Evaluation of strain circulation and the epidemiology of enteric fever caused by *Salmonella* Typhi and *Salmonella* Paratyphi A in Kathmandu, Nepal

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Abstract

Enteric fever caused by *Salmonella enterica* serovars Typhi and Paratyphi A are a major public health concern in Kathmandu. The aim of this thesis was to identify and assess the population most at risk by investigating epidemiologic trends of enteric fever within a subset population of Kathmandu. Therefore, the burden and incidence of enteric fever within the study population and the seasonal and gender distribution of enteric fever was assessed. Considerable burden of enteric fever, unrelated to population density, correlating with the seasonal fluctuations in rainfall was observed. This thesis also aimed to improve the understanding of enteric fever transmission by identifying probable transmission routes, hence various water and food samples were analysed and the extent of faecal contamination in them was determined. *S.* Typhi isolates were sequenced and genotyped and combined with GPS data to longitudinally study the local distribution and infer transmission of this human restricted bacterial pathogen. Extensive clustering of typhoid independent of population size and density and existence of an extensive range of genotypes within typhoid clusters including individual households with multiple cases was observed. These observations predict that indirect transmission had an overwhelming contribution for disease persistence, potentially through contaminated water. Consistent with this hypothesis, *S.* Typhi and *S.* Paratyphi A were detected in water supplies and it was observed that typhoid was spatially associated with public water sources and low elevation. A concurrent case-control study was also conducted which allowed for the determination of risk factors in the population at risk. These studies imply that resources should be allocated toward controlling the most important vectors of enteric fever, including food sold by vendors, chlorination of drinking water, construction of proper water distribution and sewage networks, vaccination campaigns and hygiene education.

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"I can no other answer make, but thanks and thanks"

Declaration

I, Abhilasha Karkey can confirm that the majority of work presented in this thesis is my own and was conducted under the supervision of Dr. Stephen Baker at Oxford University Clinical Research Unit in Vietnam. The only work presented in this thesis which is not my own is presented in Chapter 4, which was published by Baker et al. 2011 in Open Biology, of which I am a co-author. The spatial analyses, including the K means clustering and the enteric fever spatial risk maps, were constructed in collaboration with Dr. Archie Clements at the University of Queensland in Australia and coordinated by my supervisor Dr. Stephen Baker. Furthermore, the phylogenetic analysis of the *S.* Typhi population was performed in collaboration with Dr. Kathryn Holt at the University of Melbourne, Australia, again through an ongoing collaboration with my supervisor Dr. Stephen Baker. This thesis has not been submitted for a degree or other qualification to this or any other university.

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Abbreviations

AIDS	Acquired immune deficiency syndrome
AOR	Adjusted odds ratio
BHI	Brain heart infusion
CBS	Central bureau of statistics
CDC	Center for disease control
cfu	Colony forming units
CGIAR	Consultative group for international agriculture research
DCA	Desoxycholate citrate agar
dNTP	deoxynucleotides
E. coli	*Escherichia coli*
ELISA	Enzyme linked immunosorbent assay
ESBL	Extended spectrum beta lactamases
GDP	Gross domestic product
GIS	Geographical information system
GPS	Global positioning system
HDI	Human development index
HE	Hektoen enteric Agar
HIV	Human immune deficiency virus
IQR	Interquartile range
ITS	Internal transcribed spacer
KIA	Kligler Iion agar
kml	Keyhole Markup Language
LPS	Lipopolysaccharide
LSMC	Lalitpur sub-metropolitan city

MC	MacConkey agar
MDR	Multi drug resistant
$MgCl_2$	Magnesium chloride
MIU	Motility-indole-urease
MLST	Multi locus sequence typing
MOHP	Ministry of health and population
MOR	Matched odds ratio
MPC	Multi protein complexes
MPN	Most probable number
NASA	National aeronautical and space agency
NHRC	Nepal health research council
NTS	Non-typhoidal Salmonella
OXTREC	Oxford tropical research ethics committee
PCR	Polymerase chain reaction
PFGE	Pulsed field gel electrophoresis
RAPD	Random amplification of polymorphic DNA
RFLD	Restriction fragment length dimorphism
RVB	Rappaport vassiliadis broth
S. bongori	*Salmonella bongori*
S. boydii	*Shigella boydii*
S. flexneri	*Shigella flexneri*
S. Paratyphi	*Salmonella* Paratyphi
S. sonnei	*Shigella sonnei*
S. Typhi	*Salmonella* Typhi
S. Typhimurium	*Salmonella* Typhimurium
SDS/PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SNP	Single nucleotide polymorphisms
SOC	Super Optimal Broth with Catabolite Repression
spp	Species
TCBS	Thiosulphate-citrate-bile salts sucrose
TE	Tris EDTA
TSB	Tryptone Soya Broth
TSI	Triple Sugar Iron
UNDP-CDRMP	United Nations Development Program-Comprehensive Disaster Risk Management Program
V. cholerae	*Vibrio cholerae*
WHO	World Health Organisation
WHO-Salm	World Health Organisation Collaborating Center for Reference and Research on *Salmonella*
WHO-Salm Surv	World Health Organisation Collaborating Center for Reference and Research on *Salmonella* Surveillance
XLD	Xylose Lysine Desoxycholate Agar

1 INTRODUCTION

1.1 Introduction to Nepal

1.1.1 Geography

Nepal is a landlocked country located in the Himalayan mountain range in South Asia, and is nestled between the Republic of India to the South, East and West, and the Peoples Republic of China to the North (**Figure 1.1**). With a total land area of 147,181 km^2, the country has a diverse geography with three physiographic regions that run laterally across the country. Twenty three percent of the land area is comprised of plains in the South of the country, which are the most populated and the main agricultural region. In the North of the country, representing 42% of total landmass, rugged mountains and hills ranging from 600 to 3,000 meters cover 3/4 of the land area. The extreme North of the country, amounting to 35% of total landmass, is covered by the Himalayan region, which contains 200 peaks more than 6,000 meters in elevation and 13 peaks more than 8,000 meters high, with Mount Everest being the highest pinnacle.

Figure1.1: Geographical positioning of Nepal and the capital city of Kathmandu. (Image source: www.maps.com)

1.1.2 Climate

Nepal lies within a sub-tropical monsoon climate zone and the dramatic difference in elevations within the country results in a variety of climactic conditions and varying degrees of precipitation. The plains experience tropical conditions with temperatures ranging from 5°C to 47°C, while mountainous areas experience a spectrum of alpine to tundra conditions with temperatures ranging between 0°C to 28°C in the hills to temperatures below 0°C to 16°C in the Himalayas. The annual rainfall generally increases with elevation up to 3,000 meters and declines with elevation and latitude. Maximum precipitation is observed in the eastern-most parts of the country with a gradual decrease towards the west. Almost 80% of the precipitation is observed during the annual monsoon season that runs from June to September, when the weather is hot. The post-monsoon season that runs from mid-September to mid-October is temperate and dry, while the winter months, mid-October through March, are cold and dry. The pre-monsoon months of April and May are hot and humid.

1.1.3 Valley of Kathmandu and its Demographics

The country of Nepal is currently undergoing a demographic transition. Although there has been an increase in the contraceptive prevalence rate (41%)(WHO, 2007), the population is growing at a rate of 2.25% per annum, which is higher than that of other countries within the region such as India, Bangladesh and Sri-Lanka (The World Bank, 2001). The population of Nepal has increased steadily over the last 10 years from 23.15 million in 2001 to 26.6 million in 2011 (CBS, 2011). With approximately 2.5 million people, the Valley of Kathmandu, the country's capital region, is the geographical and urban center of Nepal and includes five major municipalities: Bhaktapur, Lalitpur, Kathmandu, Kirtipur and Thimi. While Kathmandu is nestled at an altitude

of 1,300 meters, the surrounding areas of the valley range from 1,500 to 2,800 meters in elevation. Kathmandu is the most highly and densely populated area of the country with a density of 10,560 people per square kilometer. The huge population influx into urban areas has been most prominent in Kathmandu, with many people flocking to the capital in search of employment or a better standard of living. This increasing and inevitable urbanization has had dramatic consequences, placing strain on the old infrastructure of the city and draining local resources and facilities.

1.1.4 Economics

Nepal ranks among the poorest countries in the world. Approximately 80% of the population is reliant on agriculture for their livelihood, yet the share of the agriculture sector in gross domestic product (GDP) is only 40%. Tourism, one of the main sources of income in recent decades for Nepal, has declined significantly due to the civil conflict and violence that was seen in the early part of the 21st century. Remittances from foreign employment have been a major source of income for Nepal (The World Bank, 2011). According to the World Bank (The World Bank, 2009) Nepal has a gross per capita income of $440, classifying it as the poorest nation in Asia. Nepal's human development index (HDI) in 2007 was calculated to be 0.553, giving the country a rank of 136th out of 177 countries with comparable data (United Nations Development Programmes, 2009). The most recent assessment of poverty within the general populace calculated that 24.1% of the population survives on less than $1 a day and 68.5% survive on less than $2 a day (1990-2005 figures, www.undp.org).

In addition to poverty, Nepal is a highly disaster-prone country; among 200 countries it ranks as the 11th country most prone to earthquakes and the 30th most prone to floods (UNDP-CDRMP,

2011). Several natural hazards such as floods, landslides and drought affect different geographical zones annually with a varying degree of damage to health infrastructure and the health of the populace (www.undp.org).

1.1.5 Population Health

Poverty and political instability within Nepal have had obvious detrimental effects on infrastructure; 89% of the population has access to improved water sources and 45% of the population has access to improved sanitary conditions (www.undp.org). The country continues to be afflicted by communicable diseases and recent upward trends of non-communicable diseases have added to the overall national disease burden. The incidence of preventable infections, such as diarrhoeal diseases and acute respiratory tract infections, continue to be high, being approximately 219 and 319 per 1,000 population, respectively (Ministry of Health and Population (MOHP) et al., 2007). Although maternal mortality rates have improved in recent years under-five-years and infant mortality rates remain high at 61 and 48 per 1,000 live births, respectively (Ministry of Health and Population (MOHP) et al., 2007). Neonatal mortality alone accounts for two thirds of the total infant mortality rate (Nepal demographic and health survey 2006). Malnutrition poses a serious public health problem especially among children, adolescents and females. Approximately half of the children under the age of five years are affected by stunting while 48% of them are underweight, 10% suffer acute malnutrition and 13% have a combination of stunting, vitamin A deficiency and iron deficiency (Ministry of Health and Population (MOHP) et al., 2007). Major public health concerns along with tuberculosis and HIV/AIDS include vector-borne infections such as typhus, malaria, visceral leishmaniasis, lymphatic filariasis, Japanese encephalitis and the recent emergence of dengue infections (www.who.int).

1.2 The bacterial genus *Salmonella*

1.2.1 Taxonomy and Nomenclature

The bacterial genus *Salmonella* is comprised of a large and diverse group of enteric pathogens. Currently, there are 2,610 different serotypes (Martine Guibourdenchea, 2010).The salmonellae belong to the larger bacterial family, *Enterobacteriaceae,* and the genus is comprised of two species, *Salmonella enterica* (*S. enterica*) and *Salmonella bongori* (*S. bongori*). *S. enterica* consists of six sub-species, of which *enterica* is arguably the most important, with over 2,600 serotypes. The serotypes of this subspecies are the most pathogenic to humans and include the classical invasive human pathogens, Typhi and Paratyphi serotypes and the classical non-typhoid serotypes, Typhimurium and Enteritidis. The citation/description of any the salmonellae follows a specific nomenclature, which is summarized in **Table 1.1** (Brenner FW, 2000).The antigenic formulae of *Salmonella* serotypes are available in the White-Kauffman-Le Minor scheme (Weill, 2007). This scheme is updated by the WHO Collaborating Center for Reference and Research on *Salmonella* (WHO-Salm) based at the Institut Pasteur in Paris, France. The current edition (9^{th}), was issued in 2007 and comprises of antigenic formulae that had been validated as of January 1^{st} 2007 (Martine Guibourdenchea, 2010).

Table 1.1: *Salmonella* nomenclature in use at CDC 2000. (Source: Brenner et.al., 2000)

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

1.2.2 Antigenic variation and *Salmonella* serotyping

Strains belonging to the genus *Salmonella* typically express two classical antigens, which can be used for identification and subgrouping, these are the 'O' antigen (somatic) and the 'H' antigen (flagellar). It is on the immuno-reactivity of these two main surface structures that the Kauffman-White scheme (serotyping) is based (Minor et al., 1983, Kauffmann, 1969). The long chain lipopolysaccharide is comprised of a heat stable polysaccharide that is commonly known as the 'O' antigen. These molecules are located in the outer membrane and are anchored into the cell wall by antigenically conserved lipid A and the lipopolysaccharide core regions (Mastroeni and Maskell, 2006). The 'H' antigen forms the basis of the flagella serotyping scheme and the epitopes are contained within the flagellar protein sub-units (Mastroeni and Maskell, 2006).

The majority of *S.* Typhi isolates also express a surface polysaccharide known as the virulence or 'Vi" antigen. This antigen is largely restricted to *S.* Typhi, although it is shared by some isolates of *S. enterica* serotypes Hirschfeldii, Paratyphi C and Dublin (Parry, 2003).

1.2.3 Microbiological identification of non-typhoidal and typhoidal salmonellae

The salmonellae are facultative anaerobic, flagellated, Gram-negative bacilli that can metabolise glucose (but not lactose) with the production of acid and gas. They are also incapable of producing indole and hydrolyzing urea (Hart, 2002). The presumptive identification of salmonellae can be performed through biochemical testing using the differential media Kligler Iron Agar (KIA) or Triple sugar iron (TSI) agar in conjunction with motility-indole-urease (MIU) media (Fraile et al., 1980)

A definitive diagnosis for any suspected salmonellae infection is necessary for better management of the affected individual and for increased public health awareness (WHO, 2003). The most conclusive confirmation is dependent on the isolation of salmonellae from blood, faeces, bone marrow or a specific anatomical lesion (WHO, 2003). Hence, microbiological culture should be considered as the gold standard for all salmonellae infections and should be used as the gold standard for evaluating all diagnostic tests, irrespective of their level of sophistication (WHO, 2003). Bacterial isolation not only allows confirmation of the clinical diagnosis but also allows antimicrobial sensitivity testing which can help to determine appropriate therapy (Parry et al., 2011b). The majority of clinical laboratories use one medium with low selectivity, such as MacConkey (MC) agar, and one with higher selectivity, such as Hektoen enteric (HE) agar, desoxycholate citrate agar (DCA), or xylose lysine desoxycholate (XLD) agar for the primary isolation of salmonellae from a faecal specimen (Hohmann, 2001). Less than 1% of non-typhoidal salmonellae are lactose positive, but use of a highly selective media, such as the HE agar, permits hydrogen sulphide production by the salmonellae. Fluid enrichment media, such as tetrathionate or selenite broth are also useful to detect small numbers of salmonellae in faeces or environmental samples (Hart, 2002). Even-though various enrichment media are used, the sensitivity of culture is generally low due to various factors, including the volume of blood used, the period of illness during which the sample is collected, overgrowth with other less-fastidious organisms and pretreatment with antimicrobials (Hohmann, 2001, Stuart and Pullen, 1946).

