidal activity by interfering with the later stages of the bacterial cell wall synthesis (17). The target site of the β -lactam antibiotics including the cephalosporines are the penicillin-binding proteins (PBPs). Production of β -lactamases is the most common mechanism of bacterial resistance. In the late 90s, non-Typhi *Salmonella* producing extended spectrum β -lactamases (ESBL) have been reported in numerous countries. Resistance to extended spectrum cephalosporins has been reported in isolates of *S. typhi* from Bangladesh and Italy and *S. paratyphi* A from Pakistan and Nepal (18, 19). In 2009, a *S. typhi* isolate with ESBL phenotype caused by *bla*_{CTX-15} has been described in a patient returning from Iraq (20). The cephalosporines exhibit time dependent bactericidal activity.

Overall, the cephalosporines are a safe class of antibiotics, hypersensitivity reactions are the most common adverse events. Gastrointestinal reactions, including nausea, vomiting and diarrhoea are also reported frequently. The third generation cephalosporines ceftriaxone and cefixime have been used for the treatment of MDR typhoid fever. The fever clearance times in randomised trials using intravenous ceftriaxone have been 7-10 days and 10% of patients failed clinically. Relapse rates varied between 4% and 6% (5). A study in Pakistan evaluated either 7 or 14 days of ceftriaxone treatment in children with enteric fever and found a relapse rate of 14% (4 out of 28 patients) in the 7 day treatment group compared to no relapse in the 14 day group (21). The major disadvantage of ceftriaxone is the need for parenteral administration, the high cost, especially for what is often a prolonged treatment course.

Oral cefixime was a popular choice for the treatment of typhoid fever in children. In randomised controlled trials in children the mean Fever Clearance Times ranged from 5 to 8 days and clinical failure rates were reported to be below 3% (22-24). However, a typhoid treatment trial in Vietnam reported much higher failure rates of 23% (10 out of 44 patients) when cefixime was used in children (25) and a recent trial in Nepal using Cefixime was stopped by the Independent Data and Safety Monitoring Committee because of an unacceptably high failure and relapse rate in those receiving cefixime. The overall treatment failure in this trial (acute treatment failure, relapsed patients plus one death) was determined to be (95% confidence interval) 37.6 % (27.14%–50.2%) in the cefixime group (26). Both *S. typhi* and *S. paratyphi* are predominantly intracellular organisms and the cephalosporines do not penetrate well intracellularly. This may explain the prolonged fever clearance times, higher relapse and carriage rates seen when these drugs are used.

8.2.5 Azithromycin

Azithromycin belongs to the macrolide class of antibiotics. Macrolides are inhibitors of protein synthesis by impairing the elongation of the peptidyl chain. Azithromycin resistance has not yet been reported in *S. Typhi*. Azithromycin has a bioavailability of 30% to 50%. The serum peak level is typically reached after 2 hours. Azithromycin has a large volume of distribution which is related to the ability to accumulate inside eukaryotic cells. The ratio of tissue to serum concentration for azithromycin is 50 to 1150 (27). The half life is 35 to 40 hours, which allows a single daily dose and shortened treatment regimen (3 to 5 days). Macrolides are primarily metabolised through cytochrome P450 and eliminated through the bile.

Gastrointestinal adverse events are frequent with macrolides. Macrolides have been associated with prolongation of the QT interval and should not be used in patients with concurrent administration of class IA and III antiarrhythmic agents and underlying cardiac disease. Azithromycin has become a treatment option for the treatment of MDR typhoid fever. The MICs for *S. typhi* to azithromycin range from 4 to 16 µg/ml (28). The peak serum level after a single dose of 500 mg of azithromycin is 0.4 mg/L (27). However, as azithromycin is concentrated more than 100 fold inside polymorphonuclear cells and macrophages (29) and *S. typhi* is primarily an intracellular pathogen (30), effective drug concentrations are considerably above the MIC. In randomized clinical trials, azithromycin has been used for the treatment of MDR typhoid fever in children and adults in Egypt, India and Vietnam (31-35). Cure rates were good and outcomes in patients infected with nalidixic acid resistant *S. Typhi* were satisfactory (32).

8.2.6 Fluoroquinolones

Nalidixic acid, the prototype 4-quinolone antibiotic was discovered in 1962 (36), it is active against Gram negative bacteria and only achieves modest serum and tissue concentrations. Almost 20 years later, the addition of a fluorine molecule at position C6 created the fluoroquinolones. The 6-fluoro substituent confers a greater spectrum of activity against Gram negative and Gram positive pathogens, possibly by improving tissue penetration and binding to the DNA gyrase enzyme.

Ciprofloxacin and Ofloxacin (second generation fluoroquinolones) have excellent activity against Gram negative organisms (37). Due to its availability and affordability, ofloxacin has been widely used for the treatment of typhoid fever. However over the last few years strains resistant to nalidixic acid have appeared and spread widely. These strains are much less susceptible to both ciprofloxacin and ofloxacin with patients suffering from prolonged fever clearance times, clinical failures and prolonged carriage. Therefore the effectiveness of both of these drugs has declined leaving few options for treatment in regions with both multi-drug and nalidixic acid resistance.

Gatifloxacin is a broad spectrum 8-methoxy fluoroquinolone with enhanced activity against Gram positive organisms, which has received U.S. Food and Drug Administration (FDA) approval in 1999. It features a cyclopropyl group at position 1 similar to ciprofloxacin. The addition of a methoxy group at position 8 targets both topoisomerase II and IV and probably prevents (or delays) the development of quinolone resistance.

Fluoroquinolones are considered bactericidal agents and have excellent in vitro activity against a wide range of Gram negative and Gram positive organisms. The quinolones rapidly inhibit bacterial DNA synthesis, causing rapid cell death. The targets for the fluoroquinolones are the bacterial topoisomerase enzymes, DNA gyrase (topoisomerase II) and topoisomerase IV.

The main mechanism of quinolone resistance in *S. Typhi* is the accumulation of amino acid substitutions in the bacterial target enzyme DNA gyrase. The most commonly identified alteration has been a serine to phenylalanine substitution at position 83 of *gyrA* (38, 39). These mutations are focused around a region called the quinolone resistance determining region (QRDR). The QRDR of *gyrA* is close to tyrosine at position 122, the active site of the enzyme, which is covalently linked to DNA during strand breakage (40). Single point mutations in *gyrA* of *S. Typhi* leads to nalidixic acid resistance (MIC ≥ 32 µg/ml) and reduced susceptibility to the older generation fluoroquinolones. Single isolates of fully fluoroquinolone resistant *S. Typhi* and *S. Paratyphi A* have been reported from India (41). The high-level fluoroquinolone resistance seen in these *S. Typhi* (ciprofloxacin MIC ≥ 4 mg/ml) isolates was conferred by dual mutations in *gyrA* and a single mutation in *parC* (42, 43). Gatifloxacin binds with greater affinity to the QRDR and is less susceptible to these mutations remaining effective against these strains.

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The frequency of adverse reactions to quinolones is between 6 and 11% of the subjects exposed with less than 1% of adverse events being recorded as serious (44). The most frequent adverse effects reported are nausea, upper gastrointestinal discomfort and central nervous system effects such as headache, insomnia and dizziness. The adverse events are typically mild, self limited and mostly resolve when the drug is stopped. Some adverse effects do not seem to be related to specific modifications, whereas phototoxicity and CNS effects are linked to a specific structure. Each fluoroquinolone tends to produce a characteristic profile of adverse effects.

In their preclinical evaluation, all quinolones studied caused arthropathy in immature animals, especially in young beagle dogs and usually in the major weight bearing joints (45, 46). The concern that the fluoroquinolones might also cause cartilage damage in children has led to cautious use in many countries. However, extensive experience with the fluoroquinolones, especially ciprofloxacin and levofloxacin, in children suffering from cystic fibrosis, enteric fever and bacillary dysentery has provided a body of evidence suggesting that the joint damage seen in young dogs does not occur in children and these antibiotics are safe in children (5, 14, 47-49).

Fluoroquinolones have been associated with tendinitis and tendon rupture in adults, primarily affecting the Achilles tendon; risk factors were renal dysfunction and concomitant corticosteroid use (50). Severe neurotoxic reactions are rare. However, hallucinations, depression, and psychotic reaction have been reported. The quinolones should be used with caution in patients with known CNS disorders (e.g., epilepsy) or conditions predisposing to seizures (37, 44). The most common skin reactions are non-specific skin rashes, pruritus and urticaria. Phototoxicity is a rare dermatologic complication of quinolone therapy which is inextricably related to the chemical structure, a halogen grouping at position C8 (50).

A study based on post marketing surveillance data reported that the crude incidence rate (95% confidence interval) of cases of Torsades de Pointes (TdP) per 10 million prescriptions in the United States was 0.3 (0.0-1.1) for ciprofloxacin, 2.1 (0.3-7.6) for ofloxacin, 5.4 (2.9-9.3) for levofloxacin and 27 (12-53) for gatifloxacin (51). However questions regarding the validity of both the numerators and denominators used in these incidence calculations remain (52). Preclinical and clinical data indicate that levofloxacin, moxifloxacin, and gatifloxacin prolong the QTc interval. The potential for TdP to develop as a result of this is rare and is influenced by many independent variables, especially by concurrent administration of class IA and III antiarrhythmic agents, genetic susceptibility, underlying cardiac disease, electrolyte imbalance and organ impairment. Therefore gatifloxacin, levofloxacin, moxifloxacin or gemifloxacin should not be used in patients with risk factors predisposing them to TdP (52).

The quinolones as a class have demonstrated the ability to close K⁺-ATP channels in the β cells of the pancreas, resulting in the release of insulin and subsequent hypoglycaemia. However the mechanism for hyperglycaemia remains poorly understood and might be caused by overexposure (failure to adjust the dose in patients with renal failure) (52). Product labels for ciprofloxacin, gatifloxacin, levofloxacin, and moxifloxacin mention the possibility of hypoglycaemia and hyperglycaemia. Although glucose disturbances appear to be a class effect, the odds of hypo- and hyperglycaemia appear to vary among the agents (53). A retrospective study in Texas reviewed records of dysglycaemia in hospitalised patients receiving gatifloxacin, levofloxacin, ciprofloxacin or ceftriaxone (54). Dysglycemic events were more likely to occur in patients receiving gatifloxacin (relative risk, 3.29; 95% CI, 2.33-4.65) or levofloxacin (relative risk, 1.55; 95% CI, 1.29-1.88) versus ceftriaxone.

In another study of elderly in-patients who received gatifloxacin or levofloxacin, gatifloxacin was independently associated with hypoglycaemia (OR, 2.4; 95% CI, 1.1-5.6) and hyperglycaemia (OR, 2.5; 95% CI, 1.6-3.9) versus levofloxacin (55). In diabetic patients treated with gatifloxacin, the overall incidence of hypoglycaemia was 0.4%, 0.7%, and 1.6%

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for patients below 65 years, 65 to 69 years and 80 years and above, respectively. The corresponding incidences of hyperglycaemia were 1.0%, 1.6%, and 3.3%, respectively (50).

When exposure to gatifloxacin was simulated in patients with severe hyperglycemia, who were often also older Type-2 diabetics with renal dysfunction, AUC values were 2 to 3 times those observed in patients with normal renal function (56). Therefore the authors suggested to empirically adjust the dose of gatifloxacin to 200 mg daily for patients aged above 65 years with community acquired respiratory tract infections. Only ciprofloxacin, clinafloxacin, enoxacin, grepafloxacin, pefloxacin, and tosufloxacin can inhibit the hepatic cytochrome P₄₅₀ isoform CYP 1A4 isoenzymes. Few drugs are metabolized by these isoenzymes, but important drugs include the methylxanthines (theophylline and caffeine) and warfarin.

8.2.7 Summary of treatment options

In regions of the world where MDR and Nalidixic Acid strains of *S. typhi* and *S. paratyphi* are common azithromycin and gatifloxacin are now arguably the best options for treatment. Intravenous antibiotics are not appropriate in most settings where patients are treated as out-patients. In most parts of the world where enteric fever is common the sensitivity of the strains is not known as microbiological confirmation of the infection is lacking and formal testing of sensitivities is not undertaken. Hence most patients are treated empirically. In such circumstances a 7-day regimen of azithromycin or gatifloxacin are excellent choices for all strains of *S. typhi* and *S. paratyphi*. The added value of these antibiotics is that they are effective against other pathogens which may cause a clinical syndrome similar to enteric fever. (see Section 9.2)

9 Treatment details

9.1 Dosage regimen and duration

The data presented in this application support the use of gatifloxacin at <u>10 mg/kg/d for 7 days</u> (not to exceed 600 mg/day.)
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This Section presents the pharmacological basis for this regimen. Efficacy results from randomised controlled studies are in Section 10)

We also present in this Section practical dosing schedules with existing formulations and prospective dosing with improved formulations.

9.2 Current clinical guidelines

There have been no formal WHO Guidelines published on the specific treatment for Enteric Fever. In 2003 the WHO Department of Vaccines and Biologicals produced an expert committee report "*Background document: The diagnosis, treatment and prevention of typhoid fever*" (14) in which the following recommendations were made:

Table 1. WHO recommendations from 2003 on optimal and alternative treatments for typhoid fever (NOTE: this table pre-dates the updated Cochrane Reviews and recent trials)

Susceptibility	OPTIMAL THERAPY			ALTERNATIVE EFFECTIVE DRUGS		
	Antibiotic	Daily dose (mg/kg)	Days	Antibiotic	Daily dose (mg/kg)	Days
Fully sensitive	Fluoroquinolone e.g. ciprofloxacin or ciprofloxacin ^a	15	5-7 ^b	Chloramphenicol	50-75	14-21
				Amoxicillin	75-100	14
				Trimethoprim- sulfamethoxazole	8-40	14
Multidrug resistance	Fluoroquinolone or cefixime	15	5-7	Azithromycin	8-10	7
		15-20	7-14	Cefixime	15-20	7-14
Quinolone (nalidixic acid)	Azithromycin or ceftriaxone	8-10	7	Cefixime	20	7-14
		75	10-14			

^a Three day courses are also effective and are particularly so in epidemic containment.

^b The optimum treatment for quinolone resistant typhoid fever has not been determined. Azithromycin, the third generation cephalosporines, or a 10-14 day course of high-dose fluoroquinolones is effective. Combinations of these are now being evaluated.

There have been a series of reviews since that date (5, 8, 57). The treatment options depend on local knowledge of the sensitivity patterns of the circulating strains of *S. typhi* and *S. paratyphi* (see Table 1). When culture facilities are not available and knowledge of the sensitivity patterns are unknown treatment decisions must be made empirically and consideration also given to the potential other causes and the differential diagnosis.

9.3 Summary target product profile

The ideal therapy would be an oral regimen; the drug would cure the patient quickly preferably as an outpatient, prevent the development of complications, and reduce the incidence of both short and long term carriage. The regimen would be easy to administer to enhance adherence, be effective against all strains of *S. typhi* and *S. paratyphi*, with no need for an antibiogram; it would have minimal adverse events and be affordable. As so much enteric fever is managed empirically it would be ideal if the therapy is also potentially effective against the common bacterial illnesses that can present like enteric fever.

Of all the treatments currently available the two drugs that fit this profile are gatifloxacin and azithromycin.

9.4 Pharmacological basis of gatifloxacin treatment regimen for enteric fever

<p>Work presented in the Section provides evidence that</p> <ul style="list-style-type: none"> ▪ the main determinant of gatifloxacin is the AUC₀₋₂₄:MIC. A ratio >92.7 predicts favourable response in enteric fever. ▪ This ratio is achieved with a daily dose of 10mg/kg which produces consistent levels of exposure (little inter-individual variability) both in children and adults. ▪ Nalidix acid resistant organisms remain susceptible to gatifloxacin. ▪ Susceptibility screening and in vitro <i>Salmonellae</i>-specific breakpoints are not required for gatifloxacin.
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9.4.1 Summary of antimicrobial drug resistance

In the late 1980s and early 1990s outbreaks of typhoid fever occurred that were resistant against all "first line" antimicrobials (multidrug resistance (MDR) defined as resistance to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole) (5). These MDR *S. Typhi* isolates have been responsible for numerous outbreaks in countries in the Indian subcontinent, southeast Asia and Africa (8). All MDR strains so far examined have plasmids of the *incHI1* incompatibility group.

Consequently, the fluoroquinolones have become the treatment of choice for typhoid fever especially in areas of the world with MDR strains. The fluoroquinolones show excellent tissue penetration, accumulation in monocytes and macrophages and high drug levels in the gall bladder. However, there have been reports from Vietnam, India and Tajikistan of the emergence of *S. Typhi* isolates that respond less well to the fluoroquinolones (5, 8). In 1997, a typhoid epidemics in Tajikistan caused by such isolates caused more than 10000 illnesses and 108 deaths (58). Technically these isolates remain within the breakpoints set for fluoroquinolone susceptibility by the Clinical Laboratory Standard Institute (CLSI) (59), but they are resistant to nalidixic acid (the prototype quinolone) and show higher MICs to the fluoroquinolones. Patients infected with these isolates show a poor clinical response when treated with ciprofloxacin or ofloxacin. Of all the fluoroquinolones assessed, gatifloxacin showed the lowest minimum inhibitory concentrations (MICs) for nalidixic acid resistant *S. typhi* from Nepal (60) and Vietnam (38). In vitro time-kill experiments showed a reduction in the efficacy of ofloxacin against strains harbouring a single amino acid substitution at codon 83 or 87 of GyrA, this effect was more marked against a strain with a double substitution. The 8-methoxy fluoroquinolone gatifloxacin showed rapid killing of *S. typhi* harbouring both the single and double amino acid substitutions (38).

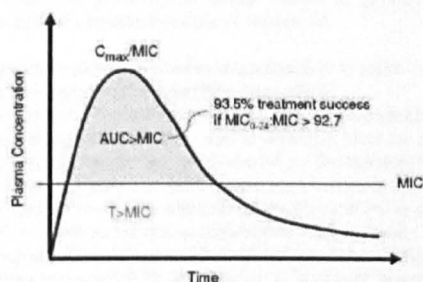
9.4.2 Pharmacodynamics and Pharmacokinetics of gatifloxacin in patients with enteric fever

Pre-clinical PK/PD models have long served as a basis for dose regimen selection in early drug development and, subsequently, PK/PD analyses of clinical data have served to confirm or refine pre-clinical PK/PD model predictions (61). The pre-clinical and clinical PK/PD of fluoroquinolone are better understood than perhaps any other class of antibacterial agents. The PK-PD relationship between exposure and response are understood in a wide range of indications, including community-acquired pneumonia, acute exacerbations of chronic bronchitis, acute maxillary sinusitis, urinary tract infections, hospital-acquired pneumonia and typhoid fever (61).

Figure 2 presents the PK/PD indices that are used as surrogate markers for clinical and antimicrobial efficacy are the ratio of peak plasma concentration (C_{max}) of the antimicrobial to the minimum inhibitory concentration (MIC) of the pathogen (C_{max}/MIC), the ratio of the area under the concentration time curve 0 to 24 hours to the MIC (AUC>MIC) and the time above MIC (T>MIC). For the fluoroquinolones family in general antibacterial activity depends on the C_{min}/MIC and the AUC>MIC.

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Figure 2. Concentration versus time curve with minimum inhibitory concentration superimposed and pharmacokinetic and pharmacodynamic markers.



Pharmacodynamics of gatifloxacin in patients with typhoid fever (see Appendix 3)

Clinical data from patients (randomised controlled trial of gatifloxacin versus azithromycin; see Section 10.4.) with typhoid fever were used to investigate the exposure-response relationship of gatifloxacin and the positive- and negative-predictive value of the nalidixic acid screening test. There are few non-clinical PK-PD data available and essentially no clinical PK-PD data for fluoroquinolones and *S. typhi* among patients with typhoid fever. If available, such data could be used to evaluate the adequacy of dosing regimens and *in vitro* susceptibility breakpoints. In an effort to clarify these issues, gatifloxacin exposure-response relationships were modelled for patients with enteric fever.

Abstract

Background. The pharmacodynamics of gatifloxacin in patients with typhoid fever and the positive- (predicts clinical cure) and negative- (predicts clinical failure) predictive value of the nalidixic acid screening test were evaluated in a randomized clinical trial.

Methods. Gatifloxacin-treated (10 mg/kg/day given orally for 7 days) patients with typhoid fever were analyzed. Previously validated population pharmacokinetic models were used in conjunction with patient-specific demographics to estimate individual patient drug exposures, as measured by the area under the concentration-time curve at 24 hours (AUC_{0-24}). Analyses included all patients with sufficient data to estimate AUC_{0-24} and who had a defined minimum inhibitory concentration (MIC) value ($N = 124$). Fever was evaluated every 6 hours. Favourable clinical response was defined as the resolution of fever and symptoms within 48 hours of the end of therapy. Relapse was defined as the recurrence of fever and symptoms and/or the isolation of *S. typhi* from blood after completion of therapy and discharge from hospital. A medical history, physical examination and stool cultures to determine chronic faecal carriage were performed at 1, 3 and 6 months after the end of therapy.

Findings. Statistically significant relationships between drug exposure intensity and clinical response were detected. In patients with a AUC_{0-24}/MIC ratios of greater than

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92.7, 93.5% had a favourable response; while for those with $AUC_{0-24}:MIC$ ratios ≤ 92.7 , only 75% had a favourable response (odds ratio = 4.81, 95% CI 1.23, 18.9; $P = 0.02$). The positive- (predicts cure) and negative- (predicts failure) predictive value of the nalidixic acid screening test was 100.0% and 9.3%, respectively.

Interpretation. The exposure-response relationships identified provide a paradigm for dose regimen evaluation of existing and new fluoroquinolones for the treatment of typhoid fever. The results of this study also indicate that the nalidixic acid screening test was not predictive of clinical failure for gatifloxacin and *Salmonellae*-specific susceptibility breakpoints may be warranted.

Population pharmacokinetics of gatifloxacin in south east Asian adult and paediatric patients with typhoid fever (see Appendix 4)

Background: An understanding of patient pharmacokinetics (PK) is critical for the rational use of antibiotics. This is especially true for pathogens such as *Salmonella typhi* in South East Asian countries where the development of multi-drug resistance is an increasing concern. Gatifloxacin is a commonly used treatment in South East Asia for typhoid fever. We investigated gatifloxacin PK in paediatric patients and adult patients from Nepal with uncomplicated typhoid fever.

Methods: PK data were collected during routine clinical care. Each patient had ≤ 3 plasma samples for PK drawn after 3 - 6 days of oral gatifloxacin therapy. Separate candidate models for adults and children were fit to the data using Monte Carlo parametric expectation maximization with S-ADAPT. Due to the sparse nature of the PK sampling, the structure and covariate relationships from previous gatifloxacin adult and paediatric population PK models derived from infected North American patients were retained but were revised to fit the data from this population.

Results: 68 PK samples from 36 patients (aged 3 - 54 years) were analyzed. Gatifloxacin PK were best fit by a linear 1-compartment model. Fits of data were excellent ($r^2 > 0.9$ for children and adult data); interindividual variability in PK was modest. Compared to North American paediatric patients, the Nepalese paediatric patient population had ~50% slower clearance (Table 2).

Conclusions: As drug clearance was markedly lower in South East Asian typhoid fever vs infected North American patients, these data demonstrate the importance of evaluating PK in varying patient populations. The PK models described herein will be used in future pharmacokinetics-pharmacodynamics (PK-PD) analyses of efficacy in South East Asian populations with typhoid fever.

Table 2. Main parameters of gatifloxacin derived from population kinetics of Asian enteric fever patients

Patient population	PK parameter	Mean (%SEM) parameter estimates	
		Previous models	Current data
Paediatric	CL/F (L/h/m ²)	8.46 (3.50)	4.41 (5.65)
	Vc (L/kg)	2.15 (3.30)	1.21 (13.8)
Adult	CL/F, nonrenal (L/h)	8.11 (35.3)	2.91 (21.9)
	CL/F, renal-slope* (L/h/mL/min)	0.0629 (37.8)	0.0629 (---)
	Vc (L/kg)	1.45 (7.9)	1.28 (17.7)

*This parameter was not fit due to the narrow range of renal function

9.5 Proposed dosing regimens

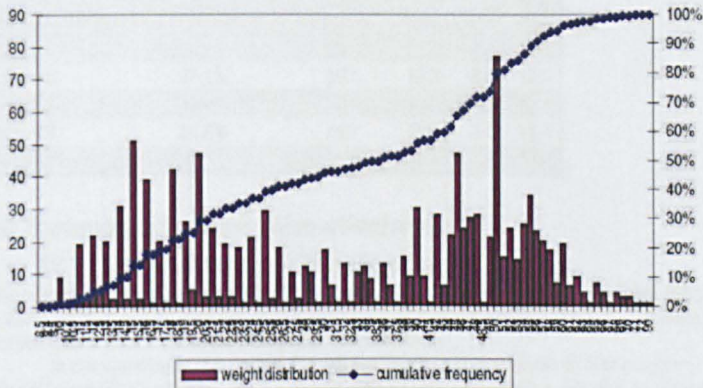
Gatifloxacin is currently formulated as 200mg and 400mg strength non scored tablets. Tablet fractionation is custom both in clinical practice and clinical trials.

We present here proposed practical dosing regimens using the current formulations and prospected improved formulations. The target dose was set at 10mg/kg and the therapeutic window at 7-13.5mg/kg/d not to exceed 600mg/d. This range is consistent with how gatifloxacin was originally developed by Bristol-Meyers Squibb and with the pharmacokinetic/pharmacodynamic data and safety margins in children and adults.

The objective was to administer whole tablets and minimize tablet crushing.

We also wanted to predict what proportion of the typical enteric fever patient population will be receiving which dose. The proportions of the overall population in the tables below refer to the weight frequencies found in the Nepal plus Vietnam database of 1208 enteric fever patients (weight distribution in Figure 3 below.)

Figure 3. Weight distribution of Asian enteric fever patients



Option 1 uses the current non scored 200mg and 400mg tablets. It was not possible to give whole tablets for patients under 15kg body weight (*) for whom tablet crushing remains the only option. Patients weighing 29kg taking one 200mg tablet will be receiving 6.9mg/kg instead of the 7mg/kg. Because of the 600mg maximum dose patients weighing =>87kg will received <7mg/kg.

Table 3. Practical dosing of gatifloxacin with current non scored 200mg and 400mg tablets

Option 1 200mg and 400mg strength not scored					
weight band	% population	mg/kg/d	mean	min	max
<15*	9.8%				
15 to 29	34.2%	200	9.5	6.9	13.3
30 to 49	29.6%	400	10.4	8.2	13.3
>= 50	26.5%	600	8.8	6.7	12.0

Option 2 refers to the possibility that manufacturers will accept to develop 200mg and 400mg tablets scored in half (or 400mg tablets scored to give four 100mg units). (*) patients weighing less than 15kg the dosing range displayed is for the weight range 8.5kg to <15kg, below which tablet crushing is required to avoid overdosing. Because of the 600mg maximum dose, patients weighing =>87kg will received <7mg/kg.

Table 4. Practical dosing of gatifloxacin with scored 200mg and 400mg tablets

Option 2 200mg strength scored & 400mg strength scored					
weight band	% population	mg/kg/d	mean	min	max
<15kg*	9.8%	100	9.4	7.1	12.5
15 to 24	27.1%	200	10.5	8.3	13.3
25 to 34	12.0%	300	10.3	8.8	12.0
35 to 49	24.6%	400	9.6	8.2	11.4
>= 50	26.5%	600	8.8	6.7	12.0

10 Summary of comparative effectiveness

10.1 Identification of clinical evidence

The current Cochrane review (57) is being updated. We completed a comprehensive search in October 2010 and have screened this and retrieved full text articles. The inclusion criteria remain the same as the current published Cochrane review.

In the updating of this review, we will use the Cochrane new risk of bias assessment. We will use GRADE to summarize the results. This will be using relative risk with confidence intervals across meta-analysis of comparisons for standard outcomes where this is appropriate. (the updated review will be submitted at a later stage as Appendix 5.)

10.2 Recent randomised comparative clinical trials

Recent clinical trials compared gatifloxacin to:

Azithromycin:

A multi-center randomised controlled trial of gatifloxacin versus azithromycin for the treatment of uncomplicated typhoid fever in children and adults in Vietnam.
Dolecek C, Tran TP, Nguyen NR, Le TP, Ha V, Phung QT, Doan CD, Nguyen TB, Duong TL, Luong BH, Nguyen TB, Nguyen TA, Pham ND, Mai NL, Phan VB, Vo AH, Nguyen VM, Tran TT, Tran TC, Schultz C, Dunstan SJ, Stepniewska K, Campbell JI, To SD, Basnyat B,

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Nguyen VV, Nguyen VS, Nguyen TC, Tran TH, Farrar J. *PLoS ONE*. 2008 May 21;3(5):e2188. PMID: 18493312.)

Background: Drug resistant typhoid fever is a major clinical problem globally. Many of the first line antibiotics, including the older generation fluoroquinolones, ciprofloxacin and ofloxacin, are failing.

Objectives: We performed a randomised controlled trial to compare the efficacy and safety of gatifloxacin (10 mg/kg/day) versus azithromycin (20 mg/kg/day) as a once daily oral dose for 7 days for the treatment of uncomplicated typhoid fever in children (above 6 months) and adults in Vietnam.

Methods: An open-label multi-centre randomised trial with pre-specified per protocol analysis and intention to treat analysis was conducted. The primary outcome was fever clearance time, the secondary outcome was overall treatment failure (clinical or microbiological failure, development of typhoid fever-related complications, relapse or faecal carriage of *S. typhi*). Patients were followed up at 1, 3 and 6 months.

Principal findings: We enrolled 358 children and adults with suspected typhoid fever, 186 patients were treated with gatifloxacin and 172 with azithromycin. There was no death in the study. 287 patients had blood culture confirmed typhoid fever, 145 patients received gatifloxacin and 142 patients received azithromycin. The median FCT was 106 hours in both treatment arms (95% Confidence Interval [CI]; 94-118 hours for gatifloxacin versus 88-112 hours for azithromycin), (logrank test $p = 0.984$, HR [95% CI] = 1.0 [0.80-1.26]).

Overall treatment failure occurred in 13/145 (9%) patients in the gatifloxacin group and 13/140 (9.3%) patients in the azithromycin group, (logrank test $p = 0.854$, HR [95% CI] = 0.93 [0.43-2.0]). 96% (254/263) of the *Salmonella enterica* serovar Typhi isolates were resistant to nalidixic acid and 58% (153/263) were multidrug resistant.

Conclusions: Both antibiotics showed an excellent efficacy and safety profile. Both gatifloxacin and azithromycin can be recommended for the treatment of typhoid fever particularly in regions with high rates of multidrug and nalidixic acid resistance. The cost of a 7-day treatment course of gatifloxacin is approximately one third of the cost of azithromycin in Vietnam.

Trial registration: Current Controlled Trials ISRCTN 67946944

Cefixime:

This trial was stopped early by the independent Data Safety and Monitoring Board due to the inferior performance of cefixime. An open randomized comparison of gatifloxacin versus cefixime for the treatment of uncomplicated enteric fever.

Pandit A, Arjyal A, Day JN, Paudyal B, Dangol S, Zimmerman MD, Yadav B, Stepniowska K, Campbell JJ, Dolecek C, Farrar JJ, Basnyat B. *PLoS One*. 2007 Jun 27;2(6):e542

Objective. To assess the efficacy of gatifloxacin versus cefixime in the treatment of uncomplicated culture positive enteric fever.

Design. A randomized, open-label, active control trial with two parallel arms. Setting. Emergency Room and Outpatient Clinics in Patan Hospital, Lalitpur, Nepal.

Participants. Patients (aged two to sixty-five years) with clinically diagnosed uncomplicated enteric fever meeting the inclusion criteria. Interventions. Patients were allocated to receive one of two drugs, Gatifloxacin or Cefixime. The dosages used were Gatifloxacin 10 mg/kg, given once daily for 7 days, or Cefixime 20 mg/kg/day given in two divided doses for 7 days.

Outcome Measures. The primary outcome measure was fever clearance time. The secondary outcome measure was overall treatment failure (acute treatment failure and relapse). Patients were followed up for 6 months.

Results. Randomization was carried out in 390 patients before enrollment was suspended on the advice of the independent data safety monitoring board due to

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significant differences in both primary and secondary outcome measures in the two arms and the attainment of a priori defined endpoints.

Among all randomized patients, **187 patients were assigned to receive cefixime and 203 to gatifloxacin**. 77 patients assigned to receive cefixime were blood culture positive for enteric fever whilst 92 of those assigned to receive gatifloxacin were culture positive.

Median (95% confidence interval) fever clearance times were 92 hours (84–114 hours) for gatifloxacin recipients and 138 hours (105–164 hours) for cefixime-treated patients (Hazard Ratio [95%CI] = 2.171 [1.545–3.051], $p=0.0001$). 19 out of 70 (27%) patients who completed the 7 day trial had acute clinical failure in the cefixime group as compared to 1 out of 88 patients (1%) in gatifloxacin group (Odds Ratio [95%CI] = 0.031 [0.004 – 0.237], $p=0.001$). Overall treatment failure patients (relapsed patients plus acute treatment failure patients plus death) numbered 29. They were determined to be (95% confidence interval) 37.6 % (27.14%–50.2%) in the cefixime group and 3.5% (2.2%–11.5%) in the gatifloxacin group (HR[95%CI] = 0.084 [0.025–0.280], $p=0.0001$). There was one death in the cefixime group. **This trial was stopped early by the independent Data Safety and Monitoring Board due to the inferior performance of cefixime.**

Conclusions. Based on this study, gatifloxacin is a better treatment for uncomplicated enteric fever than cefixime.

Trial Registration. Current Controlled Trials ISRCTN75784880

Chloramphenicol (Appendix 4):

A randomised controlled trial of gatifloxacin versus chloramphenicol for the treatment of uncomplicated enteric fever in Nepalese children and adults:

Background: It is unclear whether chloramphenicol is a reliable therapy for enteric fever or whether gatifloxacin, a newer generation and affordable fluoroquinolone, would be the better choice.

Objectives: To determine the efficacy of chloramphenicol versus gatifloxacin in the treatment of uncomplicated enteric fever.

Participants: Patients (aged two to sixty-five years) from Patan Hospital, Kathmandu, Nepal with clinically diagnosed with enteric fever who met the inclusion criteria.

Intervention: Patients received either gatifloxacin (10 mg/kg) once a day for 7 days or chloramphenicol (75 mg/kg/day) in four divided doses for 14 days.

Outcome measures: The primary outcome measure was treatment failure which comprised of persistent fever at day 10, need for rescue treatment, microbiological failure, relapse until day 31, and enteric fever related complications. The secondary outcome measure was fever clearance time, late relapse, and faecal carriage. Patients were followed up for 6 months.

Results: One thousand one hundred and fifty one patients were assessed for eligibility of which **853 were randomized and 844 were analyzed. Of these 418 were in the chloramphenicol arm and 426 were in the gatifloxacin arm.** Out of the 844 patients, 352 patients had blood culture confirmed enteric fever, 175 in the chloramphenicol arm and 177 in the gatifloxacin arm. There were 14 treatment failure patients in the chloramphenicol arm and 12 in the gatifloxacin arm (Hazard Ratio [95% CI]= 0.86 [0.40 to 1.86], $p=0.70$). Major side effects for chloramphenicol (bone marrow suppression) or gatifloxacin (dysglycemia) were not encountered although, nausea, dizziness, and diarrhea were worse in the chloramphenicol group. Only 0.5% (2/352) of the isolates were multidrug resistant (MDR), but 71 % (251/352) were nalidixic acid resistant.

Conclusion: This large clinical trial of culture confirmed enteric fever showed that both chloramphenicol and gatifloxacin had an excellent efficacy in this young population, in

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a region with full sensitivity to chloramphenicol and over 70% resistance to nalidixic acid. Treatment duration, ease of administration, decreased side effects and expense favours the usage of gatifloxacin.

Trial Registration. Current Controlled Trials ISRCTN 53258327. Funding: Wellcome Trust.

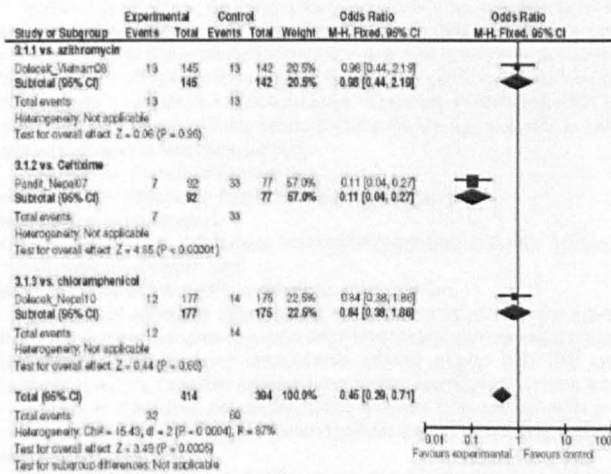
Ofloxacin:

An open randomised comparison of gatifloxacin versus ofloxacin for the treatment of uncomplicated enteric fever; ISRCTN63006567). This is an on-going trial. As of November 2010 approximately 500 patients have been enrolled.

10.3 Meta-analysis of the above RCTs of gatifloxacin for enteric fever

Two of the above RCTs (gatifloxacin vs. azithromycin and vs. cefixime) were already included in the previous Cochrane systematic review. A third study (vs. chloramphenicol) is being submitted for publication and will be included in the updated systematic review. These studies total 414 gatifloxacin and 394 active control patients.

This preliminary meta-analysis is based on overall failures (primary failure and relapse). No difference is found between gatifloxacin and azithromycin and between gatifloxacin and chloramphenicol; gatifloxacin is significantly more effective than cefixime. The latter comparison explains the heterogeneity found on aggregate (I-square = 87%).



11 Summary of comparative evidence on safety

- The class and product-specific safety liabilities are known
- The safety profile presented here is derived from RCTs of enteric fever and pulmonary tuberculosis
- There is no evidence from studies at the target dose in the target population that patients on gatifloxacin will be at a particular risk of dysglycaemia. This is further confirmed by data from tuberculosis patients exposed to ~25 times the total dose used for enteric fever.
- The drug is well-tolerated in patients with enteric fever; the gatifloxacin safety profile is similar to that of the comparator drugs.

11.1 Class and product-specific safety liabilities

Gatifloxacin is a fourth-generation fluoroquinolone antibiotic that, like other members of that family, inhibits the bacterial enzymes DNA gyrase and topoisomerase IV. Gatifloxacin is generally well tolerated. In common with all broad-spectrum antibiotics, gastrointestinal disturbances may be encountered. The fluoroquinolones are also known to have a number of adverse effects that are considered to be common to the class, although the severity of these varies considerably between compounds. These include effects on cardiac conduction, collagen formation (e.g. tendon rupture), photosensitivity, and dysglycaemia. While another 4th generation fluoroquinolone, moxifloxacin, has significant effects on cardiac repolarisation (prolongation of QT interval), this appears to be absent with gatifloxacin. In contrast, dysglycaemic effects (hypoglycaemia at the beginning of treatment, followed later by hyperglycaemia) are most commonly reported with gatifloxacin. (see also Section 8.2.6)

Hints of gatifloxacin-associated effects on glucose homeostasis were first noted during preclinical development. Preclinical studies which administered up to 19 times the 400 mg dose for up to 6 months demonstrated a dose related decrease in insulin release in pancreatic β -cells in all species studied (62). Shortly after gatifloxacin was introduced, case reports of effects on glucose homeostasis began to emerge. Patients identified as "at risk" included those with non-insulin-dependent diabetes on therapy and elderly patients with age-related decreases in renal function (56).

In summary, these data demonstrate

- a mechanism of gatifloxacin-associated dysglycemia that is
- dose- (exposure) dependent,
- patients especially at risk include non-insulin-dependent diabetics treated with oral hypoglycemic medications, and
- elderly patients with age-related changes in renal function.

Gatifloxacin-associated dysglycaemia was not noted during the course of the paediatric development programme and a large trial in children with otitis media followed for one year demonstrated no dysglycaemia adverse effects (63). The concerns of dysglycaemia in elderly Canadian patients were further heightened with the retrospective report in 2006 in the NEJM, concerning older, severely ill in-patients with a history of diabetes (64). That study received worldwide publicity leading to the drug being withdrawn in many countries.

Subsequent work (far less publicized) questioned whether these effects were any more severe or common compared to other fluoroquinolones and comparable antibiotics in an outpatient setting (65). In addition, patients with enteric fever are typically children and young adults and thus are very rarely non-insulin-dependent diabetics and have good kidney function. Clearly in these two very different populations gatifloxacin's dysglycaemia adverse effect profile is very different.

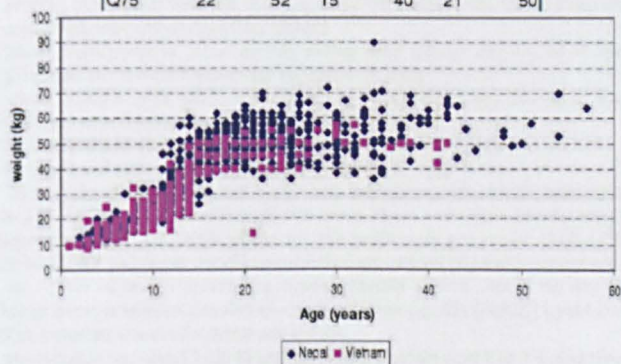
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Between Nepal and Vietnam in recent years in registered clinical trials almost 1500 young patients suffering from enteric fever, shigellosis and TB meningitis were treated with gatifloxacin with no problems noted in glucose control. Of these, over 600 patients were followed up for six months and no episodes of dysglycaemia have been seen (26, 66) and ISRCTN53258327 (An open randomised study to assess the efficacy of gatifloxacin versus chloramphenicol for the treatment of uncomplicated typhoid fever in Kathmandu, Nepal; manuscript submitted as appendix) and ISRCTN63006567 (An open randomised comparison of gatifloxacin versus ofloxacin for the treatment of uncomplicated enteric fever; ongoing). In addition, dysglycaemia has not emerged as a side effect attributable to gatifloxacin in 917 patients receiving gatifloxacin daily for four months as part of a drug combination regimen for the treatment of pulmonary tuberculosis in a multicentre RCT in Africa (Senegal, Benin, Guinea, Kenya and South Africa). Blood glucose analysis from the above studies is presented below.

As compared to the elderly Canadian patients, the profile of the enteric fever patients as derived from 1211 patients with age recorded enrolled in two RCTs in Nepal and Vietnam is that of a younger population (overall: median age 13 year, interquartile range 8-21 years; median weight 36kg, interquartile range 19.5-50kg).

Table 5. Age(n=853) and weight(n=850) profile of enteric fever patients from two RCTs in Nepal and Vietnam.

	Nepal		Vietnam		Combined	
	Age	Weight	Age	Weight	Age	Weight
count	853	850	358	358	1211	1208
median	16	42	11	23.3	13	36
Q25	9	21	7	18	8	19.5
Q75	22	52	15	40	21	50



11.2 Analysis of blood glucose in RCTs of enteric fever and pulmonary tuberculosis

We present here an analysis of blood glucose as dysglycaemia has been identified as a particular safety liability for this compound (see above.) We have detailed data from randomised controlled trials (RCTs) on enteric fever (~10mg/kg/d x 7d, n=422 gatifloxacin-treated patients) and tuberculosis (400mg/d 6 days a week in combination with rifampicin,

isoniazid and pyrazinamide for initial 2 months and in combination with rifampicin and isoniazid for the following 2 months, n=917 gatifloxacin-treated patients).

11.2.1 Blood glucose levels in enteric fever

Blood glucose levels were monitored during the trial of gatifloxacin versus chloramphenicol in Nepalese children (above 2 years) and adults (ISRCTN53258327). Random blood glucose (RBG) was measured daily on days 1 to 8, on day 15 and at one month. On days 2 to 7, during the evening visit, the blood glucose was measured by finger-prick testing (OneTouch SureStep™, Johnson & Johnson, USA) by the Community Medical Assistants. HbA1c was measured at 3 months.

For the analysis and grading of adverse events including blood glucose, the NIH Division of AIDS Table for Grading the Severity of Adult and Pediatric Adverse Events (“DAIDS AE grading table”) was used.

Table 6. Parameters used in the analysis of severity of disglycaemia

PARAMETER	GRADE 1	GRADE 2	GRADE 3	GRADE 4
	MILD	MODERATE	SEVERE	POTENTIALLY LIFE-THREATENING
Nonfasting Glucose, serum, high HYPERGLYCEMIA	116 – 180 mg/dL	161 – 250 mg/dL	251– 500 mg/dL	> 500 mg/dL
Nonfasting Glucose, serum, low HYPOGLYCEMIA	55 – 84 mg/dL	40 – 54 mg/dL	30 – 39 mg/dL	< 30 mg/dL

The following information regarding RBG and HbA1c was summarized:

- Median (IQR) levels for each visit (e.g. daily until day 8, day 15, and month 1 for random glucose and at day 90 for HbA1c)
- Worst hyperglycemia grade overall, during days 1-8, at day 15, or at month 1 (contingency tables of frequencies by treatment arm)
- Worst hypoglycemia grade overall, during days 1-8, at day 15, or at month 1 (contingency tables of frequencies by treatment arm)
- Median (IQR) levels of HbA1c at month 3.
- Proportion of patients with HbA1c >6% at month 3.

Table 7 shows the numbers of hyper- and hypoglycaemias in both treatment arms and Table 8 shows the median random glucose levels. There were more non-fasting grade 2 hyperglycaemias (160-250 mg/dl) in patients in the gatifloxacin group with 42/414 (10.14%) compared to 25/407 (6.14%) in the chloramphenicol group (p=0.04) during treatment (day 2 to day 7). There were no non-fasting hyperglycaemia events noted on day 15. The proportion of patients with an elevated glycosylated Haemoglobin (HbA1C) level between 6 and 7.5% at 3 months was similar in the two groups.

Median glucose levels (Table 8) tended to be higher on days 2 to 7 during treatment in both arms. Median glucose levels on days 4 to 8 were slightly, but significantly, higher in the gatifloxacin arm compared to the chloramphenicol arm.

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Table 7 Disglycaemia. Patients with abnormal non-fasting blood glucose. Numbers with (% of those tested)

	Chloramphenicol	Gatifloxacin	Comparison (p-value)
Hyperglycemia, Grade 2#			
At Baseline	1/414 (0.24)	2/422 (0.47)	-
On Day2 to Day7*	25/407 (6.14)	42/414 (10.14)	0.04
On Day8	0/402 (0)	1/400 (0.25)	0.5
On Day15	1/368 (0.27)	0/351 (0)	1
On Month1	1/375 (0.27)	0/383 (0)	0.50
Hypoglycemia, Grade2@ or worse			
At Baseline	4/414 (0.97)	4/426 (0.95)	-
On Day2 to Day7*	1/407 (0.25)	1/414 (0.24)	1
On Day8	2/402 (0.50)	2/400 (0.50)	1.00
On Day15	3/368 (0.82)	3/351 (0.85)	1.00
On Month1	3/375 (0.80)	4/383 (1.04)	1
HbA1c >6			
On Month3	22 (6.27)	20 (5.57)	0.75

* On Days 2 to 7 all patients were monitored with fingerstick glucose testing.

Hyperglycemia grade 2 defined as non-fasting plasma glucose level between 161 and 250 mg/dL. No grade 3 hyperglycaemias were observed.

@Hypoglycaemia grade 2 defined as non-fasting plasma glucose between 40 and 54 mg/dL

Table 8 Median daily random blood glucose level in enteric fever patients treated with chloramphenicol or gatifloxacin. Median glucose levels in mg/dl. n = Number of patients. Comparisons based on Wilcoxon test.

	All patients Glucose			Chloramphenicol Glucose			Gatifloxacin Glucose			p-value
	n	mg/dl	IQR mg/dl	n	mg/dl	IQR mg/dl	n	mg/dl	IQR mg/dl	
Day 1	836	87	77-98	414	86	77-98	422	87	76-98	0.581
Day 2	805	105	92-119	396	106	93-121	409	103	92-118	0.092
Day 3	784	106	94-119	389	104	94-118	395	107	95-120	0.18
Day 4	776	108	97-121.25	383	105	95-118	393	110	100-123	< 0.001
Day 5	767	107	96-121	383	104	95-114	384	111	99-125	< 0.001
Day 6	769	105	96-119	380	102	95-114	389	109	98-122	< 0.001
Day 7	754	106	96-117	379	102	93-114	375	110	99-121	< 0.001
Day 8	802	84	74-95	402	83	73-92	400	86	75-98	0.007
Day 15	717	81	73-91	366	81	73-92	351	81	72-90	0.412
Day 30	758	83	74-93	375	83	74.50-92	383	84	74-94.50	0.51

11.2.2 Blood glucose levels in pulmonary tuberculosis (Appendix 7)

Context

The data analysed are from a randomised open-label controlled trial of a 4 month gatifloxacin-containing regimen versus standard 6 month regimen for the treatment of adult patients with pulmonary tuberculosis. This trial has been conducted in 5 countries in Africa (Benin, Guinea, Kenya, Senegal and South Africa). The recruitment started in January 2005 and the study will end in April 2011.

Method

Male or female patients, aged 18 to 65 years, currently suffering from recently diagnosed microscopically proven pulmonary tuberculosis and providing informed consent were eligible for inclusion in the study. In the Gatifloxacin arm, patients took Gatifloxacin at the dose of 400mg daily (6 days per week), irrespective of weight, for 4 months; in association with Rifampicin, Isoniazid and Pyrazinamide during the first 2 months of TB treatment and Rifampicin, Isoniazid for the last 2 months.

Initially blood glucose measurements were taken at baseline (pre-randomisation; visit 1), week 4 (visit 3), 8 (visit 4) and end of treatment visits (either visit 6 or 8 depending on treatment arm), by finger-prick or full blood tests. During the study recruitment period additional sampling times were incorporated based on the recommendation from the Data Monitoring Committee. These were: 4 hours following first treatment dose, day 7, 14 and week 12 (visit 5) from randomisation. The measurement taken 4 hours after first dose was not taken when fasting as patients were allowed to eat 30 minutes after first drug intake.

This report provides a summary, by treatment arm, of the (i) frequency of hypoglycaemic (less than or equal to 3.5 mmol/L), normal (3.51-6.39 mmol/L) and hyperglycaemic (greater than or equal to 6.4 mmol/L) events, (ii) severity of these events, and (iii) incidence rates of hyperglycaemic events.

Results and conclusion

A total of 1,836 patients have been recruited in this trial of whom 917 were randomised to the Gatifloxacin arm. In the Gatifloxacin arm the approximate mean age and weight were 30 years and 55kg, respectively. Based on the data analysed, the incidence rates of dysglycaemic events were similar in the Gatifloxacin and control arms.

11.3 Summary of comparative safety against comparators in RCTs of enteric fever

In randomized controlled trials comparing gatifloxacin to azithromycin (66), cefixime (26) and chloramphenicol; gatifloxacin was extremely well tolerated.

Gatifloxacin versus azithromycin

Both treatments were well tolerated. One adverse event related to azithromycin was reported, a maculopapular rash that occurred after the first dose of treatment. Azithromycin was discontinued immediately and the patient was treated with ceftriaxone.

Gastrointestinal side-effects (change in consistency and frequency of stools) that were probably typhoid fever related were relatively frequent in both treatment arms at the start of treatment. In the gatifloxacin group, one patient experienced vomiting on day 2 and day 3 and one patient had diarrhoea (4 episodes/ day) on day 4 and day 5 of treatment. These episodes were self-limiting and did not require the interruption of therapy.

The median levels of serum AST and ALT fell in both groups after 7 days of therapy. In the culture positive group, the median post-treatment AST was 46.35 U/L (range 12.8 – 217.5) in the gatifloxacin arm and 45 U/L (range 5 – 358) in the azithromycin arm.

Gatifloxacin versus cefixime

There was one death in the cefixime group. This might have been due to the development of disease-related complications during treatment. The 15 year old patient was enrolled on the fourteenth day of his illness. On day 6 of treatment the patient complained of reddish stool and petechiae and was immediately admitted to hospital where he developed severe thrombocytopenia and gastrointestinal bleeding. He developed acute respiratory distress syndrome and was mechanically ventilated. He developed disseminated intravascular coagulation and succumbed to his illness on day 21 of entry into the trial. His pretreatment

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blood culture grew *S. Paratyphi A* which was sensitive to cefixime with an MIC of 0.38 mg/mL. One patient developed erythematous skin rash which needed two doses of oral antihistamine.

Among all patients who received gatifloxacin there were 2 patients with excessive vomiting, which required intravenous anti-emetics and fluids and observation in the hospital emergency room for up to 6 hours. There was an additional 23 patients who complained of excessive nausea and occasional vomiting after ingestion of the drug. Of these, two needed oral antiemetics; in the remaining 21 patients no intervention was required.

Gatifloxacin versus chloramphenicol

A comparison of adverse events in the two randomized arms is shown in Table 9. Adverse events were mild (grades 1 and 2). Anorexia, diarrhoea, nausea and dizziness were significantly more common in the chloramphenicol arm of the study. Three patients in the chloramphenicol arm who had a white cell count between 1500-1999/mm³ on day 5 to 8 and had their chloramphenicol stopped. The fever had already defervesced when the drug was stopped and they did not relapse for 6 months. There were no life-threatening complications of enteric fever in this study.

Table 9. Adverse events in enteric fever patients treated with chloramphenicol or gatifloxacin.

Adverse Event	Chloramphenicol (n=418)		Gatifloxacin (n=426)		Comparison (p value)#
	Number of Patients with event (%)	Number of Events	Number of Patients with event (%)	Number of Events	
Any selected AE	99 (23.63%)	168	59 (13.85%)	73	<0.001
Abdominal pain	11 (2.63%)	12	8 (1.88%)	8	0.5
Acne	2 (0.48%)	2	0 (0%)	0	0.26
Anorexia	9 (2.15%)	10	1 (0.23%)	1	0.01
Diarrhoea	24 (5.74%)	26	5 (1.17%)	5	<0.001
Dizziness	11 (2.63%)	11	2 (0.47%)	2	0.01
Nausea	26 (6.22%)	29	9 (2.11%)	9	0.003
Oral candidiasis	4 (0.96%)	4	0 (0%)	0	0.06
Vomiting	36 (8.61%)	39	35 (8.22%)	35	0.9
Weakness	4 (0.96%)	4	0 (0%)	0	0.06

Note: All adverse events in this list were non-severe, ie Grade I or Grade II

Fisher's exact test comparing the number of patients with an event.

12 Summary of available data on comparative cost and cost-effectiveness within the pharmacological class or therapeutic group

A variety of generic products is on the market, all generally cheap. We retrieved information and compared costs with alternative treatment options. We also used enteric fever patient data to produce specific estimates of cost of drug when enteric fever is treated with gatifloxacin. Other direct costs and indirect costs could not be quantified for the moment. The cost of illness of enteric fever is poorly characterised.

This section shows that:

- Gatifloxacin is the cheapest of all treatments of enteric fever. Azithromycin (the only alternative that could be used in areas of MRD and NAR) is 2-5 times as expensive.
- Treating 1000 patients with the current products (200 and 400mg tablets) will cost 4150\$.

12.1 Economic burden of enteric fever

There have been very few studies of the economic burden of illness in regions where enteric fever is endemic. In 2004 from India the estimated direct and indirect costs to the family of an episode of enteric fever ranged between 150US\$ - 550US\$ depending on whether the patient was treated as an out-patient or required admission to hospital (67). Neither of these estimates included the costs incurred during relapses or the costs associated with patients who develop long term carriage and pass on the infection to others in their community.

12.2 Direct costs: comparison of drug costs for treatment of enteric fever with gatifloxacin vs. other options

Cost of treatment of enteric fever with gatifloxacin and several other antibiotics was estimated using data obtained from an on-line pharmacy database in India <<http://chemistparadise.com>>. Generally accepted dosage regimens for adults of 50 kg and 75 kg body weight, and children of 15 kg and 25 kg body weight were used to estimate the overall cost of treatment for these different classes. For gatifloxacin, the maximum daily dose was set at 600mg.

Table 10. Dose and duration of therapy of enteric fever with gatifloxacin and other regimens. Examples of total dose for a 15kg and 25kg child and a 50kg and 75kg adult are provided.

	Dose (mg/kg/d)	Days	75 Kg (adult)	50 Kg (adult)	25 Kg (child)	15 Kg (child)
Gatifloxacin	10	7	600	500	250	150
Chloramphenicol	75	14	5625	3750	1875	1125
Cefixime	20	14	1500	1000	500	300
Ceftriaxone		14	4000	2000	2000	1000
Ciprofloxacin	20	7	1500	1000	500	300
Ofloxacin	20	7	1500	1000	500	300
Azithromycin		7	1000	1000	500	500

All gatifloxacin products available in India on 1st November 2010 were identified, and per tablet costs obtained in US\$. Similarly, the costs of chloramphenicol, ceftriaxone, ciprofloxacin, ofloxacin, and azithromycin were obtained. These were wholesale costs to pharmacy of single packs of, in general, 10 tablets. Since there were a large number of products available, with a wide price range, the mean cost was obtained for each compound and strength. Only solid dosage forms (capsule/tablet) were considered, although examination of the costs of specific paediatric formulations and parenteral formulations, where available, showed similar price structures. The mean single tablet costs were estimated, and applied to the different dosage regimens to give per treatment costs, using the most appropriate dose sizes for the different weight classes. The derived data (in US\$) are tabulated below.

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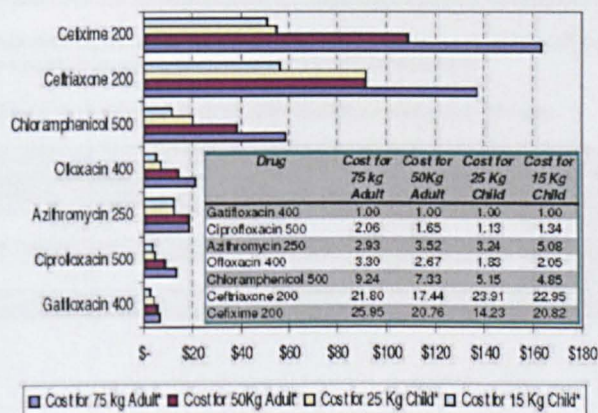
Table 11. Cost of treating children and adults with enteric fever with gatifloxacin and other regimens

Drug	Cost range per tablet	Mean cost per tablet	Cost for 75 kg Adult*	Cost for 50Kg Adult*	Cost for 25 Kg Child*	Cost for 15 Kg Child*
Gatifloxacin 400	0.07-1.00	\$ 0.55	\$ 6.30	\$ 5.25	\$ 3.83	\$ 2.44
Ciprofloxacin 500	0.09-0.81	\$ 0.46	\$ 12.96	\$ 8.64	\$ 4.32	\$ 3.28
Azithromycin 250	0.09-3.28	\$ 0.89	\$ 18.47	\$ 18.47	\$ 12.40	\$ 12.40
Ofloxacin 400	0.08-0.99	\$ 0.62	\$ 20.80	\$ 14.00	\$ 7.00	\$ 5.00
Chloramphenicol 500	0.17-0.73	\$ 0.38	\$ 58.22	\$ 38.48	\$ 19.74	\$ 11.84
Ceftriaxone 200	0.65	\$ 0.65	\$ 137.34	\$ 91.56	\$ 91.56	\$ 56.00
Cefixime 200	0.08-4.08	\$ 1.56	\$ 163.50	\$ 109.00	\$ 54.50	\$ 50.80

* usually half strength tablets/capsules used for these estimates

Gatifloxacin appears to be cheapest option. The next least expensive drug is ciprofloxacin, which is 13%-69% more expensive. Ofloxacin is 1.83-2.05 and azithromycin is 3-5 times as expensive. Chloramphenicol is 5-9 times as expensive. The most costly options are ceftriaxone (17-24) and cefixime (14-26) times as expensive.

Figure 4. Comparative costs of treatment with gatifloxacin vs. other regimens for enteric fever (in the Table: relative costs, Gatifloxacin = 1)



A very large number of products (often in excess of 100 alternatives for each dose formulation) are available, with widely differing costs, except for ceftriaxone where only a single product was listed. Costs for the better-known manufacturers were in general higher, but not always so. Although product costs have been estimated for treatments, this is a

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basic analysis, and no attempt has been made to assess quality of product, which would be a significant factor in determining true cost and efficacy of treatment. While single tablet costs of other products are often lower than gatifloxacin, cost of treatment is determined by the regimen used, and when this is considered, the cost of gatifloxacin treatment, using Indian derived material, is lower than with other accepted treatments. Some limited data are available for other countries and roughly follows that of the Indian data (much of the material used is of Indian origin, especially in the sub-continent), but detailed information on costs of all products within a country are not available to allow a similar analysis.

12.3 Direct costs of treatment of enteric fever with gatifloxacin practical dosing

Using the anthropometric database of Nepalese and Vietnamese patients, and applying the practical dosing schedule with the current 200mg and 400mg tablets, we calculated the costs of treating 1,000 cases of enteric fever. These amount to 4,150 \$. Using the Vietnamese patients' profile, the projected cost of drug for treating all estimated ~4500 cases occurring in one year in Vietnam will cost ~15,800\$.

Table 12. Cost of drug for treating 1000 enteric fever patients with gatifloxacin using the practical dosing schedule with the current non-scored 200mg and 400mg tablets

body weight class (kg)	No. per 1000 pts	total No. tablets required	cost/Rx	cost/1000 Rx
<15*	97	7 X 200mg	\$2.66	\$258.02
15 to 29	342	7 X 200mg	\$2.66	\$909.72
30 to 49	296	7 X 400mg	\$4.06	\$1,201.76
>=50	265	7 X 600mg	\$6.72	\$1,780.80
TOTAL	1000			\$4,150.30

When applying these calculations to Vietnam, the total costs of treating all the ~4500 cases per annum of the entire country will be ~15,800\$ for drug costs.

Table 13. Cost of drug to treat all cases occurring in one year in Vietnam

body weight class (kg)	% population	total No. tablets required	cost/1000 Rx
<15*	14%	7 X 200mg	\$1,671.79
15 to 29	49%	7 X 200mg	\$5,817.82
30 to 49	25%	7 X 400mg	\$4,593.02
>=50	12%	7 X 600mg	\$3,716.65
TOTAL			\$15,799.27

12.4 Total direct and indirect costs

We are not in a position at present to submit a complete cost-effectiveness analysis. Cost of illness has not been entirely quantified for enteric fever. To account for all direct costs, we are collecting information on cost of care in Nepal and Vietnam. We are also attempting to collect more anthropometric data on enteric fever patients from different settings to generate more complete projections of the applicability of the practical dosing and costs of drug.

Using gatifloxacin will also mean that an antibiogram is not needed - this cost should also be factored in.

13 Summary of regulatory status of the medicine

Gatifloxacin was originally granted licences in the USA (Bristol-Myers Squibb) and Japan (Kyorin) in 1999. The indications included general infections sensitive to fluoroquinolones and community acquired pneumonia. In 2002, there were indications that some patients might suffer dysglycaemia, and warnings were added to the SPC. Following publication of a review of dysglycaemia related deaths (64) and an editorial (68) in March 2006 in NEJM, the US FDA required a 'Black Box' warning¹ to be added to the SPC. Subsequently, in May 2006, Bristol-Myers Squibb stopped manufacture of Tequin®. Similar action was taken in 2008 in Japan. In addition, The US FDA in September 2008 decided that, since "Tequin (gatifloxacin) Tablets, Injections and Oral Suspensions were withdrawn from sales for reasons of safety or effectiveness" gatifloxacin had become ineligible to the abbreviated new drug application (ANDA) should a new submission be filed for any of the previously approved dosage forms and indications of Tequin®(69).

Although the licence for oral products has not been renewed or has been voluntary withdrawn in countries such as the USA and Japan, the product remains approved in many countries where enteric fever is endemic and drug resistant strains are present - including India, Vietnam, Nepal, Bangladesh and China. It also remains widely available as an ophthalmic solution for eye infections.

14 Availability of pharmacopoeial standards (British Pharmacopoeia, International Pharmacopoeia, United States Pharmacopoeia)

A 'Pending USP Standard' was approved May 23 2008 (70). API and analytical standards exist in the Indian and Chinese Pharmacopoeias.

15 Proposed (new) text for the WHO Model Formulary

Gatifloxacin

Tablet, 200mg, 400 mg (as sesquihydrate)

General information

Gatifloxacin is a synthetic fluoroquinolone that acts as a specific inhibitor of bacterial DNA gyrase and topoisomerase IV. It has a broad spectrum of efficacy against both Gram-

¹ The US Food and Drug Administration requires that - when serious adverse events are identified by the agency as being of particular concern - the drug's printed materials carry a warning about those adverse effects surrounded by a printed black box - thus the name. As an example, since July 2008 fluoroquinolones as a family are required to have a black box warning for tendon rupture.

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negative and Gram-positive aerobic bacteria. Transfer of genes containing DNA coding for antimicrobial resistance has been reported but as yet is of little clinical significance. Gatifloxacin is rapidly absorbed from the gastrointestinal tract. Peak plasma levels occur 1-2 hours after dosing. It is widely distributed in body tissues and concentrated in the bile. It has a plasma half-life of approximately 8 hours and is excreted in the urine mainly as unchanged drug. Elimination half-life 7-14 hours, with more than 70% of a given dose being excreted in 48 hours. Bioavailability 96%; protein binding 20%.

Clinical information

Uses

Treatment of typhoid and paratyphoid fever and infectious enteritis due to *Salmonella enteritidis*.

Dosage and administration

Children and adults: 10mg/kg orally (maximum 600mg/d) every 24 hours for 7 days. A practical dosing with 200mg and 400mg strength tablets will be as follows:

weight band	daily dose
<15kg	10mg/kg
15kg to 29kg	200mg
30kg to 49kg	400mg
>= 50kg	600mg

Contraindications

- Hypersensitivity to any quinolone antibiotic.
- Diabetes mellitus.
- Pregnancy and lactation

Precautions

DISTURBANCES OF BLOOD GLUCOSE, INCLUDING SYMPTOMATIC HYPOGLYCEMIA AND HYPERGLYCEMIA, HAVE BEEN REPORTED WITH GATIFLOXACIN, USUALLY IN DIABETIC PATIENTS. HOWEVER, HYPOGLYCEMIA AND PARTICULARLY HYPERGLYCEMIA HAVE OCCURRED IN PATIENTS WITHOUT A HISTORY OF DIABETES. IN ADDITION TO DIABETES, OTHER RISK FACTORS ASSOCIATED WITH DYSGLYCEMIA WHILE TAKING GATIFLOXACIN INCLUDE OLDER AGE, RENAL INSUFFICIENCY AND CONCOMITANT GLUCOSE-ALTERING MEDICATIONS (PARTICULARLY HYPOGLYCEMIC MEDICATIONS). PATIENTS WITH THESE RISK FACTORS SHOULD BE CLOSELY MONITORED FOR GLUCOSE DISTURBANCES. IF SIGNS AND SYMPTOMS OF EITHER HYPOGLYCEMIA OR HYPERGLYCEMIA OCCUR IN ANY PATIENT BEING TREATED WITH TEQUIN, APPROPRIATE THERAPY MUST BE INITIATED IMMEDIATELY AND GATIFLOXACIN SHOULD BE DISCONTINUED.

Reduced dosage should be considered in patients with hepatic or renal impairment.

Gatifloxacin has the potential to prolong the QTc interval of the electrocardiogram in some individuals, with an increased risk of ventricular arrhythmias. Care should be taken in individuals taking Class 1A or Class 3 anti-arrhythmic agents.

Gatifloxacin has been rarely associated with tendon rupture, usually in elderly patients and those receiving corticosteroids.

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Gatifloxacin should be administered cautiously to patients with epilepsy or raised intracranial pressure since seizures have been reported with other drugs of this class.

Use in pregnancy and early childhood

Gatifloxacin should not be used during pregnancy or during lactation. Use in children is controversial, since quinolones have been shown to induce arthropathy in the weightbearing joints of young animals. Although damage to growing cartilage has not been demonstrated in humans, use of quinolones is not generally recommended in children and adolescents. However, in severe infections such as enteric fever the benefits are considered to outweigh the risk.

Adverse effects

Gatifloxacin is generally well tolerated. The most frequently reported adverse effects are nausea, diarrhoea, vomiting, dyspepsia, abdominal pain, headache, restlessness, tremor, confusion, rash, dizziness and pruritus.

Myalgia, tendinitis, and hepatic and renal disturbances have also been reported. Rapid heartbeat, mental confusion, hallucinations, agitation, nightmares, depression; photophobia; tendon rupture; insomnia, chills, fever; back pain; constipation, inflammation of the tongue, mouth sores; abnormal vision, ringing in the ears occur occasionally. Hyperosmolar nonketotic hyperglycaemic coma, diabetic ketoacidosis, and hypoglycaemic coma have been reported and are potentially fatal if untreated.

Drug interactions

Systemic exposure to gatifloxacin is increased with concomitant administration of probenecid and reduced with concomitant administration of oral iron compounds and antacids containing aluminium or magnesium salts. Significant interactions are not seen as a result of affects on major cytochrome P450 enzymes (3A, 1A2, 2C9, 2C19, and 2D6).

Overdosage

Gastric lavage is of value if performed promptly. Electrolyte balance must be maintained and cardiac function monitored. Serum concentrations of gatifloxacin may be lowered by dialysis.

Storage

Tablets should be stored in well-closed containers.

APPENDICES

Appendix 1. Patent status

Appendix 2. Individual presentations of generic gatifloxacin in India

Appendix 3. ACCESS RESTRICTED TO REVIEWERS UNTIL PUBLISHED - Pharmacodynamics of gatifloxacin in patients with typhoid fever

Appendix 4. ACCESS RESTRICTED TO REVIEWERS UNTIL PUBLISHED - Population pharmacokinetics of gatifloxacin in south east Asian adult and pediatric patients with typhoid fever

Appendix 5. Updated Cochrane systematic review and meta-analysis of fluoroquinolones for enteric fever- will be submitted later

Appendix 6. ACCESS RESTRICTED TO REVIEWERS UNTIL PUBLISHED - A randomised controlled trial of gatifloxacin versus chloramphenicol for the treatment of uncomplicated enteric fever in Nepalese children and adults

Appendix 7. ACCESS RESTRICTED TO REVIEWERS UNTIL PUBLISHED - Blood glucose levels in pulmonary tuberculosis patients treated with gatifloxacin

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Temporal Fluctuation of Multidrug Resistant *Salmonella* Typhi Haplotypes in the Mekong River Delta Region of Vietnam

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Abstract

Background: Typhoid fever remains a public health problem in Vietnam, with a significant burden in the Mekong River delta region. Typhoid fever is caused by the bacterial pathogen *Salmonella enterica* serovar Typhi (*S. Typhi*), which is frequently multidrug resistant with reduced susceptibility to fluoroquinolone-based drugs, the first choice for the treatment of typhoid fever. We used a GoldenGate (Illumina) assay to type 1,500 single nucleotide polymorphisms (SNPs) and analyse the genetic variation of *S. Typhi* isolated from 267 typhoid fever patients in the Mekong delta region participating in a randomized trial conducted between 2004 and 2005.

Principal Findings: The population of *S. Typhi* circulating during the study was highly clonal, with 91% of isolates belonging to a single clonal complex of the *S. Typhi* H58 haplogroup. The patterns of disease were consistent with the presence of an endemic haplotype H58-C and a localised outbreak of *S. Typhi* haplotype H58-E2 in 2004. H58-E2-associated typhoid fever cases exhibited evidence of significant geo-spatial clustering along the Sông Hậu branch of the Mekong River. Multidrug resistance was common in the established clone H58-C but not in the outbreak clone H58-E2, however all H58 *S. Typhi* were nalidixic acid resistant and carried a Ser83Phe amino acid substitution in the *gyrA* gene.

Significance: The H58 haplogroup dominates *S. Typhi* populations in other endemic areas, but the population described here was more homogeneous than previously examined populations, and the dominant clonal complex (H58-C, -E1, -E2) observed in this study has not been detected outside Vietnam. IncH11 plasmid-bearing *S. Typhi* H58-C was endemic during the study period whilst H58-E2, which rarely carried the plasmid, was only transient, suggesting a selective advantage for the plasmid. These data add insight into the outbreak dynamics and local molecular epidemiology of *S. Typhi* in southern Vietnam.

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Introduction

The Mekong river delta is located in the south of Vietnam (Figure 1) in an area of 40,000 square kilometres (12% of Vietnam's land mass) and is home to over 20% of Vietnam's population. It is the area where the Mekong river divides into multiple channels and drains into the South China sea. The low-lying nature of the land and the seasonal fluctuation in water level make the region particularly vulnerable to flooding. The human-restricted disease typhoid fever is endemic to the Mekong delta

region [1,2], with a mean incidence of ~80 cases per 100,000 people per year [1,2,3,4]. *Salmonella* Typhi (*S. Typhi*), the bacterium causing typhoid fever, is transmitted human-to-human in areas with poor sanitation.

The first multidrug resistant (MDR; defined as resistance to chloramphenicol, ampicillin and co-trimoxazole) typhoid outbreak in Vietnam occurred in Kien Giang in the Mekong river delta in 1993 [5], and since then the fluoroquinolones have become the first choice for the treatment of typhoid fever. MDR *S. Typhi* is usually associated with self-transferrable IncH11 plasmids carrying

Author Summary

Typhoid fever remains a serious public health issue in some parts of Vietnam, including the Mekong delta region. Typhoid is caused by the bacterium *Salmonella* Typhi, which is frequently multidrug resistant and shows reduced susceptibility to fluoroquinolone-based drugs. We assayed single nucleotide variation in the genomes of *S. Typhi* organisms isolated from 267 patients with typhoid fever in the Mekong delta between 2004 and 2005, and identified genetically distinct *S. Typhi* strains. We also detected the presence of genes or mutations that confer drug resistance in those strains. We found that the vast majority of typhoid cases were caused by one of two subgroups of H58 *S. Typhi*, referred to as H58-C and H58-E2. The H58-E2 group appeared to cause an outbreak in 2004, affecting patients living in a small zone near the Mekong River. The other group, H58-C, was present throughout the study period and affected patients living in a broader area of the Mekong River delta. Most of the H58-C strains were resistant to multiple drugs and carried a plasmid encoding multiple resistance genes. However very few H58-E2 strains were multidrug resistant, which may explain why the strain did not persist after the initial outbreak.

multiple resistance genes encoded within mobile genetic elements [6,7,8,9,10]. Between 1994 to 1998, over 80% of *S. Typhi* strains isolated in the Mekong delta region were reported to be MDR [11], and declined to approximately 50% between 2002 and 2004

[5,11,12]. This decline may have been catalysed by the change in treatment policy and the widespread use of fluoroquinolones (such as ciprofloxacin and ofloxacin), which are effective against MDR strains [13,14].

While high-level resistance to fluoroquinolones remains uncommon in Vietnam and other endemic typhoid regions, there has been a sharp increase in the proportion of *S. Typhi* isolates that are resistant to nalidixic acid [11]. Nalidixic acid (Nal) is a quinolone antimicrobial (the precursor of fluoroquinolones) and the main mechanism for Nal resistance in *S. Typhi* is mutation of the DNA gyrase gene, *gyrA* [11,15]. *S. Typhi* strains with Nal resistance-conferring mutations in the *gyrA* gene usually have elevated minimum inhibitory concentrations (MIC) to fluoroquinolone antibiotics such as ciprofloxacin (MIC ≥ 0.125 $\mu\text{g}/\text{ml}$) [16]. However, these organisms are not resistant according to CLSI guidelines, which are currently defined by MIC ≥ 4 $\mu\text{g}/\text{ml}$ to ciprofloxacin [17]. Even though these strains are not classified as resistant, they are of clinical importance since typhoid patients infected with such strains respond less well to fluoroquinolone therapy [14,15,18,19]. Such patients frequently have a protracted fever and an increased rate of relapse, compared to those infected with strains that do not have an elevated MIC to fluoroquinolones (MIC < 0.125 $\mu\text{g}/\text{ml}$ to ciprofloxacin and < 0.25 $\mu\text{g}/\text{ml}$ to ofloxacin) [15,18,19]. Resistance to Nal is therefore often used as a marker to predict how well a patient will respond to therapy with fluoroquinolones.

The incidence of typhoid fever has declined in Vietnam. Between 1991 and 2001 approximately 17,000 cases of typhoid fever (blood culture confirmed and syndromic cases) were reported

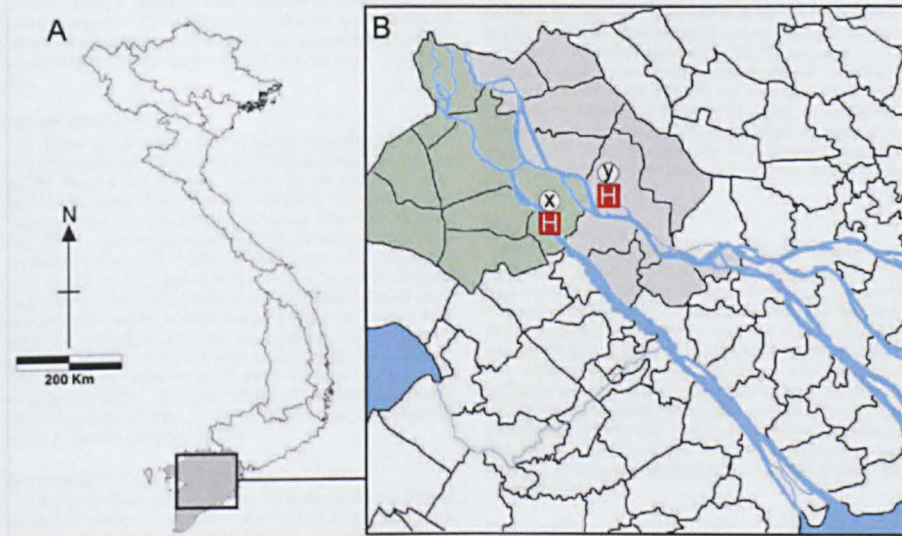


Figure 1. Location of hospitals in the Mekong river delta of Vietnam. (A) Map showing the 8 Vietnamese regions stretching from the Peoples Republic of China in the north to the Mekong river delta in the south. Highlighted in grey is Mekong river delta (Đồng Bằng Sông Cửu Long) region, which is the southernmost of the eight regions and covers 40,000 km². The dotted box corresponds to the area magnified in (B). (B) Map showing a ~22,000 km² of the Mekong river delta; highlighted are An Giang province (green) and Dong Thap province (grey). Also highlighted are the provincial hospitals of An Giang province (x) and Dong Thap (y). The direct distance between the two hospitals is 22.5 km. doi:10.1371/journal.pntd.0000929.g001

annually through the Vietnamese national surveillance system [1,2], while only 4,323 and 5,030 annual typhoid fever cases were reported in 2004 and 2005, respectively (Source: National Institute of Health and Epidemiology, Ministry of Health, Vietnam). However, 75% of these cases occurred in the Mekong delta [1,2], likely associated with high population density and the propensity of the land to become saturated with floodwaters. In this region, the occurrence of *S. Typhi* isolates that are MDR and Nal resistant severely limits treatment options. More than 95% of *S. Typhi* isolated in the Mekong delta are now resistant to Nal, placing a considerable pressure on the effective use of fluoroquinolones [11,12]. To compare alternative therapies for typhoid fever patients infected with strains that are MDR and Nal resistant, a randomized controlled trial comparing gatifloxacin (a newer 8-methoxy fluoroquinolone) and azithromycin (a macrolide) was conducted during 2004–2005 in the Mekong delta region [20]. Typhoid patients (adults and children) were recruited into the study at three hospitals in the south of Vietnam (details in Materials and Methods, locations are highlighted in Figure 1B). Here, we used a high-throughput single nucleotide polymorphism (SNP) typing assay to investigate the population structure of *S. Typhi* collected during the study [20], and to determine the genetic mechanisms of drug resistance in this *S. Typhi* population.

Materials and Methods

Ethics statement

The study was conducted according to the principles expressed in the Declaration of Helsinki and approved by the Institutional Review Board of the Hospital for Tropical Diseases and the Oxford Tropical Research Ethics Committee (OXTREC). All patients provided written informed consent for the collection of samples and subsequent analysis (written informed consent was provided by the parents or guardian of children under 18 years of age).

Patient recruitment

S. Typhi isolates were collected during a multicenter clinical trial [20] conducted between January 2004 and December 2005 at (a) the Hospital for Tropical Diseases in Ho Chi Minh City ($n=10$), (b) Dong Thap Provincial Hospital, Cao Lanh, Dong Thap province ($n=25$) and (c) An Giang Provincial Hospital, Long Xuyen, An Giang province ($n=232$). Locations of (b) and (c) are shown in Figure 1B. Adults and children over 6 months of age were eligible to be included in the study if they had clinically suspected or culture-confirmed uncomplicated typhoid fever and if fully informed written consent had been obtained. Patients were tested for typhoid carriage (via stool culture) during follow-up appointments at 1, 3 and 6 months after discharge from hospital. The 267 isolates presented in this study constitute nearly the full complement of 287 *S. Typhi* isolated from culture-confirmed typhoid patients enrolled in the trial; the recruitment flow for which is described in detail in [20].

Antimicrobial susceptibility testing

Antimicrobial susceptibility testing was performed at the time of initial isolation by disc diffusion according to Clinical Laboratory Standards Institute (CLSI) guidelines [17]. Antimicrobial agents tested were: ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole (co-trimoxazole), nalidixic acid, ofloxacin, ciprofloxacin and ceftriaxone (Oxoid, Basingstoke, UK). Minimum Inhibitory Concentrations (MICs) for amoxicillin, chloramphenicol, nalidixic acid, ofloxacin, ciprofloxacin, gatifloxacin, ceftriaxone and azithromycin were determined by E-test (AB Biodisk, Solna,

Sweden). Multidrug resistance (MDR) of isolates was defined as resistance to chloramphenicol (MIC ≥ 32 $\mu\text{g/mL}$), ampicillin (MIC ≥ 32 $\mu\text{g/mL}$) and trimethoprim-sulfamethoxazole (MIC $\geq 8/152$ $\mu\text{g/mL}$). Nalidixic acid resistance was defined by an MIC ≥ 32 $\mu\text{g/mL}$.

Bacterial isolation and DNA preparation

After initial isolation, *S. Typhi* was stored at -70°C in a 20% glycerol solution until required for further analysis and DNA extraction. To revive frozen organisms, MacConkey and Xylose Lysine Decarboxylase (XLD) agar plates were inoculated from the glycerol solution and incubated at 37°C overnight. To ensure correct identification, colonies were checked using slide agglutination with serotype specific antisera (V3, O9) and an irrelevant antisera as a negative control (O4) (Murex, Dartford, United Kingdom). Two mL of nutrient broth were inoculated with single *S. Typhi* colonies and incubated overnight. Overnight cultures were centrifuged and *S. Typhi* DNA was extracted using Wizard Genomic DNA Purification kit (Promega, USA) as recommended by the manufacturer's guidelines. DNA was stored at -20°C . DNA was quantified using the Quant-IT PicoGreen dsDNA Reagent and Kit (Invitrogen, UK). *S. Typhi* DNA concentrations were adjusted to 50 ng/mL and 250 ng of DNA were pipetted into 96-well plates. Each 96-well plate contained two isolates in duplicate and the sequenced *S. Typhi* isolate CT18 as control for assay reproducibility.

Determination of chromosomal and plasmid haplotypes

The chromosomal haplotype of *S. Typhi* isolates was determined based on alleles present at 1,485 chromosomal SNP loci identified previously from genome-wide surveys [12,21] and listed in [22,23]. IncHI1 plasmid haplotypes were determined based on eight SNPs identified previously [22,24] and resistance gene sequences were interrogated using additional oligonucleotide probes (listed in Table S1). All loci were interrogated using a GoldenGate custom oligonucleotide array according to the manufacturer's standard protocols (Illumina), as described previously [22,23]. A maximum-likelihood phylogenetic tree based on chromosomal SNPs was constructed using the RAxML software [25].

Statistical analysis

Clinical data were entered into an electronic database (Epi Info 2003, CDC, Atlanta, USA). For comparison of patient characteristics according to infecting *S. Typhi* haplotypes, Kruskal-Wallis tests were used for analysis of continuous variables (age, length of stay in hospital, fever clearance time) and logistic regression was used for categorical variables (presence of symptoms). Odds ratios were adjusted for duration of fever prior to admission and use of antibiotics prior to admission by including these variables in the logistic regression model. Where data was missing for a particular patient and variable, that patient was excluded from analysis of that variable ($N \leq 35$ patients). Two-tailed p -values are reported; statistical analysis was performed using the R package (<http://www.r-project.org/>).

PCR amplification and sequencing of *gyrA* gene in *S. Typhi*

Oligonucleotide primers for the amplification of the quinolone resistance determining regions in the *S. Typhi gyrA* gene were as follows [11]: GYRA/P1 5'-TGTCCGAG ATGGCCTGAAGC-3' and GYRA/P2 5'-TACCGTCATAAGTTATCCACG-3'. Predicted PCR amplicon size was 347 bp. PCR was performed

under the following conditions; 30 cycles of 92°C for 45 seconds, 45 62°C for 45 seconds and extension at 74°C for 1 minute, followed by a final extension step at 74°C for 2 minutes. PCR products were purified and directly sequenced using the CEQ DTCS - Quick Start Kit (Beckman Coulter, USA) and the CEQ 8000 capillary sequencer. The resulting DNA sequence was analyzed using CEQSequence Investigator CEQ2000XL (Beckman Coulter, USA). All sequences were verified, aligned and manipulated using Bioedit software (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>) and compared to other *gtfA* sequences by BLASTn at NCBI.

Spatial data collection and analysis

Patient addresses were recorded at the time of hospital admission. The latitude and longitude of the residences of typhoid fever patients (to the hamlet/village level) was assigned from the collected address data using 1/50,000 scale maps (Source: Cartographic Publishing House and VinaREN, Ministry of Natural Resources and Environment, Vietnam) and cross-checked using the websites <http://www.basao.com.vn> and <http://ciren.vn>. Location data was analysed using Quantum GIS version 1.4.0 (<http://www.qgis.org/>). Locations were colour-coded according to *S. Typhi* haplotype and clustering of specific haplotypes was calculated using the nearest-neighbour analysis function. Nearest-neighbour analysis examines the distances between each point and the closest point to it, and then compares these to expected values for a random sample of points from a CSR (complete spatial randomness) pattern. Significant clustering was inferred by Z-score

value (standard normal variable) of less than 0; a positive score was interpreted as dispersion of locations.

Results

S. Typhi population structure

A recently developed typing system, based on the simultaneous interrogation of 1,485 *S. Typhi* chromosomal single nucleotide polymorphisms (SNPs) using a custom Illumina GoldenGate array [22,23], was used to analyse each of the *S. Typhi* isolates. This approach facilitates the unequivocal assignment of isolates to haplotypes, allowing closely related strains to be distinguished phylogenetically based on single nucleotide changes. From 287 patients with culture confirmed typhoid fever recruited between January 2004 and December 2005 [20], 267 *S. Typhi* were available for SNP typing. These included 264 *S. Typhi* isolated from blood culture at admission [20] one relapse isolate and two faecal carriage isolates. A total of 24 *S. Typhi* (23 isolated from An Giang and one from Dong Thap, randomly distributed throughout the study period) were not available for SNP typing.

A total of 261 *S. Typhi* isolates (98%) were of the common H58 haplogroup. The remaining isolates were of haplotypes H1 (isolates BJ105, BJ63, BJ64), H45 (isolate BJ264), H50 (isolate BJ9) and H52 (isolate BJ3; see Figure 2 and Table 1). The H58 *S. Typhi* isolates displayed variation at 10 SNP loci (detailed in [23]), which differentiated seven distinct sub-H58 haplotypes, shown in Figure 2. However, 242 (93%) of these isolates belonged to just three closely related H58 haplotypes, designated C, E1 and E2 in

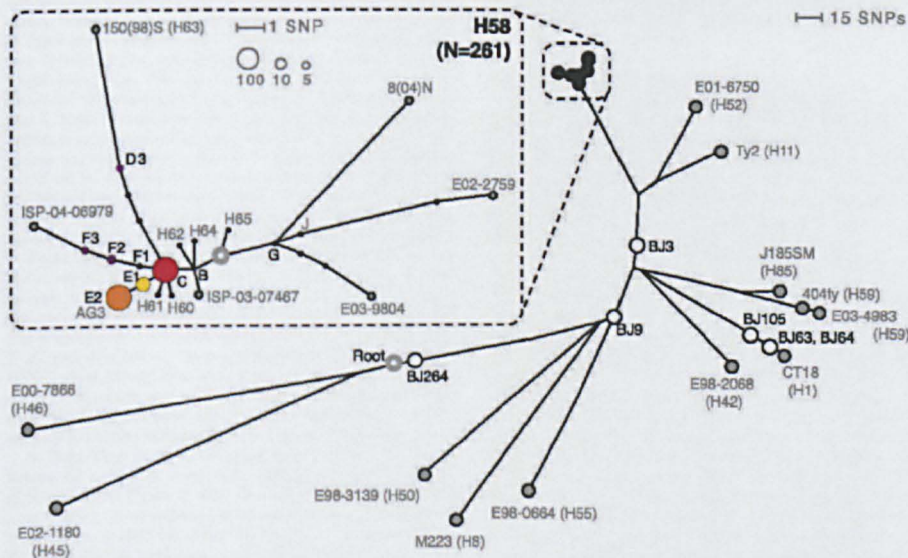


Figure 2. Phylogenetic distribution of *S. Typhi* isolates. Grey nodes represent control isolates (labelled by isolate code and haplotype group), unfilled grey circle indicates tree root, white nodes correspond to non-H58 *S. Typhi* isolated in this study (labelled with isolate code), black nodes show H58 isolates. Inset: zoom-in on the H58 haplogroup; grey nodes represent control isolates (labelled by isolate code or haplotype code), unfilled grey circle indicates tree root, coloured circles indicate nodes corresponding to H58 *S. Typhi* isolated in this study, node labels are as in the text, node colours are as in Figures 3–4, node sizes indicate the number of isolates on the scale as indicated by numbered circles. doi:10.1371/journal.pntd.0000929.g002

Table 1. Antimicrobial resistance pattern of *S. Typhi* haplotypes.

<i>S. Typhi</i> Haplotype	Total (% of all isolates)	Nal resistant (% of haplotype)	MDR (% of haplotype)	IncHI1 plasmid (% of haplotype)	MDR/Nal (% of haplotype)
H58-C	118* (44%)	117 (99%)	102 (86%)	92 (78%)	102 (86%)
H58-E1	15 (6%)	15 (100%)	15 (100%)	14 (93%)	15 (100%)
H58-E2	109* (41%)	109 (100%)	21 (19%)	17 (15%)	21 (19%)
Other H58	19 (7%)	16 (84%)	16 (84%)	16 (84%)	13 (68%)
Non-H58	6 (2%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)
Total	267	257 (96%)	154 (58%)	139 (52%)	151 (56%)

All *S. Typhi* were isolated from blood culture, except two that were isolated from the stools of two chronic carriers.

*Includes one faecal carriage *S. Typhi* isolate. Nal = Nalidixic acid; MDR = multidrug resistant. Presence of IncHI1 plasmid was inferred from GoldenGate assay results (all isolates).

doi:10.1371/journal.pntd.0000929.t001

Figure 2 (numbers given in Table 1). The genome of *S. Typhi* strain AG3, isolated during the study (March 2004) from a typhoid fever patient living in An Giang province, was sequenced previously [21]. AG3 belongs to the H58-E2 haplotype, and the SNPs separating E2 from haplotypes E1 and C were originally identified by analysis of the AG3 genome. Therefore, the ability to differentiate within the cluster of 242 *S. Typhi* isolates was dependent on the inclusion of strain AG3 in the initial genome sequencing study used to identify SNP loci [21].