For invasive (typhoidal) salmonellae, such as *S.* Typhi, the volume of blood taken is critical as it translates directly to the bacterial load in the blood. A median bacterial load of less than 1 cfu/ml

in adults and children with mild to moderate typhoid fever have been documented (Wain et al., 1998). Yet, the quantity of bacteria in the bloodstream has been shown to be higher among children as in comparison to adults and bacterial load is higher in the first week of the illness as compared to later weeks (Coleman and Buxton, 1909).

The type of culture media used for the isolation of *Salmonella* pathogens also plays a significant role - bile containing Oxgall media (BD Difco, UK), though not suitable as a general purpose blood culture media due to its inhibition of other infecting bacteria, is a suitable media for the recovery of *S.* Typhi and *S.* Paratyphi from blood and other sterile samples (Coleman and Buxton, 1909, Escamilla et al., 1984, Kaye et al., 1966, Wain et al., 2008). The advantage of Oxgall for recovery of *Salmonella* pathogenesis is attributed to its capacity to inhibit the antibacterial activity of fresh blood caused by the lysis of blood cells, rather than direct enhancement of growth by the bile salts (Cummins, 1911). Various types of media, which contain tryptone soya broth (TSB) or brain heart infusion (BHI) broth, with additional polyethanol sulphonate or bespoke media for automated blood culture systems are commonly used nowadays for the growth of invasive salmonellae from blood. Yet, adequate dilution of the blood in broth, and the length of the incubation period of the culture are additional important factors and still hinder overall sensitivity (Parry et al., 2011a). Manipulations to blood cultures, such as centrifuging the specimen prior to culturing, culturing of the buffy coat, introduction of a lysis step to release the intracellular organisms have been attempted to optimize the blood culturing methodology (Kaye et al., 1966, Escamilla et al., 1984, Wain et al., 1998). Yet, one of the most significant barriers inducing impaired sensitivity remains pre-treatment with antimicrobials (Rubin et al., 1990). To resolve the problem of antimicrobial treatment manufacturers produce

blood culture bottles for automated blood culturing systems that contain resins that are capable of absorbing and inactivating residual antimicrobials, which potentially increases the sensitivity of the culture. For invasive salmonellae, in comparison to blood cultures, bone marrow cultures are known to have a higher sensitivity. This increase in sensitivity is principally related to the fact that the bone marrow has a concentration of viable organisms, which is approximately an order of magnitude higher than in blood (Dance et al., 1991). However, it has been argued that sensitivity of blood culture is comparable to that of bone marrow culture provided a sufficient volume of blood is cultured (Wain et al., 2008, Wain and Hosoglu, 2008).

Cultures from other sites such as skin biopsy cultures from rose spots; faeces or rectal swabs and urine can also give positive results for *S.* Typhi. However, a positive culture from faeces or duodenal contents warrants a cautious interpretation as it could represent chronic carriage, with the acute infection syndrome caused by a different etiological agent (Parry et al., 2011c).Colonies from the culture plates can be tested directly for the presence of *Salmonella* somatic 'O' antigens by the slide agglutination method using specific antisera (Hart, 2002). The flagellar 'H' antigen can also be detected through slide agglutination after being sub-cultured to peptone water. The organism can also be recovered from blood clots through streptokinase digestion and incubation in broth (Hart, 2002).

1.2.4 Pathogenicity

Salmonella species comprise of a large group of enteric pathogens that are capable of causing a wide range of diseases in a broad spectrum of warm and cold-blooded hosts (Dougan et al., 2011). In humans, the majority of infections are caused by serovars belonging to *S. enterica.*

S. enterica serovars Typhi, Paratyphi A, Paratyphi B, Paratyphi C and Sendai cause the disease syndrome known as typhoid or paratyphoid fever, which is collectively referred to as enteric fever. The remaining salmonellae serovars are generally "non-typhoidal" and cause self-limiting acute diarrhoea, which is clinically indistinguishable from gastroenteritis caused by other, unrelated gastrointestinal pathogens (Parry, 2003). While *S.* Typhi is host adapted, infecting humans and higher primates only, non-typhoidal serotypes are capable of infecting a multitude of hosts and can exhibit a variety of disease phenotypes depending on the host they infect (Hapfelmeier et al., 2004).

The vast majority of *Salmonella* infections occur via the faecal-oral route, usually through the consumption of contaminated food or water (Crump and Mintz, 2010). Salmonellae are capable of surviving for several days in groundwater or sea-water (Jang-Cheon Cho, 1999, Wait and Sobsey, 2001) and for months in food such as, contaminated eggs (Humphrey TJ, 1991) and frozen oysters (Nishio T, 1981). The consumption of ice cream, flavored ice drinks, food sold by street vendors, raw fruit and raw vegetables have also been implicated as risk factors (Bhan et al., 2005). The minimal bacterial load required to be ingested in order to initiate an infection is uncertain for the majority of serotypes, yet probably varies greatly between serovars (Blaser and Newman, 1982). The infective dose for most serotypes including *S.* Typhi ranges from 10^3 to 10^6 bacteria (Hornick RB, 1970, Hart, 2002). Yet, achlorhydria due to aging, previous gastrectomy, or treatment with histamine H_2 receptor antagonists, proton-pump inhibitors, or large amount of antacids can have the effect of lowering the infective dose of the organism to $<10^3$ (Parry et al., 2002, Blaser and Newman, 1982).

Salmonella virulence requires the coordinated expression of complex arrays of virulence factors that allow the bacterium to evade the host's immune system (Ohl and Miller, 2001). The ability to invade the host by inducing their own uptake into the cells of the intestinal epithelium is shared by all *Salmonella* serotypes. While non-typhoidal serotypes orchestrate an intestinal inflammatory and secretory response, typhoidal serotypes try to avoid triggering an inflammatory response in the gut of the human host (Merrel and Falkow, 2004, Weinstein et al., 1998). Current enteric fever pathogenesis is largely based on the murine model in which *Salmonella* Typhimurium (*S.* Typhimurium) causes a systemic infection similar to typhoid (Bhan et al., 2005). After oral ingestion of the bacterium, a proportion of the salmonellae are capable of withstanding the acidic pH of the stomach and colonizing the small intestine. The bacteria are then believed to translocate across the follicular epithelium through the use of three distinct mechanisms:

i. Invasion of antigen-sampling the M cells of the Peyer's patches (Clark et al., 1994, Clark et al., 1996)

ii. Invasion of the adjacent enterocytes (Bradley D. Jones, 1994)

iii. Phagocytosis by $CD18^{+}$ phagocytes(Vazquez-Torres A, 1999)

The bacteria then colonize the underlying lamina propria and mesenteric lymph nodes (Carter and Collins, 1974, Cirillo et al., 1998), where large quantities of phagocytic cells engulf the bacteria. It is the ability of the invasive salmonellae to survive within the dendritic cells and macrophages that enable it to gain access to the lymphatics and blood where a transient bacteraemia occurs. The organisms then spread throughout the body of the infected host to major sites for systemic colonization, such as the spleen and liver where they replicate (House et al., 2001a, Guzman et

al., 2006). A large number of neutrophils infiltrate the liver and spleen during the initial stages of colonization (Cheminay et al., 2004). Salmonellae are associated with the formation of neutrophil rich lesions that develop into distinct macrophage rich granulomas that increase in number as the disease progresses (Sheppard et al., 2003). Thereafter there is an influx of innate immune effector cells to the colonized organs, which results in inflammation leading to the hepatosplenomegaly, which is characteristic of systemic salmonellae infection. If the bacteria are not controlled at this phase, a second bacteremia ensues whereby the host succumbs to the infection either through re-infection and perforation of the gut as in enteric fever, or septicemia and multi-organ failure as in typhoid like disease induced by *S.* Typhimurium in mice models (Russell et al., 2009).

Bacterial adherence factors and their cognate receptors on the intestinal epithelium most likely determine host specificity among the various serotypes, and the ability of the phagocytic cells to contain the infection also plays a significant role. During human infections with *S.* Typhimurium, the pathogen is unable to replicate in the macrophages and so the infection is contained until adaptive immunity is raised, and the salmonellae are eliminated without hepatosplenic colonization (Sebastiani et al., 2002). Yet, the molecular mechanisms that underlie the host cell specificity are still not well-understood (Ohl and Miller, 2001). However, a possible underlying molecular mechanism for persistence of salmonellae in the host, as seen in asymptomatic carrier state, has been proposed (Merrel and Falkow, 2004).

Salmonellae express a large number of virulence factors, most of which are encoded by the numerous pathogenicity islands, phage elements, and plasmids that help in evading the host defenses and enable bacterial replication (Ohl and Miller, 2001). In *S.* Typhi this pathogenic

fraction of the total genetic material accounts for approximately 7% of the bacterial genome (Parkhill J et al., 2001). Observations have suggested that pathogens have acquired these gene clusters through horizontal gene transfer, which can be identified by a variable G/C nucleotide content when compared to the rest (the non-accessory fragment) of the bacterial genome, transposon insertion sequences, or other mobile DNA elements (Groisman EA, 1996). Currently12 distinct salmonellae pathogenicity islands have been identified with the total number of hypothesized gene islands or clusters rising to over 60 if smaller genomic regions are included (Russell et al., 2009). The genes carried on these pathogenicity associated regions, amongst various other elements, encode for fimbriae, pili, and other surface antigens that mediate adherence and immune evasion, secretion systems, toxins, and other factors important for cellular invasion and survival (Russell et al., 2009). Between the various serotypes of *S. enterica,* the combination and structure of the salmonellae pathogenicity islands differ in varying degrees, and it is presumed that this heterogeneity contributes to the ability of the different serotypes to cause diseases of varying severity in a range of host species (Kaur J, 2011).

1.2.5 Non-typhoidal salmonellae (NTS)

NTS are important food borne pathogens whose infections are associated with different clinical syndromes of variable severity, including, bacteraemia, endovascular infection, focal infection and most frequently gastroenteritis (Sánchez-Vargas et al., 2011). Despite improvements in hygiene and sanitation over the centuries, NTS infections continue to be a significant burden on public health in under developed locations as well as in developed countries (Westrell et al., 2009, Majowicz et al., 2010, Weinberger M, 2005). According to estimations by Majowicz *et al*. 93.8 million cases of gastroenteritis due to *Salmonella* occur worldwide annually, causing

approximately 155,000 deaths (Majowicz et al., 2010). In Central Europe alone, the estimate of incidence for NTS diseases is 690 per 100,000 person years (Majowicz et al., 2010). In the US, the Foodborne Disease Active Surveillance Network implicated NTS illnesses as the most commonly reported infection, with 17.6 cases per 100,000 population (Lynch et al., 2009).

In reports from Salm-Surv, a World Health Organization supported food borne disease surveillance network (www.who.int/salmsurv) (CDC, 2011), data collected from 2001 to 2005 showed that *S.* Enteritidis was the most common serotype isolated worldwide accounting for 65% of all isolates in 2002 , followed by *S.* Typhimurium with a 12% isolation rate and *Salmonella* Newport (*S.* Newport) with a 4% isolation rate (Galanis E, 2006). In Africa, *S.* Enteritidis accounted for 26% of the isolates and *S.* Typhimurium for 25% of the isolates from clinical specimen. In Asia, Europe, Latin America and the Caribbean *S.* Enteritidis was the most frequently serotype, while in North America *S.* Typhimurium was the most common isolate followed by *S.* Enteritidis and other *Salmonella* species (Galanis E, 2006).

Salmonellae are hardy bacteria of special concern in immuno-compromised individuals including those with malignancy, human immunodeficiency virus, diabetes and those undergoing corticosteroid therapy or treatment with other immune-modulating agents (Hohmann, 2001). In some hospital-based studies conducted in Sub-Saharan Africa, septicemia with *Salmonella* species were more frequently caused by *S.* Enteritidis and *S.* Typhimurium than with typhoidal salmonellae. On the African continent, invasive non-typhoidal salmonellae are endemic and are associated with elevated morbidity and mortality in children under three years of age and in adults with human immunodeficiency virus infections (Crump et al., 2011, Mandomando I, 2009,

Morpeth et al., 2009). In contrast, in Asia non-typhoidal salmonellae are not commonly cultured from patients with bacteraemia, except in individuals with severe immunosuppression (Dhanoa A, 2009, Khan MI, 2010). Yet new data originating from Vietnam suggests there may be a transition from typhoidal salmonellae to NTS related to economic development and increasing prevalence of HIV (Nga et al., 2012).

Exposures frequently implicated in a number of NTS outbreaks include a variety of food products, particularly raw ingredients that have been in contact with contaminated animal or people, improper storage, or incomplete cooking of food products (Lynch et al., 2009). Mass production and distribution of food products can lead to the rapid dissemination of these pathogens within communities (World Health Organization, 2008). This rapid transmission is made even more alarming by the continuing development of antimicrobial resistance among the NTS, making control and prevention of these infections even more complex (Majowicz et al., 2010).

Studies have correlated the incubation period of NTS as being dose dependent (Abe et al., 2004, Blaser and Newman, 1982, Glynn and Palmer, 1992, Mintz et al., 1994, Brooks et al., 2005). Nausea and vomiting are the most common symptoms of NTS infection initially, but other symptoms that can be observed later, include fever, chills, abdominal pain, myalgia, arthralgia and a headache (Sánchez-Vargas et al., 2011). These symptoms are generally self limiting (Sánchez-Vargas et al., 2011) but gastrointestinal complications such as appendicitis, pancreatitis, cholecystitis, cholangitis, and abdominal or perianal abscess can also develop (Hohmann, 2001). Systemic infections have variable clinical manifestations and among these

bacteraemia is the most common presentation, occurring in 5% of the infected patients and may be associated with other extra-intestinal complications (Khan MI, 2010, Fisker et al., 2003, Huang and DuPont, 2005).

Antimicrobial treatment of uncomplicated gastroenteritis is not required in patients if there is no evidence of any underlying diseases or if there is no evidence of a clinical benefit such as reducing the duration of illness (Sánchez-Vargas et al., 2011). Various studies have in fact shown that antimicrobial therapy could increase chances of adverse events and even prolong fecal shedding (Hohmann, 2001, Crump and Mintz, 2010, Ruiz et al., 2004). Treatment of NTS infections is a global concern due to increasing resistance by the organism to all currently efficacious and readily available antimicrobials. Of particular alarm is the increasing number of salmonellae capable of producing extended spectrum beta lactamases (ESBLs), which are enzymes that can be produced by the bacteria making them resistant to cephalosporins such as cefuroxime, cefotaxime and ceftazidime (HPA, 2012, Dunne et al., 2000, Gupta et al., 2003, Sow et al., 2007). It is believed that feeding of stock animal with food containing antimicrobials play a significant role in the spread of resistant salmonellae (Angulo and Molbak, 2005, Molbak, 2004). Furthermore, an increase in multi-drug resistance (MDR) which is defined as resistance to chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole among all *Salmonella* strains, raises concerns, as infections would be difficult to treat with conventional antimicrobials and as a consequence, may lead to higher mortality rates (Varma et al., 2005b, Sánchez-Vargas et al., 2011, Varma et al., 2005a).