All but one *S. Typhi* isolated from the blood culture of patients admitted to An Giang Provincial hospital (231/232), as well as the two faecal *S. Typhi* strains isolated from chronic carriers in An Giang, belonged to the *S. Typhi* H58 haplogroup. The remaining *S. Typhi* isolate BJ264 (see Figure 2) was of the H45 haplotype and was isolated from a typhoid fever patient who was resident in neighbouring Can Tho province. One patient at An Giang Provincial hospital relapsed with symptoms of typhoid fever and had *S. Typhi* isolated from blood culture 11 days after the initial treatment (gatifloxacin) had been completed. The mother of the patient was found to be a chronic *S. Typhi* carrier. All three *S. Typhi* strains - the patient's admission and relapse blood culture isolates and the mother's faecal isolate - belonged to the *S. Typhi* H58-E2 subtype. The patient's isolates were both MDR and carried the IncHI1 ST6 plasmid (see below), whereas the mother's *S. Typhi* isolate was plasmid-free and susceptible to all first line antimicrobials at the time of isolation. All three isolates were Nal resistant but sensitive to gatifloxacin (MIC 0.19 mg/ml). Stool cultures were taken at 1 month (96% of patients), 3 months (93%) and 6 months (44% of follow-up). Chronic faecal carriage of *S. Typhi* was detected in only one trial patient. This was a MDR H58-C strain isolated from stool 6 months after treatment (with gatifloxacin), which was indistinguishable at all assayed loci from the patient's original blood culture isolate. Both isolates were Nal resistant but sensitive to gatifloxacin (MIC 0.19 mg/ml).

At Dong Thap Provincial Hospital, only 3 of the 25 *S. Typhi* isolates did not belong to the H58 haplogroup. Two H1 isolates (BJ63 and BJ64; Figure 2) were identical at all assayed loci and were isolated on consecutive days from two patients resident in Dong Thap. A third H1 strain (BJ105; Figure 2) differed from BJ63 and BJ64 at 16 chromosomal SNP loci and was isolated in Dong Thap 14 months after these isolates. Two siblings from Dong Thap province were admitted on consecutive days in 2004 and were both infected with MDR *S. Typhi* of the haplotype H58-C.

Of the ten *S. Typhi* strains isolated at the Hospital for Tropical Diseases in Ho Chi Minh City, eight were members of the H58

haplogroup, with patients resident in Ho Chi Minh City ($n=4$), Long An ($n=1$), Kien Giang ($n=2$) and An Giang ($n=1$) provinces, reflecting the larger catchment area of the hospital. The remaining two *S. Typhi* were of haplotypes H52 (BJ3) and H50 (BJ9) and were isolated from patients living in Binh Hoa province and Ho Chi Minh City, respectively.

There was no simple association between *S. Typhi* haplotype and patient age, length of stay in hospital, fever clearance time, vomiting, abdominal pain, hepatomegaly or relapse (Table 2). However, upon admission, patients infected with *S. Typhi* haplotype H58-E2 tended to report lower frequencies of diarrhoea and headache and higher frequencies of constipation compared to patients infected with other haplotypes, including H58-C (see Table 2).

Plasmids and antimicrobial resistance

The GoldenGate assay incorporated probes targeting IncHI1 plasmid sequences, allowing for detection of the presence of IncHI1 plasmid within the genomic DNA extracted from each *S. Typhi* isolate. The assay indicated that a total of 139 *S. Typhi* isolates harboured an IncHI1 plasmid. All plasmids were of the IncHI1 ST6 sequence type [24] and all plasmid-bearing isolates belonged to the *S. Typhi* H58 haplogroup (see Table 1). The MDR IncHI1 plasmid was more common among H58-C isolates than H58-E2 isolates (86% vs 19%, see Table 1). Of the 139 *S. Typhi* isolates giving positive signals for IncHI1 SNP loci, 137 (99%) were classified as MDR by antimicrobial susceptibility testing conducted at the time of isolation. One other IncHI1-positive isolate tested positive by GoldenGate assay for the genes *sulI*, *sul2*, *dhfrA7*, *tetA*, *cat*, *bla* and *cat* (resistance genes; functions outlined in Table S1) like the MDR isolates, yet had low MICs for chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole. An additional *S. Typhi* isolate, BJ5, was resistant to ampicillin and trimethoprim-sulfamethoxazole but sensitive to chloramphenicol. This was consistent with GoldenGate assay results, which gave positive signals for the *repC* replication initiation gene of IncHI1, resistance genes *strAB*, *bla*, *sulI*, *sul2*, *dhfrA7*, but no signal for sequences from the *cat* gene encoding chloramphenicol resistance. A further 17 *S. Typhi* isolates were recorded as MDR according to their antimicrobial susceptibility pattern at the time of isolation, but did not test positive for IncHI1 plasmid loci. This likely reflects loss of the IncHI1 plasmid in culture or storage between the time of isolation and DNA extraction. The MDR status of the infecting *S. Typhi* isolate was not associated with fever clearance time ($p=0.3$, two-sided T-test) or treatment failure ($p=0.18$, χ^2 test).

Table 2. Selected characteristics of typhoid fever patients, based on baseline presentation history and outcomes.

Variable	S. Typhi H58-E2 n = 107	Non-H58-E2 S. Typhi n = 157	S. Typhi H58-C n = 117	H58-E2 vs all other S. Typhi (95% CI)	p-value	Missing data
Age (yrs)	11.9	12.2	12.7	Diff. -0.8 (-2.0,1.0)	0.83	0
Time in hospital (days)	13.9	13.7	13.8	Diff. 0.2 (-1.0,1.0)	0.74	0
Fever clearance (hrs)	11.6	11.5	12.1	Diff. 1 (-1.2,1.8)	0.70	2
Constipation	13.6%	5.8%	6.0%	OR 2.6 (1.1,6.1)	0.03	1
(adjusted)				OR 2.6 (1.1,6.0)	0.03	35
Headache	55.7%	70.1%	70.1%	OR 0.54 (0.32,0.90)	0.02	1
(adjusted)				OR 0.66 (0.43,1.09)	0.10	35
Diarrhoea	55.1%	72.6%	73.5%	OR 0.46 (0.28,0.78)	0.004	0
(adjusted)				OR 0.56 (0.34,0.93)	0.02	34
Abdominal pain	51.4%	56.1%	55.6%	OR 0.80	0.37	0
(adjusted)				OR 0.76 (0.47,1.24)	0.27	34
Vomiting	35.5%	35.5%	35.0%	OR 1.03	0.90	0
(adjusted)				OR 1.02 (0.61,1.71)	0.93	34
Hepatomegaly	58.3%	52.3%	51.3%	OR 1.35	0.23	2
(adjusted)				OR 1.30 (0.79,2.14)	0.30	35

Comparisons of selected characteristics among 264 typhoid fever patients (i.e. excluding carriage and relapse isolates). For continuous variables age, time in hospital and fever clearance, values shown are means and test statistic given is the difference in means (Diff; mean value for H58-E2 S. Typhi - mean value for non-H58-E2 S. Typhi). Other variables indicate frequency of symptoms self-reported at time of admission and of clinician-diagnosed hepatomegaly; test statistic is odds ratio (OR) for H58-E2 S. Typhi vs non-H58-E2 S. Typhi; both crude OR and adjusted OR are reported (adjusted for duration of fever prior to admission and use of antibiotics prior to admission, using logistic regression). All comparisons shown are for patients infected with H58-E2 S. Typhi vs those infected with other S. Typhi haplotypes (including H58-C and others); 95% confidence intervals (CI) are given in brackets.
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A total of 257 *S. Typhi* isolates were resistant to nalidixic acid (Nal). All of these isolates belonged to the H58 haplogroup (Table 1) and all were susceptible to gatifloxacin, ciprofloxacin and ofloxacin according to current CLSI guidelines [17]. *S. Typhi* haplotypes H58-C, H58-E1 and H58-E2 were uniformly resistant to Nal, with the exception of a single H58-C isolate which had an intermediate MIC of 28 µg/mL (resistance defined as MIC ≥32 µg/mL). The sequenced H58-E2 isolate AG3 harbours a mutation changing serine (TCC) to phenylalanine (TTC) at codon 83 in the *gyrA* gene (*GyrA*-Ser83Phe [21]), which is known to confer resistance to Nal [26]. In the present study we sequenced the *gyrA* gene in 223 of the Nal resistant isolates (87%) and found the same *GyrA*-Ser83Phe amino acid substitution in all isolates tested.

Spatial and temporal distribution

Figure 3 shows the spatial distribution of the residences of 160 typhoid patients (this information was not available for the remaining patients). Of the patients admitted at An Giang Provincial Hospital and Dong Thap Provincial Hospital, sufficient address detail to allow for assignment of latitude and longitude was provided in 61% and 73% of cases, respectively. This represents 50% and 20% of all blood culture confirmed typhoid fever patients at An Giang Provincial Hospital and Dong Thap Provincial Hospital, respectively, during 2004–2005. In An Giang, patients' homes clustered around the An Giang Provincial Hospital, but also around the Sông Hậu branch of the Mekong river (see Figure 3). Most *S. Typhi* isolated from patients living near this point in An Giang province were of the H58-E2 haplotype (orange in Figure 3), and this group demonstrated significant clustering using nearest-neighbour analysis ($n=57$, Z-score = -14.145). In contrast, *S. Typhi* of the H58-C haplotype

were isolated relatively frequently in neighbouring provinces and had a more sporadic clustering pattern (red in Figure 3). While isolates from An Giang Provincial Hospital are overrepresented in this spatial analysis, the apparent increase in typhoid density in An Giang is consistent with total Typhi isolation rates at the two hospitals during the study period (284 at An Giang Provincial Hospital and 90 at Dong Thap Provincial Hospital).

The temporal distribution of *S. Typhi* haplotypes over 2004 and 2005 is shown in Figure 4. Typhoid fever cases peaked just prior to the onset of the wet season in each year, as has been observed previously in this region [1,3] (see monthly rainfall, solid line in Figure 4). In 2004, H58-E2 and H58-C were both prevalent (62 C, red in Figure 4; 103 E2, orange in Figure 4), whereas few isolates of H58-E2 Typhi were observed during 2005 (55 C, 4 E2; see Figure 4). The decline of H58-E2 may be associated with selection for the IncHI1 MDR plasmid, which was much more common in H58-C (Table 1). As Figure 4 highlights, the majority of isolates collected during the second season were MDR and carried the IncHI1 plasmid ST6.

Discussion

Our data show the vast majority of *S. Typhi* isolates ($n=261$, 98%) isolated from the Mekong delta during the two-year study period belonged to the H58 haplogroup. Furthermore, 91% of isolates ($n=242$) belonged to a single clonal complex of *S. Typhi* H58 (nodes C, E1, E2 shown in Figure 2), demonstrating remarkable homogeneity in the *S. Typhi* population in this location during the study period. The observed level of clonal dominance is greater than that observed in previous haplotyping studies of local *S. Typhi* populations. Among 54 *S. Typhi* isolates from Jakarta, Indonesia between 1975 and 2005, a total of nine

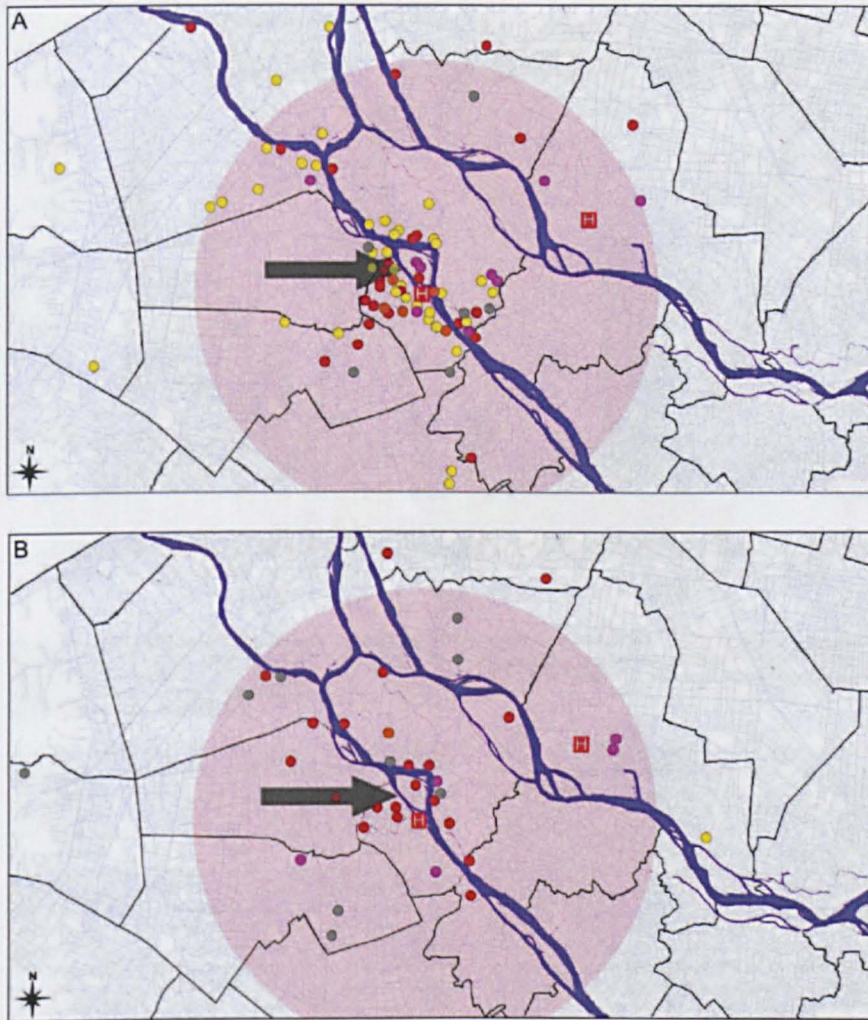


Figure 3. Spatial distribution of *S. Typhi* isolates by haplotype and year. The spatial distribution of *S. Typhi* haplotypes surrounding An Giang provincial hospital in (A) 2004 and (B) 2005. Each point corresponds to the residential location of a typhoid fever patient; colour indicates the haplotype of the *S. Typhi* isolate (with or without plasmid): dark orange = H58-E2 with MDR plasmid, light orange = H58-E2 without MDR plasmid, dark red = H58-C with MDR plasmid, pink, H58-C without MDR plasmid, grey = other *S. Typhi* haplotypes. Locations of the hospitals are indicated by a white cross on a red background; pink circle indicates a radius of 15 km from An Giang Provincial Hospital; arrow indicates the Sông Hậu branch of the Mekong river.
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haplotypes were detected, with the dominant H59 haplotype accounting for 53% of isolates; the next most frequent haplotype was genetically distant from H59 and comprised 24% of isolates

[27]. In Kathmandu, Nepal, a collection of *S. Typhi* isolated from children hospitalised with typhoid fever in 2005–2006 was dominated by the H58-G haplotype (66%) but the distant H42

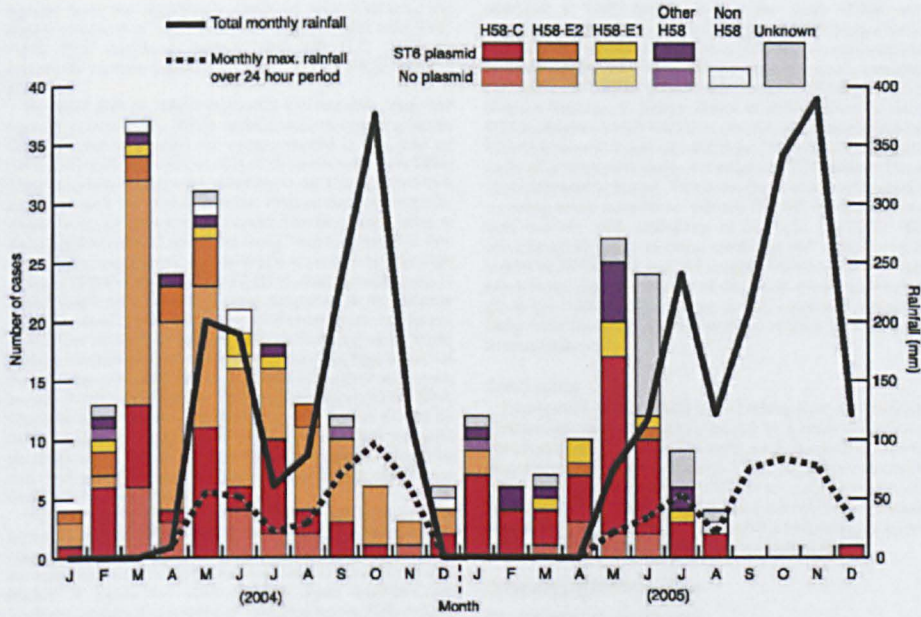


Figure 4. Monthly incidence of typhoid cases by haplotype. Bar heights indicate the total number of *S. Typhi* isolated each month during the study, according to the scale given on the left-hand y-axis; colours indicate the combination of *S. Typhi* haplotype and presence of IncHI1 ST6 plasmid as given in the legend. Solid black line = total rainfall each month recorded in An Giang, dashed line = maximum rainfall occurring in a 24 h period during each month in An Giang; rainfall scale is shown on the right-hand y-axis.
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haplotype was also present at high frequency (19%) [23]. Among *S. Typhi* isolated between 2001 and 2008 in Nairobi, Kenya, 87% were H58, although two distinct subtypes (nodes B and J, see Figure 2) were co-circulating at equally high frequencies (>40% each) between 2004 and 2008 [22]. *S. Typhi* H58 nodes B and J (co-circulating in Nairobi) represent distinct lineages, each acquiring unique SNPs since the last common ancestor of H58 (Figure 2). However H58 nodes C, E1 and E2, which account for 91% of isolates in this study in Vietnam, are closely related and formed a tight clonal complex differentiated by just two SNPs (Figure 2). Thus the overall level of clonality of the *S. Typhi* population analysed in this study was unexpectedly high. The clonal complex comprising H58-C, -E1 and -E2 was not detected in study populations in Nepal and Kenya where the same SNP typing method was used [22,23], suggesting it may have arisen locally in Vietnam.

Despite the genetic homogeneity we observed, the availability of whole-genome sequence data for *S. Typhi* H58-E2 isolate AG3 [21], collected during the study, allowed us to differentiate closely related organisms within the H58 group. Just two SNPs identified in strain AG3 subdivided the homogeneous group into three nodes C, E1 and E2 (Figure 2), of which two (C and E2) were dominant (>40% each). Isolates belonging to the H58-C node were present at a constant rate during the two years of the study (62 isolates in 2004 and 55 in 2005). However, isolates belonging to the H58-E2 node were common during 2004 (103 isolates), yet were virtually

undetected in 2005 (3 isolates). This change in both haplotype distribution and total number of typhoid cases from 2004 to 2005 is striking, and suggests an outbreak caused by *S. Typhi* H58-E2 during 2004. We additionally found that H58-C strains had a much stronger association with the ST6 IncHI1 MDR plasmid than H58-E2 (Table 1). We speculate that the persistence of H58-C strains and the corresponding disappearance of H58-E2 may be associated with a competitive phenotypic advantage conferred by the IncHI1 MDR plasmid. However, it is important to remember that node C is a precursor of node E2 and we can only differentiate E2 from C because we had whole genome sequence data for an H58-E2 strain from which to identify SNPs [21]. Thus the population of *S. Typhi* isolates assigned to node C by our SNP typing assay may be more diverse than that assigned to H58-E2. It is also important to note that since our data covers just two years, it is possible that any competitive advantage of H58-C strains may be short-lived and there is no evidence for long-term replacement of H58-E2.

We identified two cases of chronic faecal carriage of *S. Typhi* during the course of the study, one in a patient's relative and one in a patient after 6 months of follow-up. This underlines the importance of screening procedures to identify carriers and effective treatment to eliminate carriage and reduce transmission. The faecal *S. Typhi* isolates were of the dominant H58-E2 and H58-C haplotypes, respectively. In a previous case-control study performed in the Mekong delta, close contact with a patient with

typhoid fever was significantly associated with developing the disease compared to hospital controls (adjusted odds ratio (OR) = 5.2, 95% confidence interval (95% CI) [1.7, 15.9]) or community controls (adjusted OR = 11.9, 95% CI [2.3, 60.7]) [28].

We were able to collect residential location data from 160 typhoid patients (61%). While typhoid patients reporting to An Giang Provincial Hospital are overrepresented in this data set (50% of all confirmed cases vs. 20% of all confirmed cases at Dong Thap Hospital), the apparent clustering in An Giang (Figure 3) is consistent with the overall isolation rates at the two hospitals, which during the study period was more than three times higher at An Giang Provincial Hospital than Dong Thap Hospital. The data set provides roughly equal representation of patients infected with *S. Typhi* H58-C (65%) and H58-E2 (62%), thus any differences in spatial distribution between patients presenting at the different hospitals should not affect the differences between spatial distribution of these haplotypes. Spatial clustering of *S. Typhi* H58-E2 was evident particularly around the Sóng Hậu branch of the Mekong river, while other *S. Typhi* haplotypes were more broadly distributed (Figure 4). The spatial clustering of H58-E2 *S. Typhi* further supports a localised outbreak in 2004 caused by these isolates. In contrast, the broader spatial and temporal distribution of *S. Typhi* H58-C during the study suggests it may be well established in the community and can persist over longer distances and time periods.

We also observed that some symptoms reported by patients infected with H58-E2 *S. Typhi* differed from those infected with other *S. Typhi* haplotypes (Table 2). After adjusting for antibiotic use and duration of fever prior to admission, patients infected with H58-E2 *S. Typhi* were more likely to report diarrhoea and headache compared with other *S. Typhi* haplotypes (OR = 0.56, 95% CI [0.34, 0.93] and OR = 0.66, 95% CI [0.40, 1.09], respectively), but were more commonly associated with constipation (OR = 2.6, 95% CI [1.1, 6.0]). This suggests there may be some phenotypic differences between H58-E2 and other *S. Typhi* with respect to disease, however these were post-hoc analyses and no adjustments were made for multiple comparisons, hence these associations should be interpreted with caution. However if confirmed in subsequent prospective studies, it would be of interest to know whether these phenotypic characteristics were associated with specific mutations in H58-E2 *S. Typhi*. The two SNPs differentiating the E2 node from E1 and C are both synonymous mutations (C>>T in *meiA* (nt 315); G>>A in *rbsA* (nt 576)) and our earlier analysis of the AG3 sequence data detected no phase insertions and no large deletions that were not also detected in other sequenced H58 isolates [21]. However, we were unable to verify if other single-base insertions or deletions were present, which may result in gene inactivation with corresponding phenotypic effects.

Patterns of antimicrobial resistance of *S. Typhi* tend to vary markedly between different typhoid-endemic regions. In this present work, as in the recent study of Kenyan isolates [22], there were high rates of MDR associated with IncIII1 ST6 plasmids among strains of the *S. Typhi* H58 haplogroup. This suggests that the presence of the plasmid may contribute to the success of the

dominant *S. Typhi* haplotypes, and the results of our study corroborate this hypothesis. The *S. Typhi* H58-E2 subtype (which was generally not associated with a plasmid) was only transient, while the H58-C subtype (which was more commonly associated with the IncIII1 MDR plasmid) was present in 2004 and 2005 in southern Vietnam. In Kenya, almost all isolates of the dominant H58 haplotypes carried the MDR plasmid, while the plasmid-free H58-G subtype was only detected twice [22]. All the H58 isolates analysed in the present study were resistant to Nal, conferred by an identical mutation in *gyrA*. This is consistent with previous studies reporting strong associations between the Nal resistance phenotype and the H58 haplogroup of *S. Typhi* [12,22,23]. The presence of the same mutation conferring Nal resistance in all isolates of H58-C, -E1 and -E2 suggests this mutation may have arisen in the common ancestor of this clonal complex, perhaps *in situ* in the Mekong delta region, and its continued presence is likely maintained by selective pressure exerted by the use of fluoroquinolones.

Conclusions

During 2004–2005, typhoid in the Mekong river delta region of Vietnam was almost exclusively caused by a single Nal-resistant clonal complex of *S. Typhi*. This reflects a higher level of clonality than observed in other localised *S. Typhi* populations studied to date, which may be indicative of higher transmission rates in this location. The high level of Nal resistance and multidrug resistance, frequently in the same strains, is concerning and continues to pose problems for the successful treatment of typhoid fever.

Supporting Information

Checklist S1 Strobe Checklist

Found at: doi:10.1371/journal.pntd.0000929.s001 (0.09 MB DOC)

Table S1 IncIII1 plasmid and resistance-associated gene targets. Found at: doi:10.1371/journal.pntd.0000929.s002 (0.03 MB XLS)

Translation S1 Translation of the abstract into Vietnamese by Nga Tran Vu Thien. Found at: doi:10.1371/journal.pntd.0000929.s003 (0.09 MB RTF)

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Author Contributions

Conceived and designed the experiments: KEH CD NVVC TTH JF GD SB. Performed the experiments: TTC PTD TPL NVMH TVTN JIC BHM NVVC. Analyzed the data: KEH CD BHM SB. Contributed reagents/materials/analysis tools: TTPL GD. Wrote the paper: KEH CD NVVC TTH JF GD SB.

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Suitable Disk Antimicrobial Susceptibility Breakpoints Defining *Salmonella enterica* Serovar Typhi Isolates with Reduced Susceptibility to Fluoroquinolones[†]||

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Infections with *Salmonella enterica* serovar Typhi isolates that have reduced susceptibility to ofloxacin (MIC \geq 0.25 μ g/ml) or ciprofloxacin (MIC \geq 0.125 μ g/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 \geq 16 μ g/ml) identified isolates with an ofloxacin MIC of \geq 0.25 μ g/ml with a sensitivity of 97.3% (253/260) and specificity of 99.3% (552/556). For isolates with a ciprofloxacin MIC of \geq 0.125 μ g/ml, the sensitivity was 92.9% (248/267) and specificity was 98.4% (540/549). A zone of inhibition of \leq 28 mm around a 5- μ g ofloxacin disc detected strains with an ofloxacin MIC of \geq 0.25 μ g/ml with a sensitivity of 94.6% (246/260) and specificity of 94.2% (524/556). A zone of inhibition of \leq 30 mm detected isolates with a ciprofloxacin MIC of \geq 0.125 μ g/ml with a sensitivity of 94.0% (251/267) and specificity of 94.2% (517/549). An ofloxacin MIC of \geq 0.25 μ g/ml and a ciprofloxacin MIC of \geq 0.125 μ g/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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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 ($n = 40$), Hecchi 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 μ g), ampicillin (AMP) (10 μ g), trimethoprim-sulfamethoxazole (SXT) (1.25/23.75 μ g), ceftriaxone (CRO) (30 μ g), ofloxacin (OFX) (5 μ g), and nalidixic acid (NAL) (30 μ g). 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 μ g), ciprofloxacin (CIP) (5 μ g), and ofloxacin (OFX) (5 μ g). 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 μ g/ml to 4 μ g/ml), OFX (0.008 μ g/ml to 4 μ g/ml), and NAL (0.5 μ g/ml to 512 μ g/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 μ g/ml for nalidixic acid, ≤ 2 μ g/ml for ofloxacin, and ≤ 1 μ g/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 microfuge tube and centrifuged in a microfuge 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*, GYRA1 (5'-TGCCGAGATGGCTGAAGC) and GYRA2 (5'-TACCGTCATAAGTTATCCACG) (annealing temperature, 55°C); *gyrB*, SgyrB1 (5'-CAAACTGGGGGACTGTCAGG) and SgyrB2 (5'-TTCCGGCATCTGACGATAGA) (annealing temperature, 62°C); *parC*, StmparC1 (5'-CTATGCGATGT CAGAGCTGG) and StmparC2 (5'-TAA CAGCAGCTCGGCGTATT) (annealing temperature, 62°C); and *parE*, StmparE1 (5'-TCTCTCCGATGAAGTGCTG) and StmparE2 (5'-ATACGG TATAGCGCGGTAG) (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 CEQSequence 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 μ g/ml for ofloxacin and ≥ 0.125 μ g/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

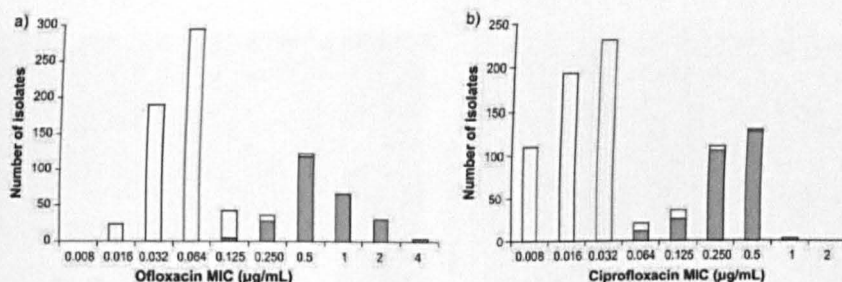


FIG. 1. Fluoroquinolone MIC histograms for 816 *S. Typhi* isolates from Asia. Histograms showing the distribution of MICs to ofloxacin (a) and ciprofloxacin (b) of 816 *S. Typhi* strains, isolated from patients with enteric fever. Each isolate used for analysis was isolated from an individual enteric 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 $\mu\text{g/ml}$), and 4 isolates had an MIC of 16 $\mu\text{g/ml}$ (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 $\mu\text{g/ml}$), 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 $\mu\text{g/ml}$ 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 MIC₅₀ (range) to ciprofloxacin of 0.03 $\mu\text{g/ml}$ (0.008 to 0.5 $\mu\text{g/ml}$) and of 0.06 $\mu\text{g/ml}$ (0.016 to 0.5 $\mu\text{g/ml}$) to ofloxacin. The 253 isolates that were resistant to nalidixic acid had an MIC₅₀ (range) to ciprofloxacin of 0.5 $\mu\text{g/ml}$ (0.064 to 1 $\mu\text{g/ml}$) and to ofloxacin of 1.0 $\mu\text{g/ml}$ (0.125 to 4 $\mu\text{g/ml}$).

Antimicrobial susceptibility test interpretive categories of *S. Typhi* to ciprofloxacin and ofloxacin. The current CLSI intermediate breakpoints are 2 $\mu\text{g/ml}$ and 4 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$) for the detection of isolates with an MIC of ≥ 0.25 $\mu\text{g/ml}$ 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 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 $\mu\text{g/ml}$, using nalidixic acid resistance (MIC of ≥ 16 $\mu\text{g/ml}$), 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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$, with the least num-

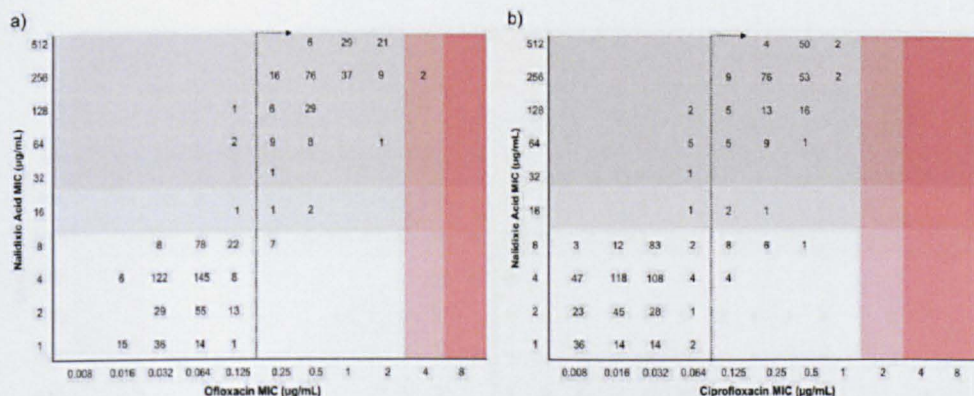


FIG. 2. Scatter plots relating ofloxacin and ciprofloxacin MICs to nalidixic acid MIC for 816 Asian *S. Typhi* isolates. Scatter plots comprised of MIC data from 816 *S. Typhi* isolates from Nepal ($n = 104$), India ($n = 25$), Indonesia ($n = 27$), Bangladesh ($n = 40$), Pakistan ($n = 53$), China ($n = 51$), 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 $\mu\text{g/ml}$ and ≥ 32 $\mu\text{g/ml}$; ofloxacin MIC, ≤ 2 $\mu\text{g/ml}$ and ≥ 8 $\mu\text{g/ml}$; and ciprofloxacin MIC, ≤ 1 $\mu\text{g/ml}$ and ≥ 4 $\mu\text{g/ml}$). The red broken line corresponds to the proposed MIC breakpoint identifying strains with reduced susceptibility to fluoroquinolones (ofloxacin MIC of ≥ 0.25 $\mu\text{g/ml}$ and ciprofloxacin MIC of ≥ 0.125 $\mu\text{g/ml}$).

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 $\mu\text{g/ml}$, 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 $\mu\text{g/ml}$ to nalidixic acid, 0.008 to 6 $\mu\text{g/ml}$ to ciprofloxacin, and 0.03 to 12 $\mu\text{g/ml}$ 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/S80L, 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 $\mu\text{g/ml}$ and 0.33 $\mu\text{g/ml}$ to ofloxacin and ciprofloxacin, respectively. Figure 4 also shows the current CLSI breakpoints and the suggested ofloxacin breakpoint of 0.25 $\mu\text{g/ml}$ and ciprofloxacin breakpoint of 0.125 $\mu\text{g/ml}$. An MIC of 0.25 $\mu\text{g/ml}$ to ofloxacin and an MIC of 0.125 $\mu\text{g/ml}$ 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-

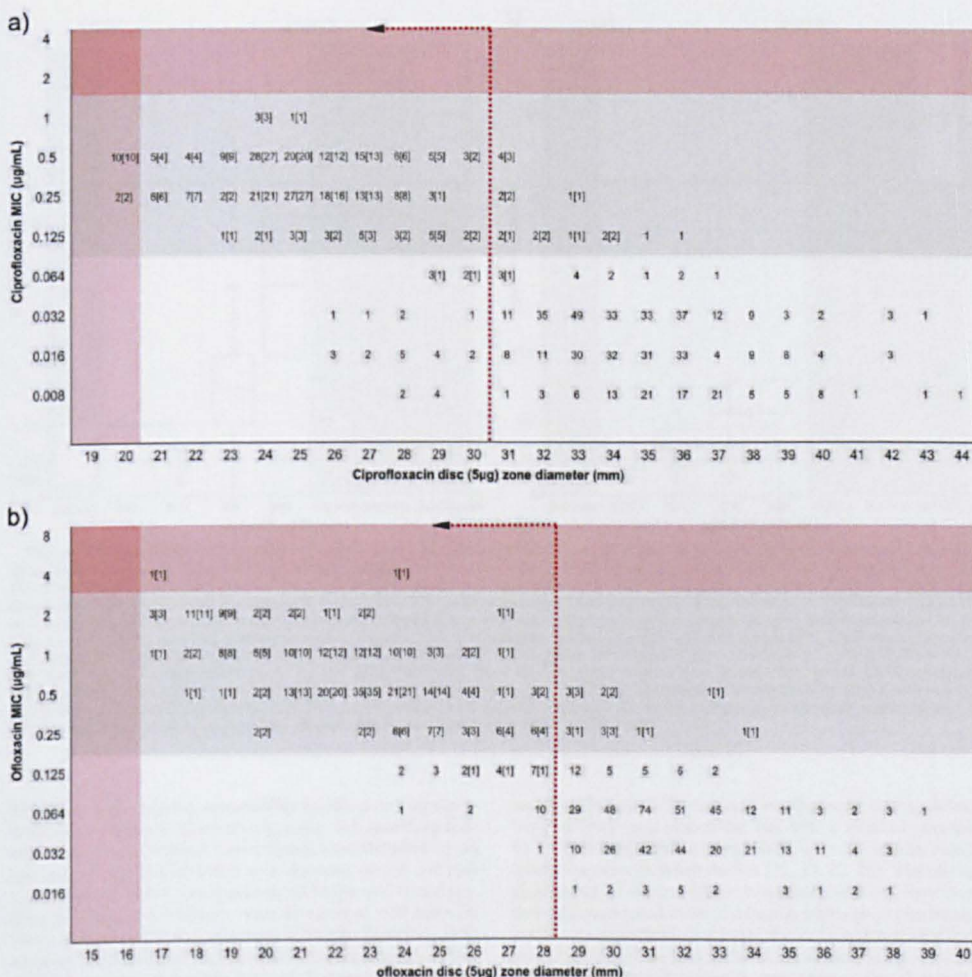


FIG. 3. Scatter plots relating ofloxacin and ciprofloxacin MIC to inhibition zone diameter for 816 Asian *S. Typhi* isolates. Scatter plots for 816 *S. Typhi* isolates comparing the inhibition zone diameters using a 5-µg ciprofloxacin disc (a) and a 5-µg ofloxacin disc (b) (x axis) and the corresponding 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

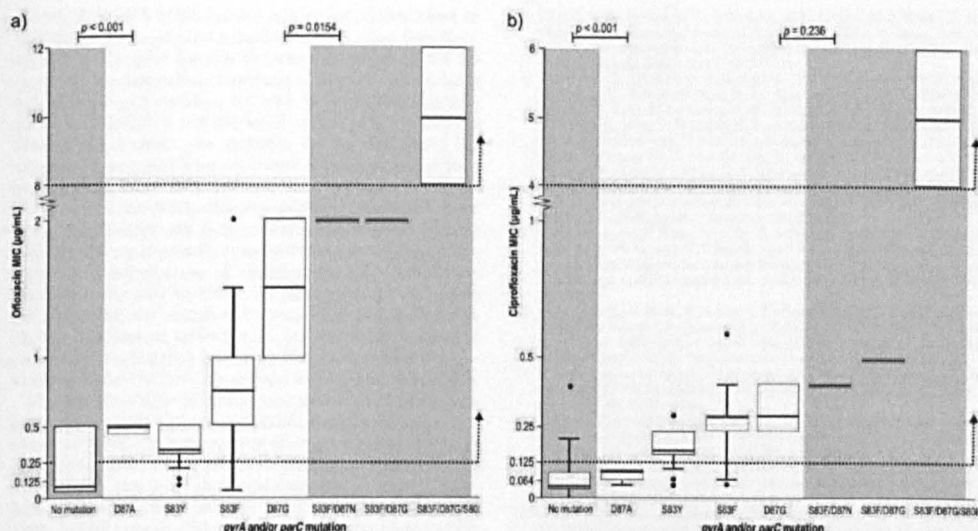


FIG. 4. The relationship of *gyrA* and *parC* mutations and the MICs to ofloxacin and ciprofloxacin in the *S. Typhi* strain isolated in Asia. Box plots (boxes relate to the 25th and 75th percentiles) relating mutations in the *gyrA* and the *parC* genes (the S80I mutation is in the *parC* gene, and 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 $\mu\text{g}/\text{ml}$, and those to ciprofloxacin from 0.008 to 6 $\mu\text{g}/\text{ml}$. Median values for each mutant group are identified 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.

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We declare that we have no competing interests.

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A Randomised Trial Evaluating the Safety and Immunogenicity of the Novel Single Oral Dose Typhoid Vaccine M01ZH09 in Healthy Vietnamese Children

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Abstract

Background: The emergence of drug resistant typhoid fever is a major public health problem, especially in Asia. An oral single dose typhoid vaccine would have major advantages. M01ZH09 is a live oral single dose candidate typhoid vaccine containing *Salmonella enterica* serovar Typhi (Ty2 *aroC*⁻ *ssaV*⁻) ZH9 with two independently attenuating deletions. Studies in healthy adults demonstrated immunogenicity and an acceptable safety profile.

Objectives: We conducted a randomised placebo controlled, single-blind trial to evaluate the safety and immunogenicity of M01ZH09 in healthy Vietnamese children aged 5 to 14 years.

Methods: Subjects were randomly assigned to receive either a nominal dose of 5×10^9 CFU of M01ZH09 or placebo and were followed up for 28 days. The primary safety outcome was the proportion of subjects with any adverse event attributed to M01ZH09. The primary immunogenicity endpoint was the proportion of subjects who showed a positive immune response to M01ZH09 in the *Salmonella* Typhi lipopolysaccharide (LPS) specific serum IgA and IgG ELISA.

Principal Findings: One hundred and fifty-one children were enrolled, 101 subjects received M01ZH09 and 50 subjects received placebo. An intention to treat analysis was conducted. There were no serious adverse events and no bacteraemias. In the M01ZH09 group, 26 (26%; 95% CI, 18–5%) of 101 subjects experienced adverse events compared to 11 (22%; 95% CI, 12–36%) of 50 subjects in the placebo group (odds ratio (OR) [95%CI] = 1.23 [0.550–2.747]; $p=0.691$). Faecal shedding of *S. Typhi* (Ty2 *aroC*⁻ *ssaV*⁻) ZH9 was detected in 51 (51%; 95% CI, 41–61%) of 100 M01ZH09 subjects. No shedding was detected beyond day 3. A positive immune response, defined as 70% increase (1.7 fold change) in LPS specific serum IgG (day 14 or 28) and/or 50% increase (1.5 fold change) in LPS specific serum IgA (day 7 or 14) from baseline was detected in 98 (97%; 95% CI, 92–99%) of 101 M01ZH09 recipients and 8 (16%; 95% CI, 7–29%) of 50 placebo recipients. Twenty-eight (100%; 95% CI, 88–100%) of 28 vaccine recipients who were evaluated in the LPS specific IgA ELISPOT assay showed a positive response compared to none of the 14 placebo recipients tested.

Conclusions: This was the first phase II trial of a novel oral candidate typhoid vaccine in children in an endemic country. M01ZH09 had an appropriate safety profile and was immunogenic in children.

Trial Registration: Controlled-trials.com ISRCTN9111837

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Competing Interests: WL, FM, JPM, AU and RB are employed by Emergent Product Development UK Ltd, which provided the vaccine and sponsored the clinical trial. Emergent Product Development UK Ltd. owns numerous patents and pending patent applications throughout the world relating to this technology, examples of these are EP 0796341, US 5,876,931, EP 1180034, US 6,936,425, EP 1183269, and US 6,756,042. There are no conflicts of interest for the other authors.
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Introduction

Typhoid fever remains a major public health burden in developing countries with approximately 21 million cases and more than 210 000 deaths worldwide per year [1].

Drug resistant typhoid fever has emerged and spread globally narrowing the treatment options [2–4]. The World Health Organization therefore recommends that countries should consider the programmatic use of typhoid vaccines for controlling endemic diseases and that the immunization of school age and/or preschool age children should be undertaken particularly in areas where antibiotic resistant *Salmonella enterica* serovar Typhi (*S. Typhi*) is prevalent [5].

Two licensed and safe typhoid vaccines are available. The oral live attenuated Ty21a vaccine is moderately immunogenic and needs to be administered in three to four doses. Ty21a enteric-coated capsules and Ty21a liquid formulation (which is currently not manufactured) are licensed for children above 6 years and 2 years, respectively. The single dose injectable Vi polysaccharide vaccine is licensed for children above 2 years. The liquid formulation of Ty21a and Vi vaccine provide about 55 to 70% protection from culture confirmed typhoid fever and protection lasts for 3 to 5 years [5,6].

From a public health perspective, a single dose oral typhoid vaccine would have major advantages [7,8]. M01ZH09 (*S. Typhi* (Ty2 *aroC*⁻ *ssaV*⁻) ZH9) is a promising candidate of such a novel typhoid vaccine, it has a well-defined dual mechanism of attenuation [9] and has been safe and immunogenic in a single dose in Western [9–11] and Vietnamese adult volunteers (Hien TT, unpublished).

Historically, oral live vaccines often showed reduced immunogenicity in developing country populations compared to Western populations [12], therefore M01ZH09 was evaluated at an early stage of its development in children in an endemic country.

We describe here the results of a randomised placebo controlled trial that evaluated the safety and immunogenicity of M01ZH09 in 151 healthy Vietnamese children aged between 5 and 14 years.

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information, see Protocol S1 and Checklist S1.

Study design and objectives

The study was designed as a randomised placebo controlled single blind trial to evaluate the safety and immunogenicity of the novel oral single dose live typhoid vaccine M01ZH09 in Vietnamese children aged 5 to 14 years (inclusive).

The study site and ethical approval

The trial was conducted at the Hospital for Tropical Diseases in Ho Chi Minh City, Vietnam. Ethics approval for the trial and all trial related documents was obtained by the Oxford Tropical Research Ethics Committee (OXTREC) and by the Institutional Review Board of the Ministry of Health, Hanoi, Vietnam. The trial was conducted in accordance with the Declaration of Helsinki and its amendments and according to Good Clinical Practice guidelines and was monitored by Matrix Contract Research Ltd,

UK (now Novella Clinical). The trial was also conducted under an US Investigational New Drug (IND) license.

Participants

Healthy Vietnamese children aged 5 to 14 years (inclusive) were invited to participate in the trial. Recruitment was carried out by word of mouth and flyers. Families who were interested in the trial were invited to attend one of several information evenings at the Hospital for Tropical Diseases. At these meetings the study was presented by the principal investigator (TTH) and all questions could be discussed and answered. Families who remained interested in the trial were invited to attend the screening visit. Children were eligible if they were available during the trial period and at least one of their parents gave written informed consent for their child to participate after the trial procedures and potential risks were carefully explained by the study investigators. All children were invited to give their assent to the study and written informed assent was obtained from subjects starting at the age of 6 years. After informed consent was obtained, screening tests were performed. Children were screened by history, physical examination (including height, weight and vital signs), blood tests (biochemistry, haematology and HIV test), urine dipsticks and pregnancy tests (for female subjects of 11 years and above). Stool cultures were performed to check for the presence of *Salmonella* species.

Subjects with a history of typhoid fever, Ty21a vaccination in the last 10 years or any other typhoid vaccine in the last 5 years, any clinically significant illness, abnormal blood test results, immune suppression, positive HIV or pregnancy test were excluded. Also excluded were subjects whose body weight was under 17 kg in the 5 to 10 year old group or under 27 kg in the 11 to 14 year old group and subjects who suffered from an acute febrile illness at the time of dosing (the complete list of exclusion criteria is available in the trial protocol). Only one child per family was allowed to participate in the trial.

The results of the screening tests were reviewed and subjects who continued to meet the inclusion criteria were invited to continue in the trial.

The M01ZH09 vaccine and dose

S. Typhi (Ty2 *aroC*⁻ *ssaV*⁻) ZH9 was constructed with a rational attenuation strategy. Two defined independently attenuating deletion mutations were introduced into *S. Typhi* Ty2. Deletion of *aroC*, encoding chorismate synthase, prevents the biosynthesis of aromatic amino acids and deprives the live vaccine bacterium of essential nutrients. Deletion of *ssaV*, encoding a structural component of the *Salmonella* pathogenicity island-2 (SPI-2) type III secretion system, prevents systemic spread of *S. Typhi* [9]. The vaccine was manufactured according to Good Manufacturing Practice protocols by Eurogentec S.A. and SynCo Bio Partners B.V; batch number M-STZH9-F16 was shipped to Vietnam. The vaccine kits were stored at 2–8 °C.

Previous studies in adult volunteers demonstrated that a nominal dose of 5×10^9 CFU of the vaccine strain was immunogenic and safe [9–11]. The Ty21a oral typhoid vaccine capsules are licensed for adults and children above 6 years using the same dose and immunization schedule and large Ty21a field trials in children used the same dose and regimen as in adults [13]. It was

therefore determined that the appropriate dose for the children's study was a nominal dose of 5×10^9 CFU of *S. Typhi* (Ty2 *araC⁻ssaV⁻*) ZH9.

The vaccine (containing 5×10^9 CFU of vaccine strain plus excipients) and the placebo (vaccine excipients only) were supplied as freeze-dried formulations in single dose vials, which were labeled identically, containing "M01ZH09 oral typhoid vaccine or placebo" but with a unique subject number corresponding to the randomisation list. The bicarbonate solution was prepared by dissolving one effervescent bicarbonate tablet (provided in the vaccine kit and containing 2.6 g sodium bicarbonate, 1.65 g ascorbic acid and 30 mg aspartame) in 150 ml of bottled drinking water (final concentration: 1.75% wt/vol sodium bicarbonate, 1.1% wt/vol ascorbic acid, and 0.02% wt/vol aspartame). The lyophilised vaccine or placebo was reconstituted in either 150 ml (for children above 10 years) or in 75 ml of the bicarbonate solution (the other 75 ml were discarded) for children below 10 years and was administered immediately.

The study used two age group specific randomisation lists, one for the 11 to 14 year old and one for the 5 to 10 year old children to ensure at least 70% children were between 5 to 10 years old.

Intervention

On the day of vaccination (day 0) which took place within 28 days of the screening, inclusion and exclusion (including history of antibiotic medication in the last 2 weeks) criteria were reviewed. Pregnancy tests (female subjects of 11 years and above only), urine dipstick test and stool cultures were performed. Blood samples for haematology, biochemistry, ELISA and ELISPOT assays (only in children 11 years and above) were obtained. After the subjects had fasted for at least 2 hours (with the exception of drinking water), the candidate typhoid vaccine or placebo was administered.

Subjects were allocated the next age-group specific subject number and the medication pack bearing the same number was prepared and issued by the pharmacist, who was otherwise not involved in the trial. The subjects were randomly assigned to receive either M01ZH09, consisting of 5×10^9 CFU of *S. Typhi* (Ty2 *araC⁻ssaV⁻*) ZH9 or the placebo reconstituted in bicarbonate solution as described above.

Volunteers were observed for at least 90 minutes at the hospital. During this time pulse and blood pressure were recorded periodically and only drinking water was provided.

Diary cards were issued for all the volunteers and all subjects received a basic hygiene kit containing soap, gloves and spatulas for the collection of stool samples. The subjects and their parents were instructed to measure and record the oral temperature of the children twice daily (morning and evening) and to record any adverse events (including headache, fever, nausea, vomiting, abdominal pain, frequency and consistency of stools and any other symptoms) for 14 days.

Follow up procedures and monitoring of adverse events

Children were followed up daily from days 1 to 14 and again on day 28 after dosing. At these appointments diary cards were checked and adverse events and concomitant medication reviewed. A history of the last 24 hours with special emphasis on temperatures of 38.5°C and above and adverse events (diarrhoea, loss of appetite, vomiting, headache and chills) was obtained. Oral temperatures and vital signs were recorded and children were examined for signs of splenomegaly. Stool cultures were performed daily from day 1 to day 14. Blood samples for biochemistry and haematology were obtained on days 7, 14 and 28; for the LPS specific serum IgA ELISA on days 7 and 14; for the IgG ELISA on days 14 and 28 and for the LPS specific IgA antibody secreting cell

(ASC) ELISPOT assay (only in subjects aged 11 years and above) on day 7. The total amount of blood taken during this study was approximately 28 ml from the 5 to 10 years old and 44 ml from the 11 to 14 years old children.

Unscheduled visits

Subjects and parents were instructed to make additional visits to the clinic, if the child felt unwell and/or had a fever of $\geq 38.5^\circ\text{C}$. At these visits the subject was assessed and samples taken for culture as clinically indicated. Blood cultures to investigate for the presence of *S. Typhi* in blood would be obtained if a fever of $\geq 39.0^\circ\text{C}$ was recorded twice over a 48 hours period, or a severe fever of $\geq 39.5^\circ\text{C}$ was recorded once.

Definition and reporting of serious adverse events and definition of stopping rules

There was no Data Safety and Monitoring Committee for this trial. Data from all children were reviewed daily and there were *a priori* defined stopping rules which would trigger a suspension of the trial and a safety review (Protocol S1). Serious adverse events were reported to AKOS Ltd (Hitchin, UK), a pharmacovigilance company within 24 hours.

Detection of *Salmonella* in stool samples at the screening visit and day 0

The detection of *Salmonella* species at the screening visit and on day 0 was performed according to microbiological standard procedures. In brief, stool samples were inoculated onto MacConkey agar and xylose lysine deoxycholate (XLD) agar plates, and in 10 ml of selenite F broth. Plates and broth were incubated at 37°C overnight and the broth was sub-cultured on MacConkey and XLD agar plates the next morning. Isolates were screened using standard biochemical tests and *Salmonella* were identified by slide agglutination with specific antisera (Oxoid Ltd., UK) and API20E profiling (bioMérieux, UK).

Detection of *S. Typhi* in stool samples

Stool samples were collected daily between days 1 and 14. Stool samples were cultured directly on deoxycholate citrate agar (DCA) Hynes plates (direct method) and in selenite F broth (enriched method), both of which were supplemented with aromatic compounds (DCA-aro and selenite F-aro, respectively) to detect *S. Typhi*, including the auxotrophic vaccine strain, in stools. Following overnight incubation at 37°C , an aliquot of the inoculated selenite F-aro broth was sub-cultured on DCA-aro Hynes plates. Suspected *S. Typhi* colonies were inoculated on brain heart infusion agar plates supplemented with aromatic compounds (BHI-aro). Oxidase negative colonies were evaluated by agglutination with Hd, Vi and O9 anti-sera (Oxoid Ltd., UK) and API20E profiling (bioMérieux, UK). Stool samples containing isolates that were positive in at least 2 out of 3 agglutinations and identified as *S. Typhi* by API20E profiling were considered to be positive for *S. Typhi*. All isolates were stored in 10% (v/v) glycerol at -80°C .

Detection of *S. Typhi* in blood samples

Blood samples were collected into either Bacter Peds Plus/F culture bottles (1–3 ml blood; BD, USA) or Bacter Plus Aerobic/F culture bottles (4–10 ml blood; BD, USA) and supplemented with aromatic compounds. Blood cultures were incubated at 35°C in the Bacter detection system and monitored for up to 5 days. Gram stain was performed on all bottles triggering a positive reaction. Positive cultures and all cultures that were negative after 5 days of

incubation were sub-cultured on XLD agar plates. Suspected *S. Typhi* colonies were sub-cultured onto BHI-aro agar plates. Oxidase negative isolates were evaluated by agglutination and API20E profiling (bioMérieux, UK) as above.

PCR identification of *S. Typhi* isolates

Genomic DNA was isolated from glycerol stocks of *S. Typhi* isolates using a DNeasy blood and tissue kit (Qiagen, UK). Multiplex PCRs were performed using a Taq PCR core kit (Qiagen, UK). Each reaction mixture contained 200 μ M dNTPs, 0.4 μ M *ssaV4* (5' ATCCCCACGACTTCAGCAAG 3') and *ssaV7* (5' CTTTCTGGGTCATCATGAGG 3'), and 0.1 μ M *aroC.Z1* (5' GACAACCTCTTCGCGTAACC 3') and *aroC.Z3* (5' TTACATCCGATTCTGTGCC 3'), 10 ng genomic DNA and 1.25 u Taq DNA polymerase in a total volume of 50 μ l reaction buffer. PCRs were performed for 25 cycles as follows: 94°C for 30 sec, 57°C for 30 sec and 72°C for 2.5 min. The PCR products were visualised by ethidium bromide staining and UV transillumination after electrophoresis on a 0.8% (w/v) TAE agarose gel. The expected sizes of the PCR products were 1.04 kb (*aroC*) and 2.59 kb (*ssaV*) for *S. Typhi* wild-type strains and 0.45 kb (*aroC*) and 0.70 kb (*ssaV*) for *S. Typhi* (Ty2 *aroC* *ssaV*) ZH9.

Detection of antibody secreting cells producing *S. Typhi* LPS specific IgA antibodies by ELISPOT assay

ELISPOT assays to detect antibody secreting cells (ASC) producing *S. Typhi* LPS specific IgA antibodies were performed on days 0 and 7 as described previously [10,14]. In brief, whole blood was collected in heparinised cell preparation tubes (Vacutainer CPT; BD, UK) and centrifuged. Peripheral blood mononuclear cells (PBMCs) were washed, resuspended in culture medium and adjusted to three cell concentrations (1×10^7 /ml, 5×10^6 /ml and 2.5×10^5 /ml). One hundred microlitres of each concentration were added to LPS coated and uncoated wells (for subtraction of non-specific results) of nitrocellulose microtiter plates (Millipore, USA) and incubated overnight at 37°C in a 5% CO₂ incubator. PBMC collected from a healthy volunteer who had received three doses of Ty21a (Vivotif, Berna, Switzerland) were included as positive control and PBMC from a non-vaccinated person as negative control. Plates were washed and an alkaline phosphatase-conjugated anti-human IgA antibody (Immune Systems Ltd., UK) was added and incubated for one hour. Plates were washed and spots were visualised by the addition of 5-bromo-4-chloro-3-indolyl-phosphate/nitro blue tetrazolium (BCIP/NBT) substrate. Antibody secreting cell (ASC) spots were counted manually using an inverted microscope. If more than 100 spots per well were present, the result was described as "too many spots to be counted". A positive response in the ELISPOT assay on day 7 was defined as ≥ 4 IgA ASC specific for LPS per 10^6 PBMCs and a negative response as < 4 IgA ASC specific for LPS per 10^6 PBMCs. Subjects with a day 0 result of ≥ 4 ASC per 10^6 PBMC were excluded from the ELISPOT analysis. ELISPOT assays were performed at the Hospital for Tropical Diseases

Analysis of *S. Typhi* LPS specific serum IgG and IgA by ELISA and definition of a positive immune response

Quantitative ELISA methods for measuring *S. Typhi* LPS specific serum IgG and IgA were developed and qualified by Emergent Product Development UK Ltd using serum samples from recipients of M01ZH09 who participated in prior clinical trials and who had given informed consent for retention and usage of their samples. For the IgG ELISA, serum which demonstrated more than 4-fold increase from pre-dose in a previously described

end point titre assay [11] were pooled and used as reference standard. The LPS specific IgG concentration in the standard serum was set arbitrarily at 30000 units/ml. For the IgA ELISA, serum from past recipients of M01ZH09 who demonstrated positive response in IgA ELISPOT were pooled and used as reference standard. The LPS specific IgA concentration in the standard serum was set arbitrarily at 100 units/ml.

Precision was evaluated as part of the assay qualification exercise. Variance component analysis was carried out (PRISM Training & Consultancy Ltd, UK) to calculate the standard error of measured sample means and the least significant difference (LSD) between two samples at the 1% significance level; this was used as the cut-off value for a positive result in the respective assay. A positive serum IgG response was defined as a 70% increase (fold change of 1.7) as compared to the corresponding baseline sample, whereas a positive serum IgA response was defined as a 50% increase (fold change of 1.5) as compared to baseline.

Serum samples for measurement were frozen at -20°C and shipped to Emergent Product Development UK Ltd for the ELISA analyses. For the IgG ELISA, microtiter plates were coated with *S. Typhi* LPS, washed and then blocked. Washing occurred between each step. Calibration standards and diluted test samples were added, and the plates were incubated. Bound IgG was detected using an anti-human IgG antibody conjugated to horseradish peroxidase (HRP) (Dako, Denmark) followed by the addition of 3,3',5,5'-Tetramethylbenzidine (TMB) substrate. The plates were read at 450 nm within 30 minutes of stopping the reactions with 0.3 mol/L sulphuric acid. The standard curve was constructed by plotting the optical densities (ODs) of standards against concentrations and fitted by a 4-parameter logistic equation (SoftMax[®] Pro 4.6, Molecular Devices, USA). The concentration of LPS specific IgG in each sample was determined from the standard curve. The IgA quantitative ELISA was performed in a similar manner, except using a double detection system of biotinylated anti-human IgA antibody (Southern Biotech, USA) followed by streptavidin-HRP conjugate (Dako, Denmark).

Outcomes of the study

Safety Outcomes. The primary safety endpoint was the proportion of subjects with any adverse events attributed to M01ZH09. The secondary safety endpoint was the proportion of subjects with any serious related adverse events; any related or unrelated adverse events; persisting faecal shedding of *S. Typhi* (Ty2 *aroC* *ssaV*) ZH9 after day 7; and/or had a fever of 38.5°C or greater in the 14 days post vaccination, withdrew from the trial due to adverse events, including bacteraemia, and/or had clinically significant changes in laboratory parameters related to the candidate vaccine.

All subjects who received a dose of the vaccine or placebo were analysed in the safety population. Post-vaccination adverse events were categorised according to body system and preferred term using the Medical Dictionary for Regulatory Activities (MedDRA, Version 9.1), allocated before unblinding. Adverse events were graded by severity (mild, moderate, severe) and judged for the relatedness to the study vaccine (unlikely, possibly, probably) by the investigator. Only possibly and probably related adverse events were attributed to the vaccine. Moderate fever was defined as an oral temperature of $\geq 38.5^\circ\text{C}$ and severe fever as an oral temperature of $\geq 39.5^\circ\text{C}$. Moderate diarrhoea was defined as more than 4 unformed stools and severe diarrhoea as more than 6 unformed stools in a 24 hour period or evidence of significant dehydration. All adverse events were recorded in the CRFs and monitored until return to normal.

The numbers and proportion of subjects reporting adverse events were listed by body system. A per subject analysis of adverse events was performed e.g., if a subject reported the same adverse event on three occasions that adverse event was only counted once. Subjects reporting more than one adverse event per body system were counted only once in that body system total.

Immunogenicity Outcomes. The primary immunogenicity endpoint was the proportion of subjects who developed a positive immune response to *S. Typhi* LPS defined by an increase of 70% (1.7 fold change) in LPS specific serum IgG on day 14 or 28 and/or an increase of 50% (1.5 fold change) in LPS specific serum IgA on day 7 or 14 compared to baseline.

The secondary immunogenicity endpoints were defined as the proportion of subjects who developed a positive immune response in each of the following assessments: *S. Typhi* LPS specific IgA ELISA assay on days 7 or 14, *S. Typhi* LPS specific IgG ELISA assay on days 14 or 28 and *S. Typhi* LPS specific IgA ELISPOT on day 7. A positive ELISPOT was defined as ≥ 4 IgA antibody secreting cells specific for *S. Typhi* LPS per 10^6 PBMCs.

Sample Size

The planned sample size was 150 subjects, of whom at least 70% should be aged 10 years or younger, as this was the target age of the vaccine, randomised to M01ZH09 or placebo in a 2:1 ratio.

No formal sample size calculation was considered appropriate; it was aimed to include a sufficient sample size to assess safety and immunogenicity based on previous observations in adult studies and immunogenicity rates of licensed typhoid vaccines in children.

Randomisation procedures and assignment of intervention (sequence generation, allocation concealment, implementation)

Randomisation codes were computer generated in blocks of 9 by Statwood Ltd, UK. The vaccine and the placebo were labeled identically but with a unique subject number corresponding to the randomisation list. The study used two age group specific randomisation lists, one for the 11 to 14 year old and one for the 5 to 10 year old children to ensure at least 70% children were between 5 to 10 years old.

Subjects were allocated the next age-group specific subject number in strict numerical sequence from this list and the medication pack bearing the same number was prepared by the pharmacist.

Blinding

This study was formally a single blind study due to slight differences in taste and aroma between the treatment preparations but it was conducted under the principles of a double blind study. M01ZH09 and placebo were packaged and labeled identically but with a unique sequential number. Possible sources of unblinding could have been the preparation of the vaccine, therefore the study pharmacist was otherwise not involved in the trial. The subjects were asked to not report the taste of the vaccine. Microbiology results were not reviewed by the investigators for at least 14 days after vaccination to avoid potential unblinding through shedding in stools. Immunology results were not reviewed by the investigators. The study site received code break envelopes in case an emergency made unblinding for a single subject necessary. No codes were broken during this study. The unblinding of treatment allocations took place after the trial had been completed and the whole database had been entered and locked.

Data collection, data entry and statistical methods

All data were recorded in Case Record Forms (CRFs). CRFs were reviewed and collected by the study monitor. Data entry, data management and statistical analysis were conducted by Statwood, UK using SAS® software (version 9.1). Data were double entered and analysed according to an *a priori* defined statistical analysis plan which included the definition of all subject populations and the trial endpoints. The safety population included all subjects who received the study medication. The intention to treat (ITT) population comprised all dosed subjects who had any post-dose immunogenicity data available. The per protocol (PP) population excluded major protocol violators (failure to meet the inclusion/exclusion criteria, to comply with the study medication or use of other vaccinations or antibiotics two weeks before until 2 weeks after vaccination, or use of antacids or proton-pump inhibitors prior to vaccination and/or did not provide samples for the ELISAs). The protocol stated that a confirmatory analysis of the primary immunogenicity endpoint in the PP population was planned if more than 5% of subjects were excluded.

The proportion of subjects who experienced post-dose adverse events was presented together with their two-sided 95% confidence intervals (95%CI). Post dose adverse events, adverse events considered to be related to the vaccine and adverse events that occurred in more than 10% of the trial population were tested using a two-sided Fisher's exact test to compare between the two groups.

The proportion of subjects who developed a positive immune response was presented together with their two-sided 95% confidence intervals calculated by using an exact Binomial distribution. The treatment difference and associated 95% confidence interval were presented as above. All available data from withdrawn subjects was included in the analysis.

Results

Participant flow and recruitment

The trial was conducted between April and July 2007. In total, 205 healthy Vietnamese children between 5 and 14 years (inclusive) were screened for eligibility. Fifty-four children were not eligible (Figure 1), the most common reasons were unavailability for the whole study period ($n=10$) and a positive stool culture for *Salmonella* species at screening (non-typhoid *Salmonella*, $n=22$). No *S. Typhi* or *S. Paratyphi A* were detected in stools at the screening visits.

One hundred and fifty-one children were randomised, 101 children received the candidate typhoid vaccine M01ZH09 and 50 children received placebo. All subjects fulfilled the inclusion and exclusion criteria at screening and dosing, however two subjects (both in the M01ZH09 group) had clinically significant elevated white blood counts (16.3 and $18.2 \times 10^9/L$ respectively) on day 0, these results were only available after dosing.

One subject in the M01ZH09 group vomited after taking approximately 50% of the required vaccine dose. The subject agreed to take another dose, but failed to retain it. Three subjects withdrew from the study, one placebo recipient withdrew due to non-compliance (refused to provide stool samples) after day 2 and two vaccine recipients left the study after day 6 (one wished to withdraw, the second subject went on holiday). All three subjects attended the day 28 visit. The remaining subjects attended all study visits. Two subjects had unscheduled visits. One subject in the vaccine group attended the clinic on day 16 because of fever of $38.1^\circ C$ and one subject in the placebo group returned on day 26 with a temperature of $38.0^\circ C$. Blood cultures were obtained from

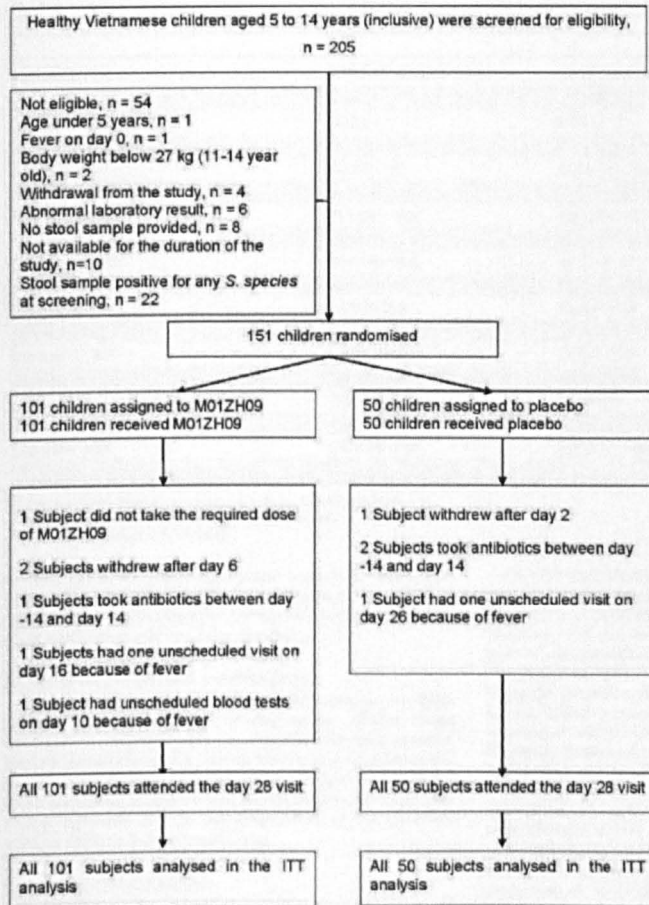


Figure 1. Flow of subjects.
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both subjects and both cultures were negative. One M01ZH09 recipient had unscheduled tests performed. The subject presented on day 10 with a temperature of 38.6°C and reported diarrhoea, vomiting and fever on the previous day. The white blood count was elevated with $14.2 \times 10^9/L$ and the blood culture result was negative.

Numbers analysed

All 151 children who were randomised and received either M01ZH09 ($n=101$) or placebo ($n=50$) constituted the intention to treat (ITT) population. Seven subjects, 4 in the vaccine group and 3 in the placebo group were protocol violators (see Fig. 1) and were excluded from the per protocol (PP) population. The analysis

of the primary endpoints in the PP population was planned if more than 5% of subjects were excluded from the ITT. The PP population comprised 95% (144/151) of subjects and therefore no per protocol analysis was conducted.

All outcomes were evaluated for the ITT population.

Baseline data

The two groups did not differ significantly at enrolment in terms of sex, age and laboratory parameters (Table 1). One hundred and seven (71%) children were aged 10 years or younger.

Two subjects in the M01ZH09 group had clinically significant elevated white blood counts on day 0 (see above). Twelve subjects

Table 1. Baseline characteristics of the subjects on day 0 (Intention to Treat population).

Characteristics	M01ZH09 group (n = 101)	Placebo group (n = 50)	Overall (n = 151)
Age in years	9 (5–14)	9 (5–14)	9 (5–14)
Number of males (%)	54 (53)	27 (54)	81 (54)
Weight in kilograms	28 (17–53)	26.5 (17–66)	27 (17–66)
Height in cm	132 (97–165)	130.50 (100–165)	132 (97–165)
Oral temperature in °C	36.80 (36.1–37.5)	36.75 (35.1–37.4)	36.80 (35.1–37.5)
Haemoglobin, g/dl ^a	13.3 (10.5–15.3)	13.4 (10.9–15.5)	13.3 (10.5–15.5)
White cell count, 10 ⁹ /L ^a	7.2 (5–18.2)	8.0 (4.9–10.7)	7.4 (4.9–18.2)
Lymphocytes, % ^a	39.6 (14.9–55.5)	39.9 (21.4–67.4)	39.7 (14.9–67.4)
Neutrophils, % ^a	48.6 (29.2–70)	50.5 (19.5–73.8)	49.9 (19.5–73.8)
Monocytes, % ^a	5.3 (2.3–12.3)	5.0 (1.4–13.2)	5.2 (1.4–13.2)
Basophiles, % ^a	0.3 (0–0.7)	0.3 (0–0.7)	0.3 (0–0.7)
Eosinophiles, % ^a	4.0 (0.1–18.4)	3.0 (0.4–15.2)	3.8 (0.1–18.4)
Platelet count, 10 ⁹ /L ^b	295 (190–503)	311 (190–467)	300 (190–503)
Serum Aspartate Aminotransferase AST, U/L	27 (14–52)	26 (14–51)	27 (14–52)
Serum Alanine Aminotransferase ALT, U/L	16 (6–58)	17 (5–43)	16 (5–58)
Creatinine, mL/L	0.47 (0.33–0.70)	0.47 (0.27–0.73)	0.47 (0.27–0.73)
Stool culture positive for <i>Salmonella</i> species	7	5	12

All data are presented as median (range) unless otherwise specified.
Data from one^a, two^b and seven^c subjects not available.
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in total, five in the placebo group and seven in the M01ZH09 group (this included the subject who vomited on the day of dosing) had a positive stool culture for non-typhoid *Salmonella* on day 0, these results were only available after dosing.

Protocol deviations and modifications

The protocol stated that the primary safety endpoint would be the proportion of subjects reporting serious adverse events attributed to M01ZH09. Due to concerns that these numbers might be small and would not be sufficient to detect a difference in safety between the two groups, the primary endpoint was changed to the proportion of subjects with any related adverse event. This change was made after the completion of the clinical phase but prior to database lock and unblinding.

Outcomes and estimation

Safety Outcomes. There were no serious adverse events, no bacteraemia and none of the subjects withdrew due to adverse events in this trial.

Similar proportion of subjects reported adverse events in both treatment groups during the 28 days of follow up, but the total number of events was higher in the M01ZH09 group. In the vaccine group, 26 (26%; 95%CI, 18–35%) of 101 subjects reported 64 adverse events compared to 11 (22%; 95%CI, 12–36%) of 50 subjects in the placebo group who reported 17 adverse events (odds ratio (OR) [95%CI] = 1.23 [0.550–2.747]; $p = 0.691$) (Table 2). Repeated occurrences of a particular adverse event in the same subject were included in the total number of 64 and 17 adverse events, respectively. Of the 64 adverse events reported by M01ZH09 recipients, 55 were mild (8 of these were considered to be related to the candidate vaccine), 8 moderate (one related) and one was severe and related to M01ZH09 (Table 2). Of the 17 adverse events in the placebo group, 12 were mild, 5 moderate and none was related.

Four (4%) M01ZH09 recipients experienced 10 adverse events that were related to the candidate vaccine compared to none in the placebo group ($p = 0.302$). Of these, 8 were mild, one moderate (diarrhoea) and one event of pyrexia was severe. The moderate and the severe related adverse events occurred in the same subject. This subject had a normal temperature on day 0, but the pre-dose blood test showed an elevated white blood cell count ($16.3 \times 10^9/L$). The subject experienced five post vaccination adverse events occurring on day 0, including fever of 38.5 and 39.0°C, diarrhoea, headache, abdominal pain and anorexia. The subject received paracetamol and recovered.

Similar proportions of subjects experienced fever post vaccination (Table 2), only one subject reported fever related to M01ZH09 (see above).

Adverse events classified as gastrointestinal disorders, nervous system disorders and investigations were experienced by a higher proportion of M01ZH09 recipients (Table 2). Twelve (12%) vaccine recipients experienced gastrointestinal disorders compared to 1 (2%) placebo recipient ($p = 0.061$). Nervous system disorders (headache) occurred in 9 (9%) vaccine recipients compared to 1 (2%) placebo recipient ($p = 0.166$) and investigations were reported by 4 (4%) vaccine recipients compared to none of the placebo recipients ($p = 0.302$).

Cough was the most frequently reported adverse event, occurring in 6 (6%) M01ZH09 recipients versus 7 (14%) placebo recipients ($p = 0.124$).

On day 1 after vaccination, faecal shedding of *S. Typhi* occurred in 47 (49%) of 95 vaccine recipients; shedding was detected by the direct method in 11 (12%) subjects and by the enriched method of culturing stools in 36 (38%) subjects. On day 2 after vaccination, faecal shedding was detected in 12 (12%) of 97 subjects (in 1 (1%) subject by direct and in 11 (11%) subjects by enriched method). Only one (1%) of 98 subjects experienced shedding on Day 3 (detected by enrichment method). In total, 51 (51%; 95% CI, 41–61%) of 100 M01ZH09 subjects experienced

Table 2. Incidence of adverse events after vaccination during 28 days of follow up (Intention to Treat population).

Event	M01ZH09 (n = 101)		Placebo (n = 50)	
	Number of subjects	Percent (95% CI)	Number of subjects	Percent (95% CI)
Any adverse event	26	26 (18–35)	11	22 (12–36)
Gastrointestinal disorders (%)	12	12 (6–20)	1	2 (0–11)
<i>related to vaccine</i>	3	3	0	0
Abdominal pain (%)	8	8	0	0
<i>related to vaccine</i>	2	2	0	0
Constipation (%)	2	2	0	0
Diarhoea	5	5	1	2
<i>related to vaccine</i>	3	3	0	0
Nausea	3	3	0	0
<i>related to vaccine</i>	1	1	0	0
Vomiting	3	3	0	0
General disorders and administration site conditions	9	9 (4–16)	5	10 (3–22)
Chills	1	1	1	2
Fatigue	0	0	2	4
Pyrexia	8	8	3	6
<i>related to vaccine and severe*</i>	1	1	0	0
Infections and infestations	1	1 (0–5)	0	0 (0–7)
Viral infection	1	1	0	0
Investigations	4	4 (1–10)	0	0 (0–7)
Urine colour abnormal	2	2	0	0
White blood cell count increased	2	2	0	0
Metabolism and nutrition disorders	3	3 (1–8)	0	0 (0–7)
Anorexia	1	1	0	0
<i>related to vaccine</i>	1	1	0	0
Decreased appetite	2	2	0	0
Nervous system disorders	9	9 (4–16)	1	2 (0–11)
Headache	9	9	1	2
<i>related to vaccine</i>	2	2	0	0
Respiratory, thoracic and mediastinal disorders	6	6 (2–12)	7	14 (6–27)
Cough	6	6	7	14
Rhinorrhoea	1	1	0	0
Skin and subcutaneous tissue disorders	1	1 (0–5)	1	2 (0–11)
Rash	1	1	1	2
Vascular disorders	1	1 (0–5)	0	0 (0–7)
Hypertension	1	1	0	0

Per subject analysis of adverse events (unlikely, possibly and probably related to the vaccine) reported during 28 days of follow up. Subjects could experience more than one adverse event. Each adverse event was only counted once for each subject and system class. There were 56 adverse events in the M01ZH09 group and 16 in the placebo group, when repeated occurrences of a particular event in the same patient were only counted once. Adverse events that were possibly or probably related to the vaccine are presented in *italics*.

*One severe adverse event was reported.

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shedding on either days 1, 2 or 3 and no subjects experienced shedding on day 4 after vaccination or later.

The presence of *S. Typhi* was detected in the stools of 1 (2%; 95%CI, 0–11%) of 50 subjects in the placebo group. This occurred on day 2 and was detected using the enriched method. No fever or adverse events were recorded for this subject. The finding of a positive stool culture for *S. Typhi* in a placebo subject was only available after unblinding of the trial. All previous and all sequential stool cultures up to day 14 of this subject were negative.

This isolate was identified as the vaccine strain *S. Typhi* (Ty2 *araC⁺ issA⁺*) ZH9 by subsequent PCR analysis.

Seven (7%) of 101 M01ZH09 recipients and 3 (6%) of 50 placebo recipients were detected to have a positive stool culture for non-typhoid *Salmonella* between day 1 and 14 after vaccination.

Immunogenicity Outcomes. Ninety-eight (97%; 95%CI, 92–99%) of 101 subjects in the M01ZH09 group and 8 (16.0%; 95%CI, 7–29%) of 50 subjects in the placebo group developed a positive immune response in either the *S. Typhi* LPS specific

serum IgG or IgA ELISA, defined as the primary endpoint (Table 3). The difference in proportions of responders between the vaccine group and the placebo group was 81.0% (95% CI: 68–89%), the lower limit of the 95% CI of this difference was greater than 50% and fulfilled the *a priori* defined criterion for an acceptable immune response.

Median baseline LPS specific antibody levels were comparable in both groups (Figure 2). In the M01ZH09 group, median IgA antibody levels increased from 3 (IQR: 3–7.2) units/ml at baseline to 94 (IQR: 19.8–231.5) units/ml and 103 (IQR: 23.9–253.5) units/ml on days 7 and 14 respectively. On day 7, the 88 immune responders in the vaccine group (Table 3) displayed a median 16.4 (IQR: 3.75–60.25) fold rise in serum IgA antibodies relative to baseline (Table 4, Supplements).

In the vaccine group, median LPS specific IgG antibody levels were 66650 (IQR: 31075–123900) units/ml and 55700 (IQR: 25450–106800) units/ml on day 14 and 28, respectively, compared to median baseline levels of 6300 (IQR: 3620–16560) units/ml. On day 14, the 91 immune responders in the M01ZH09 group (Table 3) showed a median 8.18 (IQR: 3.57–20.63) fold increase in serum IgG antibodies relative to baseline.

Forty-two out of 44 eligible subjects provided samples for the ELISPOT assay on day 7. All baseline ELISPOT samples were negative (defined as <4 ASC per 10^6 PBMC). On day 7, 28 (100%) of 28 M01ZH09 subjects who provided samples showed a positive ELISPOT response compared to none (0%) of the 14 evaluable subjects in the placebo group. Sixteen (57%) of 28 M01ZH09 recipients displayed results of >100 spots per 10^6 PBMC and among the remaining 12 vaccine subjects numbers of spots ranged from 8 to 128 per 10^6 PBMC. The median number of spots in the M01ZH09 recipients was >100 (IQR: 46.5–>100) spots per 10^6 PBMC, as counting stopped above 100 spots, this was recorded as "too many spots to be counted". All 14 placebo recipients showed <4 spots per 10^6 PBMC, this was recorded as "too few spots to be counted."

There was strong correlation between the results of the IgA ELISA and the IgA ELISPOT assays: on day 7, Twenty-eight (100%) of 28 M01ZH09 recipients showed a positive immune response and 14 (100%) of 14 placebo recipients showed a negative response in both assays.

Discussion

Interpretation

This is the first evaluation of a novel oral typhoid vaccine in school children in an endemic country. *S. Typhi* (Ty2 *aroC⁻isaF⁻*) ZH9 (contained in M01ZH09) is characterised by two well defined deletion mutations, one in an aromatic amino acid biosynthesis pathway gene and one in a functional gene of the type III secretion system encoded by SPI-2 [9]. A single dose of 5×10^8 CFU of the vaccine strain was well tolerated and had an acceptable safety profile. There were no serious adverse events, no withdrawals due to adverse events and none of the subjects experienced bacteraemia.

In general, adverse events were mild. Similar proportions of subjects, 26% (26 of 101) in the candidate vaccine group and 22% (11 of 50) in the placebo group reported adverse events during the 28 day follow up period ($p = 0.691$). The overall number of adverse events tended to be higher in the M01ZH09 group, especially those classified as gastrointestinal disorders, nervous system disorders and investigations.

There was one severe related adverse event in this trial, a high fever of 39.0°C which occurred on day 0 after vaccination in a subject who had a pre-dose elevated white blood count ($18.2 \times 10^9/L$) and might have suffered from an underlying infection. One other subject vomited after drinking approximately half of the vaccine dose, this subject was found to have a positive stool culture for non-typhoid *Salmonella* on day 0.

S. Typhi was isolated from the stools of one placebo recipient on day 2 after vaccination which was later identified as *S. Typhi* (Ty2

Table 3. Proportions of responders to the candidate typhoid vaccine M01ZH09 (intention to Treat population).

	M01ZH09 group n = 101			Placebo group n = 50		
	No.	Positive Immune response, no. (%)	95% CI	No.	Positive Immune response, no. (%)	95% CI
Detected in IgA ELISA assay						
Day 7	99	88 (89)	81–94	49	1 (2)	0–11
Day 14	99	92 (93)	86–97	49	1 (2)	0–11
Day 7 or day 14	99	94 (95)	89–98	49	2 (4)	1–14
Detected in IgG ELISA assay						
Day 14	99	91 (92)	85–97	49	6 (12)	5–25
Day 28	101	90 (89)	81–94	50	6 (12)	5–24
Day 14 or 28	101	93 (92)	85–97	50	8 (16)	7–29
Detected in either IgA or IgG ELISA assay						
Day 7, 14 or 28	101	98 (97)	92–99	50	8 (16)	7–29
Detected in IgA ELISPOT*						
Day 7	28	28 (100)	88–100	14	0 (0)	0–23

No., number of subjects who provided samples.

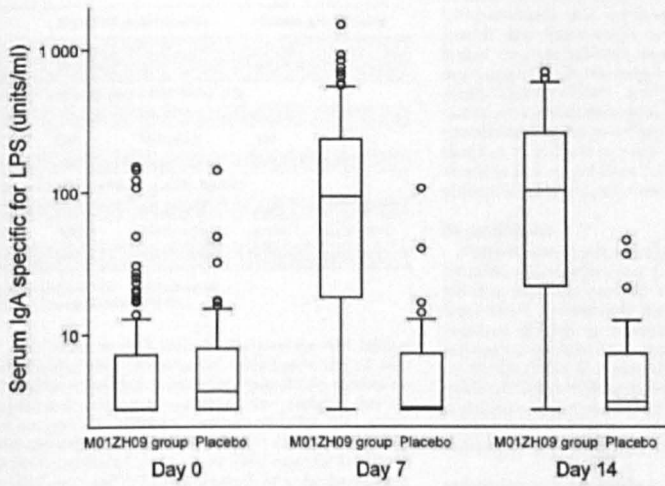
A positive immune response in the ELISA assay was defined by an increase of 50% (1.5 fold change) in LPS specific serum IgA and/or an increase of 70% (1.7 fold change) in LPS specific serum IgG compared to baseline.

*44 subjects aged 11 years and above (29 subjects in the M01ZH09 group and 15 subjects in the placebo group) were eligible for the ELISPOT.

A positive ELISPOT result was defined as ≥ 4 IgA antibody secreting cells specific for *S. Typhi* LPS per 10^6 PBMCs. None of the subjects had a positive day 0 ELISPOT result.

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A



B

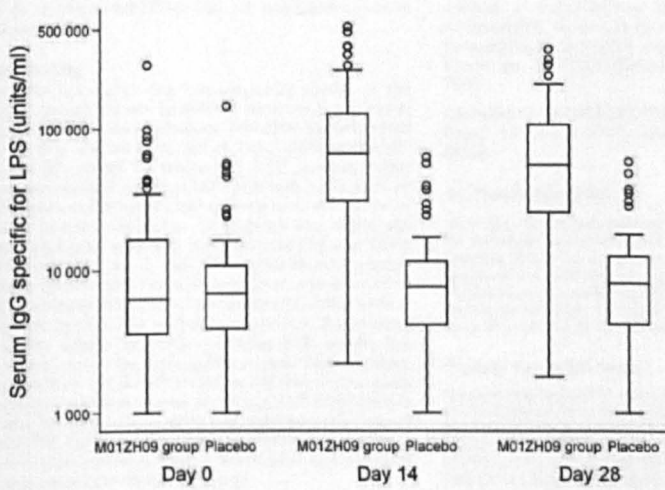


Figure 2. Time course of LPS specific serum IgA (A) and IgG (B) antibody levels according to vaccination groups (intention to Treat population). Box and whisker plots showing the distribution of antibodies according to time point and vaccination groups. The horizontal line within each box represents the median, the top and bottom of each box represents the 75th and 25th percentiles, respectively, and the bar represents the highest and lowest values within 1.5 times the interquartile range. Circles show outliers. doi:10.1371/journal.pone.0011778.g002

Table 4. Serum IgA and IgG antibody levels specific for *S. Typhi* LPS (Intention to Treat population).

Day	M01ZH09 group n = 101		Placebo group n = 50	
	Median units/ml	IQR	Median units/ml	IQR
Serum IgA antibody levels specific for LPS				
Day 0	3	3–7.2	4	3–7.9
Day 7	94 ^Δ	19.8–231.5	3.1*	3–7.4
Day 14	103 ^Δ	23.9–253.5	3.4*	3–7.4
Serum IgG antibody levels specific for LPS				
Day 0	6300	3620–16560	6925	3950–10762.5
Day 14	66650 ^Δ	31075–123900	7680*	4170–11500
Day 28	55700	25450–106800	8175	4402.5–12437.5

Data from one* and two^Δ subjects missing.
doi:10.1371/journal.pone.0011778.t004

aroC *ssaV*) ZH9 by PCR analysis. The previous stool cultures and all following stool cultures of this subject up to day 14 were negative. After a thorough check which included the randomisation codes and vaccination paperwork, the possibility that the subject received M01ZH09 by error was excluded. The subject also did not display any positive results in the immunogenicity assays. It was concluded that the most likely cause for isolating *S. Typhi* (Ty2 *aroC* *ssaV*) ZH9 in the stools of a placebo recipient was the mislabelling or mismatch of stool samples.

The candidate vaccine elicited a positive immune response in 97% (98/101) of the M01ZH09 recipients by ELISA and in 100% (28/28) of M01ZH09 recipients who were evaluable by ELISPOT assay. In conclusion M01ZH09 was safe and immunogenic in Vietnamese children.

Generalisability

The observed safety and immunogenicity profile of the candidate typhoid vaccine in children compares favourably to that seen in Western adult volunteers. M01ZH09 has been tested so far up to a nominal dose level of 5×10^9 CFU in nine UK volunteers [9] and 80 US volunteers [10,11]. Immunogenicity results from previously published M01ZH09 trials used a 4 fold or higher increase in LPS specific IgG antibody levels as definition of a positive immune response in the endpoint titre ELISA and seroconversion rates were 50% (8/16 subjects) [10] and 77.4% (24/31 subjects) [11]. In this study, allowing for these different cut-offs, the magnitude of the immune response seen in the children was approximately 30 fold and 10 fold increase of median levels of LPS specific IgA and IgG antibodies, respectively. Furthermore, the median number of ASCs producing LPS specific IgA antibodies, a measure for priming of the mucosal immune system, was greater than 100 per 10^6 PBMC in this trial, this compares favourably to an arithmetic mean of 118 ASC/ 10^6 PBMC seen in a previous M01ZH09 trial in adults [10] and a geometric mean of 119 ASC/ 10^6 PBMC (producing IgA and IgG) seen in American volunteers who received 4 doses of the licensed Ty21a typhoid vaccine at a dose of $2-6 \times 10^9$ CFU [15].

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This is encouraging as one major concern for the development of many oral vaccines has been their reduced immunogenicity when tested in developing country populations compared to Western volunteers [12,16]. For oral vaccines a brisk colonisation of the intestine is necessary to become immunogenic, it might be possible that drug resistant commensals, bacterial overgrowth, enteric viruses or helminths interfere with the colonisation of the new vaccine [17]. In this study, 51% (51/100) of vaccine recipients shed *S. Typhi* (Ty2 *aroC* *ssaV*) ZH9 in stools after vaccination, one subject excreted the vaccine strain on day 3, but no shedding was observed on day 4 and beyond. In Western adult volunteers shedding of *S. Typhi* in stools was reported for slightly longer durations and ranged from 1–6 days and 1–7 days in a small number of volunteers, respectively [9,11].

Generalisation

Typhoid fever is still a major health problem in developing countries, with high incidence [1,18] and high rates of antimicrobial drug resistance, especially in Asia [4,18]. The World Health Organisation recommends the immunisations of school and preschool children in endemic areas, especially where drug resistant typhoid fever is prevalent as well as in epidemic situations [5,19]. M01ZH09 is a promising novel oral one-dose typhoid vaccine and large trials are necessary to evaluate vaccine efficacy. If protection from typhoid fever is demonstrated, M01ZH09 may facilitate large vaccination campaigns due to its simpler logistic and broader acceptance from children.

Supporting Information

Protocol S1. Trial protocol. The trial protocol is as a true and correct copy of the original document (PDF version) minus redacted lines (personal information, names and telephone numbers of employees have been removed to maintain their confidentiality). No part in the content of the trial protocol with the exception of the vaccine recipients has been redacted. Found at: doi:10.1371/journal.pone.0011778.s001 (0.23 MB PDF)

Checklist S1. CONSORT Checklist.

Found at: doi:10.1371/journal.pone.0011778.s002 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: TTH ES CS CPS WL FM JPM AU RB GD JF NVVC CD. Performed the experiments: TTH NTD NTT NTTV TNBC NVMB TPTN CPT PVM NTCB TTDH PVT TSD JIC ES CS CPS WL FM JPM AU JF NVVC CD. Analyzed the data: TTH CG WL AU RB GD JF NVVC CD. Wrote the paper: TTH TSD JIC CS CPS CG WL FM JPM AU RB GD JF NVVC CD.

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High-throughput sequencing provides insights into genome variation and evolution in *Salmonella* Typhi

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Isolates of *Salmonella enterica* serovar Typhi (Typhi), a human-restricted bacterial pathogen that causes typhoid, show limited genetic variation. We generated whole-genome sequences for 19 Typhi isolates using 454 (Roche) and Solexa (Illumina) technologies. Isolates, including the previously sequenced CT18 and Ty2 isolates, were selected to represent major nodes in the phylogenetic tree. Comparative analysis showed little evidence of purifying selection, antigenic variation or recombination between isolates. Rather, evolution in the Typhi population seems to be characterized by ongoing loss of gene function, consistent with a small effective population size. The lack of evidence for antigenic variation driven by immune selection is in contrast to strong adaptive selection for mutations conferring antibiotic resistance in Typhi. The observed patterns of genetic isolation and drift are consistent with the proposed key role of asymptomatic carriers of Typhi as the main reservoir of this pathogen, highlighting the need for identification and treatment of carriers.

Typhoid fever, along with plague, cholera and smallpox, is one of the classical infectious diseases of humans. The disease, which is spread via oral ingestion of contaminated food or water, is caused by *Salmonella enterica* serovar Typhi (Typhi), a Gram-negative bacterium classified as a serovar of the species *S. enterica*¹. *S. enterica* is a broad and promiscuous species with isolates able to cause gastroenteritis in a range of animals, including humans². In contrast to most other *S. enterica* serovars, Typhi has forsaken the promiscuous lifestyle to become a human-restricted pathogen causing both acute systemic infections (typhoid fever) and chronic infections (asymptomatic carriers). A number of evolutionary processes have been implicated in the adaptation of Typhi to this specialized niche, including the horizontal acquisition of several *Salmonella* pathogenicity islands (SPIs)^{3,4} and extensive loss of gene function⁵.