1.2.6 Typhoidal salmonellae

The typhoidal serovars of *S. enterica* are *S.* Typhi, Paratyphi A, Paratyphi B and Paratyphi C, collectively the disease these serovars cause is called enteric fever (Bhan et al., 2005). Enteric fever is a systemic infection and *S.* Typhi and *S.* Paratyphi A are highly adapted and human specific. *S.* Typhi in particular has evolved remarkable mechanisms for persistence in its host, which contribute to its survival and ongoing transmission in endemic environments (Parry, 2004b). Enteric fever is of growing public health concern in the developing world as the emergence of antimicrobial resistance threatens to render current interventions ineffective (Schlipkoter and Flahault, 2010). Although the majority of enteric fever cases are attributed to the bacterium *S.* Typhi, *S.* Paratyphi A is now recognized as an emerging agent of enteric fever in regions where enteric fever is endemic (Ochiai et al., 2005). *S.* Typhi has become human restricted through the acquisition of virulence factors and a series of gene inactivation events (Pickard et al., 2003, Parkhill J et al., 2001, Holt et al., 2009). The inability of *S.* Typhi to interact with other hosts appears to have instigated a selective pressure resulting in the organism becoming genetically isolated and dependent on asymptomatic carriage for long-term survival in the human population (Levine et al., 1982, Holt et al., 2008a, Khatri et al., 2009). These salmonellae are important causes of febrile illness, particularly in crowded and impoverished populations where poor sanitary conditions remain and facilitate transmission (Parry et al., 2002). Exposure to contaminated food and water also pose a risk of contracting enteric fever to travelers visiting endemic areas (Whitaker et al., 2009). The issue of infection within the community is particularly poignant with respect to enteric fever, as *S.* Typhi is known to be able to survive in the gall bladder for prolonged periods which is believed to play a pivotal role in the transmission of the pathogen (Levine et al., 1982). The role of chronic *S.* Paratyphi A carriage, however, is less

well defined and remains largely non described (Karkey et al., 2008, Khatri et al., 2009).

1.3 Enteric fever

1.3.1 Clinical features of enteric fever

S. Paratyphi A is historically thought to cause a milder disease compared to the syndrome caused by *S.* Typhi (Bhan et al., 2005). However, work in Nepal has demonstrated that the clinical syndrome caused by *S.* Paratyphi A are indistinguishable from those caused by *S.* Typhi (Maskey et al., 2006).

By comparison to NTS induced gastroenteritis, enteric fever has a longer incubation period (median of 5 to 9 days for enteric fever compared to 12 to 72 hours for NTS gastroenteritis), and also a longer duration of symptoms (fever can persist for up to 3 weeks in enteric fever compared to less than10 days for NTS induced gastroenteritis) (Raffatellu et al., 2008). The clinical features of typhoid fever are extremely variable, ranging from fever with few other morbidities to marked multisystem toxaemia and associated complications such as infective endocarditis, pericarditis, empyema, osteomyelitis, meningitis, bone marrow infiltration, hepatitis and pancreatitis among many (Bhan et al., 2005, Chauhan et al., 2009).

The most common presenting symptoms of acute uncomplicated typhoid fever are prolonged low-grade fever with a dull frontal headache (Maskell, 2006). Additional symptoms include anorexia, nausea, malaise, myalgia, abdominal pain, chills, and a dry bronchitic cough (Maskell, 2006, Parry et al., 2002). The fever characteristically rises progressively, developing into a

persistent high-grade fever by the second week of the illness (WHO, 2003). If left untreated, this persistent high grade fever can continue for up to 4 weeks, which is typically followed by a return to normal temperature in those that clear the infection, while malaise and lethargy can persist for longer (Bhan et al., 2005). Gastrointestinal disturbances, ranging from constipation in adults to diarrhoea in children, are also a commonly observed (Maskell, 2006, Parry et al., 2002). The most frequent physical findings on examination are a coated tongue, splenomegaly and abdominal tenderness (Parry et al., 2002, Vinh et al., 1996, Maskey et al., 2006). Small erythematous maculopapular lesions, known as rose spots, are observed on the back, arms and legs in approximately a quarter of the cases late in the first week of fever (Maskell, 2006). Increased serum bilirubin and alanine transferase levels due to colonization of the liver by the salmonellae is a common observation and in places where the circulation of these salmonellae are endemic, enteric fever should be considered in the differential diagnosis for individuals presenting with fever and jaundice (Shetty et al., 1999). Furthermore, enteric fever and viral hepatitis are frequently endemic in the same regions, both are associated with poor sanitation and both are transmitted faeco-orally, posing potential diagnostic problems (Schwartz et al., 1994, Mishra et al., 2008, Pandey et al., 2002).

Approximately 10-15% of those infected with typhoidal salmonellae and admitted to a health care facility develop severe disease (Parry et al., 2002). Severity is dependent on a number of factors, including the duration of illness before therapy, the choice of antimicrobial therapy, strain virulence, inoculum size, previous exposure or vaccination, and various host factors such as type of human leucocyte antigen (HLA), immunosuppression, or antacid consumption (WHO, 2003). Gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy are also

common complications observed during severe typhoid (Parry et al., 2002, WHO, 2003). Approximately 10-20% of hospitalised cases develop gastrointestinal bleeding due to the erosion of the Peyer's patch into an intestinal vessel, this phenomenon is normally manifested by occult blood in the stool or malaena (Bhan et al., 2005). Rapid death occurs with presentation of severe complications such as disseminated intravascular coagulation or haemorrhage as a result of gastrointestinal bleeding.

Approximately 1-3% of hospitalized cases develop intestinal perforation, with the ileum being the most common site for perforation (Ukwenya et al., 2011, Basten and Stockenbrügger, 1994).

Various other rare complications have been reported that include hepatic, splenic and bone marrow granulomas, splenic and liver abscesses, pleural effusions, multiple organ dysfunction syndrome, pseudotumour cerebri, hemolytic uraemic syndrome and peritonitis (Mert et al., 2004, Snyder et al., 2004, Albaqali et al., 2003, Bhan et al., 2005, Chaudhary et al., 2003).

Febrile relapse occurs in 5-10% of typhoid fever patients, this relapse generally occurs 2-3 weeks after the resolution of fever (Parry et al., 2002) and in 8% of paratyphoid patients (Goh, 1981). Relapses are usually milder than the original attack, and the salmonellae isolated from the case in relapse generally have the same susceptibility pattern as of the isolate during the original episode (Cammie and Miller, 2005). Approximately 10% of convalescent patients with untreated enteric fever excrete typhoidal salmonellae in their feces for up to 3 months post infection; approximately 1-4% of enteric fever cases become long-term carriers and may shed organisms in

their stool or urine for more than a year (Gonzalez-Escobedo et al., 2011, Levine et al., 1982, Parry et al., 2002). Up to 25% of long term carriers are asymptomatic and have no history of enteric fever and are asymptomatic (Crawford et al., 2010, Parry et al., 2002). The rates of chronic carriage rates are higher among women and the elderly with biliary abnormalities such as cholelithiasis (Levine et al., 1982). Patients with structural abnormalities of the urinary tract, and those suffering from schistosomiasis, are capable of excreting organisms in the urine for long periods (Parry et al., 2002).

With the administration of early and appropriate antimicrobial therapy, the case fatality rate for enteric fever has fallen to less than 1%, however fatality rates vary in different regions of the world (Parry et al., 2002). Fatality rates are higher among hospitalised cases of enteric fever and the rates vary from less than 2% as reported from Vietnam (Tuyet Hoa et al.) and Pakistan (Bhutta, 1996) to as high as 30-50% from Indonesia (Hoffman et al., 1984, Punjabi et al., 1988) and Papua New Guinea (Rogerson et al.). Case fatality rates are known to be highest among children under one year of age and among the elderly (Butler et al., 1991, Bhutta, 1996). However, the most important contributor to a poor outcome is a delay in instituting effective antimicrobials (Parry et al., 2002).

1.3.2 Diagnosis of enteric fever

Differentiating enteric fever from other causes of febrile illnesses such as malaria, dengue, leptospirosis and rickettsial infections in endemic countries is difficult as many of the symptoms are non-specific and tend to overlap with other infections. For patients in countries where enteric fever is not endemic, a travel history is crucial (Basnyat et al., 2005). Though the culture of blood

or bone marrow is the principle method for diagnosis, culture facilities are commonly unavailable in areas where the disease is endemic (Peacock and Newton, 2008, WHO, 2003). Additionally cultures maybe positive in only 40-50% of the presumed cases (Gasem et al., 1995, Akoh, 1991, Farooqui et al., 1991, Gilman et al., 1975, Guerra-Caceres et al., 1979). The time taken to obtain a culture report is always more than 24 hours, and a blood culture can take up to a week to generate results. To overcome these shortcomings various approaches, such as, antibody detection (Gupta et al., 2006, House et al., 2001b, Losonsky et al., 1987, Nardiello et al., 1984, Pulickal et al., 2009, Shaheen et al., 1995) or antigen detection (Gupta and Rao, 1979, Nguyen et al., 1997, Pandya et al., 1995, Rockhill et al., 1980, Taylor et al., 1983), genomics (Ou et al., 2007, Parkhill J et al., 2001), proteomics (Ansong et al., 2008), transcriptomics (Slonim and Yanai, 2009, Sturdevant et al., 2010), *in vivo* induced antibody technology (IVIAT) (Harris et al., 2006) and immune-affinity proteomics-based technologies (Charles et al., 2010) are being employed to identify new antigens, gene targets and metabolic products that could be used as a basis for an improved and effective diagnosis of enteric fever (Parry et al., 2011c).

Several serologic tests have been developed to detect antibodies for *S.* Typhi and *S.* Paratyphi A. The role of the Widal test that measures the agglutinating antibodies against the somatic (O) and flagellar (H) antigens of both the serovars remains controversial. Though easier, less expensive to perform, and more widely available, it lacks sensitivity and specificity, particularly when used as a single screening test for patients with fever (Parry et al., 1999, House et al., 2001b, Buck et al., 1987).

Infection with various serovars of salmonellae induces specific serum antibody responses in the

host, and thus serological techniques such as enzyme linked immunosorbent assay (ELISA) and immunoblotting have been widely applied for the diagnosis of enteric fever. ELISA using the LPS, flagella, Vi or outer membrane protein antigens have been used to more precisely define the normal antibody response (Nardiello et al., 1984, Pulickal et al., 2009, Shaheen et al., 1995). Though ELISAs measuring anti-LPS and anti-Vi antibodies were observed to be comparatively more sensitive than the Widal test, these methods face similar limitations to the Widal test in terms of specificity (Parry et al., 2011a). SDS-PAGE/immunobloting techniques are claimed to be highly efficient for the detection of human antibodies to *S.* Typhi (Chart et al., 2007), however these methods require additional validation (Parry et al., 2011c).

Rapid diagnostics tests that are simple, reliable and easy to use at the point of care have been a long felt need of health care workers in endemic countries. Several attempts have been made to package serological tests to answer these needs; those that are currently available are based on differing methods and formats. Rapid serological tests such as the Typhidot® and Typhidot-M®, IDL Tubex® test and the IgM dipstick are available but these require further more intense evaluations before they become routinely used (Choo et al., 1999, Tam and Lim, 2003, Abdoel et al., 2007, Pastoor et al., 2008).

Antigen detection methods, through the use of polyclonal and monoclonal antibodies have also been developed to detect *S.* Typhi in body fluids through co-agglutination and ELISAs (Banchuin N, 1987, Rockhill et al., 1980, West et al., 1989, Chaicumpa et al., 1988). The antibodies for these tests are directed against the Vi, O9 and Hd antigens similar to the serological tests. Though the greatest level of sensitivity has been found with an ELISA method detecting the Vi antigens

in urine, the method shows limited specificity (Chaicumpa et al., 1992, Fadeel et al., 2004). However, as with serological tests against antibodies, these tests also require further validation.

Nucleic acid amplification tests, namely conventional PCR or real time PCR have both been explored for the detection and diagnosis of *S.* Typhi and *S.* Paratyphi A from several sterile sites, typically blood and bone marrow (Yan et al., 2004, Nga et al., 2010). Various genes have been targeted and PCR variants such as nested PCR, multiplex PCR, and PCR amplification of the blood culture after a short incubation period have also been explored with varying degrees of success (Song et al., 1993, Nizami et al., 2006, Ali et al., 2008, Chaudhary et al., 1997, Zhou and Pollard, 2010).

Further understanding of the genomes of *S.* Typhi and *S.* Paratyphi A could lead to new and better targets for nucleic acid amplification (Ou et al., 2007). *S.* Typhi and *S.* Paratyphi A are known to have limited genetic diversity within their populations (Parkhill J et al., 2001, Roumagnac et al., 2006). This fact could aid DNA test specificity over other Gram-negative organisms. Between 1 and 3% of the gene content of *S.* Typhi and *S.* Paratyphi are unique and a significant proportion of the genes remain without known function. This lack of diversity in genome architecture could be an indication for further genomic exploration through functional genomics.

There have been rapid developments in DNA based tests for detecting bacterial pathogens, but their utility for diagnosing enteric fever remains doubtful. The time of illness when the sample is

collected and the type of sample are of the utmost importance, as the limited quantity and the transient nature of the bacteria in blood hinders their detection (Baker et al., 2010a). Further, diagnosis by DNA amplifications in endemic areas will be limited for reasons of cost and overall utility, it may play a role in research studies where a variety of tests could be used to provide confirmed diagnosis of study patients.

1.3.3 Treatment of enteric fever and antimicrobial resistance

Optimal antimicrobial treatment of patients with enteric fever depends on simultaneous understanding of the local antimicrobial resistance patterns and the antimicrobial susceptibility of the *Salmonella* isolate obtained from individual patients (Crump and Mintz, 2010).

Supportive measures such as oral or intravenous hydration, use of antipyretics and appropriate nutrition or blood transfusion when indicated are essential for the management of severe enteric fever. Yet, antimicrobials are the mainstay of enteric fever treatment. The majority of enteric fever patients can be treated as outpatients if provided with appropriate antimicrobials, reliable care and close medical observation in the case of complications. However, if there is persistent vomiting, severe diarrhoea, and abdominal distention then hospitalization with parenteral antimicrobial therapy is required (WHO, 2003).

Since the late 1940s, chloramphenicol, a drug that inhibits bacterial growth by binding to the 50s ribosomal subunit and stopping protein synthesis, was the standard treatment for enteric fever until the emergence of resistance (Parry et al., 2002, Bhan et al., 2005, Woodward et al., 1948).