Typhi, together with other human pathogens such as *Yersinia pestis*, *Bordetella pertussis* and *Bacillus anthracis*, is regarded as a monomorphic organism, as the genomes of individual Typhi isolates are highly conserved and clonally related. A recent study involving the DNA sequencing of 199 gene fragments from a global collection of 105 Typhi isolates detected only 82 SNPs⁶. Analysis of the SNP data resolved Typhi into a rooted, fully parsimonious phylogenetic tree defining 85 genetically distinct haplotypes (H1–H85, **Supplementary**

Fig. 1 online). The availability of a robust phylogenetic tree proved to be a useful framework against which to investigate the recent evolution of phenotypic traits such as the acquisition of resistance to fluoroquinolones, a class of antibiotics used to treat typhoid fever^{7–7}.

Because Typhi shows such low levels of genetic variation, further studies require a whole-genome approach. Complete genome sequences are available for two Typhi isolates, CT18 and Ty2 (refs. 3,8). However, several new sequencing technologies have been developed that make draft genome sequencing simpler and more cost effective⁹. In order to capture variation in Typhi at the whole-genome level and minimize sampling bias¹⁰, we sequenced an additional 17 Typhi isolates dispersed in the phylogenetic tree, using a combination of 454 (Roche) and Solexa (Illumina) sequencing technologies.

RESULTS

Choice of Typhi isolates for whole-genome sequencing

In order to capture as much information as possible about the distribution of genomic variation in the Typhi population, we prepared DNA from CT18, Ty2 and 17 other isolates for a combination of 454 and Solexa sequencing (see Methods and Table 1). To limit selection bias as much as possible, we chose Typhi isolates from central haplotype clusters together with selected isolates from radial haplotype

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Table 1 Typhi isolates sequenced in this study

Isolate	Country	Year	Haplotype	454 coverage	Solexa coverage	Plasmid
E00-7866	Morocco	2000	H46	10.5×	—	n.d.
E01-6750	Senegal	2001	H52	8.16×	—	n.d.
E02-1180	India	2002	H45	13.1×	—	n.d.
E98-0664	Kenya	1998	H55	10.8×	—	n.d.
E98-2068	Bangladesh	1998	H42	10.9×	—	n.d.
J185SM	Indonesia	1985	H85	13.5×	—	n.d.
M223	Unknown	1939	H8	11.1×	—	n.d.
404y	Indonesia	1983	H59	8.49×	24.6×	pBSSB1
AG3	Vietnam	2004	H58	10.1×	13.1×	n.d.
E98-3139	Mexico	1998	H50	11.1×	5.40×	n.d.
150981S	Vietnam	1998	H63	—	8.60×	n.d.
8104N	Vietnam	2004	H58	—	13.1×	n.d.
CT18	Vietnam	1993	H1	—	9.30×	pHCM1, pHCM2
E02-2759	India	2002	H58	—	65.5×	pHCM2
E03-4983	Indonesia	2003	H59	—	7.42×	pBSSB1
E03-9804	Nepal	2003	H58	—	8.19×	pAKU1
ISP-03-07467	Morocco	2003	H58	—	7.87×	pAKU1
ISP-04-06979	Central Africa	2004	H58	—	72.9×	pAKU1
Ty2	Russia	1916	H10	—	8.60×	n.d.

Country and year of isolation are shown. Haplotypes correspond to those previously defined⁵. Coverage refers to oversampling in sequence data. n.d., none detected.

groups and subjected these to 454 sequencing (Supplementary Fig. 1). To gain additional insight into SNP variation among recently expanding haplotypes, we used Solexa sequencing to generate sequence information from an additional six isolates from the H58 group, which has undergone recent clonal expansion in Southeast Asia^{5,7}, and a second isolate from the H59 group, known to be circulating in Indonesia¹¹. Three isolates, including one H58 and one H59 isolate, were sequenced using both 454 and Solexa, and the results were compared.

SNPs and phylogenetic analysis

We detected high-quality SNPs by mapping 454 contigs or Solexa reads to the finished CT18 sequence (see Methods). Our analysis focused on the nonrepetitive component of the genome, and we did not attempt to identify single-base insertions or deletions. Repetitive sequences, including VNTRs, exact repeats of ≥ 20 bp, $> 95\%$ identical repeats of > 50 bp and phage and insertion sequences (IS), account for 7.4% of the CT18 genome (Supplementary Table 1a online). Here, we excluded these classes of repetitive sequences from SNP analysis as (i) non-identical repeats can appear indistinguishable from SNPs, particularly with short sequencing reads (100–250 bp for 454, 25 bp for Solexa), (ii) assembly and mapping of short reads are unreliable in repetitive regions and (iii) repeated regions may be subject to different selective pressures compared to the rest of the genome, for example, recombination between repeat copies. After excluding these sequences, we identified a total of 1,964 SNPs in the nonrepetitive genome, approximately 1 every 2,300 bp. Details of these SNPs are given in Supplementary Table 2 online.

We determined complete allele data for 1,787 SNPs (missing data was due to low coverage or deletion of SNP loci in one or more isolates). These SNPs traced the same phylogenetic tree as previously defined⁵ (Supplementary Fig. 1) but provided better estimates of branch lengths and greatly increased resolution, particularly within the H58 and H59 groups (Fig. 1). By comparing sequence data from 454, Solexa and published sequences, we determined cut-offs for quality filtering (Supplementary Fig. 2 online) and estimated a false-positive rate of 2.7% and SNP detection sensitivity of 82–99.7% for both

sequencing platforms (see Methods and Supplementary Table 1b). This apparently high false-positive rate is due to the extreme paucity of true SNPs; the actual error rate of the sequencing technologies is very low (around 7 to 10 errors in 4.45×10^6 base pairs for Solexa resequencing on CT18 and Ty2; see Supplementary Methods online). Only ten SNPs (0.56%) did not fit the previously determined phylogenetic tree, two of which are confirmed examples of convergent evolution at sites under adaptive selection in *gyrA* (see below). Thus, we have little reason to suspect high error rates among allele assignments, or to doubt the phylogenetic tree structure shown in Figure 1.

Using the resulting rooted phylogenetic tree, we were able to group mutations into relative age groups: (i) recent mutations, furthest from the root and lying on intra-haplotype branches, (ii) intermediate mutations, lying on haplotype-specific branches, and (iii) older mutations, lying on branches closest to the root and shared by

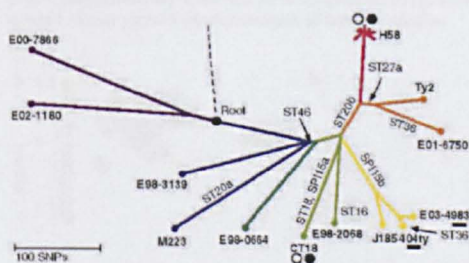


Figure 1 Phylogenetic tree based on SNP data. Branch colors indicate different lineages of typhi; branch lengths are measured in number of SNPs, scale as indicated. Central, small black circle indicates the ancestral root, dashed line represents *Salmonella* lineage; phage (ST) and SPI15 insertion events are shown along branches; plasmids detected in each isolate are indicated by filled circles (IncHI1 multidrug resistance plasmids), open circles (cryptic plasmid) and filled lines (linear plasmid carrying z66 flagella variant).

Table 2 Genetic variation detected in 19 Typhi genomes

	Intrahaplotype	Interhaplotype	Conserved	Total
Deletions	5	8	7	20
Phage insertions	n.a.	5	4	9
Plasmids	3	2	0	5
SNPs (complete allele data)	93	1,356	338	1,787
- Intergenic	6 (6.5%)	177 (13.1%)	44 (13.0%)	227
- Synonymous	21 (22.6%)	477 (35.2%)	106 (31.4%)	604
- Nonsynonymous	61 (65.6%)	663 (48.9%)	176 (52.1%)	900
- Nonsense	5 (5.4%)	39 (2.9%)	12 (3.6%)	56
- dN/dS	0.98	0.46	0.52	0.49
SNPs (incomplete allele data)	19	122	35	176
- Intergenic	4 (21.1%)	24 (19.7%)	6 (17.1%)	34
- Synonymous	3 (15.8%)	41 (33.6%)	12 (34.3%)	56
- Nonsynonymous	12 (63.2%)	57 (46.7%)	17 (48.6%)	86
- Nonsense	0 (0.0%)	0 (0.0%)	0 (0.0%)	0
- dN/dS	1.24	0.44	0.44	0.48

Frequencies of mutations according to age (recent, intrahaplotype, intermediate, interhaplotype, oldest, conserved). Percentages indicate the relative frequencies within each age group, separately for alleles that could be reliably determined for all isolates (complete allele data) and those that could not (incomplete allele data). n.a., not applicable.

multiple haplotypes. The distribution of SNPs and other variants in each group is shown in Table 2.

dN/dS as a measure of stabilizing selection

The ratio of nonsynonymous to synonymous SNP rates, dN/dS, is a common measure of stabilizing selection. A dN/dS ratio close to 1 indicates no selection against nonsynonymous SNPs, whereas dN/dS close to 0 indicates strong stabilizing selection. The mean dN/dS of each isolate compared to the last common ancestor was 0.66 ± 0.053 (s.d.), indicating either a weak trend in the direction of stabilizing selection since the last common ancestor of Typhi, or a combination of stabilizing selection in some genes and diversifying selection in others. As there is little evidence of diversifying selection in any Typhi genes (see below and Supplementary Table 3a online), weak stabilizing selection is most likely. The weakness of the signal for stabilizing selection observed here may be due to too little time for selection to act, and/or genetic drift due to low effective population size. It has been previously shown that in closely related bacteria, the reciprocal of dN/dS, or $1/(dN/dS)$, is related to time¹²; simulations indicated that when population size was large, this relationship was linear, but when effective population size was small, genetic drift became more important and $1/(dN/dS)$ reached a plateau. The relationship of $1/(dN/dS)$ to time (measured by the number of intergenic SNPs) for the sequenced Typhi isolates was nonlinear (Fig. 2a). Intergenic SNPs serve as an approximation of time, as they are less likely to be under purifying selection than SNPs in coding regions. However, intergenic SNPs may have regulatory or other functions that may be under selection, so as an alternative measure, we also calculated dN/dS among SNPs of different relative ages, which confirmed the same trajectory (Fig. 2b). In the light of the previously described model¹², these patterns are suggestive of genetic drift in Typhi due to a small effective population size, which seems likely, as Typhi has no known reservoir outside of humans. A small effective population size ($N_e = 2.3 \times 10^5 - 1.0 \times 10^6$) has been calculated previously using Bayesian skyline plots based on 82 SNPs in 105 Typhi isolates⁵.

Potential signals of selection

We found very little evidence of adaptive selection in Typhi genes, which would be represented by an overabundance of nonsynonymous SNPs or independent changes in the same or nearby amino acid residues. We found that 72% of genes contained no SNPs and that the distribution of 0–6 SNPs per gene followed a Poisson distribution in the range of 0–6 SNPs per gene (Fig. 3). However, there were a few exceptions (Supplementary Table 3a). Three genes (*yehU*, *trtE* and *STY2875*) contained more than six SNPs, which deviates from the Poisson model. *STY2875* is an exceptionally large gene (3,625 bp compared to the genome mean of 910 bp), which may account for the high number of SNPs. However, *yehU* and *trtE* are small (562–579 bp) and thus the high number of SNPs may be evidence of diversifying selection in these genes, the second of which is encoded within SPI7 and is involved with Vi synthesis⁴. Ten SNPs did not fit the phylogenetic tree, which may indicate either recombination or convergent evolution, whereby the same mutation arose independently in different lineages. If the latter explanation is true, it would suggest the possibility of adaptive selection at these sites, which include nonsynonymous SNPs in three genes encoding membrane proteins (*STY1204*, *gadG* and *tsuC*) and two nonsynonymous SNPs in *gyrA* that are known to increase resistance to fluoroquinolones, a class of antibiotics used to treat typhoid fever^{15,16}. Fifteen genes contained clusters of nonsynonymous SNPs, whereby two residues within five amino acids were mutated, which may indicate adaptive selection in localized regions of the encoded protein (Supplementary Table 3a).

Of the 26 genes in which we detected potential signals of adaptive selection, half encode proteins that are surface-exposed, exported or secreted, or affect synthesis of such proteins (Supplementary Table 3a). These weak signals may reflect selective pressures stemming from interactions with the human host¹⁴, including selection for more virulent mutants or those with antigenic variants that better evade the human immune system. The genes identified here as potentially under selection warrant further investigation, illustrating the value of this approach, which could potentially be adapted to genetic association studies in pathogenic bacteria similar to those carried out routinely in eukaryotes¹⁵. However, most genes whose products are secreted or are surface-exposed showed no evidence of adaptive evolution. For example, with the exception of *sfaA*, which encodes a SPI1 effector protein (Supplementary Table 3a), no other genes encoding known secreted effector proteins showed evidence of immune selection.

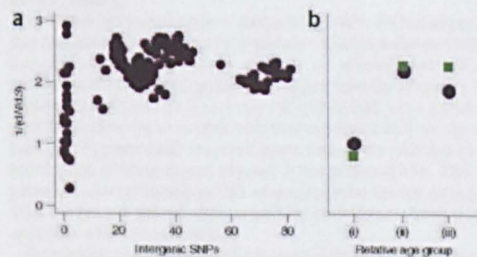


Figure 2 Trajectory of dN/dS over time. y axis is the reciprocal of dN/dS, or $1/(dN/dS)$. (a) Pairwise dN/dS between 19 Typhi isolates. (b) dN/dS for SNPs in three relative age groups (i–iii, youngest–oldest), calculated from SNPs with complete allele data in 19 isolates (purple circles) and SNPs with incomplete allele data (green squares).

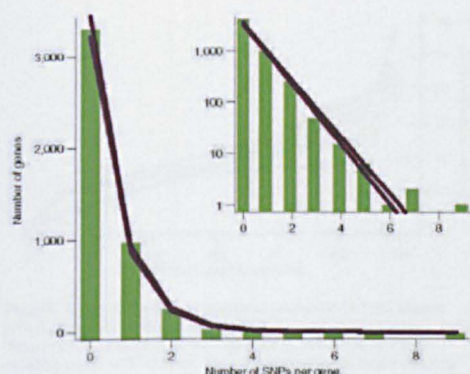


Figure 3 Distribution of number of SNPs per gene. Lines indicate 95% confidence interval of mean predicted values under a Poisson distribution fitted to the data shown in green. Inset shows gene count on a log scale to better show deviation from the Poisson model at high numbers of SNPs per gene.

Recombination

Other than the ten SNPs that do not fit the phylogenetic tree, which are potentially a result of convergent evolution, we found no evidence of recombination between Typhi isolates and very little evidence of recombination with other bacteria (see Methods, **Supplementary Note** and **Supplementary Table 1c** online). Imports from *S. enterica* serovar Typhimurium (Typhimurium) have been reported in two Typhi isolates including 404ty², but reanalysis of the history of the isolates affected revealed that these were introduced in the laboratory during the production of *araA*-knockout mutants and do not represent wild-type variation. Large-scale recombination has been proposed between Typhi and the human-restricted *S. enterica* serovar Paratyphi A¹⁶. However, this most likely occurred before the evolution of the common ancestor of extant Typhi, which seems to be genetically isolated.

IS elements, phage, pathogenicity islands and plasmids

As 454 reads were long enough to be assembled, DNA insertion events could be identified among 454-sequenced Typhi isolates and confirmed by PCR and capillary sequencing (see Methods). Known IS1 insertions in the CT18 genome were not present at the same sites in any other isolates, although we detected an IS1 element at a different genomic site within H58 isolates (see **Supplementary Note**).

CT18 harbors seven well-defined prophage-like elements^{1,17}, and while all sequenced isolates showed conservation in most of these, some isolates harbored new phages. **Figure 1** shows the occurrence of phage insertion events in the phylogenetic tree, and the number of insertion events occurring in each relative age group is shown in **Table 2**. The new phages are discussed further in the **Supplementary Note**.

We also observed variation in the 6-kbp genomic island SPI15 (ref. 18). This region includes an integrase gene adjacent to four hypothetical genes and was inserted within tRNA-Gly, generating direct flanking repeats. The region seemed to exist in three forms among the sequenced Typhi: (i) CT18; (ii) J185SM, 404ty and E03-4983; and (iii) all other isolates. In each case, the insertion site and

direct repeats were identical, but three distinct but related alleles were present for the integrase gene (95% amino acid identity between forms i and ii, 70% between all three forms). Each of the three forms contained a unique set of cargo genes. The function of these genes is unknown, with no matches to known protein domains in the Pfam database (accessed July 3, 2008). These genes merit further investigation because of their potential contribution to virulence.

Plasmids detected in seven of the sequenced Typhi isolates (see Methods) fell into three classes (**Table 1** and **Fig. 1**), which are discussed in more detail in the **Supplementary Note**.

Genomic deletions

Genomic insertions were rare in the sequenced isolates, but deletions were twice as common and more conserved (**Table 2**). Note that in many comparative studies, insertions and deletions are indistinguishable, but we were able to separate these events by using the rooted phylogenetic tree. The deletions range in size from 60 bp to 6.5 kbp, and some correspond to variant regions previously identified using DNA microarrays¹⁹ (**Supplementary Table 3b**). Most of the deleted regions include protein-coding sequences, resulting in partial or total deletion of 42 Typhi genes.

In addition, SPI7, which harbors genes required for synthesis of the polysaccharide Vi capsule⁴, was missing from 404ty and 150(98)S. The isolate E98-3139 seemed to be a mixed population in regards to SPI7, as its coverage in both 454 and Solexa reads was ~25% that of genomic coverage (**Supplementary Fig. 3** online). Note that the low mapping coverage in this region is most likely due to deletion of SPI7 rather than replacement with a similar island, as deletion is known to occur during culture^{20,21} and no alternative island could be assembled from 454 reads. No other SPIs were deleted from the sequenced Typhi, indicating that they are relatively stable in the genome (although we observed three variants of the 6-kbp SPI15, as described above).

Ongoing functional gene loss

In addition to identifying 42 genes affected by deletion events, we detected 55 nonsense SNPs that had occurred since the last common ancestor of Typhi. These introduce stop codons into protein-coding genes, thereby cutting short translation. Read-through of stop codons has been reported²²; however, the described mechanism applies to only two of the nonsense SNPs we detected. There was evidence of selection against nonsense SNPs, with a lower rate of occurrence than nonsynonymous SNPs. Nevertheless, many nonsense SNPs were fixed, making up 2.9% of SNPs in the intermediate and oldest age groups (**Table 2**).

CT18 and Ty2 each contain ~200 pseudogenes^{3,8}, defined as genes that are putatively inactivated by mutations including nonsense SNPs, frameshifts and truncation by deletion or rearrangement. This constitutes 4.5% of Typhi genes, much higher than the frequency in Typhimurium (0.9%) or *Escherichia coli* K12 (0.7%). High pseudogene frequencies are associated with host restriction in a variety of bacteria^{23–26}, presumably as certain genes required for infection in a broad range of hosts are not required in the preferred host. This is potentially also attributable to high rates of mutation fixation resulting from accelerated genetic drift caused by evolutionary bottlenecks associated with host adaptation.

By mapping the deletions and nonsense SNPs to the phylogenetic tree, we found that 92 new pseudogenes have accumulated among the sequenced Typhi isolates since their last common ancestor (**Supplementary Table 3c**), which itself harbored ~180 pseudogenes^{3,8}. Many of these genes fall into gene categories (metabolism, cobalamin utilization, peptide or sugar transport, fimbriae) previously associated

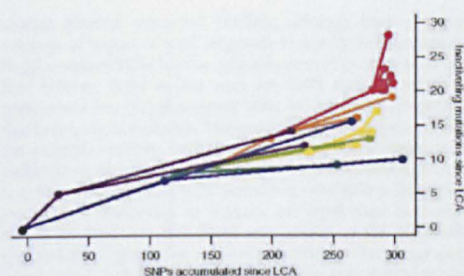


Figure 4 Accumulation of gene-inactivating mutations in Typhi lineages. Points correspond to bifurcations in the phylogenetic tree in **Figure 1**; y axis shows the total number of genes inactivated by deletion or nonsense mutation up to that bifurcation. Each line represents the accumulation of mutations in a particular isolate; different lineages of Typhi are colored as in **Figure 1**. LCA, last common ancestor.

with pseudogenes in host-restricted pathogens²³ (**Supplementary Table 3c**). **Figure 4** shows the rate of accumulation of inactivating mutations in each branch of the phylogenetic tree. Nearly all of these genes showed evidence of expression in Typhi according to microarray data accessible at the NCBI GEO database (**Supplementary Methods and Supplementary Table 3c**), thus most of the nonsense and deletion mutations we observed probably result in true inactivation of previously functional genes. Because the losses have occurred independently in different lineages, Typhi isolates at different points in the phylogenetic tree have varying complements of functional genes and may have different pathogenic potential. This may contribute to the differences observed in clinical manifestations of typhoid fever in different regions¹. Of note, different lineages show variation in the relative rates of accumulation of SNPs and inactivating mutations (line slopes in **Figure 4**). This may be attributable to variation in mutation rates or different selective pressures for or against pseudogene formation in particular lineages.

As only 3% of possible SNPs in the Typhi genome are nonsense SNPs, we expect only 1–2 false nonsense SNP calls overall (3% of the estimated total of 53 false SNP calls). This constitutes ~2% of genes inactivated by nonsense or deletion mutation and thus would make little difference to conclusions regarding the continuous accumulation of pseudogenes. In addition, we did not attempt to analyze frameshift mutations, as single-base insertions or deletions are currently difficult to detect reliably from 454 and Solexa sequence data. However, most of the genes identified as differentially inactivated between CT18 and Ty2 were due to frameshift mutations (20 frameshifts versus 4 nonsense SNPs and 2 deletions)³⁸, thus we hypothesize that many more pseudogenes may have accumulated in the Typhi population than those caused by nonsense SNPs or deletions. Therefore, although our analysis demonstrates that gene loss is ongoing in Typhi, we most likely underestimate the extent of this phenomenon.

DISCUSSION

Few whole-genome intraspecies comparisons of this scale exist for pathogenic bacteria^{27,28} and none at this level of subspecies resolution. In addition, the choice of isolates for sequencing is usually driven by clinical phenotype or simply availability, rather than unbiased sampling from reliable phylogenies. However, isolate selection is critically important for comparative analysis, which can only uncover

mutations that differ between the sampled isolates. Sampling from one part of the phylogenetic tree will overlook much of the variation present in the population and collapse all isolates outside the sequenced subpopulation into a single type¹⁰. For example, when SNPs detected between CT18 and Ty2 were typed in a larger Typhi collection²⁹, most isolates were assigned to the same genotype even though they were probably far more variable than the scheme suggests. By sequencing isolates from major nodes in the previously defined phylogenetic tree (**Supplementary Fig. 1**), we expect to have captured much of the variation present in the Typhi population. We also anticipate that the SNPs we have detected among these sequences will serve as genotypic markers providing phylogenetic information at high resolution in future genotyping studies.

Our whole-genome analysis supports the proposals of small population size and genetic drift in Typhi. Although we detected signals of selection in *gyrA*, we did not detect signals of the same magnitude in other Typhi genes, suggesting that this level of selection is exceptional in Typhi. Furthermore, our whole-genome comparisons provide the opportunity to gain broad insight into the spectrum of genetic variation in Typhi, including SNPs, insertions, deletions and recombinations as well as plasmid and phage content (although we did not analyze insertion or deletion of single nucleotides). The patterns of genome-wide variation we detected demonstrate that pseudogene formation is ongoing in Typhi (**Fig. 4**) and support the hypothesis that evolution in this host-restricted pathogen is dominated by genetic drift and loss of gene function rather than by diversifying selection or gain of function through point mutation, recombination or acquisition of new sequences. Although gain of function seems to be rare, it may be occurring in a few genes through point mutations.

The lack of evidence for adaptive selection in general is in contrast with the known adaptive selection for mutations in *gyrA* associated with fluoroquinolone resistance. We detected the signal of selection in *gyrA* as clustered, homoplasic nonsynonymous SNPs in neighboring codons 83 and 87. Three other genes contained homoplasic nonsynonymous SNPs, one of which (*yadG*) encodes the membrane component of an efflux protein in *E. coli*³⁰ and may therefore be associated with antibiotic resistance in Typhi (efflux proteins can act as pumps to remove antibiotics from the bacterial cell³¹). However, no genes besides *gyrA* contained multiple homoplasic SNPs, and few contained multiple nonsynonymous SNPs at all, consistent with the hypothesis of genetic drift in the Typhi genome. The adaptive mutations evident in the *gyrA* gene highlight the strong selective pressure on the Typhi genome associated with antibiotic use in the human population. This is not particularly surprising, as the fitness advantage associated with increased antibiotic resistance is likely to be very strong. However, the lack of similar evidence for other adaptive mutations suggests that Typhi is under relatively little selective pressure from its host or the environment in general.

The limited evidence of selection in Typhi gene sequences is particularly notable when compared to patterns observed among other human bacterial pathogens, which show a variety of mechanisms for antigenic variation. For example, antigenic variation is achieved by extensive recombination in the *Helicobacter pylori* and *Chlamydia trachomatis* populations^{32,33}, whereas in *Mycobacterium tuberculosis*, antigenic variation is associated with duplication and diversification of antigen-associated gene families³⁴. In contrast, only 3 Typhi genes contained more than 6 SNPs, and 16 genes contained independent nonsynonymous SNPs in the same or neighboring amino acids (see **Supplementary Table 3a**). Although these may represent cases of antigenic variation, the level of variation is low, with most of the SNPs unique to a single haplotype and therefore most haplotypes

sharing identical sequences. Similarly, although there was some evidence of import of small fragments of non-Typhi sequences (see **Supplementary Table 1c**), the only indication of possible recombination between Typhi isolates were ten SNPs that do not fit the phylogenetic tree (**Supplementary Table 3a**), which could equally be due to convergent evolution. The sparsity of direct sequence evidence for antigenic variation in Typhi suggests that this pathogen is not under strong selective pressures from the human immune system and may interact with its host in a different way, possibly favoring immune evasion and localization to immune privileged sites. However, it cannot be ruled out that Typhi may possess as yet unidentified mechanisms of generating antigenic diversity or that phage genes, which were excluded from SNP analysis in this study, may have a role.

It has long been suspected that human carriers provide the main reservoir driving the transmission of Typhi^{35,36}. The bacterium is relatively difficult to isolate from water and the environment even in endemic regions^{37,38}, and it is generally believed that Typhi has a limited survival time outside the human host³⁹. If human carriers provide the main persistent reservoir for Typhi, this could account for the patterns of genetic drift and lack of recombination or gene acquisition we observed, as the human reservoir is likely to be small and physiologically isolated^{35,36}. Furthermore, adaptive mutations arising during symptomatic typhoid infections may have no fitness advantage in the carrier state and may therefore not persist in the long-term Typhi population. All nodes of the Typhi phylogenetic tree shown in **Supplementary Figure 1** were detected among a set of approximately 450 extant Typhi isolates⁵, suggesting that the Typhi population is not shaped by clonal replacement. These patterns are well described by the source-sink model of evolutionary dynamics, which distinguishes permanent 'source' and transient 'sink' populations and predicts that adaptive mutations arising in the sink (individuals with typhoid) may be short lived in the population if they provide no fitness benefit in the long-term source (carriers of Typhi)⁴⁰. Similar dynamics may be occurring in other human-restricted bacterial populations.

An understanding of the evolutionary dynamics of the Typhi population has important implications for the control of typhoid. The SNP typing of individual Typhi isolates into distinct genotypes may lead to improved methods for tracking the spread of Typhi between human hosts¹¹. Vaccination may be a crucial long-term strategy for disease control, as it could not only reduce the level of typhoid infections but also the level of asymptomatic Typhi carriage in the population, a key reservoir of typhoid infections.

METHODS

Bacterial strains. Details of Typhi isolates used in this study are provided in **Table 1**. Isolates were provided by the Oxford University Clinical Research Unit (CT18, J185SM, AG3); B. Holmes at the National Collection of Type Cultures (M223); the Wellcome Trust Sanger Institute (404ty, Ty2); and E-X.W. (all other isolates).

DNA sequencing. We pelleted bacterial cells by centrifugation and prepared DNA using the Wizard Genomic DNA Kit (Promega) according to the manufacturer's instructions. We sequenced eight Typhi isolates using a 454 Life Sciences GS-20 sequencer (Roche), and an additional two isolates (M223, E02-1180) using the 454 Life Sciences GS-FLX sequencer (Roche). Twelve isolates were sequenced using the Illumina/Solexa Genome Analyzer System according to the manufacturer's specifications. In all cases, we generated single-end reads. Two isolates, E02-2759 and ISP-04-06979, were each sequenced over seven Solexa lanes during protocol optimization and thus have much higher coverage than other isolates, which were sequenced in one Solexa lane each.

We used Sanger sequencing of PCR products to confirm insertion and deletion sites. Primers used for PCR and sequencing are provided in

Supplementary Table 3d. PCR was done in a 25 μ l volume using PCR Supermix Taq Polymerase (Invitrogen) and cycled on an MJ Research thermal cycler. Products were checked on a 0.8% agarose gel and purified using QIAquick PCR Purification Kit (QIAGEN).

Plasmid identification. In order to verify the presence and size of plasmids within Typhi isolates, we prepared plasmid DNA from Typhi isolates, as described in **Supplementary Methods**. All plasmids detected in this way were represented in the sequence data for their host isolates and were identified by mapping to known plasmid sequences (using *blastn* for 454 contigs and *Maq* v0.6.0 for Solexa reads).

SNP detection from sequence data. We assembled 454 reads *de novo* into contigs (that is, without reference to any other sequence) using *newbler* (v1.1, Roche). We used *MUMmer* (v3.19, nucmer algorithm)⁴¹ to align contigs to the finished CT18 sequence and to generate primary SNP calls. Solexa reads were too short to be assembled effectively using current software and thus were mapped directly to the CT18 reference sequence using *Maq* v0.6.0, which was also used to generate primary SNP calls. We filtered SNP calls according to quality criteria determined by comparison of data from multiple sequencing platforms, as described in **Supplementary Methods**. We combined filtered SNP calls into a single list of SNP loci and determined the allele at each locus in each of the 19 Typhi sequences and additional *S. enterica* serovars (using *fast3* search for 454 contigs or finished sequences, and *Maq* consensus base calls for Solexa data). This allowed recovery of some SNPs that were initially rejected in one isolate because of low confidence but detected with high confidence in a second isolate. Nonsense SNPs were verified by manually inspecting multiple alignments of all 454 and Solexa reads mapping to each nonsense SNP locus.

Estimation of sensitivity and specificity of SNP calls. We estimated SNP detection accuracy and sensitivity for 454 and Solexa by comparing results from three isolates sequenced using both platforms, as described in **Supplementary Methods**. Additional estimates for Solexa data were determined by comparing Solexa data from CT18 and Ty2 to the published sequences (see **Supplementary Methods**).

Phylogenetic analysis. SNPs lying within recombined regions (see below) or within repeat regions were excluded from analysis, leaving 1,964 SNP calls. We checked alleles against an independent whole-genome multiple alignment of all 454 and published Typhi sequences generated using *Kodon* (Applied Maths). Alleles could be confirmed in all nineteen Typhi isolates for 1,787 (90%) SNPs. These support a single maximum parsimony tree, determined using the *mix* algorithm in the *PHYLIP* package (**Fig. 1**), consistent with the reference phylogenetic tree (**Supplementary Fig. 1**).

dN/dS calculations. We calculated dN/dS according to the formula $(N/n)/(S/s)$, where N = sum of nonsynonymous SNPs, n = nonsynonymous sites in nonrepetitive protein-coding sequences, S = sum of synonymous SNPs, s = synonymous sites in nonrepetitive protein-coding sequences. The mean dN/dS since the last common ancestor was calculated by weighting dN/dS by 1/2 for H59 isolates, 1/7 for H58 isolates and 1 for all other isolates, so that each haplotype is represented equally. The error reported (0.053) is 1 s.d. of this weighted mean.

Detection of recombination events. We checked SNP calls from each Typhi isolate for SNP clusters (defined as >3 SNPs within 1,000 bp) and searched these regions against the EMBL database using *blastn* in order to identify potential recombination events (**Supplementary Note**).

URLs. *Maq*, <http://maq.sourceforge.net> *PHYLIP*, <http://evolution.genetics.washington.edu/phylip.html>; mapped assemblies of all 454 and Solexa datasets, http://www.sanger.ac.uk/Projects/S_typhi; 454 and Solexa reads data, <ftp://ftp.era.ebi.ac.uk/ERA000001>; Enteritidis strain PT4 sequence, <http://www.sanger.ac.uk/Projects/Salmonella>.

Accession codes. EBI Whole Genome Shotgun database raw sequence data (454 *de novo* assembled contigs) are available with accession codes CAAQ-CAAZ. European Short Read Archive: Solexa and 454 reads, ERA000001. GenBank: Typhi strain CT18, AL513382; Typhi strain Ty2,

AEO14613; Typhimurium strain LT2, AE006468; Paratyphi A strain, CP000026; Choleraesuis strain SC-B67, AEO17220; *E. coli* K12, NC_000913; Shigella flexneri 5 strain 8401, CP000266; pHCM1, AL513383; pHCM2, AL513384; pBSSB1, AM419040; pAKU1, AM412236.

Note: Supplementary information is available on the Nature Genetics website.

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AUTHOR CONTRIBUTIONS

G.D., J.P., M.A., P.R. and J.W. designed the study; E.-X.W. and G.D. contributed isolates for analysis; J.G. and R.R. performed 454 and Solexa sequencing; K.E.H. and S.J. performed validation experiments; D.J.M. co-supervised the PhD studies of K.E.H. and contributed to experimental design; K.E.H. and C.J.M. analysed data and K.E.H., J.P., P.R. and G.D. wrote the manuscript.

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A Multi-Center Randomised Controlled Trial of Gatifloxacin versus Azithromycin for the Treatment of Uncomplicated Typhoid Fever in Children and Adults in Vietnam

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Abstract

Background: Drug resistant typhoid fever is a major clinical problem globally. Many of the first line antibiotics, including the older generation fluoroquinolones, ciprofloxacin and ofloxacin, are failing.

Objectives: We performed a randomised controlled trial to compare the efficacy and safety of gatifloxacin (10 mg/kg/day) versus azithromycin (20 mg/kg/day) as a once daily oral dose for 7 days for the treatment of uncomplicated typhoid fever in children and adults in Vietnam.

Methods: An open-label multi-centre randomised trial with pre-specified per protocol analysis and intention to treat analysis was conducted. The primary outcome was fever clearance time, the secondary outcome was overall treatment failure (clinical or microbiological failure, development of typhoid fever-related complications, relapse or faecal carriage of *S. typhi*).

Principal Findings: We enrolled 358 children and adults with suspected typhoid fever. There was no death in the study. 287 patients had blood culture confirmed typhoid fever, 145 patients received gatifloxacin and 142 patients received azithromycin. The median FCT was 106 hours in both treatment arms (95% Confidence Interval [CI]: 94–118 hours for gatifloxacin versus 88–112 hours for azithromycin), (logrank test $p = 0.984$, HR [95% CI] = 1.0 [0.80–1.26]). Overall treatment failure occurred in 13/145 (9%) patients in the gatifloxacin group and 13/140 (9.3%) patients in the azithromycin group, (logrank test $p = 0.854$, HR [95% CI] = 0.93 [0.43–2.0]). 96% (254/263) of the *Salmonella enterica* serovar Typhi isolates were resistant to nalidixic acid and 58% (153/263) were multidrug resistant.

Conclusions: Both antibiotics showed an excellent efficacy and safety profile. Both gatifloxacin and azithromycin can be recommended for the treatment of typhoid fever particularly in regions with high rates of multidrug and nalidixic acid resistance. The cost of a 7-day treatment course of gatifloxacin is approximately one third of the cost of azithromycin in Vietnam.

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Introduction

There are approximately 21 million cases of typhoid fever annually, with more than 210 000 deaths [1]. The emergence of

antimicrobial drug resistance in *Salmonella enterica* serovar Typhi (*S. typhi*) is a major problem particularly in South East Asia and the Indian sub-continent and challenges our current treatment options [2–4]. There is a need for an efficacious, safe and affordable oral

treatment, particularly in regions with a high proportion of both multidrug and nalidixic acid resistant *S. typhi*.

In Vietnam, multidrug resistant (MDR) isolates of *S. typhi* (resistant to ampicillin, chloramphenicol and trimethoprim-sulfamethoxazol) first appeared in 1993 [5]. From this time the fluoroquinolones became the treatment of choice for typhoid fever [4], and were simultaneously sold widely over the counter to treat fever of various aetiologies. The extensive antibiotic pressure lead to the selection of single point mutations in the DNA Gyrase A of *S. typhi*, causing resistance to nalidixic acid (the prototype quinolone) and reduced susceptibility to the fluoroquinolones (but formally these isolates are still within the Clinical Laboratory Standard Institute (CLSI) breakpoints for susceptibility) [6]. This resulted in a poor clinical response to treatment with the older generation fluoroquinolones, ofloxacin or ciprofloxacin [7,8].

The World Health Organisation recommends the fluoroquinolones or cefixime for the treatment of MDR typhoid fever and azithromycin, the third-generation cephalosporins, or a 10–14 day course of high-dose older generation fluoroquinolones (e.g. ofloxacin or ciprofloxacin) for the treatment of nalidixic acid resistant typhoid fever [9].

Azithromycin, an azalid antibiotics, has achieved excellent clinical results in the treatment of MDR and nalidixic acid resistant typhoid fever [7,8]. However azithromycin is expensive. Cefixime has recently failed in the treatment of nalidixic acid resistant typhoid fever in Nepal (these data were not available at the start of this trial) [10].

A recent trial from southern Vietnam used ofloxacin at the maximum recommended dose of 20 mg/kg/day for 7 days for the treatment of MDR and nalidixic acid resistant typhoid fever and showed high clinical failure rates (36%), high immediate post-treatment faecal carriage (19%), which may lead to transmission in the community after discharge from hospital, and prolonged mean fever clearance times of 8.2 days (95% CI, 7.2–9.2 days) [8].

These results underline that the older generation fluoroquinolones are clearly failing in the treatment of nalidixic acid resistant typhoid fever.

Of the newer fluoroquinolones, gatifloxacin is available and affordable in South and South East Asia including Vietnam [10]. Of all the fluoroquinolones, gatifloxacin showed the lowest minimum inhibitory concentrations (MICs) for nalidixic acid resistant *S. typhi* from Nepal [11] and Vietnam and a rapid bactericidal effect in time-kill experiments involving *S. typhi* isolates with single and double mutations in the *GyrA* of *S. typhi* [6].

We conducted a randomised controlled trial comparing the efficacy of gatifloxacin to azithromycin in southern Vietnam, an area characterised by a very high proportion of MDR (88%) and nalidixic acid resistant (93%) *S. typhi* isolates [8].

Methods

The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1.

Study design and objectives

The study was designed as a multicenter, open-label randomised controlled trial to compare the efficacy and safety of gatifloxacin versus azithromycin for the treatment of uncomplicated typhoid fever in children and adult in-patients in southern Vietnam.

The overall objective of the study was to identify an efficacious, safe, available and affordable oral treatment for MDR and nalidixic acid resistant typhoid fever.

Participants

Patients were eligible to be included in the study if they had clinically suspected or culture confirmed uncomplicated typhoid fever and if fully informed written consent had been obtained. For children, consent was obtained from the parent. Exclusion criteria were pregnancy, age under 6 months, history of hypersensitivity to either of the trial drugs, any signs of severe typhoid fever (shock, deep jaundice, encephalopathy, convulsions, bleeding, suspicion or evidence of gut perforation), or previous reported treatment with a fluoroquinolone antibiotics, a third generation cephalosporine or macrolide antibiotics within one week prior to hospital admission.

The study sites and ethical approval

The study was conducted at three hospitals in the south of Vietnam.

Adult and paediatric patients were recruited at the Hospital for Tropical Diseases in Ho Chi Minh City, at the Dong Thap Provincial hospital in Cao Lanh, Dong Thap province and at the An Giang Provincial hospital in Long Xuyen, An Giang province.

The study was approved by the Ethical and Scientific Committee of the Hospital for Tropical Diseases in Ho Chi Minh City and the Oxford University Tropical Research Ethics Committee (OXTREC), UK for all three study sites. The clinical and microbiological data from the first 40 patients recruited to each arm of the study were sent to the independent Data Safety and Monitoring Committee for their advice regarding the continuation of the study. The study was not stopped.

Intervention

According to their randomisation number patients were assigned to oral treatment with either 20 mg/kg azithromycin (Zithromax® suspension, Pfizer, USA; 200 mg/5 mL or Zithromax® tablets, Pfizer, USA; 500 mg/tablet) or 10 mg/kg gatifloxacin (Tequin®, Bristol-Myers Squibb, USA; 400 mg/tablet) once daily for 7 days. Tablets were cut to obtain the appropriate study dosage and administered with water. Inevitably, the dose administered was an estimate of 10 mg/kg/day of gatifloxacin or 20 mg/kg/day of azithromycin (number of tablets or proportions of tablets were documented in the CRFs). Gatifloxacin was only available as tablets, which were cut to obtain the appropriate dosage and crushed if necessary for children.

The maximum dose of azithromycin was 1 g per day. All drugs were purchased commercially.

Procedures

In-patient procedures. On admission to the hospital the patient's full history was taken, a standard clinical examination was performed and axillary temperature, weight and height were measured. Before treatment, full blood counts including white blood differential counts, serum aspartate transaminase (AST), serum alanine transaminase (ALT) and bilirubin were checked and blood cultures were obtained. For adult patients, creatinine, blood urea nitrogen (BUN) and glucose levels were additionally measured. In some patients bone marrow cultures were obtained. Urines were checked with dipstick and pre-treatment stool cultures were obtained. Chest X-ray and abdominal ultrasound were performed and repeated as clinically indicated. Randomisation and initiation of therapy took place either immediately on admission to hospital or patients were observed until results of blood tests including blood cultures were available and then randomised. Vital signs including measurement of axillary temperatures were measured and recorded every 6 hours (at 6, 12, 18 and 24 hours) until discharge. Patients were examined

daily until discharge from hospital, with particular reference to clinical symptoms, FCT, side effects of the drug and any complication of the disease. Additionally laboratory tests were scheduled if clinically indicated. All adverse events were recorded. On day 7 to 9 after the start of treatment full blood counts, liver function tests, blood and stool cultures were checked. In case of insufficient response to therapy, development of complications or drug-associated adverse events, the initial treatment was suspended and parenteral ceftriaxone (2 g per day) in two divided doses was used as rescue treatment for 10 days.

Follow-up procedures. Out-patient follow-up appointments were scheduled at 1 month, 3 months and 6 months after discharge from hospital to seek evidence for relapse (1 month visit) and check for chronic typhoid carriage (all visits). At these appointments a full history was taken, relevant examinations performed and stool cultures obtained. Blood or bone marrow cultures were only obtained if clinical symptoms were indicative of acute infection. If patients did not attend their follow up appointment, they were reminded by letter or a member of the study team visited their home. If stool samples were not available, a rectal swab was obtained.

Patients with convalescent stool carriage of *S. typhi* or *S. paratyphi* A were retreated according to the sensitivity of the isolate and were further followed up. Ultrasound was performed to exclude biliary or kidney stones if carriage was persistent.

Microbiology

Five to 8 mL of blood was collected from adults and inoculated into Bactec Plus Aerobic Blood bottles, and 3 to 5 mL of blood from children was inoculated into Bactec Peds Plus culture bottles (Becton Dickinson, New Jersey, USA). The bottles were incubated at 37°C in the BAGTEC 9050 automated analyser for 7 days and sub-cultured according to standard methods when the machine indicated a positive signal, or incubated at 37°C in a standard laboratory incubator (An Giang hospital) and examined daily.

Stool samples or rectal swabs were inoculated onto MacConkey agar and Xylose Lysine Decarboxylase (XLD) agar plates, and in 10 mL of selenite F broth. Plates and broth were incubated at 37°C overnight and the broth was sub-cultured on MacConkey and XLD agar plates the next morning.

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

Antimicrobial susceptibility testing was performed by disc diffusion according to Clinical Laboratory Standards Institute (CLSI) guidelines [12], using CLSI breakpoints [13]. Antimicrobial agents tested were: ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, ofloxacin, ciprofloxacin and ceftriaxone (Oxoid, Basingstoke, UK). Minimum Inhibitory Concentrations (MICs) for amoxicillin, chloramphenicol, nalidixic acid, ofloxacin, ciprofloxacin, gatifloxacin, ceftriaxone and azithromycin were determined by E-test (AB Biodisk, Solna, Sweden). Multidrug resistance (MDR) of isolates was defined as resistance to chloramphenicol (MIC \geq 32 μ g/mL), ampicillin (MIC \geq 32 μ g/mL) and trimethoprim-sulfamethoxazole (MIC \geq 8/152 μ g/mL). Nalidixic acid resistance was defined as an MIC \geq 32 μ g/mL. The CLSI breakpoints for ofloxacin and gatifloxacin were \leq 2 μ g/mL susceptible and \geq 8 μ g/mL resistant, for ciprofloxacin \leq 1 μ g/mL susceptible and \geq 4 μ g/mL resistant and for ceftriaxone \leq 8 μ g/mL susceptible and \geq 64 μ g/mL resistant. There were no CLSI MIC breakpoints for azithromycin [13]. The control strains used for all susceptibility tests were *E. coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, *Staphylococcus aureus* ATCC 29213.

All cultures, identification of *S. typhi* and *S. paratyphi* A and disc diffusion were performed at the three study sites. All isolates were sent to the Hospital for Tropical Diseases, Ho Chi Minh City, for confirmation of identity, susceptibility testing and MIC testing.

Outcomes of the study

The primary endpoint of the study was the resolution of fever (fever clearance time, FCT), which was defined as the time from the start of the antibiotic treatment to when the axillary temperature first fell \leq 37.5°C and remained there for at least 48 hours. Secondary endpoints were the overall failure to treatment, which was defined a priori as any of the following: clinical failure (persistence of fever and symptoms two days after the end of treatment, i.e. on day 10) or need for re-treatment due to insufficient treatment response as judged by the treating physician; microbiological failure (positive blood culture on day 7 to 9 after the start of treatment); the development of typhoid fever-related complications during hospital-stay; the occurrence of relapse (symptoms and signs suggestive of typhoid fever) within 1 month after completion of treatment or the detection of faecal carriage of *S. typhi* at the follow-up visits at 1, 3 and 6 months (to exclude faecal carriage a minimum of two consecutive follow-up visits had to be attended).