In the early 1970s, with outbreaks in Mexico, India, Vietnam, Thailand, Korea and Peru, chloramphenicol resistant typhoid fever raised serious public health concerns (Bhutta, 2006, Mirza et al., 1996). In addition, these strains were also found to be resistant to the sulfonamides, tetracycline, and streptomycin but were sensitive to amoxicillin and trimethoprim-sulphomethoxazole. Resistance to chloramphenicol was attributed to a high molecular weight, self-transferable, *Inc*HI plasmid (Parry et al., 2002). In the late 1980s and 1990s *S.* Typhi developed MDR strains (Mirza et al., 1996) and the subsequent spread of these strains led to major outbreaks in India, Pakistan, Bangladesh, Vietnam, the Middle East and Africa (Parry et al., 2002).

Surveillance studies have demonstrated that there are significant differences in the proportion of MDR isolates within the same region, for example MDR *S.* Typhi is more prevalent in India, Pakistan and Vietnam than in comparison to China and Indonesia (Ochiai et al., 2008). Longitudinal studies have also demonstrated that the proportion of MDR strains can decrease over time following changes in antimicrobial use (Maskey et al., 2008, Lakshmi et al., 2006). In endemic areas where chloramphenicol resistance has been high enough to restrict the use of the antimicrobial, the re-emergence of sensitive strains has been reported (Wain and Kidgell, 2004). This is assumed to be in response to a decrease in selective pressure as the removal of any selective advantage conferred by the plasmid results in the less-fit resistant isolates being reduced in the bacterial population to be replaced by plasmid free strains (Wain and Kidgell, 2004, Sood et al., 1999b). Although the re-emergence of chloramphenicol sensitive *S.* Typhi and *S.* Paratyphi A may ensure that this inexpensive drug will remain in use in developing countries of Asia, the drug can induce a higher rate of relapse particularly if the full 14 day course is not completed

(Thaver et al., 2009, Parry et al., 2002) and prolonged treatment exposes patients to rare but serious toxicity such as bone marrow depression (Butler, 2011).

Effective antimicrobials are essential for appropriate clinical management of enteric fever, yet the emergence and rapid dissemination of MDR strains in endemic areas limited the options for treatment (Chau et al., 2007, Chuang et al., 2008). With the advent of MDR strains, fluoroquinolones became the drugs of choice for treatment of uncomplicated typhoid fever (Parry et al., 2002). Fluoroquinolones are a subgroup of quinolones that interact with the bacterial topoisomerases, DNA gyrase and topoisomerase IV (Khodursky and Cozzarelli, 1998, Shen et al., 1989). Both these enzymes are composed of two pairs of subunits that participate in DNA replication, transcription, recombination, and repair. The quinolones bind to these targets and arrest DNA replication, leading to cell death (Hernandez et al., 2011). The first quinolone, nalidixic acid was introduced in 1962 and since then structural modifications have resulted in four generations of fluoroquinolones that have improved coverage for bacterial infections (Oliphant and Green, 2002).

Second generation fluoroquinolones such as ofloxacin and ciprofloxacin were selected for enteric fever treatment due to their potent bactericidal activity against *S.* Typhi and *S.* Paratyphi A. *In vivo*, both drugs have plasma levels considerably in excess of the prevailing MICs and excellent intracellular penetration (Bethell et al., 1996). In endemic areas these antimicrobials are frequently sold over the counter to treat febrile illnesses of various aetiologies. This overuse of fluoroquinolones has lead to extensive selection pressure, which has resulted in the emergence of strains with elevated MIC, which, in turn have been associated with major epidemics (Murdoch et al., 1998, Threlfall, 2002). The current WHO guidelines recommend fluoroquinolones as the

optimal drug of choice for the treatment of uncomplicated enteric fever in adults caused by fully sensitive and MDR organisms (WHO, 2003). Since the detection of *Salmonella* strains with reduced susceptibility to fluoroquinolones, the occurrence of these strains has increased in Asia and are becoming increasingly common in Africa (Chau et al., 2007, Chuang et al., 2008, Cooke et al., 2007, Lynch et al., 2009 , Smith et al., 2012, Parry and Threlfall, 2008). For effective treatment, the identification of salmonellae with low susceptibility to fluoroquinolones is important but according to current guidelines these strains are still categorized as sensitive (Parry et al., 2011b). *Salmonella* strains with low susceptibility to fluoroquinolones can be identified by using resistance to nalidixic acid as a surrogate marker for fluoroquinolone susceptibility (Cooke et al., 2007, Kapil and Das, 2002).

A third generation fluoroquinolone, gatifloxacin, has been shown to be effective in the treatment of nalidixic acid resistant enteric fever in randomized control trials performed in Nepal (Arjyal et al., 2009, Pandit et al., 2007) and Vietnam (Dolecek et al., 2008). Though the WHO states that all available fluoroquinolones, with the exception of norfloxacin which has inadequate oral bioavailability, are highly active and equivalent in efficacy, differences in efficacy between available fluoroquinolones or between different doses or durations of treatment with an individual fluoroquinolone have not yet been demonstrated hence, clinicians need to take into account current, local resistance patterns (WHO, 2003, Effa Emmanuel et al., 2011).

A consensus for the oral antimicrobial regimen for uncomplicated typhoid fever caused by strains that are MDR and also have a low susceptibility to fluoroquinolones does not exist (Parry et al., 2007). Second-line therapies that have been considered for resistant bacteria include the 3rd

generation cephalosporins, such as ceftriaxone and cefixime, other beta lactams such as aztreonam and imipenem, and the azalide, azithromycin. Trials of ceftriaxone showed the antimicrobial to be a credible alternative drug, particularly in comparison to chloramphenicol (Islam et al., 1993, Acharya et al., 1995, Islam et al., 1988). However, it has been observed that a shorter duration of treatment with ceftriaxone led to a higher relapse rate (Bhutta et al., 2000, Tatli et al., 2003). There have been few studies looking at the efficacy of cefixime for the treatment of enteric fever, but existing trials show higher rates of failure and relapse than fluoroquinolones (Pandit et al., 2007, Phuong 1999, Girgis et al., 1995b). Furthermore, the potential emergence of ESBL expressing strains of *S.* Typhi and *S.* Paratyphi A, are a serious concern (Pfeifer et al., 2009, Pokharel et al., 2006)

Clinical trials studying the efficacy of aztreonam have demonstrated mixed results, and imipenem is typically used only as third line therapy. Both drugs are expensive and require parenteral administration that limits their desirability (Parry, 2004a).The azalide, azithromycin, has proven to be the most promising antimicrobial for the treatment of uncomplicated enteric fever caused by MDR strains with low susceptibility to fluoroquinolones. It has proved to be efficacious in clinical trials for both children and adults and in prospective comparative studies has proven to be more efficacious than the ofloxacin in adults (Parry, 2004a, Frenck et al., 2000, Butler et al., 1999, Chinh et al., 2000, Dolecek et al., 2008, Parry et al., 2007, Frenck et al., 2004, Girgis et al., 1995a, Girgis et al., 1999, Gotuzzo et al., 1994). Other than the higher cost, it outperforms other antimicrobials with regard to cure rates, fever clearance times against infections caused by bacteria with reduced susceptibility to fluoroquinolones, and prevention of faecal carriage and relapse (Dolecek et al., 2008, Parry et al., 2007, Frenck et al., 2000, Frenck et al., 2004, Girgis et

al., 1999, Tribble et al., 1995, Butler et al., 1999). However, there have been some reports of resistance to azithromycin (Harish and Menezes, 2011) and hence further studies with this antimicrobial are warranted and monitoring of resistance emergence is required.

1.4 Epidemiology of enteric fever

1.4.1 Global epidemiologic trends

An accurate assessment of the true global burden of enteric fever is difficult to estimate, due to reporting rates, a lack of diagnostics and a lack of disease understanding. Our best current estimates originates from the year 2000, when an estimated 21.7 million illnesses and 217,000 deaths due to typhoid fever and 5.4 million illnesses due to paratyphoid fever were calculated from available data (Crump and Mintz, 2010). Historically, prior to the beginning of the twentieth century, enteric fever was a significant cause of morbidity and mortality in the overcrowded and unsanitary conditions of European and North American cities (Cutler D, 2005, WILSON, 1978). Advances in public health, such as the provision of reliable and clean water supplies and sewage systems, led to a decrease in the incidence of enteric fever in these places. Today the vast burden of disease is encountered in the developing world where poor sanitary conditions remain and in addition facilitate transmission. While enteric fever in rural communities is not uncommon, the overwhelming focus is akin to pre-twentieth century socio-economics of the disease and affects the impoverished living in urban centers. There are major limitations on the current epidemiologic data available, but some epidemiologic trends in enteric fever have been observed in the African, Asian, and Latin American regions.

1.4.2 African epidemiologic trends

In sub-Saharan Africa the overall burden of enteric fever is not well characterized, hospital based studies have shown that the major cause of bloodstream infections are attributable to *S.* Enteritidis and *S.* Typhimurium (Mweu and English, 2008, Reddy et al., 2010). Outbreaks of typhoidal salmonellae are, however, reported from the African continent, surprisingly with a large number of people reporting with complications, predominantly intestinal perforations (Akinyemi et al., 2005, Clegg-Lamptey et al., 2007, Muyembe-Tamfum et al., 2009, Weeramanthri et al., 1989).

1.4.3 Latin American epidemiologic trends

In Latin America, with economic growth and introduction of adequate water and sanitation measures, there is evidence that incidence of typhoid fever has reduced dramatically. Though enteric fever still remains a public health problem in the region, it provides insight into what can be accomplished for countries where the illness is epidemic (Crump and Mintz, 2010, Crump et al., 2004, Clemens et al., 1999, Levine et al., 1982, Black et al., 1990).

1.4.4 Asian epidemiologic trends

The majority of the morbidity and mortality attributable to typhoidal salmonellae occurs in Asia and is predominantly observed among infants, children and adolescents (Crump and Mintz, 2010, Ochiai et al., 2008). A large population based prospective study estimating enteric fever incidence in five Asian countries not only confirmed the high incidence of the illness in this region but demonstrated substantial variation in incidence between surveillance sites across the

continent (Ochiai et al., 2008). Similarly, enteric fever attributable to *S.* Paratyphi A has been seen in an increasing proportion of the cases in various Asian countries (Kanungo et al., 2008, Ochiai et al., 2005, Woods et al., 2006). Enteric fever is an ongoing public health problem, particularly in South Asia, and Nepal is known to have a high burden (Karkey et al., 2008, Maskey et al., 2006). Kathmandu, the capital city of Nepal, has previously been coined an enteric fever capital of the world (Karkey et al., 2008). Unsanitary conditions remain apparent in some parts of twenty-first century Kathmandu, where living conditions appear to be optimal for constant transmission. Accurate estimates of the burden of enteric fever in the context of Nepal and Kathmandu are difficult to obtain as diagnostic facilities tend to be limited within the city and are non-existent in many rural areas. In endemic countries such as Nepal, the adolescent population is considered to be the most at risk (Sharma et al., 2006). However, in a recent study conducted in Kathmandu, it was demonstrated that natural immunity to *S.* Typhi increased with age, inferring that the burden of typhoid fever amongst young children may have been underestimated (Pulickal et al., 2009). Studies have suggested that parts of the Kathmandu valley have some of the highest prevalence of enteric fever in the South Asian region (Karkey et al., 2008, Maskey et al., 2006). With indiscriminate use of antimicrobials, cases of resistant salmonellae are increasing in Kathmandu (Koirala et al., 2012, Neopane et al., 2008).

Reported risk factors include contact with enteric fever patient, not washing hands with soap, living in crowded households with poor housing and past evidence of *Helicobater pylori* infection (Vollaard, 2004, Bhan et al., 2002, Luxemburger et al., Gasem et al., 2001).

1.4.5 Prevention and control measures

Enteric fever is transmitted through the faecal-oral route and contaminated water and food are thought to be important vehicles for transmission (Vollaard, 2004, Sur et al., 2007). Based on the available evidence, the prevention of enteric fever should be focused on improving sanitation, ensuring availability of clean food and water, identifying and treating chronic carriers of *S.* Typhi and *S.* Paratyphi, and the use of available typhoid vaccines to reduce the susceptibility of hosts to infection (Crump and Mintz, 2010). Studies analysing measures of intervention for the prevention of diarrhoeal diseases have observed that improving the quality of drinking water could be more important for the prevention of enteric fever than sanitation measures (Esrey et al., 1985, Esrey et al., 1991). A growing body of research suggests that improving water quality at the household level, as well as the source, can significantly reduce diarrhoea (Clasen et al., 2007). Yet, extending the benefits of sanitation and availability of uncontaminated food and water to low and middle-income countries has proved to be difficult (Crump and Mintz, 2010). Though the identification and treatment of *S.* Typhi carriers, particularly those involved with food production has proven to be an important strategy for the control of typhoid fever in low incidence countries, identification of carriers at the community level in endemic areas has proved to be a challenge (Crump and Mintz, 2010).

Licensed, safe and efficacious vaccines against typhoid fever, the injectable Vi polysaccharide and the oral Ty21a, are available at a low price (Vi polysaccharide cost US $0.50 per dose) (Ochiai et al., 2008). Yet these vaccines are not implemented as routine public health measures in endemic countries despite the high cost of treatment (Bahl et al., 2004). Lack of updated data on enteric fever incidence or local epidemiological patterns in endemic areas has hampered the

implementation of effective vaccination programs (DeRoeck et al., 2007). In addition, these vaccines are not effective against paratyphoid fever and there are currently no vaccines effective against *S.* Paratyphi, which is a matter for concern given the evidence for the ongoing emergence of the pathogen (Ochiai et al., 2005). The considerable geographic heterogeneity of typhoid fever burden in endemic areas of Asia, such as Nepal, underscores the importance of evidence on disease burden towards making policy decisions about interventions to control the disease (Ochiai et al., 2008).

1.4.6 Population structure of *S.* Typhi and *S.* Paratyphi A

S. Typhi and *S.* Paratyphi A are human restricted pathogens, yet whilst they are members of the same genus they are genetically distinct. They have convergently evolved to harbor many common characteristics, including the ability to cause clinically indistinguishable infections (Jacobsen et al., 2011). Yet, one of the classical characteristics they share is clonality, the populations of both organisms are monophyletic, i.e. they lack extensive genetic (and phenotypic) variation (Kidgell et al., 2002). This lack of diversity hinders epidemiological examinations and limits our ability to investigate evolution, transmission and regional colonisation. Our overall understanding of the population structure of *S.* Paratyphi A is limited due to a lack of studies assessing the global and temporal diversity of isolates. This is not the case for *S.* Typhi as genomic variation was originally assessed through Single Nucleotide Polymorphisms (SNPs) detected in strains compared to the original *S.* Typhi CT18 genome sequence (Holt et al., 2008a). Roumagnac et al. created a highly parsimonious phylogenetic structure comprised of data from 105 strains isolated over 84 years on three continents (Roumagnac et al., 2006). This work identified almost no genetic homogeneity and demonstrated the persistence of multiple

haplotypes in a single country over decades, indicating a stable population rather than clonal replacement by new lineages. Latterly, the granularity of this population structure was further enhanced by genome sequencing of multiple *S.* Typhi isolates, which corresponded to specific locations within the phylogenetic structure (Holt et al., 2008a). The consequence of these two major studies was to explain the overriding nature of the diversity within this clonal pathogen. We now know that there is no evidence of purifying selection, antigenic variation or recombination between differing *S.* Typhi isolates. This lack of evidence for antigenic variation driven by immune selection is surprising for a human restricted pathogen that has an intimate association with the immune system. In contrast there appears to be a strong adaptive selection for mutations conferring antimicrobial resistance (Holt et al., 2008a). Unsurprisingly, the organism is in genetic isolation, which is consistent with long-term carriage of *S.* Typhi in the gallbladder.