Sample Size

The primary outcome measure for the study was the fever clearance time (FCT).

Previous studies that used azithromycin to treat typhoid fever patients, reported a mean fever clearance time of 130 hours [7] and 139 hours [8]. For gatifloxacin, clinical observations from a small number of typhoid fever patients were available and indicated a mean FCT of 76 hours. We calculated that 139 patients with culture-confirmed typhoid fever would be needed in each treatment arm to detect a Hazard Ratio of 1.40 with two-sided alpha of 0.05 and power of 0.80 [14]. Therefore, assuming a median fever clearance time of 130 hours for azithromycin, the sample size of 140 patients with culture-confirmed typhoid fever in each arm would give power of at least 0.80 to detect a difference between treatments if the fever clearance time in the gatifloxacin group was 92 hours or less.

Randomization procedures and assignment of intervention (sequence generation, allocation concealment, implementation)

An administrator independent from the study generated the random number sequence in Excel using RAND function. These randomised codes were blocked in a size of 50. Treatment assignments were folded and kept in opaque, sealed, sequentially numbered envelopes at all three study sites. Due to logistic reasons, randomisation was not stratified by centre.

After all inclusion and exclusion criteria were checked, and informed consent given, the study doctor opened the envelope to determine which treatment the subject would receive. The sealed envelopes were opened in strict numeric sequence.

Blinding

This study was conducted as an open study.

Statistical methods

Binary outcomes (clinical failure, microbiological failure, typhoid fever-related complications) were compared between the two treatment groups using Fisher's exact test, assuming the worst case scenario (all lost to follow up treated as failures). The un-

adjusted Odds Ratio (OR) and Cornfield's 95% confidence interval [15] were calculated to show the relative risk of developing individual secondary outcomes (clinical, microbiological failure, typhoid fever-related complications) in the gatifloxacin group compared to the azithromycin group.

Fever clearance time, time to relapse and time to overall failure were analysed using survival methods. The time to overall failure equaled the earliest time individual failure was recorded. Kaplan-Meier estimates of probabilities of each event were calculated at any time-point, and they were compared between the two treatment groups using the log-rank test. Data of patients who were lost to follow-up were censored at the time of the last recorded outcome. The Hazard Ratio was derived from Cox proportional hazard model [16].

All patients with positive blood or bone marrow culture for *S. typhi* and *S. paratyphi* A (per protocol analysis) and separately all randomised patients (intention to treat analysis) were analysed.

All data were recorded prospectively into individual Case Record Forms (CRF) and entered into an electronic database (Epi Info 2003, CDC, Atlanta, USA) and double-checked.

Analysis was performed using STATA version 8.0 (Stata Corporation, Texas, USA) statistical software program.

Results

Participant flow and recruitment

During the study period, 460 patients were assessed for eligibility (Figure 1). One hundred and two patients were non-eligible, the main reason was the reported previous use of fluoroquinolone, macrolid or third generation cephalosporin antibiotics (41 patients) in the week before hospitalisation.

Between April 2004 and August 2005, 358 patients with suspected typhoid fever were randomised to receive either gatifloxacin or azithromycin. Two hundred eighty-eight of these patients had blood or bone marrow confirmed typhoid fever and 70 patients were culture negative for *S. typhi*. One culture positive patient was excluded from the per protocol analysis (PP), because he had received ciprofloxacin before entry to the trial. The PP group consisted of 287 patients, 145 in the gatifloxacin group and 142 in the azithromycin group. All PP patients, except two in the azithromycin group, finished the full course of treatment.

The total number patients visiting the follow-up at 1 month was 275 out of 287 (96%), at 3 months 268 out of 287 (93%), at 6 months 128 out of 287 (44%) patients.

Numbers analysed

All 358 randomised patients were analysed in the intention to treat (ITT) analysis. Two hundred and eighty-seven patients with culture confirmed typhoid fever, 145 treated with gatifloxacin and 142 with azithromycin, were analysed in the pre-specified PP analysis.

Baseline Data

The median age of patients recruited in this trial was 11 years (range 1–41) in the PP group.

The baseline characteristics of the patients were similar in the two treatment groups and in the culture negative patients (Table 1).

Patients with suspected and blood culture confirmed typhoid fever were eligible for this trial. In the PP group, the median delay in time between hospital admission and randomisation was 3 days (interquartile range 1–4) in the gatifloxacin group and 3 days (interquartile range 2–4) in the azithromycin group. In the ITT group, the median delay in time between hospital admission and randomisation was 2 days (interquartile range 0–4) in the

gatifloxacin group and 3 days (interquartile range 1–4) in the azithromycin group.

Protocol deviations and modifications

At one study site, the An Giang Provincial Hospital, the follow-up visit at 6 months was not possible for logistic reasons. It was therefore agreed to carry out two follow-up visits at 1 and 3 months and to schedule additional (cross-sectional) follow-up dates to invite as many patients as possible to a third follow-up visit. From the PP population, 22 out of 91 patients in the gatifloxacin arm and 17 out of 87 patients in the azithromycin arm attended the third visit.

Outcomes and estimation

Primary outcomes. There was no significant difference in the resolution of fever (FCT) between the two treatment groups (Table 2).

By PP analysis, the median FCT was 106 hours in both treatment arms (95% Confidence Interval [CI]: 94–118 hours for gatifloxacin versus 88–112 hours for azithromycin), (logrank test $p=0.984$, HR [95% CI] = 1.0 [0.80–1.26]). The Kaplan-Meier survival curve for the fever clearance time is shown in Figure 2. At day 7, fever clearance rate was 82.8% (95% CI: 76.2%–88.4%) in the gatifloxacin group and 80.5% (95% CI: 73.6%–86.6%) in the azithromycin group.

In the ITT population, the median FCT was 100 hours in both treatment arms (95% CI: 92–106 hours for gatifloxacin versus 88–112 hours for azithromycin), (logrank test $p=0.914$, HR [95% CI] = 1.01 [0.82–1.25]). At day 7, fever clearance rate was 84.2% (95% CI: 78.5%–89%) in the gatifloxacin group and 82.6% (95% CI: 76.5%–87.9%) in the azithromycin group (Figure 3).

Secondary outcomes. There was no death in the study.

There was no significant difference in overall failure to treatment between the two groups (Table 2).

By PP analysis, the number of patients that showed overall failure to treatment was 13/145 (9%) in the gatifloxacin group and 13/140 (9.3%) in the azithromycin group (logrank test $p=0.854$, HR [95% CI] = 0.93 [0.43–2.0]), or when assuming the worst case scenario, that all dropped-out patients were failures, 15/142 (10.6%) failures in the azithromycin group (logrank test $p=0.570$, HR [95% CI] = 0.81 [0.38–1.7]). Figure 4 shows the proportion of patients failing through time after the start of treatment.

In the azithromycin arm, more than one failure event occurred in individual patients (Table 2). Clinical failure occurred in 6/145 (4.3%) patients in the gatifloxacin group and in 6/140 (4.2%) in the azithromycin group ($p=1.000$, OR [95% CI] = 0.96 [0.25–3.7]). Three patients in each study arm were re-treated with ceftriaxone, the other patients resolved their symptoms within 24 hours.

Microbiological failure was seen in 2 out of 145 patients in the gatifloxacin arm (1.4%) and in 3 out of 140 (2.2%) in the azithromycin arm ($p=0.680$, OR [95% CI] = 0.64 [0.05–5.7]). Two of the azithromycin recipients showed additionally signs of clinical failure.

There were no typhoid fever-related complications in the 145 gatifloxacin patients compared to 8 out of 140 (5.7%) patients in the azithromycin arm ($p=0.003$, OR [95% CI] = 0 [0–0.4]). Two azithromycin recipients developed signs of liver dysfunction (elevated AST and ALT, deepening of jaundice) in addition to signs of clinical failure. Study treatment was continued and symptoms resolved by the time of discharge. Four patients, three children and one adult, suffered from gastrointestinal bleeding on day 3, day 5 (2 cases) and day 7 of treatment respectively, three patients received blood transfusions. One of these patients

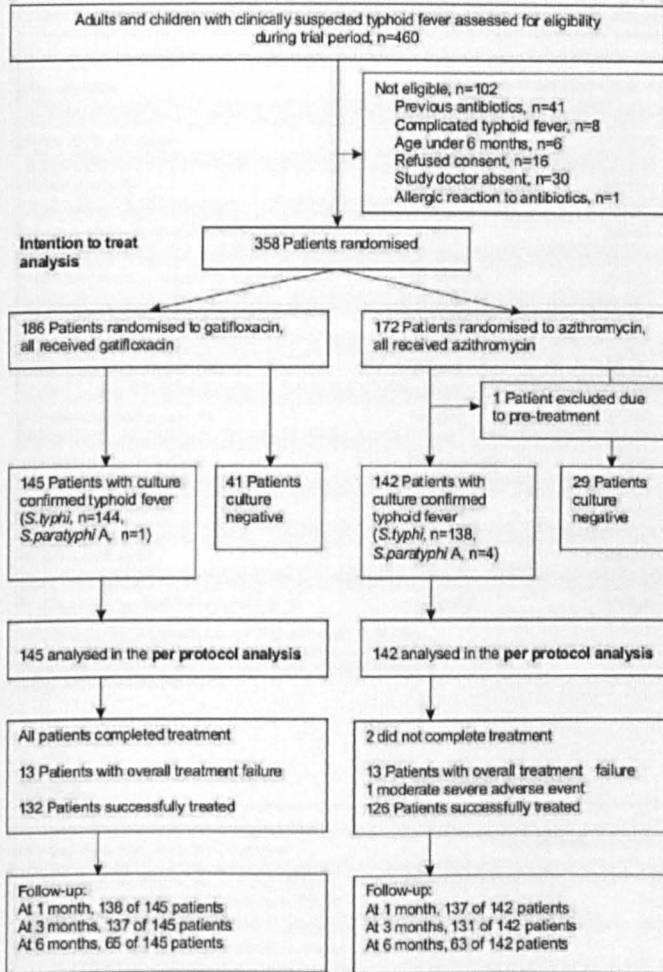


Figure 1. Profile of the Trial.
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developed shock but responded to intravenous fluids and supportive treatment. Treatment was discontinued immediately in all the patients and re-treatment with ceftriaxone was initiated. Two adult patients developed pneumonia during treatment.

Relapse was evaluated only in patients that were initially categorised as successfully treated, patients with clinical failure, microbiological failure or complications were not evaluated. Four patients out of 137 (2.9%) relapsed in the gatifloxacin group compared to 0/127 in the azithromycin group (logrank test $p=0.052$, HR [95% CI] = not estimable due to zero observations

in one group), (Figure 5). These relapses with symptoms suggestive of typhoid fever occurred on day 7, 11, 13 and 15 respectively, after completion of treatment, three patients were confirmed culture positive for *S. typhi*. One patient developed acute respiratory distress syndrome (ARDS) and needed ventilation. The patient was treated with ceftriaxone and perfloraxone and subsequently made a complete recovery.

Chronic faecal carriage was evaluated in patients who attended at least two follow-up appointments, 137 in the gatifloxacin group and 131 in the azithromycin group. Only one patient with chronic

Table 1. Baseline characteristics of culture confirmed patients (PP analysis) and culture negative patients.

Characteristics	Culture confirmed patients treated with		Blood culture negative patients, n = 70
	Gatifloxacin, n = 145	Azithromycin, n = 142	
Median age in years (range)	11 (2–30)	11 (1–41)	9 (2–42)
Number of children defined as age under 15 (%)	109 (75.2)	101 (71.1)	56 (80)
Number of males (%)	71 (49)	76 (53.5)	29 (41)
Median weight in kilograms (range)	25 (8.5–55)	24.5 (9.5–57)	19.5 (10.5–53)
Median duration of fever before admission in days (range)	7 (2–30)	7 (2–30)	7 (3–30)
Number of patients who received pretreatment (%) ^a	21 (14.5)	18 (12.7)	16 (22.9)
Median temperature at admission in °C (range)	39 (37–40.5)	39 (37.3–41)	38.75 (37–40)
Hepatomegaly, number of patients (%)	69 (47.6)	63 (44.4)	36 (51.4)
Splenomegaly, number of patients (%)	17 (11.7)	14 (9.8)	2 (2.9)
Abdominal pain, number of patients (%)	82 (56.5)	76 (53.5)	43 (61.4)
Weight loss, number of patients (%)	69 (47.6)	71 (50)	21 (30)
Vomiting, number of patients (%)	47 (32.4)	54 (38)	19 (27.1)
Diarrhoea, number of patients (%)	95 (65.5)	82 (57.7)	49 (70)
Mild jaundice, number of patients (%)	12 (8.3)	20 (14.1)	1 (1.4)
Median haematocrit in % (range)	34.3 (19.2–54.3)	34.6 (20.7–60.5)	34.2 (24.6–46.7)
Median white cell count, 10 ⁹ /L (range)	6.9 (2–17.2)	7.05 (2.4–16.8)	7.25 (2.8–11.7)
Median platelet count, 10 ⁹ /L (95% CI, range)	172 (34–500)	172.5 (45–578)	208 (51–496)
Median AST, U/L (range)	85 (16.9–773)	72 (17.6–1190)	50.1 (11–533)
Median ALT, U/L (range)	67.4 (10.3–276)	59.4 (10.2–734)	44.1 (10–375)
Numbers of <i>S.typhi</i> / <i>S.paratyphi</i> A isolated from blood cultures	144/1	138/4	0
Positive pretreatment faecal cultures, numbers (%)	11/124 (8.9)	6/118 (5.1)	0

AST, Serum Aspartate Aminotransferase AST (normal range, 12–30 U/L).

ALT, Serum Alanine Aminotransferase ALT (normal range, 13–40 U/L).

^aTreatment with amoxicillin or cotrimoxazole prior to hospital admission.

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Table 2.

Outcome Type	Outcomes Sub-Categories	Treatment group (n = 287)		
		Gatifloxacin n = 145	Azithromycin n = 142	p-Value
Primary	Fever Clearance Time in hours (95% CI)	106 (94–118)	106 (86–112)	0.964 ^a
Secondary	Overall treatment failure, numbers of patients (%)	13/145 (9)	13/140 (9.3) ^b	0.854
	Did not complete full treatment course, n (%)	0	2	
	^c Clinical failure, n (%)	6/145 (4.3)	6/140 (4.2)	1.000 ^d
	^e Microbiological failure, n (%)	2/145 (1.4)	3/140 (2.2)	0.680 ^d
	^f Typhoid fever related complications, n (%)	0/145 (0)	8/140 (5.7)	0.003 ^d
	Gastrointestinal bleeding	0	4	
	Pneumonia	0	2	
	Liver dysfunction	0	2	
	Relapse after discharge from hospital, n (%)	4/137 (2.9)	0/127 (0)	0.052 ^g
	^h Number of patients with faecal carriage at follow-up, n (%)	1/137 (0.7)	0/131 (0)	

^aPatients can fail in more than one subcategory.

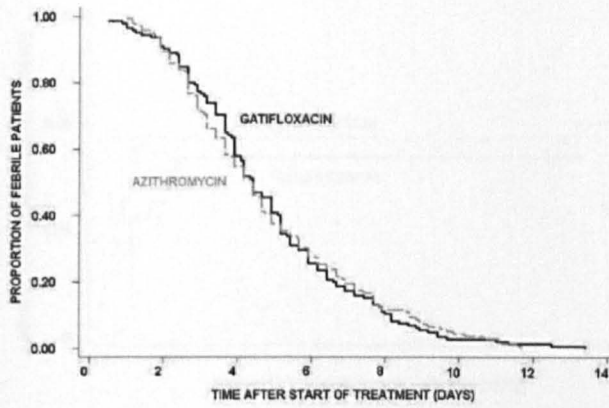
^bIn the worst case scenario: 15/142 (10.6%) showed overall treatment failure in the azithromycin group, log rank test p = 0.570.

^cThe p value is based on the log rank test.

^dThe p value is based on Fisher's exact test.

^eEvaluated in patients who attended at least two follow-up visits.

doi:10.1371/journal.pone.0002188.t002



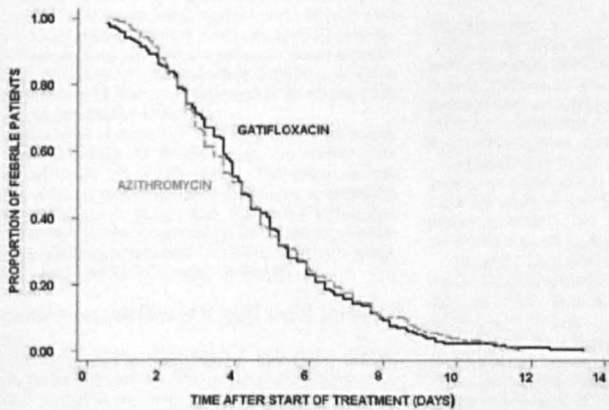
Numbers at risk							
Azithromycin	142	133	77	42	17	7	1
Gatifloxacin	145	132	92	43	16	5	3

Figure 2. Proportion of culture confirmed patients still febrile. Kaplan-Meier survival curve showing the proportion of culture confirmed patients (PP analysis) still febrile through time by treatment group. doi:10.1371/journal.pone.0002188.g002

faecal carriage was detected after 6 months (An Giang study site), the patient had received gatifloxacin.

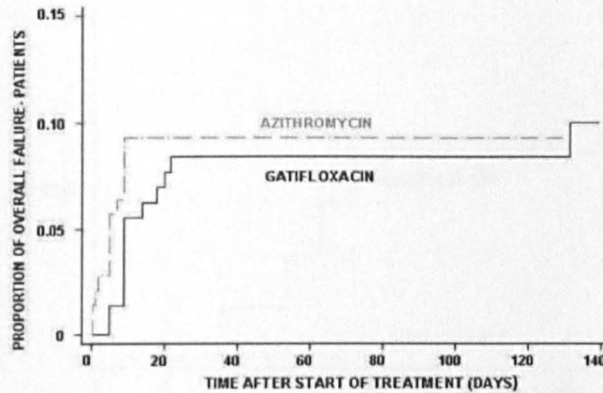
In the ITT analysis (all 358 randomised patients), overall treatment failure was reported in 13 out of 185 (7%) in the gatifloxacin group compared to 14 out of 168 (8.4%) in the

azithromycin group (logrank test $p=0.615$, HR [95% CI] = 0.82 [0.39–1.76]). One culture negative patient in the azithromycin group had a positive blood culture on day 7 after start of treatment. There were no clinical failures or typhoid fever-related complications in the culture negative patients.



Numbers at risk							
Azithromycin	172	157	86	41	18	7	1
Gatifloxacin	186	159	97	49	18	5	3

Figure 3. Proportion of all randomised patients still febrile. Kaplan-Meier survival curve showing the proportion of all randomised patients (ITT analysis) still febrile through time by treatment group. doi:10.1371/journal.pone.0002188.g003



Numbers at risk	0	20	40	60	80	100	120	140
Azithromycin	142	124	121	119	118	51	49	47
Gatifloxacin	145	130	128	128	127	60	58	52

Figure 4. Proportion of patients with overall failure in the culture confirmed population. Kaplan-Meier survival curve showing the proportion of patients with overall failure in the culture confirmed population (PP analysis) by treatment group. doi:10.1371/journal.pone.0002188.g004

Adverse events

Both treatments were well tolerated. One adverse event related to azithromycin was reported, a maculopapular rash that occurred after the first dose of treatment. Azithromycin was discontinued immediately and the patient was treated with ceftriaxone.

Gastrointestinal side-effects (change in consistency and frequency of stools) that were probably typhoid fever related were relatively frequent in both treatment arms at the start of treatment. In the gatifloxacin group, one patient experienced vomiting on day 2 and day 3 and one patient diarrhoea (4 episodes/day) on day 4 and day 5 of treatment. These episodes were self-limiting and did not require the interruption of therapy.

The median levels of serum AST and ALT fell in both groups after 7 days of therapy. In the PP group, the median post-treatment AST was 46.35 U/L (range 12.8–217.5) in the gatifloxacin arm and 45 U/L (range 5–358) in the azithromycin arm. The median post-treatment ALT fell to 46.8 U/L (range 7.4–278) and 49.9 (1.1–494), respectively. In the culture-negative patients, the median post-treatment AST was 44.8 U/L (range 12–654) and ALT was 40 U/L (range 10–424.9).

Antimicrobial susceptibilities of *S. typhi* and *S. paratyphi* A isolates

From the PP population, 282 (98%) *S. typhi* and 5 (2%) *S. paratyphi* A strains were isolated. Two hundred and sixty three *S. typhi* and 5 *S. paratyphi* A were received at the Hospital for Tropical Diseases for antimicrobial susceptibility testing.

Fifty-eight percent of the *S. typhi* isolates were MDR and 96% were nalidixic acid resistant and showed reduced susceptibility to the older generation fluoroquinolones (Table 3). However technically, using current CLSI breakpoints, all isolates remained susceptible in vitro to ciprofloxacin and ofloxacin. The MIC₉₀ of gatifloxacin was the lowest of all the fluoroquinolones tested at

0.19 µg/mL (range 0.004–0.5). All isolates were susceptible to ceftriaxone.

The 5 *S. paratyphi* A strains were fully susceptible to all the antimicrobials tested.

Discussion

Interpretation

The results of this trial show that both antibiotics worked well for the treatment of MDR and nalidixic acid resistant typhoid fever in Vietnam. A seven day oral course of gatifloxacin had similar efficacy and safety as a seven day course of azithromycin, which is recommended for the treatment of MDR and nalidixic acid resistant typhoid fever [7,9].

However, azithromycin is not available throughout much of the developing world and it is expensive. The costs of a 7-day treatment course of gatifloxacin (at 10 mg/kg/day) for an adult patient in Vietnam are approximately 25 US\$, the costs of azithromycin (at 20 mg/kg/day) are more than 90 US\$.

The results for gatifloxacin in this trial are comparable to the excellent clinical outcomes achieved with ofloxacin in Vietnam in the early 1990s, when *S. typhi* isolates were still susceptible to nalidixic acid [17–19].

Gatifloxacin has a higher affinity to GyrA and is less inhibited by the common mutations in the *GyrA* gene [20]. The gatifloxacin MIC₅₀ of the study isolates was 0.19 µg/mL compared to the ofloxacin MIC₅₀ of 0.75 µg/mL. We would not recommend the continued use of the older generation fluoroquinolones (ofloxacin and ciprofloxacin) in regions with high rates of nalidixic acid resistant typhoid fever for fear of selecting further mutations in *gyrA* [21]. This could put at risk the potential clinical benefit of the newer fluoroquinolones, including gatifloxacin.

There have been several case reports of gatifloxacin-associated dysglycemia in patients with type II diabetes mellitus, overweight

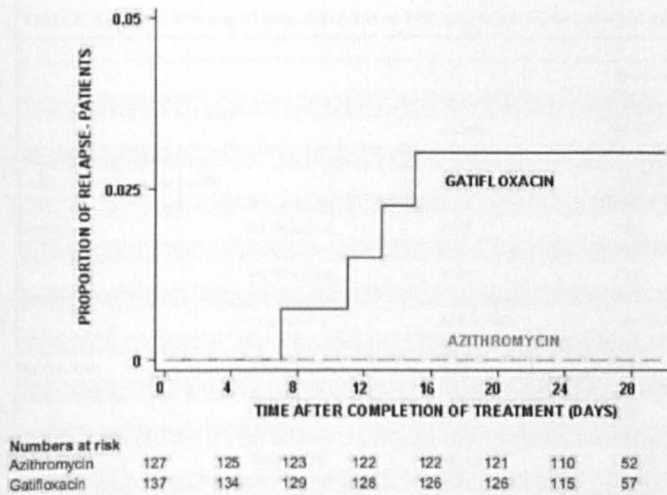


Figure 5. Proportion of patients with relapse in the culture confirmed population. Kaplan-Meier survival curve showing the proportion of patients with relapse in the culture confirmed population (PP analysis) by treatment group. doi:10.1371/journal.pone.0002188.g005

or with other comorbidity [22–24]. Recently there have been concerns about the use of gatifloxacin, after a retrospective case-control study in 1.4 million individuals over the age of 66 years (mean age 77 years) in Canada was published [25].

As our trial was completed before publication of this report, we did not systematically monitor for hypo- and hyperglycemia. Blood glucose levels taken as part of the routine care were normal. All patients were managed as in-patients and potential symptoms of hypo- and hyperglycemia would have been noted by the study physicians. No dysglycemia events were reported during the in-patient period or during the follow up period of 3 to 6 months.

The patients in our trial were healthy, young and non-obese individuals. A trial in 867 children with otitis media with glucose monitoring and a one year follow-up [26], as well as a recent enteric (typhoid and paratyphoid) fever trial in Nepal used gatifloxacin and did not report any dysglycemia [10]. In our setting and in our patient population gatifloxacin was highly effective despite very high rates of drug resistance and was well tolerated.

Other newer generation fluoroquinolones, i.e. gemifloxacin and moxifloxacin have shown low MICs for nalidixic acid resistant *S. typhi* and *S. paratyphi A* [11], unfortunately these drugs are not available in Vietnam and they are considerably more expensive. The *in vitro* results seen with these other newer generation fluoroquinolones should be evaluated in clinical trials.

Generalizability

The emergence of nalidixic acid resistant *S. typhi* and *S. paratyphi A* with reduced susceptibility to the fluoroquinolones is a widespread problem throughout Asia and therefore our study is relevant to the whole region [2,5]. Many case reports and some randomised controlled trials have described the worsening clinical response to ciprofloxacin and ofloxacin [8,27,28].

The search for effective antibiotics to treat typhoid fever is imperative.

Typically trials in typhoid fever are limited by small sample sizes, a recent Cochrane Report has stressed the need for large well-designed trials in enteric fever [29]. The evidence from our trial is strengthened by a sample size of 287 patients with culture confirmed typhoid fever (358 patients randomised), which we believe is so far the largest RCT performed in typhoid fever.

Both antibiotics also worked well for the patients with negative blood cultures. This is an important finding because the sensitivity of blood culture for the diagnosis of typhoid fever is only approximately 50 to 80% [9].

Limitations of the study

The randomisation sequence was generated with a large block size of 50, which resulted in uneven numbers in the two treatment groups (186 versus 172 patients in the ITT population).

One possible limitation was the low rate of stool cultures positive for *S. typhi*. Faecal carriage is usually characterised by intermittent shedding and the stool culture for *S. typhi* is not very sensitive. When comparing our data with other studies that demonstrate that azithromycin is highly efficacious for the treatment of typhoid fever, we find similar low rates of faecal carriage at follow-up [7,30]. It could be hypothesized that antibiotics that show high intracellular concentrations and good tissue penetration like azithromycin and the fluoroquinolones, achieve rapid bacterial killing and elimination throughout the body, which reduces faecal carriage.

The dose of gatifloxacin and azithromycin tablets was prepared by careful cutting of the tablets (proportions of the tablets administered were recorded in the CRFs). Inevitably, it was therefore an estimation of the exact dose, hence we cannot guarantee that each patient received exactly 10 mg/kg/day of gatifloxacin or 20 mg/kg/day of azithromycin.

Table 3. Antimicrobial susceptibilities and minimum inhibitory concentrations (MIC) of 263 *S. typhi* isolate.

	All isolates n = 263	Treatment with	
		Gatifloxacin n = 137	Azithromycin n = 126
Multidrug resistant, numbers (%)	153 (58)	87 (63.5)	66 (52.3)
Nalidixic acid resistant, numbers (%)	254 (96.3)	132 (96.3)	121 (96)
	MIC 50 (µg/ml)	>256	>256
Amoxicillin	MIC 90 (µg/ml)	>256	>256
	range (µg/ml)	0.125 to >256	0.125 to >256
	MIC 50 (µg/ml)	>256	>256
Chloramphenicol	MIC 90 (µg/ml)	>256	>256
	range (µg/ml)	0.38 to >256	2 to >256
	MIC 50 (µg/ml)	>256	>256
Nalidixic acid	MIC 90 (µg/ml)	>256	>256
	range (µg/ml)	1.5 to >256	1.5 to >256
	MIC 50 (µg/ml)	0.75	†
Ofloxacin	MIC 90 (µg/ml)	1.5	1.5
	range (µg/ml)	0.023–2	0.023–2
	MIC 50 (µg/ml)	0.38	0.38
Ciprofloxacin	MIC 90 (µg/ml)	0.5	0.5
	range (µg/ml)	0.004–0.75	0.004–0.38
	MIC 50 (µg/ml)	0.125	0.125
Gatifloxacin	MIC 90 (µg/ml)	0.19	0.19
	range (µg/ml)	0.004–0.5	0.004–0.5
	MIC 50 (µg/ml)	0.125	0.125
Ceftriaxone	MIC 90 (µg/ml)	0.125	0.19
	range (µg/ml)	0.064–0.25	0.064–0.25
	MIC 50 (µg/ml)	8	8
Azithromycin	MIC 90 (µg/ml)	12	12
	range (µg/ml)	1.5–16	4–16

MIC₅₀₋₉₀ concentration at which 50% and 90% of the organisms respectively are inhibited. MDR is defined as resistance to chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole. CLSI MIC breakpoints are as follows: for chloramphenicol, ampicillin and nalidixic acid resistance ≥ 32 µg/ml; ofloxacin and gatifloxacin ≤ 2 µg/ml, susceptible and ≥ 8 µg/ml resistant; ciprofloxacin ≤ 1 µg/ml susceptible and ≥ 4 µg/ml resistant; ceftriaxone ≤ 8 µg/ml, susceptible and ≥ 64 µg/ml resistant; there are none for azithromycin.
doi:10.1371/journal.pone.0002188.t003

Overall evidence. We performed a MEDLINE search for “azithromycin, clinical trial, typhoid/enteric fever” and used the recent enteric fever Cochrane report [29] to identify 6 clinical trials in the literature. In total, 251 typhoid fever patients were treated with azithromycin.

Four trials, three from Egypt and one from India, used azithromycin to treat MDR typhoid fever [30–33]. Azithromycin achieved cure rates between 88% and 100%, the mean FCT ranged from 3.8 to 4.5 days. Two trials performed in Vietnam used azithromycin at 20 mg/kg/day [7] and at 10 mg/kg/day [8] for the treatment of MDR and nalidixic acid resistant typhoid fever. In total, 107 patients with culture confirmed typhoid fever were enrolled. The cure rate was 93% and 82% and the FCT was 5.6 and 5.8 days, respectively. Our results concur with these excellent data.

A recent trial conducted in Kathmandu, Nepal used gatifloxacin at the same dose and duration for the treatment of nalidixic acid resistant typhoid fever [10]. Successful treatment was achieved in 96.5% (85 out of 88) patients and the median FCT (95% CI) was 92 hours (84–114 hours). The trial in Nepal was stopped early by

the independent Data and Safety Monitoring Committee as a result of the poor clinical response in the patients randomised to cefixime.

We believe on the basis of this and other recently published trials, that gatifloxacin or azithromycin are now the treatments of choice for enteric fever in areas of MDR and nalidixic acid resistance [7,8,10]. However it is important to use these antimicrobial agents cautiously because indiscriminate use would inevitably induce further resistance.

Supporting Information

Protocol S1 Trial Protocol

Found at: doi:10.1371/journal.pone.0002188.s001 (0.07 MB DOC)

Checklist S1 CONSORT Checklist

Found at: doi:10.1371/journal.pone.0002188.s002 (0.06 MB DOC)

Acknowledgments

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Author Contributions

Conceived and designed the experiments: TH JF SD PT NS NC KS CD BB JC TS NV TP NR LP HV DD NB ML PB VH CS. Performed the experiments: TH JF SD NS NC CD JC TS NV TP NR LP HV DD NB DL LH NB NH PD ML PB VH NH TN TC CS. Analyzed the data: TH JF PT NS NC KS CD NV TP NR LP HV. Wrote the paper: TH JF PT NS NC KS CD NV TP NR LP HV.

BJ STUDY NOTES

CASE RECORD FORM USED FOR THE GATIFLOXACIN *VERSUS* AZITHROMYCIN TYPHOID FEVER TRIAL DESCRIBED IN CHAPTER 2

1. PATIENT DETAILS

PATIENT NAME:		
FATHER NAME: (if patient child)		
MOTHER NAME: (if patient child)		
BJ STUDY NO:		
DATE OF ENTRY TO STUDY:		
HOSPITAL NUMBER:		
SEX:	MALE / FEMALE	
AGE:	___ YEARS.	
OCCUPATION:		
ADDRESS DETAILS:	House number: Road: Town/City: District/Province:	Telephone number: Mobile number:
ADDRESS DETAILS	House number: Road: Town/City: District/Province:	Telephone number: Mobile number:

INFORMATION SHEET

OXTREC No: BJ

An open randomized comparison of Azithromycin versus Gatifloxacin for the treatment of uncomplicated enteric fever

Introduction to the study

You (or your child) are being asked to be in a research study on typhoid fever. Typhoid fever is a serious infection and in Viet Nam is now becoming very difficult to treat. This study is designed to see if we can improve the treatment of typhoid fever.

Blood Tests:

Whether or not you take part in the study you will be given the same standard of care for your illness. If you wish to take part in the study we will randomize the treatment you receive to one of two alternatives. We do not know which one you will receive or which is the best treatment. We believe both treatments to be effective. We will ask to take blood and stool samples during your stay in hospital and for the next 6 months after you are discharged from hospital to ensure that you have cleared the infection. This is very important to ensure that your family and friends are not at risk of infection. We will study the cells in the blood that protect you against infection and also your genes to try and understand why you got sick. The blood will be stored in a freezer at the Hospital for Tropical Diseases in Ho Chi Minh City. Further tests on these stored samples may be undertaken in the future to further the understanding of this disease.

Confidentiality

We will keep the information we get from you as private. Your name will not be on your test results—we will use a number instead of your name. The results of your blood tests will be told only to your doctor. These results will be under the authority and supervision of the doctor responsible for your inpatient care. Your doctor will discuss these results with you. All this information will be kept confidential in your medical records. Your name will not be mentioned in any papers or speeches about the study.

Risks

There are very few risks to you from being in our study. All the drugs being used in this study are routinely used in Viet Nam. Taking blood by needle will hurt for a moment, and may leave a bruise.

Costs

There will be no cost to you.

Refusal to participate

You may refuse to be in any parts of the study. If you do not want to be in the study that decision will not in any way interfere with your ability to receive proper medical care or attention.

Questions:

If you have any other questions about the study please ask the doctor on the ward or the doctors named at the top of this sheet.

(This is the doctor you see every day. Please ask any nurse or doctor who will help you)

CONSENT FORM:

EXTREC No: BJ:

An open randomized comparison of Azithromycin versus Gatifloxacin for the treatment of uncomplicated enteric fever

Consent from patient:

I have been fully informed of the possible risks and benefits of taking part in this study and agree to take part. I agree that the samples may be stored and that further tests may be undertaken on these samples in the future to further the understanding of this disease.

Name of patient _____

Signature: _____ Date: _____

Name of physician _____

Signature: _____ Date: _____

If the patient gives verbal consent to take part in the trial but is unable to sign, the physician can record the consent here:

Name of physician: _____ Signature: _____

Date: _____

Reason for giving verbal consent: _____

Consent from relative: (Only to be used when the patient is unable to give consent because the patient is a child)

I have been fully informed of the possible risks and benefits of this study. I agree that my relative _____ may take part in the study.

Name of relative: _____

Relationship with patient: _____

Signature: _____ Date: _____

Name of physician: _____

Signature: _____ Date: _____

PLEASE NOTE, THE PATIENT CAN ONLY ENTER THE STUDY IF THE CONSENT FORM HAS BEEN SIGNED

2. HISTORY

BJ

Reasons for coming to hospital:		Duration of illness (days):
Duration of symptoms (record days, 0 = not reported)		
Fever:	Cough:	Constipation:
Headache:	Diarrhoea:	Vomiting:
Weight loss:	Abdominal pain:	Convulsions:
Any other significant symptoms:		
Past History		
Previous typhoid: Y / N Date if Yes:	Diabetes: Y / N Date if Yes:	
Family member with typhoid: Y / N Date if Yes:	TB: Y / N Date if Yes:	
Typhoid vaccination: Y / N Date if Yes:	Hepatitis: Y / N Date if Yes:	
Drug addict: Y / N	Any daily medication necessary: Y / N If yes: Drug: _____ Dose: _____ oral / i.v. / i.m.	
Allergy to drugs: Y / N / Not known If yes: Drug: _____		
Treatment in the last 2 weeks		
Y / N / Not known Date if Yes: Antibiotics (drug and dose): _____ Other treatment (drug and dose): _____ Where: _____		

3. EXAMINATION

Mental state: normal / agitated / confused	Jaundice: None / Mild / Mod / Severe
GCS (Glasgow Coma Score): ____ / 15	Hepatomegaly: Y / N If yes: ____ cm
Temperature: ____ °C	Splenomegaly: Y / N If yes: ____ cm
Blood pressure: _____ mmHg	Roseolae: Y / N
Pulse: _____ / min	Stiff neck: Y / N

Respiratory rate: _____ /min	Enlarged lymph nodes: Y / N
Weight: _____ kg	Abdominal tenderness: Y / N
Height: _____ cm	Any others:

4. CULTURE

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			

Bone marrow culture	Date: _____ (D__)	Date: _____ (D__)	Date: _____ (D__)
Result			

5. ANTIBIOGRAM

Bacteria isolated: <i>S.typhi</i> <i>S.paratyphi</i> other (specify):							
From blood/ bone marrow/ stool (please indicate)							Date: _____
Sens	Nal acid	Oflox	Cipro	Gati	Azm	Chlor	Amp
R or S							
MIC							

6. BLOOD TESTS

BJ

Date:	Result	Date:	Results	Date	Result
Haematocrit		Haematocrit		Haematocrit	
WBC		WBC		WBC	
Neutrophils		Neutrophils		Neutrophils	
Lymphocytes		Lymphocytes		Lymphocytes	
Monocytes		Monocytes		Monocytes	
Eosinophils		Eosinophils		Eosinophils	
Platelets		Platelets		Platelets	
Sodium		Sodium		Sodium	
Creatinine		Creatinine		Creatinine	
AST/ SGOT		AST/ SGOT		AST/ SGOT	
ALT/SGPT		ALT/SGPT		ALT/SGPT	
Bilirubin TT		Bilirubin TT		Bilirubin TT	
Bilirubin GT		Bilirubin GT		Bilirubin GT	
BUN		BUN		BUN	
Glucose		Glucose		Glucose	

Date:	Result	Date:	Results	Date	Result
Sodium		Sodium		Sodium	
Creatinine		Creatinine		Creatinine	
AST/ SGOT		AST/ SGOT		AST/ SGOT	
ALT/SGPT		ALT/SGPT		ALT/SGPT	
Bilirubin TT		Bilirubin TT		Bilirubin TT	
Bilirubin GT		Bilirubin GT		Bilirubin GT	
BUN		BUN		BUN	
Glucose		Glucose		Glucose	

7. CHEST X-RAY (please summarize most important finding)

Chest X-Ray	Date: ____ (D __)	Date: ____ (D __)	Date: ____ (D __)
Result			

8. OTHER TESTS

ECG, keep with notes	Date: ____ (D __)	Date: ____ (D __)	Date: ____ (D __)
Result			

Other tests	Date: ____ (D __)	Date: ____ (D __)	Date: ____ (D __)

9. MANAGEMENT

Diagnosis:		
General assessment: Severe / Average / Mild		
Treatment prescribed:		
Start of treatment: date _____ dose _____		
Complications: Yes / No		
COMPLICATIONS	Date	Duration, level of illness, management
GI bleeding		
Intestinal perforation		
Hepatitis/Cholecystitis		
Shock		
Others		

10. HAND WRITTEN SUMMARY

STUDY NOTES

BJ _____

Date of admission:

		Day 1 Date:				Day 2 Date:				Day 3 Date:				Day 4 Date:			
Antibiotic																	
Dose (po/im/iv)																	
Hours since start treatment																	
Time		6	12	18	24	6	12	18	24	6	12	18	24	6	12	18	24
Fever °C	40.0																
	39.0																
	38.0																
	37.0																
	36.0																
Pulse rate																	
Blood pressure																	
Respiratory rate																	
GCS																	
Hepatomegaly																	
Splenomegaly																	
Diarrhoea																	
Melaena																	
Roseolae																	

STUDY NOTES

BJ _____

Date of admission:

		Day 5 Date:				Day 6 Date:				Day 7 Date:				Day 8 Date:			
Antibiotic																	
Dose (po/im/iv)																	
Hours since start treatment																	
Time		6	12	18	24	6	12	18	24	6	12	18	24	6	12	18	24
Fever °C	40.0																
	39.0																
	38.0																
	37.0																
	36.0																
Pulse rate																	
Blood pressure																	
Respiratory rate																	
GCS																	
Hepatomegaly																	
Splenomegaly																	
Diarrhoea																	
Melaena																	
Roseolae																	

STUDY NOTES

BJ _____

Date of admission:

		Day 9 Date:				Day 10 Date:				Day 11 Date:				Day 12 Date:			
Antibiotic																	
Dose (po/im/iv)																	
Hours since start treatment																	
Time		6	12	18	24	6	12	18	24	6	12	18	24	6	12	18	24
Fever °C	40.0																
	39.0																
	38.0																
	37.0																
	36.0																
Pulse rate																	
Blood pressure																	
Respiratory rate																	
GCS																	
Hepatomegaly																	
Splenomegaly																	
Diarrhoea																	
Melaena																	
Roseolae																	

ADMISSION OUTCOME

BJ _____

Uncomplicated recovery? Y / N If No, describe:	Fever clearance time: (Time from the start of treatment until the temp. falls below 37.5°C and remains at or below 37.5°C for at least 48 hours)
	= _____ hours Duration of antibiotic treatment: (days)
Treatment failure:	Clinical (fever after 7 days of treatment) Yes/No _____
	Microbiological (positive stool culture on day 9) Yes/No _____
Date of death:	
Date of discharge:	

SIDE-EFFECTS OF STUDY DRUGS

SIDE-EFFECTS		Yes / No
SIDE EFFECTS	Date	Duration, level of illness, management
Allergic reaction		
Nausea		
Vomiting		
Headache		
Others		

FOLLOW-UP

Follow-up 1

after 1 MONTH

Date: _____

Time since finished treatment : _____ (days)

Complaint : _____

Clinical examination: _____

Temperature : _____ °C

Other symptoms : _____

Relapse: Yes/No

Comments: _____

Stool culture (1 sample) : _____

Faecal carriage: Yes/No

Name of doctor _____ Date: _____

Follow-up 2

after 3 MONTH

Date: _____

Time since finished treatment : _____ (days)

Complaint : _____

Clinical examination: _____

Temperature : _____ °C

Other symptoms : _____

Relapse: Yes/No

Comments: _____

Stool culture (1 sample) : _____

Faecal carriage: Yes/No

Name of doctor _____ Date: _____

Follow-up 3 **after 6 MONTH**

Date: _____

Time since finished treatment : _____ (days)

Complaint : _____

Clinical examination: _____

Temperature : _____ °C

Other symptoms : _____

Relapse: Yes/No

Comments: _____

Stool culture (1 sample) : _____

Faecal carriage: Yes/No

Name of doctor _____ Date: _____

Antimicrobial Drug Resistance of *Salmonella enterica* Serovar Typhi in Asia and Molecular Mechanism of Reduced Susceptibility to the Fluoroquinolones⁷

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This study describes the pattern and extent of drug resistance in 1,774 strains of *Salmonella enterica* serovar Typhi isolated across Asia between 1993 and 2005 and characterizes the molecular mechanisms underlying the reduced susceptibilities to fluoroquinolones of these strains. For 1,393 serovar Typhi strains collected in southern Vietnam, the proportion of multidrug resistance has remained high since 1993 (50% in 2004) and there was a dramatic increase in nalidixic acid resistance between 1993 (4%) and 2005 (97%). In a cross-sectional sample of 381 serovar Typhi strains from 8 Asian countries, Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and central Vietnam, collected in 2002 to 2004, various rates of multidrug resistance (16 to 37%) and nalidixic acid resistance (5 to 51%) were found. The eight Asian countries involved in this study are home to approximately 80% of the world's typhoid fever cases. These results document the scale of drug resistance across Asia. The Ser83→Phe substitution in *CyrA* was the predominant alteration in serovar Typhi strains from Vietnam (117/127 isolates; 92.1%). No mutations in *gyrB*, *parC*, or *parE* were detected in 55 of these strains. In vitro time kill experiments showed a reduction in the efficacy of ofloxacin against strains harboring a single amino acid substitution at codon 83 or 87 of *CyrA*; this effect was more marked against a strain with a double substitution. The 8-methoxy fluoroquinolone gatifloxacin showed rapid killing of serovar Typhi harboring both the single- and double-amino acid substitutions.

There are approximately 21 million cases of typhoid fever worldwide, with a particularly high incidence in Asia. An estimated 220,000 deaths per year occur as a consequence of the disease (1).

This article describes the extent and pattern of drug resis-

tance of *Salmonella enterica* serovar Typhi across Asia. This information is vital for guiding treatment and is also important for helping policy makers to plan vaccination campaigns. The emergence and spread of drug resistance have limited treatment options for typhoid fever in many countries.

Since the isolation of multidrug-resistant (MDR) serovar Typhi strains which show resistance to all first-line antibiotics (chloramphenicol, ampicillin, and trimethoprim-sulfamethoxazole) in the 1980s, the fluoroquinolone class of antibiotics has become the treatment of choice for enteric fever (4, 38). Unfortunately, outbreaks of serovar Typhi strains that were resis-

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tant to nalidixic acid (the prototype quinolone, which is used for *in vitro* screening tests) and showed reduced susceptibility to the fluoroquinolones have been reported subsequently in a number of countries (25). Vietnam and particularly the Mekong Delta region of Vietnam faced a series of typhoid fever epidemics over the last decade, imposed on a background of endemic disease, that reflected changes in resistance patterns and pointed to a serious problem of drug resistance (24). MDR is associated with a transferable plasmid (36), while reduced susceptibility to the fluoroquinolones in serovar Typhi is usually associated with point mutations in the bacterial target genes encoding DNA gyrase and/or DNA topoisomerase IV.