1.4.7 Genotyping

Our ability to now genotype *S.* Typhi originates from a complement of SNPs that have been identified through SNP detection, sequencing and genome re-sequencing (Baker et al., 2008, Holt et al., 2009, Holt et al., 2010, Roumagnac et al., 2006). As *S.* Typhi and *S.* Paratyphi A exhibit exceptionally low levels of genetic variation, the current gold standard for bacterial genotyping, multi-locus sequence typing (MLST), has insufficient resolution for distinguishing within groups of these organisms (Kidgell et al., 2002). Alternative techniques such as pulsed-field gel electrophoresis (PFGE), RAPD and RFLP have been used to try and discriminate between isolates, but do not generate the type of fine genomic information that can be applied to study global evolution or local transmission patterns (Baker et al., 2010b). Using such high-resolution

genotyping enables investigators to accurately and consistently distinguish between circulating *S.* Typhi strains, allowing the diversity of the organism to be precisely defined within any given temporal or geographical boundary (Baker et al., 2008). This methodology was used to identify *S.* Typhi haplotypes circulating in an urban area of Jakarta. In the study by Baker et al., the authors SNP profiled 140 *S.* Typhi strains, identifying nine haplotypes circulating in the Indonesian archipelago over more than 30 years, with eight detected in a single suburb over two years. The findings from this work demonstrated that genotypic and phenotypic differences exist within the Typhi population within a limited geographical area over a short time frame. Furthermore, SNPs were used to differentiate the population of Typhi causing infections in the Mekong river delta, highlighting a temporal linkage between MDR strain and specific haplotypes (Holt et al., 2011).

1.5 Hypothesis of the study

Enteric fever is an ongoing problem in Kathmandu, caused by the co-circulation of *S.* Typhi and *S.* Paratyphi A. The city is ever expanding and the infrastructure, water systems and sanitary systems are outdated and cannot cope with the demand. Owing to the level of national poverty, the infrastructure is unlikely to be improved over the coming years. There is a clear lack of epidemiological data related to enteric fever in locations such as Kathmandu. I aimed to address some of these gaps in the knowledge of the epidemiology of enteric fever. Owing to the lack of sanitary conditions, my primary hypothesis was that the majority of the transmission of *S.* Typhi and *S.* Paratyphi occurs through extrinsic factors such as the municipal water supply, rather than through intrinsic factors such as contact within households. The rationale for this hypothesis is that the members of the genus *Salmonella* are highly adaptable and known to be capable of surviving for several days in sea and groundwater (Wait and Sobsey, 2001). Furthermore, faecal contamination of urban water supplies in Nepal has been associated with an outbreak of *S.* Typhi infecting 5,936 people in Bharatpur in 2002 (Lewis et al., 2005). A study from the Terai region of southern Nepal found that coliforms were present in 61% of the provided water samples (Atreya et al., 2006). In an urban setting, (Bhatta et al., 2007), could isolate and identify multiple drug resistant *S.* yphi and *S.* Paratyphi in the drinking water supply of 14% of their samples (Bhatta et al., 2007).

1.6 Aims and objectives of the study

The primary aims and objectives for this investigation of epidemiology of enteric fever were:

1. To describe epidemiologic trends of enteric fever attributable to *S.* Typhi and *S.* Paratyphi within our study population in LSMC area of the Kathmandu Valley.
2. To identify probable transmission routes for enteric fever attributable to *S.* Typhi and *S.* Paratyphi within our study population in LSMC of the Kathmandu valley that will aid in the development of a rational control strategy.

The secondary aims and objectives for this investigation of epidemiology of enteric fever were:

1. To calculate the burden of enteric fever attributable to *S.* Typhi and *S.* Paratyphi at Patan Hospital.
2. To estimate the incidence of enteric fever attributable to *S.* Typhi and *S.* Paratyphi in the population residing in LSMC.
3. To define the age and gender distribution of enteric fever patients within LSMC.
4. To define any seasonal trends of enteric fever cases attributable to *S.* Typhi and *S.* Paratyphi that may infer a relationship to the local water supply
5. To identify enteric fever "hot-spots" by localization of cases attributable to *S.* Typhi and *S.* Paratyphi using geographical data.

6. To genotype *S.* Typhi cases of enteric fever attributable to this pathogen, use the data to define the local population and to infer potential transmission routes.
7. To calculate the extent of water contamination by examining microbiological, physical and chemical properties of water from various sources within the study area.
8. To elucidate any prevailing risk factors of *S.* Typhi and *S.* Paratyphi by comparing patients with enteric fever attributable to *S.* Typhi and *S.* Paratyphi to afebrile community controls.

2 Materials and Methods

2.1 Methods applicable throughout thesis

2.1.1 Ethical approval and informed consent process

All the work presented in this thesis was conducted according to the principles expressed in the Declaration of Helsinki and was approved by the institutional ethical review boards of Patan Hospital (PH), The Nepal Health Research Council (NHRC) and The Oxford Tropical Research Ethics Committee (OXTREC) in the united Kingdom. The strains used for mapping were obtained from three clinical trials. These trials were gatifloxacin versus cefixime (ISRCTN75784880) (Pandit et al., 2007), gatifloxacin versus chloramphenicol (ISRCTN53258327) (Arjyal et al., 2009) and gatifloxacin versus ofloxacin (ISRCTN63006567), all of which went through the same ethical review process. All enrollees were required to provide written informed consent for the collection of samples, residential mapping and subsequent analysis, in the case of children this was provided by the parent or guardian.

2.1.2 Study site

The site for the work presented in this thesis was Lalitpur Submetropolitan City (LSMC), one of the urban districts within the Kathmandu Valley. LSMC is divided into 22 administrative wards and is situated on an elevated plateau of land, separated from the metropolitan city of Kathmandu by the Bagmati River to the north, and has a land area of 15.43 km^2 with a

population of 162,991 living in 68,922 households, according to the 2001 Nepali census (**Figure 3.3**).

The location for enrollment to the studies presented in this thesis was Patan Hospital in LSMC. Patan Hospital is the only general hospital in LSMC, though there are numerous private clinics and physician practices where patients may seek advise. It is a 318-bed government hospital providing emergency and elective outpatient and inpatient services. There are some 200,000 outpatient visits, 35,000 emergency visits and 15,000 admissions annually. Enteric fever is a common reason for outpatient attendance at Patan Hospital (**Chapter 3**) and is one of the few locations in LSMC capable of performing a blood culture and an accurate microbiological diagnosis of enteric fever. At Patan Hospital, all febrile patients suspected of having bacteraemia have a blood culture performed. There has been no widespread implementation of a typhoid vaccine in this area, a generic typhoid Vi vaccine is available for purchase in some health care settings, yet there is limited community uptake.

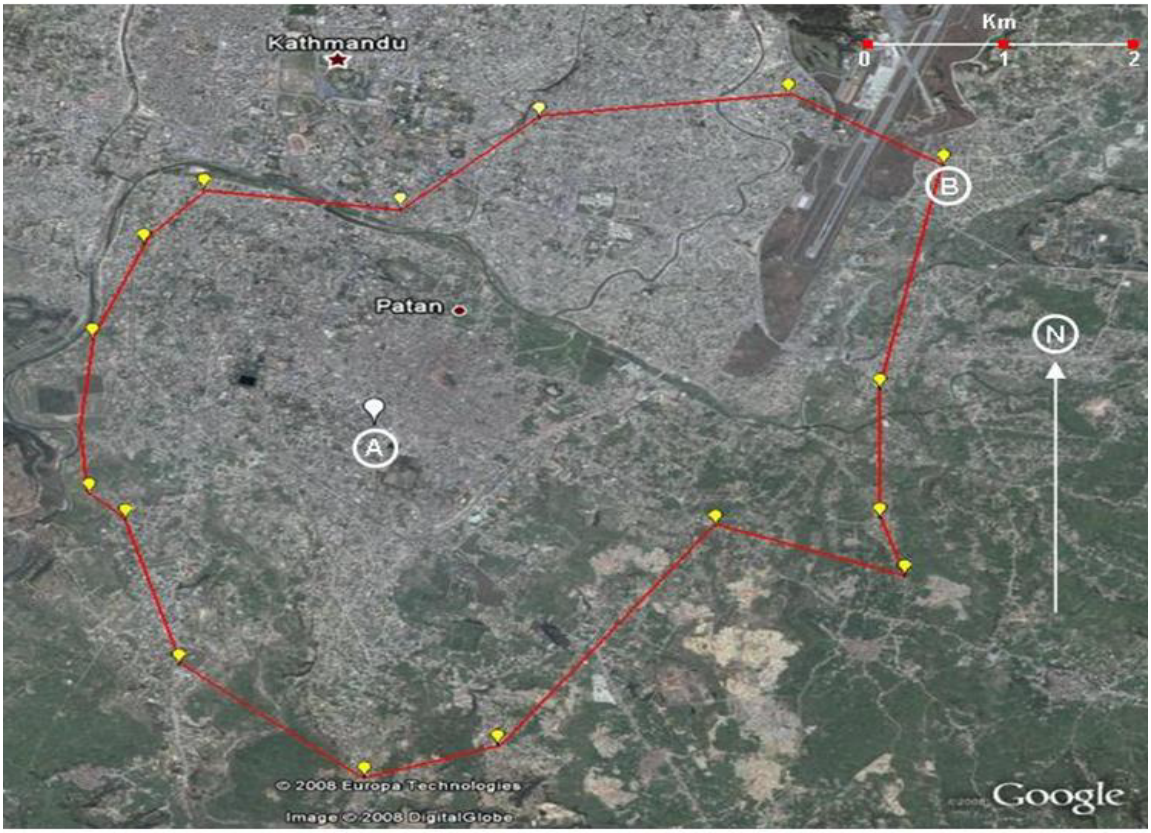


Figure 2.1: The enteric fever catchment area surrounding Patan Hospital

(Image source: Google Earth)

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

2.1.3 Study population

The population of LSMC is generally poor, overcrowding in residential buildings is common and people commonly obtain their drinking water from numerous sunken wells and municipal supplies located around the city. Antimicrobials are available with prescription in the community in a variety of outlets (clinics and pharmacies) and there are numerous private physician clinics where patients may seek advice and clinical diagnosis for febrile disease, such as enteric fever. Therefore, patients attending Patan Hospital may only represent the most severe end of the spectrum of enteric fever as many patients may seek local advice or self-treat.

The demographic data analyzed for the retrospective epidemiologic investigation (**Chapter 3**) and the GIS investigation (**Chapter 4**) were collected from patients enrolled in three consecutive randomized controlled trials as mentioned in **section 2.1.1**, at Patan Hospital for the treatment of uncomplicated enteric fever. The population enrolled in the clinical trials included patients who presented to the outpatient or emergency department of Patan Hospital between June 2005 and May 2009 and met enrollment criteria. The enrollment criteria were consistent for all three studies and are described in greater detail by Pandit *et.al.* (Pandit et al., 2007).

The patients enrolled for the case control study were patients who had a positive blood culture for either *S.* Typhi or *S.* Paratyphi presenting at either the outpatient or emergency department of Patan Hospital from April 2010 to May 2011. Patients from two of the randomized controlled trials, gatifloxacin versus ofloxacin (ISRCTN63006567) and gatifloxacin versus

ceftriaxone (NCT01421693) were also included.

All of the patients enrolled in the various studies resided within the study catchment area around Patan Hospital in LSMC (**Figure 2.1).**

2.2 Microbiological methods for diagnosing and identifying invasive *Salmonella* species

2.2.1 Blood culture

From individuals with suspected enteric fever (typically with a temperature of ≥ 37^{o}C for ≥ 5 days without a focus of infection) 10ml samples of venous anti-coagulated blood were collected in ethylene diaminetetraacetic acid (EDTA) tubes from those over the age of 12 years, and 5 ml from those 12 years of age or younger. For the culture of invasive salmonellae, 6 ml and 3ml of blood were used from those over 12 years of age and those 12 years of age or less, respectively. EDTA blood was inoculated into 30-50ml of medium containing tryptone soya broth and sodium polyethanol sulphonate. The inoculated medium was incubated at 37°C and examined daily for bacterial growth over a 7 day period. If the broth became turbid, indicative of growth, the inoculated broth was sub-cultured onto MacConkey agar medium to isolate invasive salmonellae. Any colonies presumptive of *S.* Typhi or *S.* Paratyphi A were identified using standard biochemical tests and serotype-specific antisera (Murex Biotech, Dartford, UK). All *S.* Typhi and *S.* Paratyphi strains were stored at -80°C in 20% glycerol on site. Duplicates were dispatched for secondary verification at the microbiology laboratory at Oxford University Clinical Research Unit in Ho Chi Minh City and were stored until required at -80°C in 20% glycerol.

2.2.2 API 20E

All bacterial isolates were identified using the API20E biochemical identification kit, which is a standardised identification system for *Enterobacteriaceae* and other non-fastidious, Gram-negative rods that use 21 miniaturised biochemical tests and a database. The API20E strip consists of 20 microtubes containing dehydrated substrates of O-nitrophenyl-βD-galactopyranoside (ONPG), arginine (ADH), lysine (LDC), ornithine (ODC), trisodium citrate (CIT), sodium thiosulfate (H2S), urea (URE), tryptophane (TDA), sodium pyruvate (IND), acetoin (VP), gelatin (GEL), glucose (GLU), mannitol (MAN), inositol (INO), sorbitol (SOR), rhamnose (RHA), sucrose(SAC), melibiose (MEL), amygdalin (AMY) and arabinose (ARA). The procedure was performed according to the manufacturer's recommendations (Biomérieux). Briefly, the test substrates were inoculated with a bacterial suspension in sterile saline that reconstituted the media. After filling the cupules of ADH, LDC, ODC, H_2S and URE, the microtubes were overlaid with mineral oil. The incubation box was prepared by adding 5 ml of sterile water to the honeycombed wells of the tray to create a humid atmosphere, and the API strip was placed within the incubation box and incubated at 36°C ±2°C for 24 hours. During incubation, metabolism of the organism produced colour changes in the various tests spontaneously or in the case of VP, TDA and IND through the addition of reagents **(Figure 2.2)**. Additionally oxidase test was performed to detect the ability of the microorganism to produce the enzyme cytorchrome c oxidase. Each biochemical reaction was assessed according to the set score system that created a numerical seven digit profile for the organism which was used for identification following manufacturer's instructions.

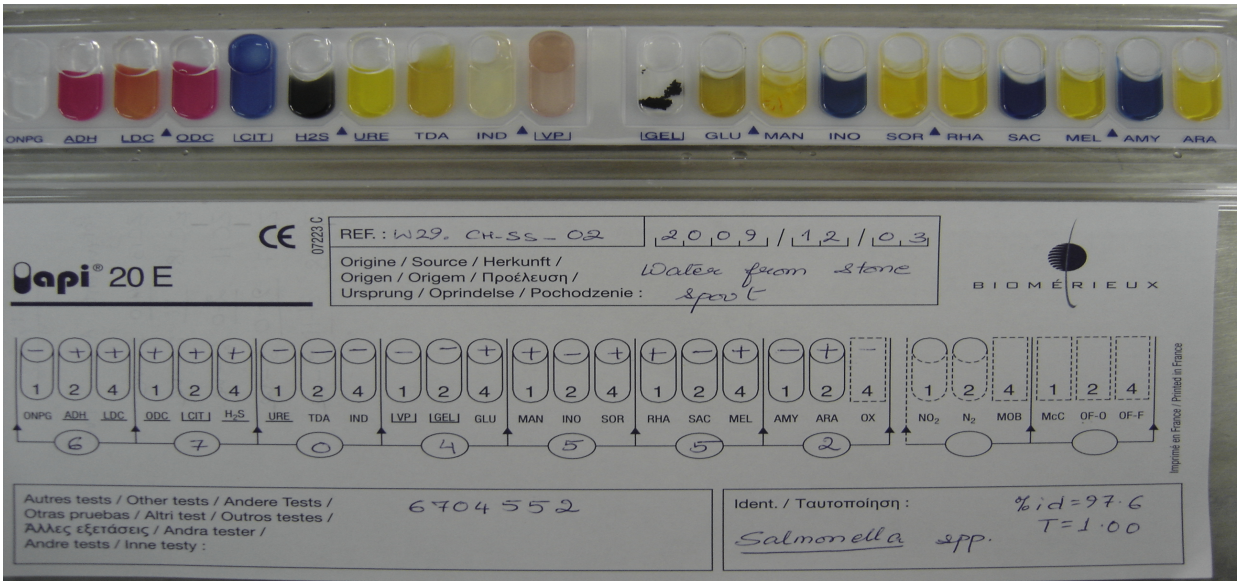


Figure 2.2: Representative biochemical characteristic of *Salmonella* species using the API 20E test.

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

2.2.3 Serological agglutination method

For isolates identified as *Salmonella*, *Shigella* or *Vibrio* species by the API 20E, confirmation of serovars was done through the use of serological agglutination with specific antisera.

For *Salmonella* serovars, *S.* Typhi strains were defined as isolates exhibiting agglutinations with the poly O, O_9 and Vi antisera (Murex, Dartford, UK). *S.* Paratyphi A strains were defined as isolates exhibiting agglutinations to poly O, and O_2 antisera. For *Vibrio* serovars, *V.* cholerae O1strains were defined as isolates exhibiting agglutinations with the polyvalent antiserum, and either or both of anitserums for serovars Inaba and/or Ogawa (Denka Seiken, Tokyo, Japan). *V.* 0139 strains were defined as isolates exhibiting agglutinations with the 0139 antisera. For *Shigella* serovars, *S. dysenteriae* strains were defined as isolates exhibiting agglutinations with the poly Group A antisera (Difco, Detroit, U.S.A.). *S. flexneri* and *S. boydii* strains were defined as isolates exhibiting agglutinations with the poly Group B and Group C antisera respectively. *S. sonnei* strains were defined as isolates exhibiting agglutinations with the Poly Group D antisera.

One drop of the appropriate agglutinating sera was placed on a clean glass slide. One colony of the test strain picked from a nutrient plate was mixed with the agglutinating serum. After thorough mixing, the slide was gently rotated for approximately 15 seconds and observed for agglutination with the naked eye (**Figure 2.3**). Positive (ATCC strains) and negative (sterile water) controls were also performed.

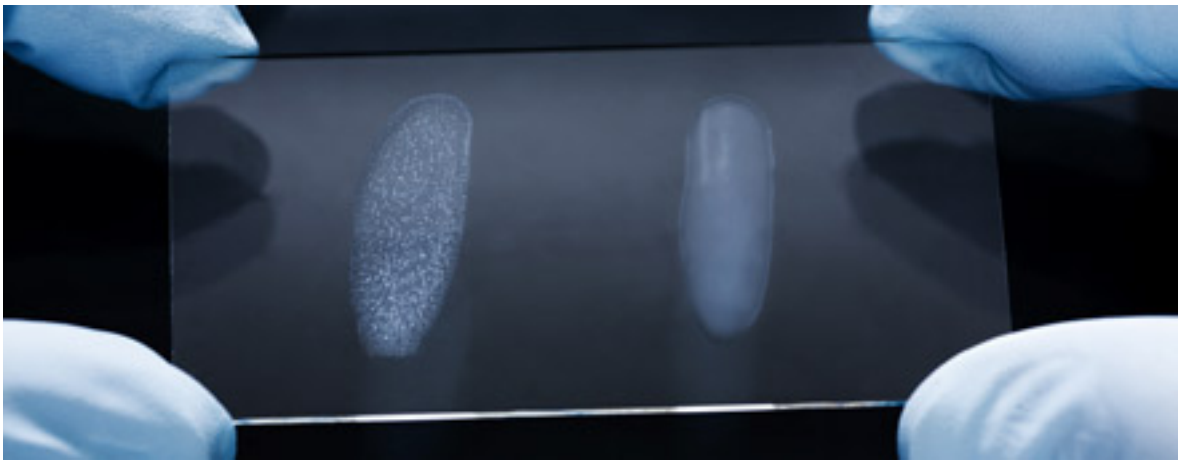


Figure 2.3: Slide showing positive (left suspension pool) and negative (right suspension pool) agglutination reactions when mixed with specific antisera.

When a bacterial culture (*S.* Typhimurium) was mixed with the specific antiserum (*Salmonella* H antigen) directed against the bacterial surface components, the cells bound together through antigen-antibody bonds to form aggregates that were visible to the naked eye as clumps in the suspension (left pool suspension). In the absence of the surface component (*Salmonella* H antiserum) on the organism (*E. coli*) there is no antigen- antibody bonding and hence no aggregate clumps are formed. (Image source: Statens Serum Institut, www.ssi.dk)

2.2.4 Bacterial DNA extraction

After identification of organisms, total nucleic acid was isolated using the Wizard ® Genomic DNA purification kit. Overnight cultures, scraped from 50 % of a media plate, were suspended in 1ml of nutrient broth. The mixture was thoroughly vortexed to form an even suspension. The suspension was centrifuged for 2 minutes at 15,000 X g (Thermo Fishcer Scientific, IEC Micro CL17, Germany) and the supernatant was discarded. To the cell pellet 600 µl of nuclei lysis solution was added and mixed by gently pipetting the suspension. The solution was incubated for 5 minutes at 80°C and then allowed to cool at room temperature. To the suspension 3µlof RNAse solution was added, mixed and incubated at 37°C for 30 minutes, and then allowed to cool to ambient temperature. To the suspension 200 µl of protein precipitation solution was added and vortexed vigorously. The suspension was then incubated at -20°C for 10 minutes before centrifugation at 15,000 X g in a bench top microfuge for 10 minutes. The supernatant was transferred to a sterile microfuge tube containing 600 µl of isopropanol and gently mixed. The suspension was centrifuged for two minutes at 15,000 X g and the supernatant was discarded. To the solution 600µl of room temperature 70% ethanol was added and mixed by gentle pipetting. The suspension was centrifuged for two minutes at 15,000 X g, the supernatant was removed and the DNA pellet was air dried at ambient temperature. The DNA pellet was rehydrated with 100 µl of rehydration solution, and stored at -20°C until required.

2.3 Methods specifically related to Chapter 3: Retrospective data analysis and demographics

2.3.1 Retrospective hospital data

For the purpose of obtaining seasonal and annual trends of enteric fever cases within the Lalitpur area, I examined retrospective data from hospital records between 2005 and 2009. These data were extracted from the microbiology database and included the total number of blood cultures performed monthly and the number that were culture positive for *S.* Typhi or *S.* Paratyphi. These data were used to assess the hospital case burden and seasonal trends at this single health care facility. Data were also extracted from the hospital database on the total number of people attending the outpatients department over the same period.

2.3.2 Demographic data

The demographic data analyzed for this study were collected from patients enrolled in three consecutive randomized controlled trials at Patan Hospital for the treatment of uncomplicated enteric fever. These trials were gatifloxacin versus cefixime (ISRCTN75784880), gatifloxacin versus chloramphenicol (ISRCTN53258327) and gatifloxacin versus ofloxacin (ISRCTN53258327). The population enrolled in clinical trials included patients who presented to the outpatient or emergency department of Patan Hospital (as described above) between June 2005 and May 2009 and met enrolment criteria. The clinical trials enrollees were, therefore, a proportion of the total enteric fever cases at this health care facility. Patients were eligible to enter the studies if they had clinically diagnosed enteric fever (including a temperature of >37.5°C). Other inclusion criteria were that patients must be aged between 2

and 65 years, 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. Patients who had received a third generation cephalosporin, fluoroquinolone or macrolide in the week prior to presentation to Patan Hospital were also excluded.

2.3.3 Analysis for retrospective and demographic data

A case of enteric fever was defined as any patient with a positive blood culture for *S*. Typhi or *S*. Paratyphi attending Patan Hospital. A primary analysis of temporal and seasonal trends in the enteric fever case burden was performed on data from patients with enteric fever at Patan Hospital. Secondary demographic analysis was performed on the subset of patients that were enrolled in clinical trials, who represented a proportion of the total enteric fever cases attending the Hospital. All patients enrolled in clinical trials were resident in LSMC.

Data from those enrolled in clinical trials was combined with population data, obtained from the 2001 Nepalese census, to calculate a minimum estimate of the incidence of enteric fever in LSMC per 1,000 persons per year and per 1,000 households per year. Data on the average monthly rainfall between 2005 and 2009 were obtained from the meteorological station at Kathmandu airport.

To test for a linear trend over time in the monthly rate of enteric fever cases, a Poisson regression with the (log-transformed) monthly number of outpatient admission as an offset was used. These data were adjusted for over dispersion using quasi-likelihood. The Chi

squared test was used to compare the seasonal distribution of *S.* Typhi and *S.* Paratyphi cases. Spearman's rank correlation coefficient was used to investigate the statistical dependence between population density and the average distance of patients' residence from Patan Hospital and the ward-level incidence of enteric fever. A 2-tailed t-test was used to compare the age distribution of male and females with enteric fever.

2.4 Methods specifically related to Chapter 4: GIS analysis and bacterial genotyping

2.4.1 Definition of cases

For the purposes of the spatial analyses, a case of enteric fever was defined as a patient from whom a *S.* Typhi or *S.* Paratyphi organism was cultured from their blood on arrival in the clinic at the outpatients department according to the methodologies previously described. Cases were all attendees of the outpatients department or emergency department at Patan Hospital between June 2005 and May 2009 and were all enrolled in one of three consecutive randomized controlled trials for the treatment of uncomplicated enteric fever as described in **section 2.3**.

2.4.2 Definition of controls

As a comparator for spatial analyses of enteric fever a control population was required. Controls were randomly selected from afebrile attendees of the outpatients department at Patan Hospital seeking medical assistance from within the same radius (5 Km) of the hospital as the cases. These controls were used to adjust the spatial estimate of *S.* Typhi and *S.*

Paratyphi risk for the distribution of the population, and to account for hospital outpatient referral bias and locality bias of health care seeking behaviour for outpatients. Using a GPS receiver I recorded the location of the residences of 2,048 afebrile outpatients attending Patan Hospital over a year from June 2008 to May 2009.

The inclusion criteria for this control group was: being afebrile, to account for enteric patients with a negative blood culture or other infections which may also cause fever and may have a similar spatial distribution to enteric fever, such as leptospirosis or rickettsial infections; aged between 2 and 65 years; and providing verbal consent to the mapping of their residence. All outpatients meeting these criteria and attending the outpatients departments between 0900 hours and 1200 hours on weekdays were invited for enrollment.

2.4.3 GIS analysis

For the purposes of GIS analysis, support was sought from Professor Archie Clements at the University of Queensland in Australia. Professor Clements performed the K means analysis and calculated the elevation and the distance to the water sources.

Addresses are unreliable in Kathmandu; consequently all locations were recorded and stored with a handheld Etrex legend GPS device (Garmin, United Kingdom). Multiple infections in the same residence were also recorded and were defined as more than one culture case in the same residence, as defined by a single dwelling with an identical GPS location recording. GPS data (decimal degrees in latitude and longitude e.g. 27.67715, 85.32606) were entered along

with patient data in Excel 2007 (Microsoft, USA). GPS locations were converted to kml format and visualized and validated in Google Earth version 5 (http://www.google.com/earth /index.html). GIS data (land usage, hydrology and transport routes) concerning LSMC and the surrounding area were kindly provided by the Ministry of Land Reform and Management, Geodetic Survey Department, Government of Nepal, Kathmandu, Nepal. These data were combined with the GPS location data from the cases, controls and the water stone spouts in Quantum GIS version 1.5.0 (http://www.qgis.org/). All resulting maps were created from screenshots of the required datasets in Quantum GIS version 1.5.0.

Distance to nearest water spout of the cases and the controls was performed in Quantum GIS using a decimal degrees distance calculator. For elevation analysis, a global 90-metre digital elevation model dataset, originally developed by the US National Aeronautics and Space Administration (NASA), was obtained from the Consultative Group for International Agriculture Research (CGIAR) Consortium for Spatial Information (http://srtm.csi.cgiar.org/). The elevation of each case and control residence was extracted in Quantum GIS.

2.4.4 Detection of spatial clustering

The scale and significance of clustering of enteric fever cases with *S.* Typhi and *S.* Paratyphi A relative to controls was assessed using Ripley's *K*-functions, by the approach of Chetwynd and Diggle (Chetwynd et al., 2001a). The K-function produces a plot of the degree of spatial clustering of the cases relative to the controls over different distances. $K(t)$ is the number of events within distance t of an arbitrary event, divided by the overall density of events. We modeled $\hat{K}_1(t)$ and $\hat{K}_2(t)$, the homogeneous *K*-functions for cases and controls respectively,

and plotted the difference between them over different separating distances, *t*. This gives a plot of the degree of spatial clustering of the cases relative to the controls at different spatial scales. Statistical significance of the observed difference can be assessed using simulation, under the null hypothesis of no spatial clustering. For a series of iterations, the status (case or control) is randomly reallocated to each residence and the difference between $K_1(t)$ and $K_2(t)$ is calculated with the simulated dataset. An envelope, showing the maximum and minimum of the simulated values of $K_1(t)–K_2(t)$ at each separating distance can be plotted relative to the observed $\hat{K}_1(t)–\hat{K}_2(t)$. Significant clustering is deemed to occur over separating over separating distances *t* for which the observed difference between $\hat{K}_1(t)$ and $\hat{K}_2(t)$ exceeds the simulated envelope. One hundred and ninety nine simulations were performed, giving a significance threshold of $p = 0.05$ for rejecting the null hypothesis.