This study describes the magnitude and patterns of drug resistance in 1,393 serovar Typhi strains isolated from 1993 to 2005 in Vietnam and from a cross-sectional sample set of 381 serovar Typhi strains isolated in 2002 to 2004 in eight Asian countries (Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and central Vietnam). These countries are home to more than 80% of the world's typhoid fever cases (11). We defined the molecular mechanism of nalidixic acid resistance and performed *in vitro* bacterial time-kill experiments with isolates that harbored the common mutations in the *gyrA* gene. The time-kill experiments allowed us to model the impact of the *gyrA* mutations on the time course of the antimicrobial effects of older (ofloxacin) and newer-generation (gatifloxacin) fluoroquinolones.

(This work was presented in part at the American Meeting of Hygiene and Tropical Medicine, Atlanta, GA, December 2005.)

MATERIALS AND METHODS

Bacterial isolates. (i) *Serovar Typhi* strains isolated in southern Vietnam from 1993 to 2005. One thousand three hundred ninety-three serovar Typhi isolates were collected consecutively from patients with uncomplicated typhoid fever during prospective hospital-based clinical studies between 1993 and 2005 conducted at Dong Thap Provincial Hospital, Dong Nai Provincial Hospital, An Giang Provincial Hospital, and the Hospital for Tropical Diseases, Ho Chi Minh City, all located in southern Vietnam. These studies have been described previously (7, 8, 23, 26, 32–35).

(ii) *Serovar Typhi* isolates from eight Asian countries in 2002 to 2004. One hundred forty-nine serovar Typhi isolates were collected in March and April 2003 during a hospital-based descriptive study at Patan Hospital, Kathmandu, Nepal. Fifty isolates were collected consecutively during a clinical trial in 2002 and 2003 at the Wellcome Trust-Mahosot Hospital-Oxford Tropical Medicine Research Collaboration, Lao People's Democratic Republic, Laos (27). One hundred eighty-two serovar Typhi isolates were collected as part of population-based prospective surveillance studies conducted by multiple teams in collaboration with the International Vaccine Institute (IVI), Seoul, South Korea (1). These surveillance sites included whole townships (China and Vietnam), specific slum areas (Bangladesh, Pakistan, and India), and an impoverished urban sub-district (Indonesia). Forty isolates were collected from February till December 2003 in an urban slum in Dhaka, Bangladesh; the setting has been described (5); 21 isolates were collected during 2002 in Hechi city, Guang Xi, China; 23 strains were collected from May to July 2003 in slum areas in Kolkata, West Bengal, India; 17 isolates were collected from July to September 2002 in North Jakarta, Indonesia; 34 strains were isolated between January 2002 and March 2003 in one slum area in Karachi, Pakistan; and 47 isolates were collected between July 2002 and September 2004 in Hue city, central Vietnam.

All serovar Typhi isolates were collected consecutively from febrile patients during the indicated periods and came from geographically contiguous areas. The isolates were unselected and were representative of the population they came from.

Identification and antimicrobial susceptibilities. Isolates were identified using the API20E biochemical identification system (bioMérieux, Paris, France). Se-

TABLE 1. Oligonucleotide primer sequences used for PCR amplification

Gene	Primer	Primer sequence (5'→3')	Reference
<i>gyrA</i>	GYRA/P1	TGTCGAGATGGCCGTGAAGC	16
	GYRA/P2	TACCGTCATASGTTATCCACG	
<i>gyrB</i>	StygyrB1	CAAACCTGGCGGACTGTCCAGG	20
	StygyrB1	TTCCGGCATCTGACGATAGA	
<i>parC</i>	StmparC1	CTATGCGATGTCAGAGCTGG	13
	StmparC2	TAACAGCAGCTCGCGTATT	
<i>parE</i>	StmparE1	TCTCTCCGATGAAGTGCTG	13
	StmparE2	ATACGGTATAGCGCGGTAG	
<i>qnrS</i>	QnrS1	ATGGAACCTCAACATCATC	—*
	QnrS2	AAAAACCCCTGACTTAAGT	
<i>QntA</i>	QP1	GATAAAGTTTTTCAGCAA	19
		GAGG	
	QP2	ATCCAGATCGGCAAAGGITA	

* Sequences for the *qnrS* primers were designed based on the sequence of *Shigella flexneri* (17).

rology was carried out using specific antisera (polyvalent O, O9, H6, and V) (Murex, Dartford, United Kingdom).

Antimicrobial susceptibility testing with ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, nalidixic acid, ofloxacin, ciprofloxacin, gatifloxacin, and ceftriaxone was performed by disc diffusion according to Clinical and Laboratory Standards Institute (CLSI) methods (10) and interpreted following CLSI guidelines (9). The control strains used for all susceptibility tests were *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 27853, and *Staphylococcus aureus* ATCC 29213. MICs were determined by using the E test (All Biochim, Solna, Sweden). MDR of isolates was defined as resistance to chloramphenicol (MIC \geq 32 μ g/ml), ampicillin (MIC \geq 32 μ g/ml), and trimethoprim-sulfamethoxazole (MIC \geq 8/152 μ g/ml). Nalidixic acid resistance was defined as a MIC of \geq 32 μ g/ml. The breakpoints for ofloxacin and gatifloxacin were \leq 2 μ g/ml (susceptible) and \geq 8 μ g/ml (resistant), and for ciprofloxacin, \leq 1 μ g/ml (susceptible) and \geq 4 μ g/ml (resistant) (9). All tests were performed at the Hospital for Tropical Diseases (HTD), Ho Chi Minh City, Vietnam, except for the isolates from Nepal, which were tested at Patan Hospital, Kathmandu, Nepal, using identical methods.

DNA isolation. A single colony was inoculated in 6 ml of LB broth (Sigma) and incubated overnight at 37°C. DNA was extracted using the Qiagen Genomic-tip 100/G and Genomic DNA buffer set (Qiagen, Ltd., Hilden, Germany) or the cetyltrimethylammonium bromide method of DNA extraction (2). DNA stock was stored at -20 and -80 °C. Four hundred nanograms of DNA was used for each PCR.

PCR and sequencing. Oligonucleotide primer pairs are shown in Table 1. PCR amplification of *gyrA* (347 bp), *gyrB* (345 bp), *parC* (270 bp), and *parE* (240 bp) were performed with 30 cycles of denaturation at 92°C for 1 min, annealing at 62°C for 1 min, and extension at 74°C for 2 min, followed by a final extension step at 74°C for 1 min.

PCR products were purified using the QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany) and used directly as templates for sequencing, which was performed with the CEQ DTCS-Quick Start kit and analyzed using an automated sequencer, the CEQ8000 genetic analysis system (Beckman Coulter, Inc., Fullerton, CA).

Selected strains were screened for the presence of the *qnrA* and *qnrS* genes by PCR. The PCR conditions for the amplification of *qnrS* were as follows: 94°C for 2 min; 34 cycles of 94°C for 45 s, 48°C for 45 s, and 72°C for 45 s; final extension at 74°C for 5 min. PCR conditions for *qnrA* were identical except for the annealing temperature, which was 53°C. The positive control used was a *Citrobacter* sp. isolate (identified by API20E) harboring both the *qnrA* and *qnrS* genes, as confirmed by sequencing of PCR products.

In vitro time-kill analysis. All time-kill experiments were determined in duplicate. Ofloxacin powder was purchased from Sigma, Steinheim, Germany, and gatifloxacin powder was provided from Bristol-Myers Squibb, New Brunswick, NJ. Three serovar Typhi colonies were taken and inoculated in 10 ml Mueller-Hinton broth (Oxoid, Basingstoke, United Kingdom) at 37°C for 15 to 18 h. Two drops of this broth were inoculated into 10 ml of Mueller-Hinton broth and incubated at 37°C for 1 h to give 2×10^8 CFU/ml. Ten milliliters of Mueller-Hinton broth containing ofloxacin or gatifloxacin at $32 \times$ MIC was added at time zero to give a final concentration of $16 \times$ MIC; serial twofold dilutions were used

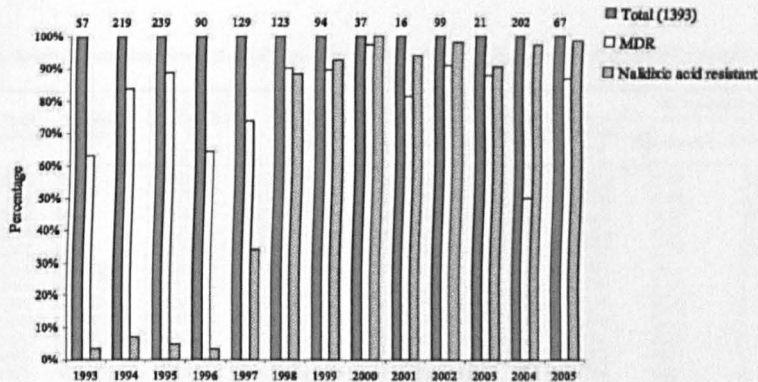


FIG. 1. Antimicrobial drug resistance of serovar Typhi strains isolated during clinical studies in southern Vietnam from 1993 to 2005. Percentages of MDR and nalidixic acid-resistant serovar Typhi isolates. The number of isolates from each year is shown on top of the bars.

to obtain $8\times$, $4\times$, $2\times$, and $1\times$ MIC. The growth control contained no antibiotic. The cultures were incubated at 35 to 37°C for 24 h. Viable counts were measured immediately prior to the addition of the antibiotic and at 30 min and 1, 2, 4, 6, 8, and 24 h after the addition of the antibiotic. Viable counts were performed by using the Miles and Misra technique on nutrient agar plates following serial dilution in maximum-recovery diluents (Oxoid, United Kingdom). The lower limit of detection was 10^2 CFU/ml.

Nucleotide sequence accession numbers. The partial DNA sequences of the *gyrA* gene of serovar Typhi AG 152 and DT 15 have been registered in the GenBank nucleotide sequence database under the accession numbers EF680460 and EF680461, respectively.

RESULTS

Antimicrobial susceptibility testing. (i) Serovar Typhi isolated in southern Vietnam from 1993 to 2005. Between 1993 and 2005, 1,393 isolates of serovar Typhi were collected (Fig. 1). The proportion of MDR serovar Typhi was 63.2% (36/57 strains) in 1993 and increased to more than 80% in the late 1990s and early 2000. During the same period, there was a dramatic increase in nalidixic acid resistance. In 1993, 2 out of 57 (3.5%) serovar Typhi isolated from patients in southern

Vietnam were nalidixic acid resistant (respective MICs of ofloxacin, 0.250 and 0.125 µg/ml) (37). Nalidixic acid resistance surged to 88.6% (109/123) in 1998. It has remained at high levels since then, with 97% (196/202) of isolates in 2004. Since 1998, a high proportion of strains show the combination of MDR and nalidixic acid resistance (Fig. 1).

The antimicrobial susceptibility data of 202 serovar Typhi isolated in 2004 in southern Vietnam are shown in more detail in Table 2.

(ii) Serovar Typhi strains isolated in eight Asian countries in 2002 to 2004. The antimicrobial susceptibilities of 381 serovar Typhi isolates collected in 2002 to 2004 from eight Asian countries were analyzed (Table 2). There were various rates of MDR across the sites, ranging from 16% (8/50) of isolates from Laos to 37.5% (15/40) from Bangladesh. China and Indonesia were exceptions, with no MDR serovar Typhi identified.

The percentages of nalidixic acid-resistant serovar Typhi isolates ranged from 0% in Indonesia and Laos and 4.8% (1/21) in China to 51% (76/149) in Nepal (Table 2). The com-

TABLE 2. Antimicrobial drug resistance of serovar Typhi isolates in 2002 to 2004 across eight Asian countries*

Country	% Nalidixic acid-resistant isolates ^a	MIC of ciprofloxacin (µg/ml)			% Ciprofloxacin-resistant isolates ^a	MIC of gatifloxacin (µg/ml)			% Chloramphenicol-resistant isolates ^a	% MDR Isolates ^a
		Range	50%	90%		Range	50%	90%		
China	4.8 (1/21)	0.008–0.25	0.015	0.03	0	0.012–0.190	0.023	0.023	0 (0/21)	0 (0/21)
Indonesia	0 (0/17)	0.002–0.03	0.015	0.015	0	0.012–0.023	0.016	0.023	0 (0/17)	0 (0/17)
Laos	0 (0/50)	0.006–0.023	0.012	0.016	0	0.012–0.047	0.016	0.023	18 (9/50)	16 (8/50)
Bangladesh	40 (16/40)	0.006–0.38	0.025	0.38	0	0.012–0.19	0.016	0.19	40 (16/40)	37.5 (15/40)
India	47.8 (11/23)	0.006–0.25	0.094	0.25	0	0.012–0.19	0.125	0.19	26 (6/23)	26 (6/23)
Nepal	51 (76/149)	0.002–32	0.125	0.5	4 (6/149)	0.012–1.500	0.094	0.25	19 (28/149)	NA ^b
Pakistan	38.3 (13/34)	0.004–0.25	0.012	0.25	0	0.012–0.190	0.023	0.19	26.5 (9/34)	26.5 (9/34)
Central Vietnam (IVI)	50 (23/47)	0.006–0.5	0.023	0.38	0	0.008–0.250	0.016	0.19	21.3 (10/47)	21.3 (10/47)
Southern Vietnam (HTD)	97 (196/202)	0.008–0.75	0.38	0.5	0	0.006–0.250	0.125	0.19	50 (101/202)	50 (101/202)

* Parenthetical numbers indicate no. of resistant isolates/no. tested.

^b NA, not available.

TABLE 3. Results of sequence analysis of the QRDR of *grrA*, *grrB*, *parC*, and *parE* and MICs of antimicrobial agents for 55 selected serovar Typhi strains

Isolate ^a	Yr of isolation	Country or province ^b	Amino acid substitution(s) in <i>grrA</i>	Nucleotide change(s) in <i>grrA</i>	QRDR profile ^c			Presence of MDR	MIC of drug (μg/ml)			
					<i>grrB</i>	<i>parC</i>	<i>parE</i>		Nalidixic acid	Ciprofloxacin	Ofloxacin	Gatifloxacin
D 43*	2004	India	S83Y	TCC→TAC	wt	wt	wt	No	>256	0.25	0.5	0.19
B 111*	2004	India	S83Y	TCC→TAC	wt	wt	wt	Yes	>256	0.25	0.5	0.19
E 86*	2004	India	S83F	TCC→TTC	wt	wt	wt	No	>256	0.25	0.5	0.19
A 102*	2004	India	S83Y	TCC→TAC	wt	wt	wt	Yes	>256	0.25	0.5	0.19
C 152*	2004	India	S83Y	TCC→TAC	wt	wt	wt	No	>256	0.25	0.5	0.19
CT 29*	1994	Tien Giang	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	1	0.094
CT 61*	1994	Tien Giang	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.125	1	0.064
nar 102*	1995	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	128	0.125	1	0.094
nar 104*	1995	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.25	1	0.125
nar 107*	1995	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.25	1	0.125
nar 108	1995	HCMC	S83F	TCC→TTC	wt	wt	wt	No	256	0.25	1	0.125
ipt 2*	1995	HCMC	D87G	GAC→GGC	wt	wt	wt	No	256	0.25	2	0.125
nar 28	1996	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	1	0.19
nar 45	1996	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	0.5	0.125
nar 46	1996	HCMC	D87A	GAC→GCC	wt	wt	wt	Yes	64	0.06	0.5	0.032
nar 50	1996	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	1	0.094
nar 51	1996	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	1	0.125
ipt 32	1997	Long An	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	1	0.125
ipt 33	1997	Long An	S83F	TCC→TTC	wt	wt	wt	Yes	256	0.5	0.5	0.125
CT 118*	2001	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.38	1	0.094
CT 142*	2001	HCMC	S83F	TCC→TTC	wt	wt	wt	No	>256	0.38	1	0.125
CT 144*	2001	Can Tho	D87G	GAC→GGC	wt	wt	wt	No	>256	0.25	1	0.094
CT 145*	2001	Long An	S83F	TCC→TTC	wt	wt	wt	No	128	0.38	0.5	0.094
DT 2*	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	2	0.125
DT 3*	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	2	0.094
DT 9	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	2	0.25
DT 15	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.094
DT 18	2002	Dong Thap	S83F and D87G	TCC→TTC and GAC→GGC	wt	wt	wt	Yes	>256	0.5	2	0.25
DT 37	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.125
DT 40	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.38	0.5	0.125
DT 42	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.125
DT 47*	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	128	0.5	1	0.125
DT 48	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.125
DT 49	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.125
DT 54	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.125
DT 60	2002	Dong Thap	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.25	1	0.125
AG 3	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	No	>256	0	2	0.25
AG 5	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.25
AG 6	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1	0.19
AG 7	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1.5	0.19
AG 8	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1.5	0.19
AG 15	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1.5	0.13
AG 16	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.38	1.5	0.13
AG 17	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.5	1.5	0.19
AG 152*	2005	An Giang	S83F and D87N	TCC→TTC and GAC→AAC	wt	wt	wt	Yes	>256	0.38	3	0.25
AG 168	2005	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.38	1	0.13
AG 169	2005	An Giang	S83F	TCC→TTC	wt	wt	wt	No	>256	0.38	1	0.13
AG 176	2005	An Giang	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.25	0.75	0.09
AG 182	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	No	>256	0.38	1	0.13
AG 258*	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	No	>256	0.5	1.5	0.19
AG 259*	2004	An Giang	S83F	TCC→TTC	wt	wt	wt	No	>256	0.38	1.5	0.13
HTD 798	2003	HCMC	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.38	1	0.13
BL 21801*	2004	Pakistan	S83F	TCC→TTC	wt	wt	wt	No	>256	0.25	0.5	0.19
BL 21095*	2004	Pakistan	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.25	0.5	0.19
BL 3769*	2004	Pakistan	S83F	TCC→TTC	wt	wt	wt	Yes	>256	0.25	0.5	0.19

^a Isolate names consist of an abbreviation for the study followed by the isolate number. *, strain screened for presence of *grrA* and *grrB* genes by PCR.^b An Giang Province, Dong Thap Province, Can Tho Province, Tien Giang Province, Long An Province, and Ho Chi Minh City (HCMC) are located in southern Vietnam.

*wt, wild type.

bination of MDR and nalidixic acid resistance was found in 4.3% (2/47) of serovar Typhi isolates from central Vietnam, 8.7% (2/23) of isolates from India, 23.5% (8/140) of isolates from Pakistan, and 30% (12/40) of isolates from Bangladesh. In Nepal, 18.1% (27/149) of serovar Typhi isolates were resistant to chloramphenicol and nalidixic acid.

However, using current CLSI breakpoints, all isolates remained susceptible *in vitro* to ciprofloxacin and ofloxacin, with the exception of one isolate from southern Vietnam, AG 152, with intermediate susceptibility (MIC, 3.0 µg/ml) to ofloxacin (Table 3) and six isolates (4%) from Nepal that were ciprofloxacin resistant. The highest MICs of gatifloxacin at which 50% and 90% of serovar Typhi isolates were inhibited were 0.125 µl/ml and 0.25 µl/ml, respectively (Table 2). All isolates were susceptible to ceftriaxone.

DNA sequence analysis of QRDR of DNA gyrase and DNA topoisomerase IV and effect of mutations on fluoroquinolone susceptibility. One hundred twenty-seven nalidixic acid-resistant serovar Typhi isolates (118 from southern Vietnam, 5 from India, and 4 from Pakistan) with reduced susceptibilities to the fluoroquinolones (MIC of ofloxacin ranging from 0.5 µg/ml to 3 µg/ml) were selected for molecular analysis of the quinolone resistance determining region (QRDR) of *gyrA*. Six different types of mutations were detected. The most prevalent amino acid substitution was Ser83→Phe (TCC→TTC) in 117/127 (92.1%) strains. Four isolates (3.1%) had an alteration at codon 83 changing Ser to Tyr (TCC→TAC). Two isolates showed the Asp87→Gly (GAC→GGC) substitution and two isolates the Asp87→Ala (GAC→GCC) substitution. Two serovar Typhi isolates had double-amino-acid substitutions in *GyrA*: isolates DT 18 (Ser83→Phe and Asp87→Gly) and AG 152 (Ser83→Phe and Asp87→Asn), as shown in Table 3.

Fifty-five of these strains were analyzed for mutations in the QRDR of *gyrB*, *parC*, and *parE* (13, 20); no mutations were detected (Table 3). Twenty-five isolates (indicated with an asterisk in Table 3) were screened for the presence of the plasmid-mediated quinolone resistance genes *qnrA* and *qnrS* (15); none were detected in these isolates.

In vitro time kill analysis. One isolate representing each mutation group was selected for *in vitro* time-kill experiments: CT 76, wild-type strain (MICs, 0.064 µg/ml for ofloxacin and 0.008 µg/ml for gatifloxacin); HTD 798 (Ser83→Phe; MICs, 1.0 µg/ml for ofloxacin and 0.13 µg/ml for gatifloxacin); CT 144 (Asp87→Gly; MICs, 1 µg/ml for ofloxacin and 0.094 µg/ml for gatifloxacin); and DT 18 (Ser83→Phe and Asp87→Gly; MICs, 2.0 µg/ml for ofloxacin and 0.25 µg/ml for gatifloxacin). The mean changes in log₁₀ CFU/ml are presented in Fig. 2. Ofloxacin showed rapid killing of wild-type strain CT 76 (Fig. 2a); viable counts of serovar Typhi HTD 798 and CT 144 decreased after 4 h at 4× MIC, but complete killing could not be achieved (Fig. 2b and c). No bactericidal activity was achieved against serovar Typhi DT 18 (Fig. 2d). Gatifloxacin at 4× MIC decreased the bacterial population of CT 76, HTD 798, and CT 144 (Fig. 2e, f, and g) in the first 30 min and showed complete killing after 6 h. Viable counts of serovar Typhi DT 18 decreased after 4 h, followed by regrowth; higher concentrations (8× or 16× MIC) showed a more pronounced bactericidal effect against this double mutant (Fig. 2h).

DISCUSSION

This study describes the trends in antimicrobial drug resistance of serovar Typhi in Vietnam between 1993 and 2005 and across Asia in 2002 to 2004.

In 1993, during the initial outbreak of MDR serovar Typhi in Kien Giang province in the south of Vietnam, the fluoroquinolone antibiotics were introduced for the treatment of typhoid fever (22). Since 1993, the proportion of MDR serovar Typhi has remained at high levels and there has been a dramatic increase in nalidixic acid resistance. In 1998, 5 years after ofloxacin and ciprofloxacin become widely available in an uncontrolled market, 87% of the isolates were resistant to nalidixic acid; this increased to 97% by 2004. The combination of MDR and nalidixic acid resistance is a particular problem in Vietnam, because it severely restricts the therapeutic options for patients with typhoid fever.

Patients infected with nalidixic acid-resistant serovar Typhi show poor clinical response, high failure rates (up to 36%), and prolonged fecal carriage when treated with an older-generation fluoroquinolone, such as ofloxacin (8, 26). The antimicrobial resistance data from southern Vietnam are complemented by the results of a cross-sectional study from eight Asian countries: Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and Vietnam. These countries are home to approximately 80% of the world's typhoid fever cases (11).

While in southern Vietnam the MDR phenotype of serovar Typhi has remained at high levels over the last 13 years, there have been reports of a return to chloramphenicol sensitivity in some regions (12, 21). However, in our study the prevalence of chloramphenicol resistance remained high in many Asian countries (18% in Laos, 19% in Nepal, 26% in India and Pakistan, and 40% in Bangladesh), with the exception of China and Indonesia.

In 2002 to 2004, all countries in the region, with the exception of China and Laos, faced a problem of nalidixic acid resistance, with southern Vietnam as a particular hot spot. Roumagnac et al. recently suggested that fluoroquinolone use has driven the clonal expansion of a nalidixic acid-resistant serovar Typhi haplotype, H58, in Southeast Asia (29). The emergence of resistance of serovar Typhi to ciprofloxacin (6/149 isolates; 4%) in Nepal, together with reports of high-level ciprofloxacin resistance in India and Bangladesh (14, 28, 30), might be the prelude to a worsening drug resistance problem in Asia.

In this study carried out across Asia, mutations associated with nalidixic acid resistance and reduced susceptibility to fluoroquinolones for serovar Typhi were defined only in *gyrA*, as single-amino-acid substitutions at either codon 83 or 87 (6, 18, 31, 37), with the exception of two isolates from Vietnam, which had double-amino-acid substitutions. There have been two recent reports of serovar Typhi with the Ser83Phe and Asp87Gly double alteration in high-level-ciprofloxacin-resistant serovar Typhi (28, 30). In our study, the isolates with double mutations in *gyrA* were less susceptible to the fluoroquinolones, and this phenotype may become more widespread in the future if continued drug pressure is applied. This is a particular problem in many parts of Asia, where antibiotics are readily available in an unregulated marketplace and inadequate doses and durations of antibiotics are often used.

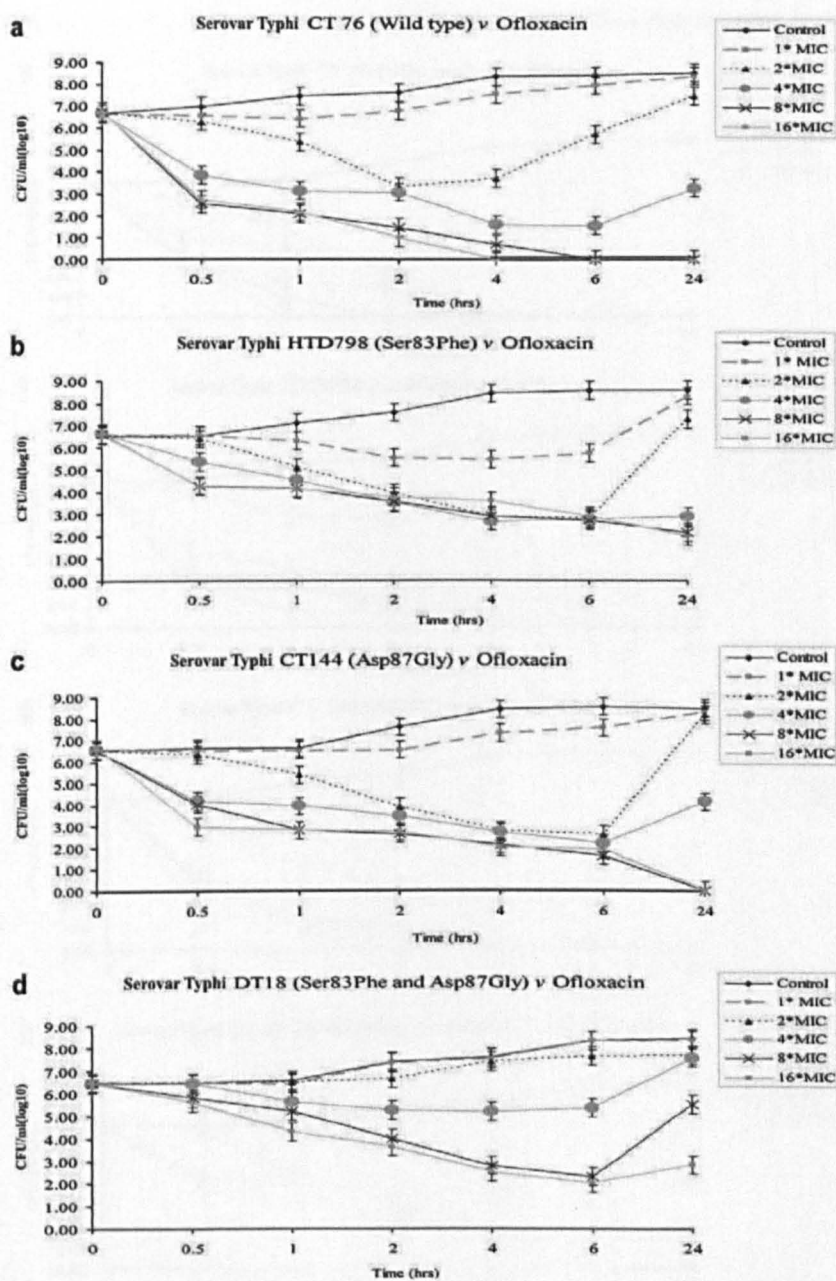


FIG. 2. In vitro time-kill experiments of wild-type serovar Typhi and serovar Typhi harboring single and double amino acid substitutions in GyrA. Figure 2a to d shows exposure to ofloxacin, and Fig. 2e to h shows exposure to gatifloxacin at concentrations of 1× to 16× MIC over 24 h. Results represent means of duplicate values; the standard deviation is indicated by error bars.

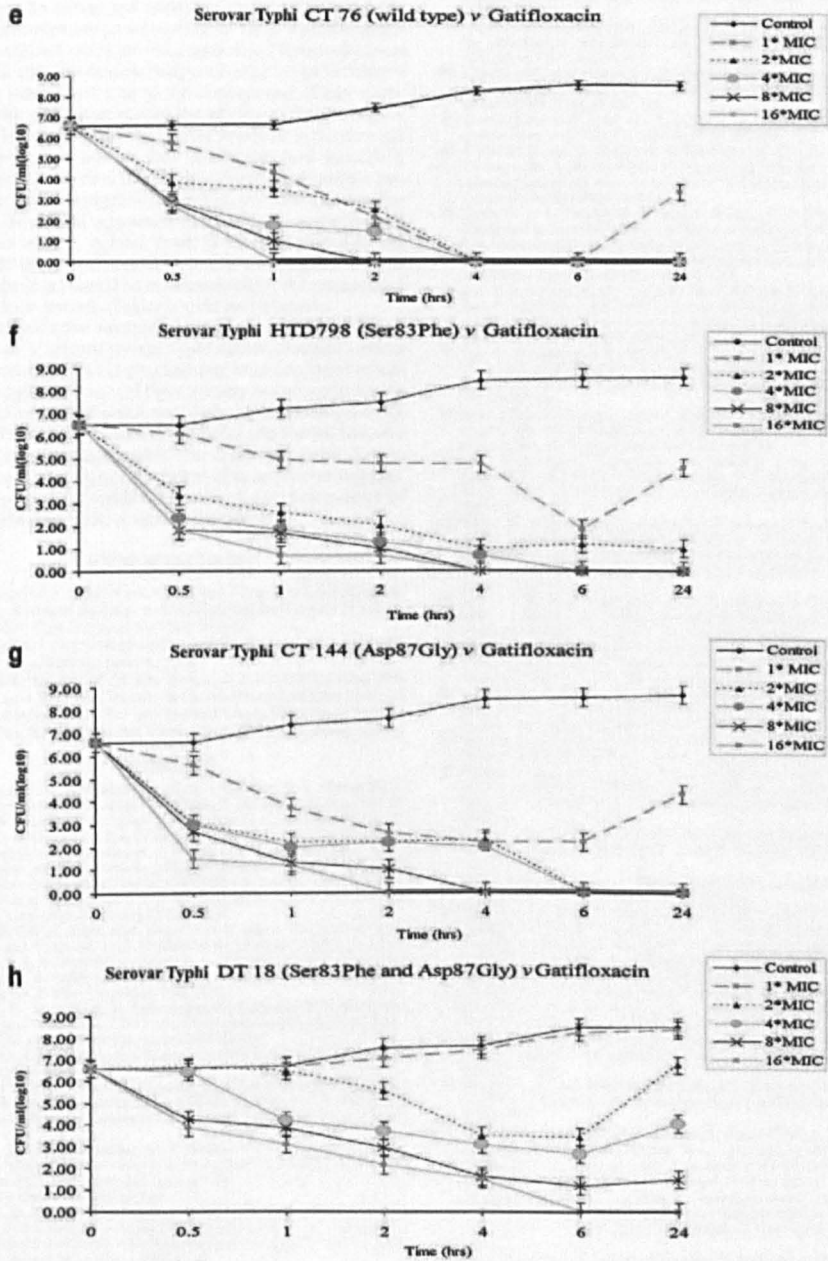


FIG. 2—Continued.

Our time-kill experiments suggest that the choice of the fluoroquinolone and the dose used for the treatment of serovar Typhi may be critical and underline that clearly not all the fluoroquinolones are as susceptible to these common mutations. Continued use of the older-generation fluoroquinolones (ofloxacin and ciprofloxacin) may encourage the persistence of resistant isolates and lead to the development of new mutations which might compromise the efficacy of the newer generation. With lower MICs and better responses in the time-kill experiments, it is possible that gatifloxacin (and potentially other newer-generation fluoroquinolones) would prove a better choice for use in typhoid fever. This provides a clear rationale for the clinical assessment of these drugs in randomized controlled trials in typhoid fever. If these *in vitro* data are supported by clinical results, then this newer generation of fluoroquinolones should be recommended for the treatment of typhoid fever instead of ciprofloxacin and ofloxacin.

In conclusion, the emergence and persistence of MDR and nalidixic acid-resistant serovar Typhi strains constitute a major problem across Asia. No drug has ever been developed specifically for typhoid fever, and there are very few potential targets in *Salmonella* against which new drugs could be designed (3). We need to use our current drugs better and use the best and most affordable drugs available in order to prevent further resistance. Knowledge of the extent of drug resistance should be an important factor when discussing the implementation of a comprehensive typhoid vaccination strategy.

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An Open Randomized Comparison of Gatifloxacin versus Cefixime for the Treatment of Uncomplicated Enteric Fever

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Objective. To assess the efficacy of gatifloxacin versus cefixime in the treatment of uncomplicated culture positive enteric fever. **Design.** A randomized, open-label, active control trial with two parallel arms. **Setting.** Emergency Room and Outpatient Clinics in Patan Hospital, Lagankhel, Lalitpur, Nepal. **Participants.** Patients with clinically diagnosed uncomplicated enteric fever meeting the inclusion criteria. **Interventions.** Patients were allocated to receive one of two drugs, Gatifloxacin or Cefixime. The dosages used were Gatifloxacin 10 mg/kg, given once daily for 7 days, or Cefixime 20 mg/kg/day given in two divided doses for 7 days. **Outcome Measures.** The primary outcome measure was fever clearance time. The secondary outcome measure was overall treatment failure (acute treatment failure and relapse). **Results.** Randomization was carried out in 390 patients before enrollment was suspended on the advice of the independent data safety monitoring board due to significant differences in both primary and secondary outcome measures in the two arms and the attainment of a priori defined endpoints. Median (95% confidence interval) fever clearance times were 92 hours (84–114 hours) for gatifloxacin recipients and 138 hours (105–164 hours) for cefixime-treated patients (Hazard Ratio[95%CI]=2.171 [1.545–3.051], $p<0.0001$). 19 out of 70 (27%) patients who completed the 7 day trial had acute clinical failure in the cefixime group as compared to 1 out of 88 patients (1%) in gatifloxacin group (Odds Ratio [95%CI]=0.031 [0.004 – 0.237], $p<0.001$). Overall treatment failure patients (relapsed patients plus acute treatment failure patients plus death) numbered 29. They were determined to be (95% confidence interval) 37.6% (27.14%–50.2%) in the cefixime group and 3.5% (2.2%–11.5%) in the gatifloxacin group (HR[95%CI]=0.084 [0.025–0.280], $p<0.0001$). There was one death in the cefixime group. **Conclusions.** Based on this study, gatifloxacin is a better treatment for uncomplicated enteric fever as compared to cefixime. **Trial Registration.** Current Controlled Trials ISRCTN75784880

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INTRODUCTION

Enteric fever (Typhoid and Paratyphoid fever) is a systemic infection caused by the bacterium *Salmonella enterica* serovar Typhi (*S. typhi*) or *Salmonella enterica* serovar Paratyphi (*S. paratyphi*) which in humans is transmitted through the fecal-oral route [1,2]. Today the vast burden of disease is encountered in the developing world where sanitary conditions remain poor. The best global estimates are of at least 22 million cases of typhoid fever each year with 200,000 deaths [3]. Crucially these are almost exclusively confined to resource poor countries. A recent Cochrane review [4] on typhoid treatments underscored the need for large sample size drug interventional trials, especially in children in whom this disease predominates.

In 1948 the introduction of chloramphenicol revolutionized the treatment of typhoid fever [5,6]. Unfortunately the emergence of resistance to the “first line” antimicrobials (for example, ciprofloxacin) has been a major setback and has given rise to the possibility of untreatable enteric fever [7,8]. Gatifloxacin, a relatively inexpensive fluoroquinolone antibiotic in South Asia with once daily oral administration, is a new broad spectrum synthetic 8-methoxyfluoroquinolone which has the lowest minimum inhibitory concentration (MIC) against *S. typhi* from Nepal [9]. This in vitro activity needs to be verified clinically before gatifloxacin can be recommended for widespread use. Cefixime, an orally administered third generation cephalosporin, is a commonly used drug in South Asia for the treatment of enteric fever. Although cefixime is recommended as a drug of choice by the

World Health Organization (WHO) for the treatment of resistant typhoid fever [10] it is relatively expensive in South Asia and has to be administered for a longer duration than the currently used fluoroquinolones. Clearly there is an urgent need for a treatment [11] that combines ease of oral administration, with speed of clinical response, reduction in secondary transmission and inexpensiveness. In this open randomized trial, we aimed to compare clinical outcomes for the treatment of uncomplicated enteric fever with gatifloxacin or cefixime in an outpatient setting.

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METHODS

Participants

The study was approved by Nepal Health Research Council and Oxford Tropical Research Ethics Committee. The protocol for this trial and supporting CONSORT checklist are available as supporting information; see Checklist S1 and Protocol S1. We enrolled patients who presented to the outpatient or emergency department of Patan Hospital, Lalitpur, Nepal from June 5, 2005 to September 8, 2005. Patan Hospital is a 313 bed hospital located in the Lalitpur district in Kathmandu Valley. Patients were eligible to enter the study if they had clinically diagnosed enteric fever and their residence was within approximately 2.5 km radius from the hospital. Other inclusion criteria were that patients must be aged between 2 and 65 years, able to take oral medications, non-pregnant and non-lactating, without a history of seizures, able to stay in the city for the duration of the treatment, not known to have contraindications to either cephalosporins or fluoroquinolones and willing to give informed written consent to take part in the study. For children enrolled into the study, written informed consent was taken from a parent. Patients were excluded from the study if they had any signs of complicated typhoid defined as the presence of jaundice, gastrointestinal bleeding, peritonism, shock, encephalopathy, convulsions, myocarditis or arrhythmia at the time of enrollment. Patients who had received a third generation cephalosporin, fluoroquinolone or macrolide in the week prior to presentation to our clinic were also excluded.

Interventions

On presentation to Patan Hospital all patients with fever without an obvious focus were referred to the enteric fever study clinic, where they were seen by the study physician. Patients who fulfilled the inclusion criteria were randomly assigned to receive Gatifloxacin (Broadband™, Novartis AG Basel, Switzerland) 10 mg/kg/day [12], in a single dose orally for 7 days or Cefixime (Cifex™, Aegis, Nicosia, Cyprus) 20 mg/kg/day [13] in two divided doses orally for 7 days. Both drugs were administered in tablet form, cut and weighed in a sensitive scale to ensure that underdosing did not occur. To children who were apprehensive of swallowing the tablet, the drug was embedded in a banana and given. All patients were asked to swallow the study drug under direct observation during each visit.

Each patient had haematocrit, total leucocyte count with differential, serum creatinine, total bilirubin, alanine aminotransferase (ALT), and aspartate aminotransferase (AST) measured, and blood and stool cultures were also performed before the start of the study intervention.

The exact location of the patient's home was recorded and the first dose of drug administered at the clinic. We employed six Community Medical Auxiliaries (CMA) who had all received at least 15 months of prior formal primary health care worker training and been registered in a government recognized institution. The CMAs visited patients twice daily at their homes to perform a simple clinical assessment, measure the oral temperature and give directly observed therapy with the study drugs. The CMA visited the patient's home every 12 hours, morning and evening, until day 10 following enrollment or complete resolution of illness, whichever came later. The oral temperature of the patient was recorded twice every day by the CMA and a note was made of the timing and dosages of acetaminophen intake. The quality of patient-visits was ensured by regular unplanned supervisory checks in which the study doctor accompanied the CMA during the visits to patients' homes.

CMAs were asked to send patients immediately to the hospital on encountering any severe symptoms, and the patients also were asked to attend clinic if they had any severe symptoms at any other time. A symptom questionnaire was used daily during each visit to monitor any adverse events. Any patient with any severe symptom was seen by the study physician. The CMAs and study physicians held daily case conferences at which all the study patients were discussed.

All patients regardless of the culture results were seen at hospital on Day 10 following enrollment. Blood and stool cultures were repeated on Day 10 in all culture positive patients and thereafter if the patient again became ill with probable enteric fever. All culture positive patients were followed up until six months after enrollment, and stool cultures were performed at the end of the first, third and sixth month.

Microbiological Procedures Blood culture was performed on media containing tryptone soya broth and sodium polyethanol sulphate, incubated at 37°C and examined daily for growth over 7 days [11]. *Salmonella enterica* serotype Typhi or Paratyphi A, B or C isolated in culture were identified using standard biochemical tests and specific antisera (Murex Biotech, Dartford, England). Antibiotic susceptibilities were determined during isolation using the Kirby-Bauer disc diffusion method involving antibiotic discs containing Nalidixic acid, Ofloxacin, Ciprofloxacin, Chloramphenicol, Ampicillin, Cotrimoxazole, Cefixime and Cefotaxime (HiMedia Laboratories, Mumbai, India). Minimum Inhibitory Concentrations (MICs) were determined later for organisms stored in glycerol (bacterial preserver) at -70°C. The MICs were determined by Chloramphenicol, Nalidixic acid, Gatifloxacin, Cefixime, Ceftriaxone and Gemifloxacin E-tests™ (AB Biodisk, Solna, Sweden), according to the manufacturer's instructions. The sensitivity tests were interpreted using Clinical and Laboratory Standards Institute criteria for Enterobacteriaceae.

Objectives

The objective of the study was to compare the efficacy of Gatifloxacin and Cefixime in the treatment of uncomplicated culture positive enteric fever.

Outcomes

The primary outcome was the fever clearance time (FCT). FCT was defined as time to first drop in oral temperature $\leq 37.5^\circ\text{C}$, remaining $\leq 37.5^\circ\text{C}$ for 48 hours. The secondary outcomes included acute treatment failure. Acute treatment failure was defined as including any severe complication; the persistence of fever ($> 38^\circ\text{C}$); the persistence of symptoms for more than 7 days after the start of treatment, requiring additional or rescue treatment. If a patient had a temperature above 37.5 and below 38 for more than 7 days, but did not need additional or rescue therapy, and subsequently their fever cleared by day 10, that patient would not qualify as an acute treatment failure. Patients who failed the study treatment were given rescue treatment.

The rescue drug was Ofloxacin 20 mg/kg/day orally in two divided doses for 14 days for the Cefixime group, and Ceftriaxone 40 mg/kg/day IV in a single daily dose for 14 days for the Gatifloxacin group. For the Cefixime group alone, if on day 8 of treatment the patient still had a fever of $\geq 38^\circ\text{C}$, the study drug was continued for 10 days and the patient categorized as acute treatment failure. If the temperature on Day 10 was >37.5 , rescue treatment was given.

A relapse was defined as fever with a positive blood culture within a month of completing treatment. All the relapses were

patients that were initially categorized as successfully treated. Any patient given rescue treatment or prolonged treatment was precluded from the "relapse" group.

Patients categorized as "overall treatment failures" included patients experiencing acute treatment failure, plus those falling into the relapsed category, plus all deaths within the trial follow up period.

Sample size

The sample size was calculated to detect a FCT difference of approximately 48 hours between gatifloxacin (assumed median FCT 156 hrs) and cefixime (assumed median FCT 204 hrs) [14] with $p = 0.05$ and power = 80%. The accrual time for recruitment was assumed to last 70 days, and that the last patient would be followed up until 8 days after recruitment. Therefore, we estimated the minimum sample size at 235 participants. Assuming a loss to follow-up of 5%, the sample size was calculated as 125 blood culture positive patients in each arm.

Before the recommended sample size had been reached, once 169 blood culture positive patients had been enrolled, the independent data safety monitoring committee (DSMC) advised the Principal Investigators to stop recruitment to the trial based on a priori defined difference ($p < 0.01$) between the two treatment arms in the primary endpoints of the study.

Randomization—Sequence generation

Patients were randomized in blocks of 100 from a computer generated randomization list, by an investigator not involved in patient recruitment or assessment.

Randomization—Allocation concealment

The randomization sequence and block size was concealed from the physicians allocating treatment and managing the patients, prior to patient enrollment. Treatment allocations were kept in sealed opaque envelopes, which were opened only on enrollment of the patient to the study after all inclusion and exclusion criteria had been checked.

Randomization—Implementation

Participants were enrolled by the study physician in the same order in which they presented to the study clinic. The sealed envelopes were opened in strict numeric sequence.

Blinding

Blinding was not feasible in this trial due to logistical reasons.

Statistical methods

All data were entered into an electronic database (Microsoft Office Access Version 2003, Wash., USA), and analyses were performed using Stata 9 (Stats Corp LP, Texas, USA). Continuous covariates were compared between groups of patients using the Mann-Whitney test, and categorical covariates were compared using the chi-square test or Fisher's exact test when appropriate. Fever clearance times and time to relapse were analyzed using Kaplan Meier survival curves and compared between the two groups using the logrank test. Binary outcomes (clinical failures) were compared between the two treatment groups using Fisher's exact test. Analysis was done in all randomized patients (intention to treat, ITT) and separately in patients with positive pretreatment culture (per protocol, PP) and negative pretreatment culture.

RESULTS

Participant flow

Of the 482 patients from the study area who were clinically diagnosed with enteric fever, 390 patients were enrolled into the study and randomized. 92 patients were ineligible, the main reason (49 patients) being a history of already having taken antibiotics (fluoroquinolone, macrolide, or third generation cephalosporin) within one week prior to study entry (Figure 1).

Among all randomized patients, 187 patients were assigned to receive cefixime and 203 to gatifloxacin. 77 patients assigned to receive cefixime were blood culture positive for enteric fever whilst 92 of those assigned to receive gatifloxacin were culture positive. There were unequal number of positive patients in each of the study arms. One possible reason for the difference in number of culture positive patients between study arms is that cultures were drawn and culture results obtained after randomization had been done.

Recruitment

We enrolled patients who presented to the outpatient or emergency department of Patan Hospital, Lalitpur, Nepal from June 5, 2005 to September 8, 2005. All enrolled patients were followed up for at least 10 days after recruitment. Patients with a positive pretreatment blood culture were followed up for 6 months after enrollment.