2.4.5 Bacteria genotyping

To identify circulating genotypes in the LSMC region, genotyping was performed by a combination of SNP typing and pyrosequencing. I performed these methods through the kind assistance of Professor Gordon Dougan's group at the Wellcome Trust Sanger Institute in Cambridge where I spent four weeks using this technology. Dr Kathryn Holt at the University of Melbourne, Australia and my supervisor Dr Stephen Baker at Oxford University Clinical Research Unit, Ho Chi Minh City, Vietnam, assisted data analysis.

2.4.6 Single nucleotide polymorphism (SNP) genotyping

DNA was extracted from *S.* Typhi isolates **(Table 9.1)** as previously described in **section**

2.2.4. DNA quality and concentration was assessed using the Quant-IT kit (Invitrogen, USA) prior to SNP typing. Alleles at 113 Typhi chromosomal loci **(Table 9.2)** were determined using the iPLEX Gold assay (Sequenom Inc, USA). Assays for all SNPs were designed using the MassARRAY Assay Design software version 3.1 (Sequenom Inc, USA). Samples were amplified in multiplexed PCR reactions before allele specific extension. Allelic discrimination was obtained by analysis with a MassARRAY Analyzer Compact mass spectrometer. Genotypes were automatically assigned and manually confirmed using MassArray TyperAnalyzer software version 4.0 (Sequenom Inc, USA). The resulting alleles were used to assign each *S.* Typhi isolate to previously defined haplotypes as described previously (Baker et al., 2008, Holt et al., 2008a, Roumagnac et al., 2006).

2.4.7 High throughput sequencing

Fourty *S.* Typhi H58G isolates were arbitrarily selected from the H58G strains with sufficient DNA for additional variant detection by whole genome sequencing **(Table 9.1)**. Index-tagged Illumina libraries were prepared for each sample, and sequenced twelve per lane using an Illumina GAII machine as described previously (Harris et al., 2010a) **(Table 9.2)**. The software package Maq (Li et al., 2008) was used to align reads to the reference genome sequence for Typhi CT18 (Parkhill et al., 2001b) (EMBL: AL513382) and identify single nucleotides that differ from the corresponding reference nucleotide (SNPs), as previously described (Holt et al., 2008b). These SNP loci were compared to those identified previously among 19 *S.* Typhi genomes (Holt et al., 2008b), to confirm that the sequenced isolates had been correctly assigned to the H58G haplotype and to identify SNPs that differentiated among the newly sequenced H58G isolates. A total of 16 such SNPs were identified in two or more

sequenced isolates. Alleles at these loci were determined for all study isolates of the H58G haplotype using an iPLEX Gold assay as described above (Sequenom Inc, USA).

2.4.8 Statistical analysis

Statistical tests used were; two-sample t tests with equal variance for case-control comparisons of elevation and mean distance between residences to the nearest water spout, the Mantel test with 1,000 simulations for comparing spatial, temporal and genetic distances i.e. Euclidean distance (latitude/longitude) vs. temporal distance (days), Euclidean distance versus phylogenetic distance and temporal distance versus phylogenetic distance and the Chi-square test for proportional quantification of individual *S.* Typhi haplotypes in a geographically defined area. To test the hypothesis of transmission within a residence, a randomization was designed for the 55 residences with multiple haplotyped *S.* Typhi isolates. Isolates were randomly placed in residences according to the true number observed in each residence and the haplotype-frequency distribution of the *S.* Typhi isolates SNP-typed for this study. In each of 1,000 randomisations, the number of residences with identical haplotypes was recorded, and the resulting distribution was compared to the observation of 11 residences with only identical isolates. Because no temporal- haplotype correlation was observed with a Mantel test, there was no requirement for different randomizations for different study periods.

2.5 Methods specifically related to Chapter 5: Water and food sampling and analysis

2.5.1 Selection of sampling sites and definitions

Within the identified spatial "hot-spots" of enteric fever through the GIS analysis (**Chapter 4**), all of the public water sources were mapped using handheld GPS receivers and the ten water sources that were most commonly used were identified. The selected locations consisted of five stone spouts, four sunken wells and one municipal piped supply.

A water stone spout is a location within LSMC where the general populace accesses the ground water for consumption and household use. Unique to the Valley of Kathmandu are the historic water stone spouts that are features throughout the city and are often highly decorated (**Figure 2.4 A**). The water is gravity dependent and replenished through rainfall and snowmelt from the surrounding mountains. Natural soft rock aquifers act as reservoirs for ground water, and ultimately the untreated water enters the stone spouts from the aquifers through a series of underground channels. The local aquifers are also recharged by conveyance canals and through ponds (UN-HABITAT, 2008).

A well is defined a structure that is created in the ground by digging or boring to access groundwater from underground aquifers. More than 1,000 wells exist within the Kathmandu Valley and are common sources of water for domestic purposes. Typically built with a brick masonry wall and circular in shape these wells collect water from shallow acquifers, normally 4 to 6 meters in depth (**Figure 2.4 B)**. Based on the water quality and depth of water in the well, some wells are popular while others are used for secondary purposes like washing and

cleaning. Unlike the water stone spouts, dug wells are not directly linked with traditional canals and ponds. However, infiltration of pond and canals are obvious from the drying-up of the well water after the recent destruction of ponds and canals (UN-HABITAT, 2008).

A municipal supply is defined as water that is centrally collected from surface water bodies such as rivers and lakes and is then treated using processes that are energy and chemical intensive. The treated water is then supplied to various homes within the city through piped water systems **(Figure 2.4 C)**.

(A) 

(B) 

(C) 

Figure 2.4: (A) Traditional water stone spouts within LSMC. (B) A community dug well within LSMC. (C) Piped water supply in a house within LSMC that receives municipal water supply

2.5.2 Collection of water for analysis

Water was collected, when permitted by water flow (dependent on rainfall in some locations), from three the different types of water source in the 10 locations **(Figure 2.5)** once a week over one year from May 2009 to April 2010. All samples were subject to various physical, chemical and microbiological analyses, which are described below. A total of 431 samples were collected from all the sources for analysis during the period. From each of the sources mid-flow water samples were collected in two sterile bottles, in volumes of 1 liter and 500 milliliters. To prevent contamination, the neck of the bottle and the inside of the cap was not touched and only came in contact with the water. From the waterspouts and the municipal supply, the stopper was aseptically removed and free flowing water was allowed to flow directly into the sterile bottle. For wells, a clean steel bucket was attached on to the end of a length of rope. The bucket was then lowered into the well until at least partially submerged. After the bucket was seen to be full, it was raised out and water from the bucket was poured into the bottles avoiding contamination. All the bottles were labeled with the source code, date and time of collection of the samples. The bottles were then put into an insulated box for transportation to the microbiology laboratory and the Water Engineering Laboratory in Kathmandu for physical and chemical analyses.

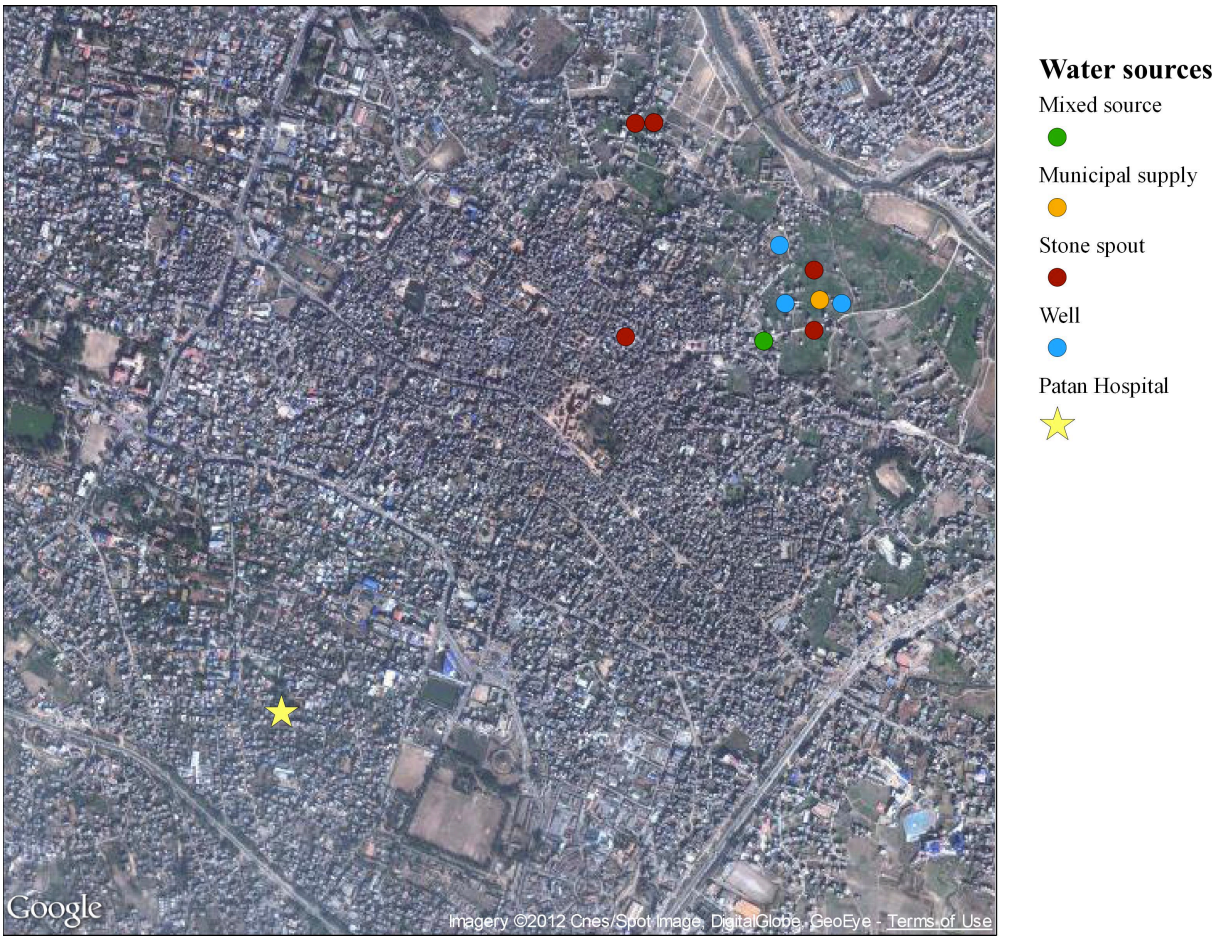


Figure 2.5: A map indicating the different locations from where water was collected and analysed for a period of one year. (Image source: Google Earth).

Yellow star highlights the location of Patan Hospital where the microbiological analysis of water was performed. Blue balloons indicate locations of sunken dug wells from where the water was analysed. Red balloons indicate location of stone spouts from where water samples were analysed. The orange balloon indicates the location from where municipal water was collected. The green balloon indicates the location where well water and municipal supplied water was mixed in the storage tank and it was this sample that was analysed.

2.5.3 Water analysis

2.5.3.1 Physical and Chemical Analysis

The physical parameters that were analyzed were temperature, pH (alkalinity), conductivity, and turbidity, methods for these tests performed at the Water Engineering Laboratory are described in **Appendix 9.3**. The chemical parameters that were analyzed were total hardness, chloride content, iron content, arsenic content, ammonia, nitrate and nitrite, again the methods for these tests performed at the Water Engineering Laboratory are described in **Appendix 9.4**.

2.5.4 Microbiological quality of water

2.5.4.1 Most Probable Number (MPN) method

To assess the microbiological quality of the water, specifically related to faecal contamination I used the most probable number (MPN) method. The MPN method is a procedure to estimate the density of viable microorganisms in a test sample. It is based upon the application of the theory of probability to the numbers of observed positive growth responses to a standard dilution series of sample inoculums placed in a set number of culture media tubes. Five ten-fold serial dilutions were made from each water sample, firstly by inoculating 1ml of undiluted water sample into 9ml of soya broth. This was continued until a dilution factor of 10^{-5} was obtained. A total of thirty tubes, 5 tubes for each dilution factor were prepared with each tube containing 9ml of MacConkey broth and an inverted Durhams' tube. MacConkey broth is used for the detection of coliform bacteria in milk and water while the Durhams' tube is used for the detection of gas that is produced by the metabolic action of microorganisms.

Sets of 5 tubes were inoculated from each of the ten fold dilutions (10^0 to 10^{-5}) with each tube being inoculated with 1ml of the corresponding diluted sample (**Figure 2.6**).

The inoculated broths were incubated at 44°C for 48 hours. After incubation, each tube was examined and those that were positive (production of acid and gas) were counted. Production of gas within the Durhams' tube indicated a positive reaction for gas production, while change in the colour of the MacConkey broths from the original red to yellow indicated a positive reaction for acid production. The first set of tube that exhibited dilution of microorganisms to extinction (tubes inoculated with one ml of 10^{-3} in Figure 2.6) was noted followed by the set with the dilution immediately before the first extinction dilution (tubes inoculated with one ml of 10^{-2} in Figure 2.6) and immediately after the first extinction dilution (tubes inoculated with one ml of 10^{-4}in Figure 2.6). The number of positive and negative tubes in each of these three sets was noted in order (5-4-0 in Figure 2.6) and this data was used for the estimation of the coliform content using the 5 tubes MPN table (**Table 2.1**).

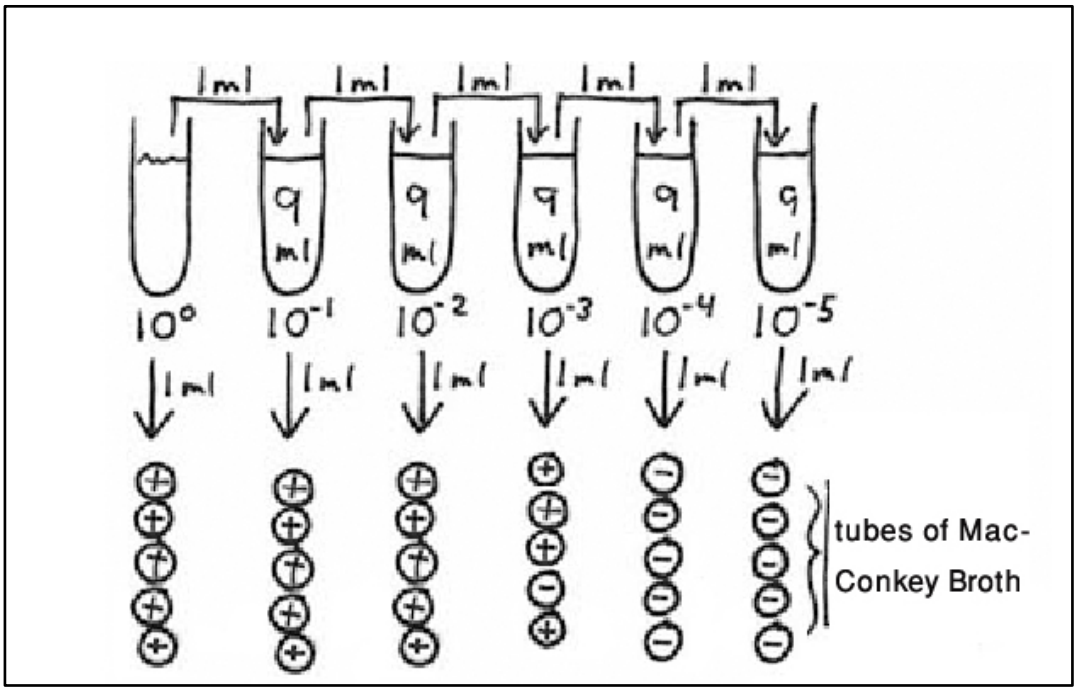


Figure 2.6: Serial dilution for MPN method.