At the point that the DSMC asked to examine the trial data for the primary outcome measure in positive pre-treatment patients, the median fever clearance time was 92 hours (95% CI, 84–114 hours) for the gatifloxacin treated patients and 138 hours (95% CI, 105–164 hours) for cefixime treated patients. The difference between the two treatment arms was 46 hours ($p < 0.0001$).

Baseline data

Admission characteristics are shown for all ITT patients (Table 1) and for all PP patients (Table 2). The median age of patients enrolled into the trial was 17 with a range of 2–64 years. There were no baseline differences in the culture positive and culture negative groups, other than temperature at presentation, AST and ALT which were higher and platelets and total WBC which were lower in the culture positive patients as compared to the culture-negative patients. Among all PP patients, there were no differences in the baseline characteristics between the two treatment groups. There were 40 patients, 15 in the gatifloxacin arm and 25 in the cefixime arm, who had taken amoxicillin up to the week before study entry. Of these 4 and 7 were culture-positive respectively.

Numbers analyzed

Analysis was done in all 390 randomized patients (ITT) and separately in 169 patients with positive pre-treatment culture (PP). All endpoints were analyzed in the ITT and PP populations, apart from relapse which was only analyzed in the PP population.

Outcomes and estimation

Primary outcome In all ITT patients, median (95% confidence interval) fever clearance time was 102 (90–117) hours for the cefixime group and 72 (62–80) hours for the gatifloxacin group, logrank test $p < 0.0001$, Hazard Ratio [95% Confidence Interval] = 1.821 [1.466–2.263]. The proportion of all patients failing through time to clear fever is shown in Figure 2. At day 7 fever clearance rate was 73.9% (67.0%–80.3%) in cefixime group and 94.2% (90.2%–96.9%) in gatifloxacin group.

In the PP group, median (95% CI) fever clearance time was 92 hours (84–114 hours) for gatifloxacin recipients and 138 hours

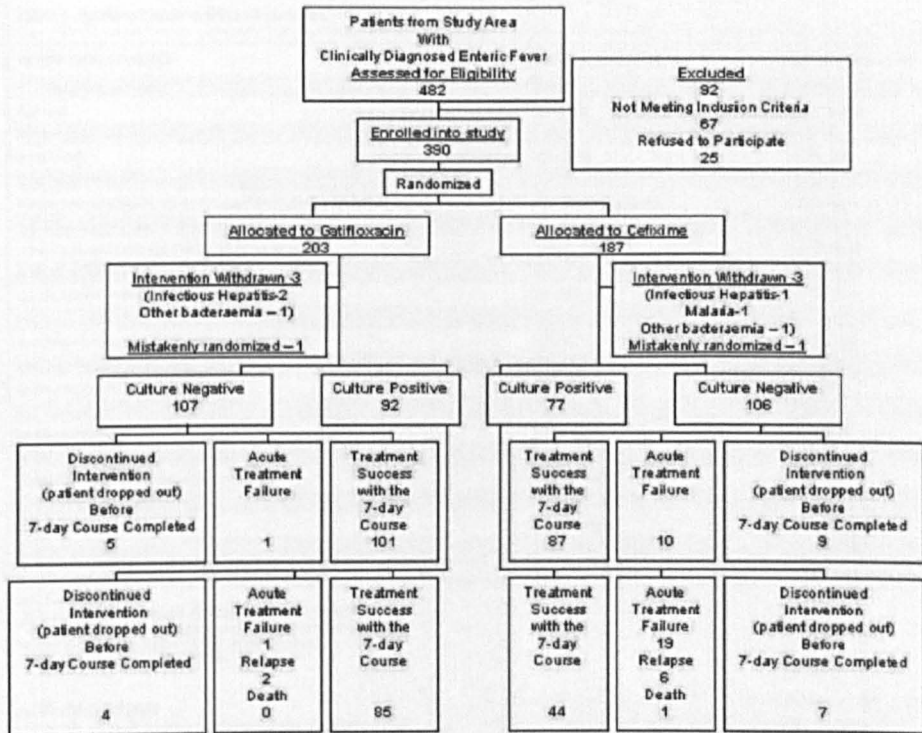


Figure 1. Profile of the Trial. The consort flow diagram showing the flow of participants through the trial. doi:10.1371/journal.pone.0000542.g001

(105–164 hours) for cefixime-treated patients (HR [95% CI] = 2.171 [1.545–3.051], $p < 0.0001$). The proportion failing to clear fever for each study drug through time after treatment is shown (Figure 3). At day 7 the fever clearance rate was 62.7% (95% CI = 51.5%–73.8%) in the cefixime group and 91.8% (95% CI = 84.8%–96.4%) in the gatifloxacin group.

In the group with negative blood culture but clinically diagnosed enteric fever (Fig 1), the FGT was 82 hours (95% CI = 44–94 hours) for the cefixime group and 39 hours (95% CI = 28–54 hours) for the gatifloxacin group (HR [95% CI] = 1.740 [1.309–2.312], $p < 0.0001$ logrank test).

Secondary Outcomes In the ITT group, overall, 30 out of 167 (18%) in the cefixime group and 2 out of 190 (1%) in the gatifloxacin group were acute clinical failures, OR [95% CI] = 0.049 [0.011–0.207], $p < 0.001$, Fisher's exact test.

In the PP group, 19 out of 70 (27%) patients who completed the 7-day trial had acute clinical failure in the cefixime recipients as compared to 1 out of 88 (1%) in the gatifloxacin recipients (Odds Ratio [95% CI] = 0.031 [0.004–0.237], $p < 0.001$). Considering all patients to be failures who dropped out of the study before completion of the seven day treatment course, 26 out of 77 (34%)

failed in the cefixime group as compared to 5 out of 92 (5%) in the gatifloxacin group (OR [95% CI] = 0.112 [0.041–0.312], $p < 0.001$).

138 patients were evaluable for relapse; 20 had acute treatment failure and 11 withdrew from the study before day 7. In total, eight relapses (Figure 1) were observed. Relapse rates were 12.4% (6/51) in the cefixime group and 3.4% (2/87) in gatifloxacin group (HR [95% CI] = 0.185 [0.037–0.915], $p = 0.0199$). The Kaplan-Meier plots for the time of relapse are shown in Figure 4.

Overall failures (acute treatment failure plus relapse plus death) were 29 in number (Figure 1). Overall failure rate at 1 month was estimated as 37.6% (95% CI = 27.14%–50.2%) in the cefixime group and 3.5% (95% CI = 2.2%–11.5%) in the Gatifloxacin group (HR [95% CI] = 0.084 [0.025–0.280], $p < 0.0001$) (Figure 5).

From patients with negative cultures, 11 had acute clinical failures, 10 (out of 97, 10%) in Cefixime group and 1 (out of 103, 1%) in the Gatifloxacin group, OR [95% CI] = 0.086 [0.011–0.686], $p = 0.004$, Fisher's exact test.

Similarly, treating drop-out as treatment failures, we had 50 out of 187 (27%) in the Cefixime group and 15 out of 203 (7%) in the Gatifloxacin group acute treatment failures, OR [95% CI] = 0.219 [0.118–0.405], $p < 0.001$, Fisher's exact test.

Table 1. Baseline characteristics all patients.

PATIENT CHARACTERISTICS	Culture negative (213)	Culture positive (169)
No of males/No of females	136/77	111/58
Age (yrs)	18 (2-64)	17 (2.75-30)
Number Aged <14 years (%)	79(37.1)	60 (35.5)
Weight (Kg)	44 (10-80)	46 (10-73)
Duration of fever before treatment (days)	5 (0-21)	5 (2-23)
Median oral temperature at presentation(95% CI, range) (in degrees C)	38.7 (38.6-39; 36.5-40.7)	39(38.8-39.2; 36.8-41)
Headache, Number with (%) (median duration (days))	204 (95.7) (4)	164 (97.0) (5)
Anorexia, Number with (%) (median duration (days))	160 (75.1) (4)	129 (76.3) (4)
Abdominal Pain, Number with (%) (median duration (days))	88 (41.3) (4)	80 (47.3) (4)
Cough, Number with (%) (median duration (days))	83 (39.0) (3)	59 (34.9) (3)
Diarrhoea, Number with (%) (median duration (days))	45 (21.1) (3)	41 (24.3) (3)
Vomiting, Number with (%) (median duration (days))	30 (14.1) (1)	27 (16.0) (2)
Abdominal tenderness (n (%))	32 (15.1)	23 (13.6)
Splenomegaly (n (%))	18 (8.5)	18 (10.6)
Hepatomegaly (n (%))	12 (5.76)	9(5.3)
Hematocrit (in%)	40 (27-53)	40 (29-50)
White Cell Count (in $\times 1000$ per microlitre)	7.2 (2.3-24.2)	6.7 (3.0-20.0)
Platelet Count (in $\times 1000$ per microlitre)	192 (66-546)	180 (65-380)
* ALT (in U/L)	30(11-240)	37 (12-200)
**AST (in U/L)	43 (20-354)	52 (21-169)
Total Bilirubin (in mg/dL)	0.8 (0.17-3.6)	0.89 (0.18-3.2)

Baseline epidemiological, clinical and laboratory features at presentation of all intention to treat patients showing a comparison between culture positive and culture negative groups.

*ALT (serum alanine aminotransferase) normal range 5-34 U/L

**AST (serum aspartate aminotransferase) normal range 5-34 U/L

All data presented as median (range) unless specified.

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Ancillary analyses

Among all culture positive patients in the cefixime group, one patient (1/70, 1%) had *S. Paratyphi A* cultured from her blood on day 10, but there were no (0/83, 0%) positive blood culture growths in the gatifloxacin group on day 10.

No patient was found to be a persistent carrier of *S. Typhi* or *Paratyphi A* in their stool. A positive stool culture for *S. Typhi* was seen for one patient on day 10 and for another on day 30. Subsequent cultures were negative for both patients. We were able to obtain stool cultures from 147 (87%), 141 (83%), and 130 (77%) pretreatment blood culture positive patients at one, three, and six months respectively.

Microbiology Antibiotic sensitivity testing revealed that all strains were sensitive to gatifloxacin, cefixime, ceftriaxone or gemifloxacin. One strain was resistant to chloramphenicol, and 136 (83%) of the pretreatment isolates were nalidixic acid resistant strains (NARST). Minimum inhibitory concentration (MIC) was determined for 161 of the pretreatment blood culture isolates. The median (range) MICs for each antibiotic were as follows: gatifloxacin 0.125 (0.006-0.5) $\mu\text{g}/\text{mL}$, cefixime 0.380 (0.016-2.0) $\mu\text{g}/\text{mL}$, nalidixic acid >256 (1.5->256) $\mu\text{g}/\text{mL}$, chloramphenicol 8.0 (1.5->256) $\mu\text{g}/\text{mL}$, ceftriaxone 0.125 (0.047-0.5) $\mu\text{g}/\text{mL}$ and gemifloxacin 0.125 (0.004-0.5) $\mu\text{g}/\text{mL}$.

Adverse events

Among all patients who received cefixime, there was one death, which might have been due to the development of disease-related complications during treatment. This patient was enrolled on the

fourteenth day of his illness. On day 6 of treatment the patient complained of reddish stool and petechiae and was immediately admitted to hospital where he developed severe thrombocytopenia and gastrointestinal bleeding. He developed acute respiratory distress syndrome and was mechanically ventilated. He developed disseminated intravascular coagulation and succumbed to his illness on day 21 of entry into the trial. His pretreatment blood culture grew *S. Paratyphi A* which was sensitive to cefixime with an MIC of 0.38 $\mu\text{g}/\text{mL}$. One patient developed erythematous skin rash which needed two doses of oral antihistamine.

Among all patients who received gatifloxacin there were 2 patients with excessive vomiting, which required intravenous antiemetics and fluids and observation in the hospital emergency room for up to 6 hours. There were an additional 23 patients who complained of excessive nausea and occasional vomiting after ingestion of the drug. Of these, two needed oral antiemetics; in the remaining 21 patients no intervention was required.

DISCUSSION

Interpretation

In this study examining fever clearance time, acute treatment failure and relapse as indicators of treatment efficacy, that the results raise doubts on the usefulness of cefixime and suggest that gatifloxacin is a potent choice for the treatment of uncomplicated enteric fever.

Febrile illness is one of the most common reasons for presentation to hospitals in many developing countries. In patients with fever, a very common clinical diagnosis is enteric fever, and *S.*

Table 2. Baseline characteristics at presentation of culture positive patients.

PATIENT CHARACTERISTICS	GATFLOXACIN (n = 92)	CEFDIOXIME (n = 77)
No of males/No of females	67/25	44/33
Age (yrs)	18 (2.75–45)	15 (3–50)
Number Aged <14 years (%)	27 (29%)	33 (43%)
Weight (Kg)	49 (10–70)	42 (11–73)
Duration of fever before treatment (days)	5.2	5.4
Median oral temperature at presentation(95% CI, range) (in degrees C)	39 (38.9–39.2; 37.5–41.0)	39 (38.8–39.2; 36.8–40.5)
Headache, Number with (%) (median duration [days])	88 (95.7%) (5)	76 (98.7%) (4.5)
Anorexia, Number with (%) (median duration [days])	75 (79.3%) (4)	56 (73%) (4)
Abdominal Pain, Number with (%) (median duration [days])	43 (46.7%) (4)	40 (52%) (4)
Cough, Number with (%) (median duration [days])	37 (40.2%) (3)	22 (29%) (3)
Diarrhoea, Number with (%) (median duration [days])	21 (22.8%) (3)	20 (26%) (3)
Vomiting, Number with (%) (median duration [days])	17 (18.5%) (2)	10 (13%) (1.5)
Abdominal tenderness (n (%))	14 (15.2%)	8 (10.4%)
Splenomegaly (n (%))	10 (10.9%)	8 (10.4%)
Hepatomegaly (n (%))	5 (5.4%)	4 (5%)
Hematocrit (in%)	41 (30–50)	40 (29–50)
White Cell Count (in $\times 1000$ per microlitre)	6.9(3.0–18)	6.7 (3.1–20)
Platelet Count (in $\times 1000$ per microlitre)	180(65–367)	186 (120–380)
* ALT (in U/L)	36 (12–180)	39(18–200)
**AST (in U/L)	53 (24–155)	49 (21–169)
Total Bilirubin (in mg/dL)	0.85 (0.18–3.2)	0.9 (0.35–2.3)
Positive pretreatment fecal cultures (n (%))	9 (9.8%)	3 (3.8%)

Baseline epidemiological, clinical and laboratory features at presentation of all blood culture positive patients showing a comparison between the gatifloxacin and cefixime arms.

*ALT (serum alanine aminotransferase) normal range 5–34 U/L

**AST (serum aspartate aminotransferase) normal range 5–34 U/L

All data presented as median (range) unless specified.

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antigen serotype Typhi or Paratyphi A are the two most commonly isolated pathogens from the blood in febrile patients in our hospital [15]. Before the advent of multi-drug-resistant (MDR) *S. Typhi*, chloramphenicol, ampicillin or cotrimoxazole were successfully used as the first line drug in the treatment of enteric fever. After the emergence of MDR strains, fluoroquinolones and third-generation cephalosporins have been suggested and used as

alternative antimicrobials [16,13]. However the emergence and spread of point mutations in the *gyrA* gene of the bacterial genome [17] has conferred resistance to nalidixic acid and reduced susceptibility to the commonly used fluoroquinolones such as ofloxacin, leading to a poorer clinical response [18,19]. A recent study in Viet Nam (CM Parry, unpublished) showed ofloxacin at the dose of 20 mg/kg/day was able to achieve a cure rate in only

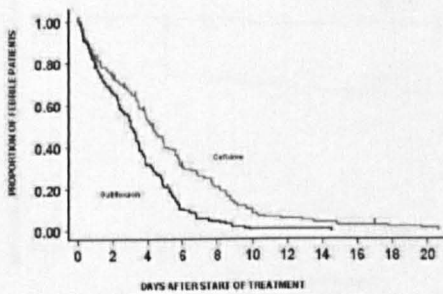


Figure 2. Proportion of all patients still febrile. Kaplan-Meier survival curve showing the proportion of all patients (TT) still febrile through time.
doi:10.1371/journal.pone.0000542.g002

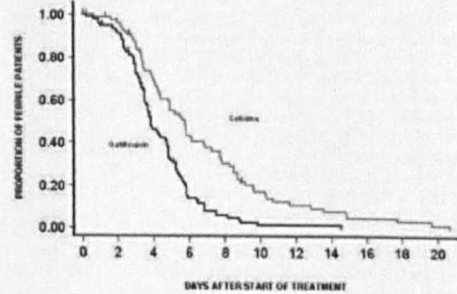


Figure 3. Proportion of culture positive patients still febrile. Kaplan-Meier survival curve showing the proportion of culture positive (PP) patients still febrile through time.
doi:10.1371/journal.pone.0000542.g003

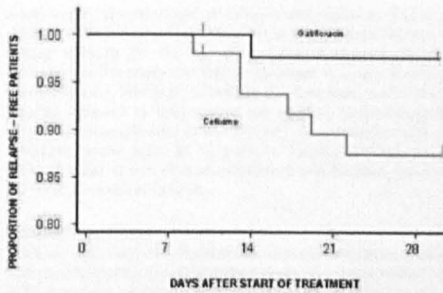


Figure 4. Proportion of relapse free patients. Kaplan-Meier survival curve showing the proportion of relapse free patients in the culture positive population.

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64% of patients. In our context of high nalidixic acid resistance, gatifloxacin is the most effective and appropriate choice for treatment of enteric fever. Gatifloxacin (Sandoz, India) is relatively inexpensive (US\$1.2 for a 7 day treatment course) and needs to be administered just once a day; both of these features are attractive in this setting. Gatifloxacin has a different binding motif than some other fluoroquinolones [20], and this characteristic enables it to retain activity against *Salmonella enterica* serovar Typhi or *Salmonella enterica* serovar Paratyphi A even in the presence of marked reduction in sensitivity to the older fluoroquinolones [17].

Cefixime, a third generation cephalosporin, is widely trusted to be effective for enteric fever as first line treatment, and is also used as second line therapy when initial treatment with a fluoroquinolone in a patient suspected to be enteric fever fails [13]. The fact that we saw a high overall failure rate associated with cefixime despite all of the strains being fully sensitive in vitro to the drug shows that the mechanism of action of cefixime [21,22] may not be suited to the eradication of *S. Typhi* or Paratyphi A from the body or blood, and the poor intracellular penetration into macrophages and reticulo endothelial tissues where the typhoid organisms colonize [16] may be the cause of high failure rates.

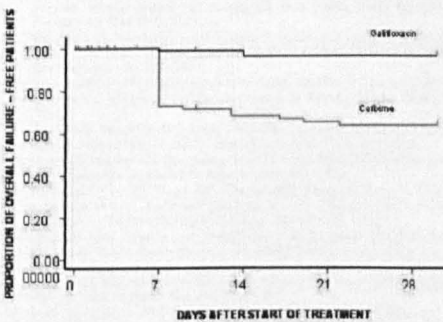


Figure 5. Proportion of overall failure free patients. Kaplan-Meier survival curve showing the proportion of overall failure free patients in the culture positive population.

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This study was unique in that we used CMAs to simulate a hospital setup in the community. CMA's directly observed patients taking the therapies, monitored fever and identified complications early; these characteristics have not been used in the past for typhoid trials although enteric fever in endemic areas [1] is treated on an outpatient basis. A major advantage of follow-up using CMAs was that the health workers knew the exact house location of the patients, and therefore follow up even after the successful completion of the initial seven-day drug trial was possible. In developing countries follow up of patients can be very difficult because of a lack of a proper address and relative unavailability of other means of communication, for example, a telephone.

In addition to its relevance to culture confirmed enteric fever, another major strength of this large randomized study is that gatifloxacin proved to be more efficacious than cefixime with respect to fever clearance time and failure rates, even in the subgroup of patients who were clinically presumed to have enteric fever but who had a negative blood culture. Antibiotic treatment for typhoid in highly endemic areas is usually started based on the presence of a "syndromic" illness (acute fever for a few days and constitutional symptoms with no known source of infection) before culture results are known. Enteric fever, which continues to be a neglected disease [23], is an important cause of morbidity and mortality, and facilities for blood culture or other reliable methods of diagnosis rarely exist in this setting.

Generalizability

Despite widespread resistance to Nalidixic acid in Kathmandu, and rising MICs to the older fluoroquinolones, ciprofloxacin and ofloxacin, gatifloxacin has proven to be a potent drug for the treatment of enteric fever. Our study has relevance to South Asia, as resistance to nalidixic acid is widely prevalent there. Inevitably there will be emergence of resistance to gatifloxacin in areas with both MDR and NARST; and in this situation alternative antibiotics like azithromycin may need to be used. Of interest, in keeping with anecdotal reports from elsewhere in South Asia, only one strain was resistant to chloramphenicol in the present study. In areas of the world where chloramphenicol susceptibility has reemerged there may be an argument for reassessing chloramphenicol.

In the present study Gatifloxacin was associated with nausea in 12% of patients and it may be important to forewarn patients of this possible side effect. There have been sporadic reports of dysglycemia caused by gatifloxacin [24-26], and a recent population-based, case controlled study examining gatifloxacin usage amongst elderly individuals in Canada (mean age 77 years) who developed dysglycemia [27] also raises possible concerns. We did not do any blood sugar testing to look for dysglycemia. However in a study involving a younger age group where blood sugar testing was done the results revealed no dysglycemia: 887 children were treated with gatifloxacin (10 mg/kg) for otitis media and were followed for a year with no signs of alteration of glucose homeostasis either acutely or otherwise [28]. Clearly, it would be prudent to treat diabetics and elderly people suffering from enteric fever with an alternative antibiotic such as azithromycin and avoid the potential problems in this specific population with gatifloxacin.

Limitations of the study The DSMG advised the Principal Investigators in this study to stop recruitment to the trial based on a priori defined difference ($p < 0.01$) between the two treatment arms in the primary endpoints of the study. It is possible that if the trial had been continued with a larger sample size, other important information could have been gathered. In addition if patients and/or investigators had been blinded to treatment assignments, the

study would have been further strengthened; however as in most typhoid trials, it was not possible to do this due to the difference in dosing schedule for the two drugs being compared. Another limitation of this study was that temperature was only measured every 12 hours. However, to address this limitation, and to avoid missing increases in temperature, we checked temperatures for 10 days after enrollment, or for 48 hours after resolution of fever, whichever came later, in all patients. Finally, a telephone or internet based system of randomization would be ideal, but such a system does not exist here.

Overall evidence

We have compared the outcomes from our trial with those of other comparable studies, identified from a recent Cochrane review [4], WHO typhoid guidelines [10], and a search of Medline using these terms: cefixime, typhoid trials. The findings in our study are consistent with those of a 1995 study done in Viet Nam which showed that cefixime (20 mg/kg/day) for 7 days was inferior to ofloxacin (10 mg/kg/day) for 5 days in the treatment of MDR typhoid fever in children [14]. However other studies have suggested cefixime can be successful in the treatment of enteric fever [29–33]. Overall these studies, both descriptive and randomized, have examining the use of cefixime in confirmed enteric fever (total of 292 patients) and with treatment durations of mostly 14 days, have found failure rates ranging from 4% to 23%. Besides the general undesirability of a longer course with cefixime with increased morbidity and possibly complications, this drug is also more expensive (a 7-day course costs US \$7 (Blue Cross Laboratories, India)). The present study is the largest randomized controlled trial ever conducted with cefixime in enteric fever and clearly shows, even in a setting with fully sensitive strains, that cefixime is a poor drug for this disease. These findings are contrary to the recommendation by many sources [13,1] including the World Health Organization [10] that cefixime can be used as first

or second line therapy in the treatment of enteric fever. Based on the present study, we believe gatifloxacin to be an optimal choice in the treatment of uncomplicated enteric fever.

SUPPORTING INFORMATION

Checklist S1 CONSORT Checklist

Found at: doi:10.1371/journal.pone.0000542.s001 (0.05 MB DOC)

Protocol S1 Trial Protocol

Found at: doi:10.1371/journal.pone.0000542.s002 (0.05 MB DOC)

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Author Contributions

Conceived and designed the experiments: JF BB AP AA JD MZ BY CD BP. Performed the experiments: JF BB AP AA JD SD MZ BY JC CD BP. Analyzed the data: KS. Contributed reagents/materials/analysis tools: JF BB AP AA JD SD MZ BY JC CD BP. Wrote the paper: JF BB AP AA JD MZ BY CD BP. Other: Helped write the paper: JC KS SD.

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Evolutionary History of *Salmonella* Typhi

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Abstract

For microbial pathogens, phylogeographic differentiation seems to be relatively common. However, the neutral population structure of *Salmonella enterica* serovar Typhi reflects the continued existence of ubiquitous haplotypes over millennia. In contrast, clinical use of fluoroquinolones has yielded at least 15 independent *gyrA* mutations within a decade and stimulated clonal expansion of haplotype H58 in Asia and Africa. Yet, antibiotic-sensitive strains and haplotypes other than H58 still persist despite selection for antibiotic resistance. Neutral evolution in Typhi appears to reflect the asymptomatic carrier state, and adaptive evolution depends on the rapid transmission of phenotypic changes through acute infections.

Many bacterial taxa can be subdivided into multiple, discrete clonal groupings (clonal complexes, or ecotypes) that have diverged and differentiated as a result of clonal replacement, selective sweeps, periodic selection, and/or population bottlenecks (1). Geographic isolation and clonal replacement can also result in phylogeographic differences between bacterial pathogens from different parts of the world (2), even within young, genetically monomorphic pathogens (3) (supporting online material text) such as *Mycobacterium tuberculosis* (4) and *Yersinia pestis* (5). Typhi is a genetically monomorphic (6), human-restricted bacterial pathogen that causes 21 million cases of typhoid fever and 200,000 deaths per year, predominantly in southern Asia, Africa, and South America (7). Typhi also enters a carrier state in rare individuals [such as Mortimer's example of "Mr. N the milker" (8)], who can shed high levels of these bacteria for decades in the absence of clinical symptoms. Genome sequences are available from strains CT18 (9) and Ty2 (10), but the global diversity, population

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genetic structure, and evolutionary history of Typhi were poorly understood. It has been speculated that Typhi evolved in Indonesia, which is the exclusive source of isolates with the z66 flagellar antigen (11).

We investigated the evolutionary history and population genetic structure of Typhi by mutation discovery (12) within 200 gene fragments (~500 base pairs each) from a globally representative strain collection of 105 strains. The 200 genes included 121 housekeeping genes, 50 genes encoding cell surface structures, regulation, and pathogenicity; and 29 pseudogenes. Size variation of a poly-T_{6,7} homopolymeric stretch within one gene fragment was inconsistent with other phylogenetic patterns (homoplasies) and this fragment was excluded from further analysis. The other 199 gene fragments cover 88,739 base pairs, or 1.85% of the genome. Sixty-six were polymorphic as a result of 88 alternative allelic states [biallelic polymorphisms (BiPs)], for a frequency of approximately one BiP per kilobase. Five of the 88 BiPs probably represent three independent recombination events: Four seem to reflect two similar imports spanning 24 to 25 kb from *S. enterica* serovar Typhimurium (fig. S1), and a gene fragment with six single-nucleotide polymorphisms (SNPs) is identical to the corresponding gene fragment in *S. enterica* serovar Paratyphi A. The other 83 BiPs consisted of 37 nonsynonymous SNPs, 3 of which resulted in premature stop codons; 33 synonymous SNPs; 12 SNPs in pseudogenes; and one deletion of 4 base pairs.

We anticipated that housekeeping genes would exhibit diminished levels of nucleotide diversity, π , as a result of purifying selection, and that pathogenicity genes would exhibit elevated levels as a result of diversifying selection. However, π did not differ significantly with gene category ($P > 0.05$, analysis of variance) (table S1). Purifying selection should result in K_a/K_s (the ratio of nonsynonymous substitutions per nonsynonymous site to synonymous substitutions per synonymous site) values that are less than 1.0 and diversifying selection should result in ratios higher than 1.0. A trend in this direction was observed (table S1), but it was not particularly strong. We therefore concluded that these 88 BiPs largely reflect the lack of strong selection and are markers of neutral population structure in Typhi. It was somewhat surprising that a supposedly obligate pathogen such as Typhi should possess a neutral population structure, but the population structure of several other bacterial species that occasionally cause disease can also be explained by neutral genetic drift (13).

The distribution pattern of the 88 BiPs within Typhi is highly unusual because it is fully parsimonious according to maximum parsimony analysis (homoplasy index = 0). The 88 BiPs defined 59 haplotypes that form a unique path within a single minimal spanning tree of length 88, except for three hypothetical nodes (Fig. 1). These observations suggest that each BiP was caused by a unique genetic event, either a single mutation (83 BiPs) or the three imports described above (5 BiPs). The tree contains 19 informative BiPs that mark the evolutionary history of Typhi plus 69 noninformative BiPs that are specific to single haplotypes. A second, highly unusual feature of this data set is that the ancestral node, haplotype H45, is represented by extant bacteria. H45 must be the ancestral "root" node, because it possesses the identical nucleotides for all 82 SNPs, as did eight genomes of *S. enterica* of other serovars, whereas all other haplotypes result from one or more mutations. The general appearance of the tree (Fig. 1) suggests descent from H45 in multiple lineages, followed by diversification during multiple, independent population expansions that resulted in radial clusters of haplotypes containing the noninformative BiPs. For example, one cluster contains all seven Indonesian isolates with the z66 flagellar variant. The z66 cluster radiates from a single haplotype, indicating that it has arisen only once. Hence, z66 isolates cannot represent the evolutionary source of Typhi (11), because the z66 cluster is distant from H45.

The haplotype tree has a third, highly unusual feature: Most links between sequential haplotypes consist of single SNPs, and many longer edges, including one hypothetical node,

were resolved into steps of single SNPs when additional strains were surveyed (fig. S2). Even within this initial sample of 105 isolates, almost half of the mutational steps during the evolutionary history of Typhi are represented by extant haplotypes, indicating long persistence of individual haplotypes. If ecotypes associated with periodic selection were to exist within Typhi, the genetic continuum between haplotypes implies that ecotypes are subdivisions of haplotypes. Furthermore, haplotypes and haplotype clusters were found in multiple continents. For example, it is unclear where H45 evolved, because it has been isolated from five locations in Asia, Africa, and North America (3). Because each BiP is associated with a single, genetic event, each haplotype or haplotype cluster that is present in multiple continents marks at least one independent wave of global transmission. Global transmission has not been previously described for Typhi but is a well-known phenomenon with other human pathogens.

To place the time scale associated with neutral evolution in context, we calculated the time since the most recent common ancestor (tmrca) and the effective population size (N_e) from the selectively neutral data in Fig. 1. These calculations were performed with the use of two estimates of the molecular clock rate, a high rate corresponding to the long-term rate of accumulation of synonymous mutations between *Escherichia coli* and *S. enterica* (5) and a clock rate one-fifth as high, corresponding to the rate of accumulation of all mutations in conserved housekeeping genes between these species (14). For Typhi, tmrca is 10 to 43 thousand years (95% confidence limits of 5.7 to 15.8 thousand years for the high clock rate and 25.5 to 71 thousand years for the low rate) according to both Bayesian skyline plots (15) and maximum likelihood trees (fig. S3). Based on the same clock rates, N_e is currently 2.3×10^5 to 1.0×10^6 (confidence limits of 1.2×10^4 to 9.3×10^5 for the high clock rate and 5.3×10^4 to 4.1×10^6 for the low clock rate) (fig. S3A). Similar values were obtained from the nucleotide variation, θ_w , by an independent method (16) (table S1). The maximum likelihood tree also suggests that H45, the ancestral haplotype, and multiple descendent haplotypes arose after human migrations out of Africa but before the Neolithic period (fig. S3B).

The existence of an asymptomatic human carrier state for typhoid is formally similar to tuberculosis, for which the reactivation of granulomas after decades results in delays of centuries between initial new infections and subsequent epidemic peaks (17). Likewise, we propose that the human carrier state allowed persistence of infection with Typhi during periods of isolation and was essential for transmission between hunter-gatherer groups. Hence, the population structure and geographical distribution of Typhi may largely reflect the frequency of carriers.

The 55 polymorphic coding gene fragments (excluding pseudogenes) were screened by mutation discovery with 59 additional strains that were isolated between 1958 and 1967 from Africa and Vietnam. All but three strains were assigned to known haplotypes from the global sample (fig. S4). Twelve haplotypes were isolated on multiple occasions over a range of 22 to 44 years from eight countries (Table 1), demonstrating that Typhi haplotypes persist in single countries for decades, or longer. For example, CT18 (9) is a multidrug-resistant (MDR) strain of haplotype H1 that was isolated in Vietnam in 1993, soon after multidrug resistance emerged. However, a Vietnamese isolate from 1967 was also of haplotype H1, showing that H1 was present in Vietnam long before multidrug resistance emerged. The long-term persistence of Typhi may also reflect the carrier state and can help explain why Typhi remains endemic in regions of the world with poor drinking-water quality and limited sewage treatment (18).

Antibiotic-resistant typhoid fever has recently become an enormous public health problem in southern Asia because of the emergence of MDR Typhi followed by nalidixic acid resistance (Nal^R) with concomitant reduced susceptibility to fluoroquinolones (19). Fluoroquinolones were first used for antibiotic therapy in southeast Asia in 1989 (20) and Nal^R Typhi were reported in 1991 (21). Such strong selection should have led to a population expansion of

Nal^R Typhi, and possibly to clonal replacement of existing haplotypes within southern Asia. We therefore performed mutation discovery with the 55 polymorphic coding fragments on 295 additional strains of Typhi that were isolated from southern Asia between 1986 and 2004.

Again, most strains were assigned to known haplotypes and only a few defined new, peripheral haplotypes (Fig. 2B). However, the relative frequencies of isolates differed from those in the global set of 105 strains (Fig. 1), because most recent isolates from southern Asia, particularly Nal^R isolates, belonged to haplotype H58 (Fig. 2B). Thus, a recent population expansion of H58 seems to have resulted from the general use of fluoroquinolones.

We also investigated the genetic diversity of a 489-base pair fragment of the *gyrA* gene encoding a DNA gyrase subunit, within which nonsynonymous mutations at codons 83 and 87 result in resistance to nalidixic acid (22). All 125 strains that were sensitive to nalidixic acid (Nal^S) and all other strains with unknown resistance to nalidixic acid possessed the ancestral *gyrA*⁺ sequence. In contrast, all 119 Nal^R strains, most of which were isolated in south central or southeast Asia (table S2), possessed one of six nonsynonymous mutations at codons 83 and 87 of *gyrA* (Fig. 2A), and no other mutations were detected in *gyrA* (or *parC*). We identified 15 independent mutational events (A through O in Fig. 2B) in distinct haplotypes that also possessed *gyrA*⁺ alleles. Assuming that they all arose between 1991 and 2004 (13 years), the identification of ≥ 15 mutations in two codons (6 base pairs) yields a minimum frequency of 0.19 per base pair per year, $\geq 2.5 \times 10^8$ greater than the long-term mutation clock rate within *E. coli* (14).

For most haplotypes with *gyrA* mutations, Nal^R strains were detected only once or twice; however, Nal^R variants of haplotype H58 and its derivative haplotypes (H34, H57, and H60 to H65) were isolated in Vietnam, India, and Pakistan, and other countries in southern Asia (Table 2). These Nal^R variants represent at least five distinct *gyrA* mutations (K, L, M, N, and O), which arose during or before the mid-1990s (Fig. 2C). The frequency of *gyrA*⁺ and mutation L has remained fairly constant since the mid-1990s, but H58 isolates with mutation K seem to have become more common in recent years, particularly in Vietnam (Table 2).

These results show that selection for antibiotic resistance has probably led to clonal expansion of H58 and its Nal^R derivatives in southern Asia. These strains have now also reached Africa, given that one MDR H58 strain (isolated in Morocco in 2003) was included among nine rare, recent MDR isolates from Africa and that the sole MDR Nal^R isolate from Africa that was tested (mutation K, isolated in central Africa in 2004) also belonged to H58 (Table 2). Thus, H58 is probably not ethnically restricted to southern Asians, and nalidixic acid-resistant typhoid fever may soon present an additional public health problem in Africa.

Despite the selection for resistance to nalidixic acid in southern Asia, the data do not show complete clonal replacement, which would be expected from periodic selection; about 20% of Typhi isolated in recent years in northern Vietnam and 5% of Typhi from southern Vietnam remain susceptible to nalidixic acid, as are many other recent H58 isolates (Fig. 2C). Furthermore, recent isolates from southern Asia also belong to other haplotypes, where mutations in *gyrA* are rare (Fig. 2B). Thus, the population structure indicative of neutral evolution has not been disrupted by strong selection for resistance to nalidixic acid during the past 15 years, except for the clonal expansion of H58. Possibly *gyrA* mutations in many haplotypes reduce fitness (23) or some cases of typhoid fever have not been treated with fluoroquinolones. But still another alternative is that the population structure of Typhi reflects two distinct epidemiological dynamics associated with different time scales: first, the human carrier state permitting slow neutral evolution (millennia), and second, infectious transmission facilitating a rapid response to selection in real time. Outbreaks of infections, similar to the recent expansion of H58 in southeast Asia, may be responsible for independent chains of intercontinental transmission. These, in turn, create a global distribution of carriers for multiple

haplotypes. According to this interpretation, it is exactly because the environment selects that everything is everywhere in Typhi, thus inverting a hotly disputed (24) tenet of microbial ecology that was proposed by L. G. M. Baas Becking in 1934 (25).

The results presented here open multiple avenues for future research. Long-term epidemiology with larger strain collections is now possible on the basis of neutral SNPs (fig. S2), whereas classical microbiological methods do not seem to provide reliable markers for such purposes (table S3). Surveillance of haplotypes is particularly appropriate to provide early warning of the continued spread of Nal^R H58. Our overview of the current global population diversity in Typhi will allow comparisons of genomic sequences from representative strains without the risk of phylogenetic discovery bias (26). Finally, we suggest that the human carrier state may be of much greater importance for neutral evolution and genetic buffering than had been previously appreciated, an interpretation that would demand major changes in public health campaigns to reduce the incidence of typhoid.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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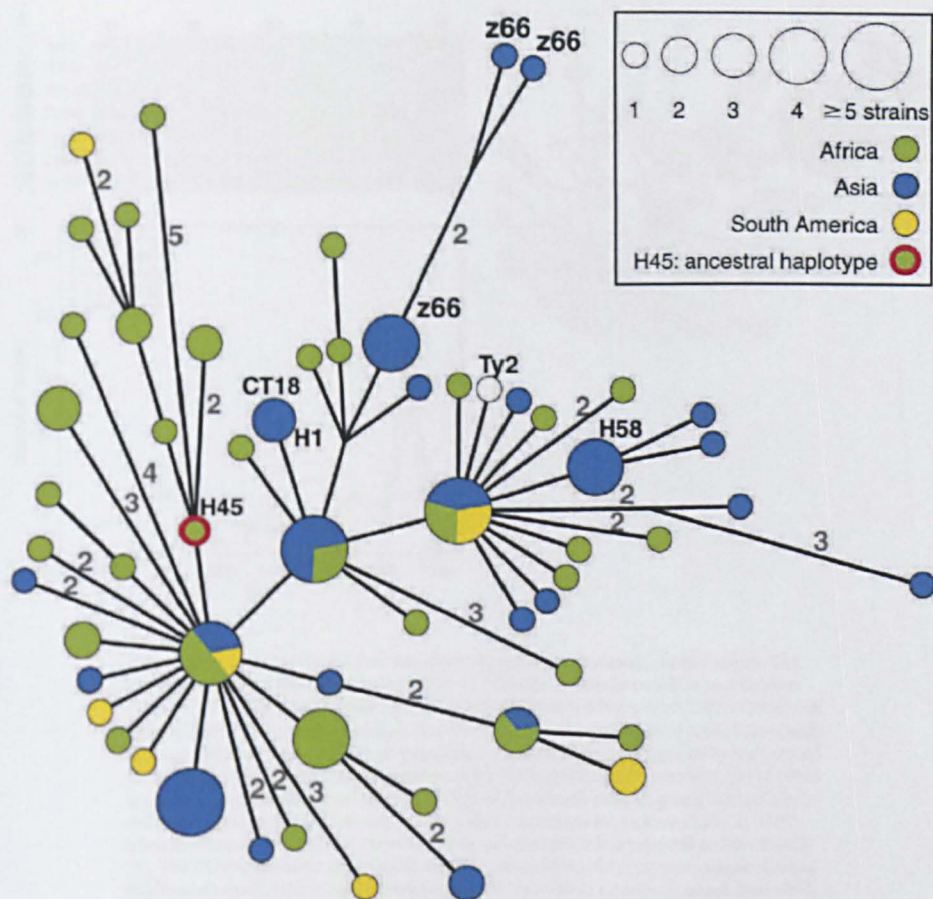
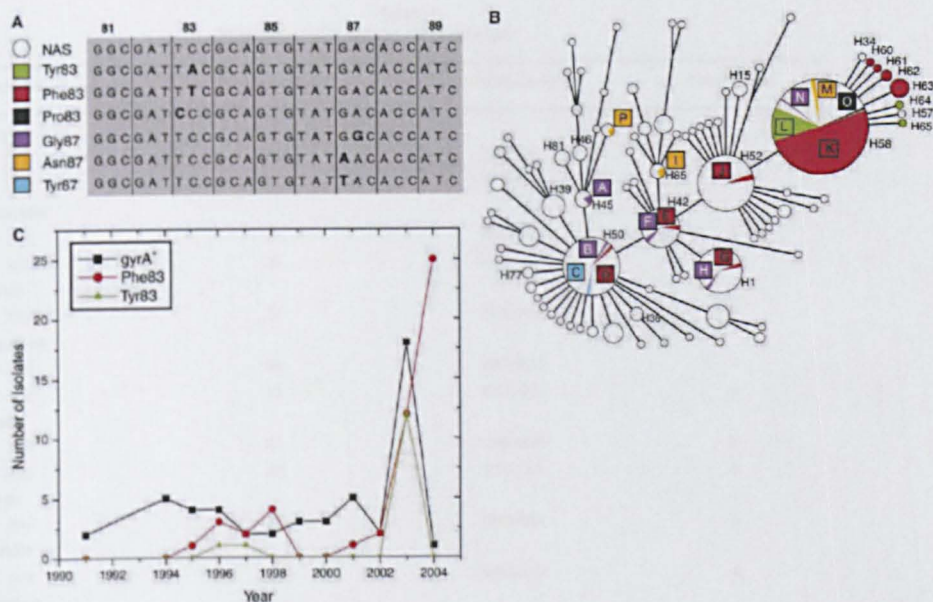


Fig. 1. Minimal spanning tree of 105 global isolates based on sequence polymorphisms in 199 gene fragments (88,739 base pairs). The tree shows 59 haplotypes (nodes) based on 88 BiPs, the continental sources of which are indicated by colors within pie charts. The numbers along some edges indicate the number of BiPs that separate the nodes that they connect; unlabeled edges reflect single BiPs. The genomes of the CT18 and Ty2 strains have been sequenced (GenBank accession codes AL513382 and AE014613, respectively). z66 refers to a flagellar variant that is common in Indonesia (11).

**Fig. 2.**

Selection for mutations in *gyrA* versus a neutral population framework in 483 strains. The strains consisted of 105 global isolates (Fig. 1), 59 older isolates from Africa and Vietnam (1958 to 1967) (fig. S4 and Table 1), and 317 isolates from southeast Asia (1984 to 2004) and other sources. **(A)** Sequence of codons 81 to 89 of *gyrA*, showing all mutated nucleotides (bold) that were detected within a 489-base pair stretch. Each mutation is designated by the name of the resulting amino acid and codon position (left). NAS, nalidixic acid sensitive. **(B)** Minimal spanning tree of 85 haplotypes based on 97 BiPs within 55 polymorphic genes. Sizes of circles and arcs reflect numbers of isolates. Strains without mutations in *gyrA* are shown in white, whereas strains with mutations are indicated by colored arcs that correspond to the colors in **(A)**. The 15 letters indicate independent mutations associated with resistance to nalidixic acid. **(C)** Time course of isolation of 118 isolates of haplotype H58 or its derivative haplotypes H34, H57, and H60 to H65. These isolates were selected for haplotyping and *gyrA* genotyping without prior knowledge of their susceptibility to nalidixic acid. Fifty-two other H58 isolates from Vietnam are not included because they were a nonrandom sample of *Nal^R* bacteria. The apparent increase of Phe83 in 2004 is based on a sample from the Mekong Delta province of Vietnam and may represent an outlier.

Table 1
Persistence of haplotypes over decades

Haplotype	Persistence (years)	Years persisted	No. of Isolates
Vietnam			
H1	37	1967-2004	25
H50	37	1959-1996	3
Madagascar			
H15	31	1965-1996	4
H50	33	1967-2000	2
Algeria			
H36	34	1966-2000	3
Ivory Coast			
H39	34	1967-2001	4
H81	35	1967-2002	2
Senegal			
H39	22	1966-1988	4
H52	39	1962-2001	4
Congo			
H46	34	1966-2000	3
Morocco			
H52	34	1966-2000	2
Cameroon			
H77	44	1958-2002	2

Table 2

Geographic sources of haplotypes with *grr* mutations by haplotype. Where more than one isolate was found in a country, the number of isolates is indicated in parentheses

Haplotype	<i>grr4</i> mutation (isolates)	<i>grr1</i> (isolates)
H15	A: India	Global (1)
H50	B: India C: China D: Mexico E: India, F: Pakistan G: Vietnam H: Vietnam	Global (55)
H12	I: India	Global (24)
H1	J: India (2)	Vietnam (23); Laos (7); Bangladesh; Indonesia
H85	K: Vietnam (98); Pakistan (5); India (4); Cambodia, Nepal, central Africa;	Morocco, Pakistan, Indonesia
H52	L: India (6); Bangladesh (6); Vietnam (5); Indonesia	Global (53)
H58	M: Pakistan (2) N: Vietnam	Vietnam (31); India (12); Laos (5); Pakistan (3); Hong Kong (2); Bangladesh, Sri Lanka, Morocco
H14, H57, and H60-65	O: Pakistan K: Vietnam (8); Bangladesh (2) L: India (2)	Nepal, Laos

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