The first tube was inoculated with 1ml of undiluted water sample and thus was said to have a dilution factor of 10^0. Following the first dilution, a set of 5 tubes with 9ml MacConkey broth was inoculated from each of the ten fold dilutions, with each tube being inoculated with 1 ml. The middle set tubes, i.e. those with dilution factors of -2, -3 and -4 were noted for interpretation through the use of the MPN table.

(Source: http://www.jlindquist.net/generalmicro/102dil3.html)

Table 2.1: 5-tube MPN table.

The first three columns denote the number of positive tubes in each set of tubes. The last column suggests the average organisms inoculated into each of the tubes of the middle set (of the three sets of tubes chosen). For example in **Figure 2.6**, the obtained combination of results in order is 5-4-0, which interpreting through the table suggests that the middle set of tubes chosen, i.e. those inoculated with one ml of 10^{-3} dilution contains an average of 1.3 organisms. Hence, the most probable number of organisms per one ml of he original undiluted samples would be 1.3 X 10^{3}. (Source: Standard Methods for Examination of Water and Wastewater, 15th edition, 1980)

No. of tubes positive in			**MPN in the inoculum of the middle set of tubes**
First set	Middle set	Last set	
0	0	0	‹0.01
0	0	1	0.02
0	1	0	0.02
0	2	0	0.04
1	0	0	0.02
1	0	1	0.04
1	1	0	0.04
1	1	1	0.06
1	2	0	0.06
2	0	0	0.05
2	0	1	0.07
2	1	0	0.07
2	1	1	0.09
2	2	0	0.09
2	3	0	0.12
3	0	0	0.08
3	0	1	0.11
3	1	0	0.11
3	1	1	0.14
3	2	0	0.14
3	2	1	0.17
4	0	0	0.13
4	0	1	0.17
4	1	0	0.17

No. of tubes positive in			MPN in the inoculum of the middle set of tubes
First set	Middle set	Last set	
4	1	2	0.26
4	2	0	0.22
4	2	1	0.26
4	3	0	0.27
4	3	1	0.33
4	4	0	0.34
5	0	0	0.23
5	0	1	0.31
5	0	2	0.43
5	1	0	0.33
5	1	1	0.46
5	1	2	0.63
5	2	0	0.49
5	2	1	0.7
5	2	2	0.94
5	3	0	0.79
5	3	1	1.1
5	3	2	1.4
5	3	3	1.8
5	4	0	1.3
5	4	1	1.7
5	4	2	2.2
5	4	3	2.8
5	4	4	3.5
5	5	0	2.4
5	5	1	3.5
5	5	2	5.4
5	5	3	9.2
5	5	4	16
5	5	5	›24

2.5.4.2 Bacteriological analysis

2.5.4.2.1 Direct plating

To detect the presence of *Enterobacteriaceae*, in particular salmonellae, shigellae, vibrionaceae and *E. coli,* 10, 20, 50, 100 and 500 µl of undiluted water was directly plated onto XLD and MacConkey agar plates. XLD is a selective media used for the isolation and presumptive identification of both salmonellae and shigellae, where shigellae do not ferment xylose and salmonellae exhaust the xylose and decarboxylate the lysine with the production of hydrogen sulphide. MacConkey agar is a differential medium for the selective isolation and differentiation of lactose fermenting and non-fermenting *Enterobacteriaceae.* Varying amounts of water samples were used so that an effective number of colonies, i.e. on average 30 to 300 for interpretation were obtained. The plates were incubated at 37°C overnight and observed for any growth.

2.5.4.2.2 Plating after enrichment

To detect the presence of salmonellae and shigellae, 100ml of undiluted water was filtered through a membrane filter with a pore size 0.45 µm (Whatman®). With sterile forceps I removed the filter paper and placed it in 90ml of soya broth. Soya broth is a nutritious general medium that supports a luxuriant growth of many fastidious microorganisms due to the inclusion of tryptone and soya peptone in the medium. The bottle was vortexed to displace the organisms on the membrane and incubated for 18 hours, ±2 hours at 37°C. After overnight incubation 1 ml of the pre-enrichment culture was transferred to 10ml of selenite broth, which is a selective medium for the isolation of salmonellae and incubated at 37°C for 24 hours. Further, 1ml of the pre-enrichment culture was transferred to 10ml of Rappaport vassiliadis broth (RVB), which is an enrichment media for the selective

isolation of *Salmonella* spp.

The incubated overnight broth was then plated onto XLD and MacConkey agar plates. The plates were incubated overnight at 37°C and observed for growth.

For the detection of vibrionaceae, 1ml of the undiluted water sample was diluted in 9ml of alkaline peptone water, which is a medium used for the enrichment of *V. cholerae* and *Vibrio* species from food, water, faeces and clinical studies. The suspension was then incubated overnight at 37°C and then plated on to MacConkey, XLD and thiosulphate-citrate-bile salts sucrose (TCBS) agar, which is a selective media for the isolation of *V. cholerae* and other enteropathogenic vibrios. After incubation, plates were observed for bacterial growth.

2.5.5 Identification of isolates

On the MacConkey and XLD plates, individual colonies of various microorganisms were observed. The colony morphology including the form, size, surface appearance, texture, colour, elevation and margin of all individual colonies were noted. Of special interest were colonies that were circular, with an entire margin and slightly raised elevation that were non-lactose fermenting on both plates, with or without the production of hydrogen sulphide on the XLD plate. Individual colonies with aforementioned characteristics were isolated and plated on to nutrient agar, which is a growth medium commonly used for the routine cultivation of non-fastidious bacteria and incubated at 37°C for 24 hours. Isolated colonies obtained on the nutrient agar plates were then subject to an API20E test as mentioned in **section 2.2.2** to identify *Enterobacteriaceae* and other non-fastidious Gram

negative rods.

2.5.6 Culture of spiked water samples for detection of salmonellae in water

Five bottles containing 500ml of sterile water were inoculated with varying concentrations of *S.* Typhi. An initial bacterial suspension of 0.5 McFarland, with a cell density of 1 X 10^8cfu/ml was prepared. Five ten-fold dilutions were prepared by inoculating 1ml of the dilution into 9ml of sterile water. 10ml of each dilution was then inoculated into an individual bottle containing 500ml of sterile water. These bottles were then incubated at 37°C overnight and then subjected to microbiological growth as in **section 2.5.4.2**.

2.5.7 Culture of popular street food for detection of faecal contamination

Over a period of 12 weeks, from February to June 2010, popular street food items were purchased from various street vendors and roadside stalls located within the study catchment area. Levels of faecal contamination were assessed and isolated bacteria were identified from each food item that was selected. The food items that were subjected to microbiological analysis were:

1. Momo. Steamed dumplings with meat filling topped with tomato sauce.
2. Kachila. Shredded raw meat with spices.
3. Chhwela. Roasted meat with spices.
4. Wa. Rice flour patties either without stuffing or with meat or egg.
5. Pani-puri. Puffed, deep-fried unleavened bread with a sweet and sour sauce comprising of water, tamarind, spices, chili, potato, onion and chick-peas.
6. Khua-baraf. Iced-popsicle high in milk content.

8. Peda. Sweet confection high in milk content.
9. Barfi. Sweet confection high in milk content.
10. Samosa. Stuffed, deep-fried pastry with fillings of spiced potatoes, onions, peas, coriander and lentils.
11. Halwa. Semolina based dense, sweet confection.
12. Chatpat. Fried dough with spices, onions and yoghurt.
13. Packaged mango drink

2.5.7.1 MPN method for food samples

To detect the level of faecal contamination in food, 50 grams of the food item was homogenized in 100ml of nutrient broth. This homogenate was considered to have the initial dilution of 10^0. Five ten-fold dilutions were then performed from this initial dilution by adding1ml of the homogenate and consecutive dilutions to successive 9ml of soya broth. Then the MPN procedure was performed as described in **section 2.5.4.1**.

2.5.8 Bacterial isolation

All food products and their sauces were tested individually. The iced popsicles were allowed to thaw and melt completely before analysis was performed. For food products with fillings, the inner filling was removed and analysed. 50 grams of the food item was homogenized in 100ml of nutrient broth and incubated at 37°C for 4 hours. After incubation, 50ml volumes of the homogenate were added to two separate bottles containing 50ml of double strength selenite F broth, which is an enrichment medium used for the isolation of salmonellae from faeces, urine, water, foods and other materials of sanitary importance. One of the inoculated bottles was incubated at 37°C and the other

bottle at 43°C. The incubated cultures were then plated onto XLD and MacConkey agar plates and incubated at 37°C for 24 and 48 hours. Individual colonies suspected to be of the *Enterobacteriaceae* family, particularly salmonellae, shigellae, vibrionaceae or *Eshcerichia coli* were picked for further investigations with the API20E kit as described in **section 2.2.2**.

2.5.9 Molecular examination of water samples

2.5.9.1 Metagenomic DNA extraction from water

Metagenomic DNA from all water samples was extracted with a commercial kit, Metagenomic DNA isolation kit for water from Epicentre® Biotechnologies.Water samples were centrifuged at 1,000 rpm (Hettich Zentrifugen, EBA 21, Germany) for 5 minutes to remove large debris from the samples and then decanted into sterile containers. After centrifugation 100ml of the centrifuged water was filtered through a pre-sterilized filter with a pore size of 0.45µm (Whatman®). Using sterile forceps and scissors pre-soaked in 70% ethanol, the membrane was removed from the filter apparatus and cut into four pieces. The cut pieces were then placed along the side (near the bottom) of a 50ml sterile conical tube. The upper surface of the filter faced the center (not the wall) of the tube. Filter wash buffer was prepared by adding 2µl of Tween-20 to 1 ml of filter wash buffer immediately before use. One mililitre of filter wash buffer containing 0.2% Tween-20 was added to the filter pieces in the tubes to remove organisms on the filter surface. The tube was vortexed initially at a low setting to rewet the filter pieces, and then gradually increased to the highest setting for approximately 2 minutes with intermittent breaks. The cell suspension was transferred to a clean micro-centrifuge tube and centrifuged at 14000 X g (Thermo Fishcer Scientific, IEC Micro CL17, Germany) for 2 minutes to pellet the

cells. The supernatant was discarded. The cell pellet was re-suspended in 300µl of TE buffer, and 2µl of ready-lyse lysozyme solution and 1µl of RNAse A was added and mixed thoroughly. The tube was incubated at 37°C for 30 minutes and 300 µl of 2 X meta-lysis solutions and 1 µl of Proteinase K was added to the tube and thoroughly mixed by vortexing. To ensure that all the solution was at the bottom of the tube, the tube was pulse centrifuged. The tubes were then incubated at 65°C for 15 minutes. The solution was cooled to ambient temperature and placed on ice for 5 minutes. Three hundred and fifty microlitres of MPC protein precipitation reagent was added to the tube and mixed thoroughly by vortexing vigorously for 10 seconds. The debris was pelleted by centrifugation for 10 minutes at 14000 X g (Thermo Fishcer Scientific, IEC Micro CL17, Germany) at 4°C.The supernatant was transferred to a clean micro-centrifuge tube and the pellet was discarded. To the supernatant, 570 µl of isopropanol was added and mixed by inverting the tube multiple times. The DNA was pelleted by centrifugation for 10 minutes at 14000 X g (Thermo Fishcer Scientific, IEC Micro CL17, Germany) at 4°C. The isopropanol was removed and the sample was briefly pulse centrifuged and any residual liquid was removed without disturbing the pellet. To the pellet 500 µl of 70% ethanol was added without disturbing the pellet. The tube was then centrifuged for 10 minutes at 14000 X g (Thermo Fishcer Scientific, IEC Micro CL17, Germany) at 4°C. Ethanol was removed without dislodging the DNA pellet and the sample was briefly pulse centrifuged and any residual fluid was removed without disturbing the pellet. The pellet was then air dried for 8 minutes at ambient temperature.

2.5.9.2 Real-time Polymerase chain reaction

Real-time PCR was performed on all extracted metagenomic DNA to detect DNA

Shigella spp. and *Vibrio cholerae* primers and conditions were as previously described, using methods published by Vu Dinh Thiem (Thiem et al., 2004) and Olivia D. Nigro (Nigro et al., 2011) respectively. The Real-time PCRs for *S.* Typhi and *S*. Paratyphi A were originally established to detect the presence of these organisms in blood and were adapted to amplify from metagenomic DNA. These assays were performed by myself in the laboratory of Dr. Stephen Baker at Oxford University Clinical Research Unit in Vietnam in collaboration with Ms. Tran Vu Thieu Nga (Nga et al., 2010). These PCRs were established and validated using the following methods.

2.5.9.3 Target sequence selection for *S.* Typhi and *S.* Paratyphi A

Sequences unique to *S.* Typhi or *S.* Paratyphi A were identified using a whole genome comparison of *S.* Typhi and strain CT18 (GenBank AL513382) (Parkhill et al., 2001a) and *S.* Paratyphi A strain AKU12601 (GenBank FM200053) (Holt et al., 2009), conducted using BLASTn and visualized using the Artemis Comparison Tool (ACT). To confirm whether these sequences were likely to discriminate more generally between members of the *S.* Typhi and *S.* Paratyphi A populations, we searched for sequences in all available *S.* Typhi (finished sequence for strain Ty2; GenBank AE014613) and 17 additional 454 shotgun sequenced strains (GenBank CAAV01000001-CAAV01003682)(Holt et al., 2008a) and *S.* Paratyphi A strains (finished sequence for strain ATCC9150 (GenBank CP000026)). Genomic data from the recent *S.* Typhi and *S.* Paratyphi A sequencing projects were mined to find genes that were specific for each serovar (Holt et al., 2008a, Holt et al., 2009). The criteria for selection was, a lack of homology with other genes in other pathogens or human sequences (to ensure no cross-reactivity) and the sequence was required to be conserved in all the re-sequenced and previously sequenced strains.

2.5.9.4 DNA manipulation, bacterial strains and construction of internal control

An *E. coli* strain containing an alien gene was used as a negative control and constructed by PCR amplifying the gB gene from Phocid herpes virus using the primers phHV-1 forward and reverse (van Doornum et al., 2003). The gB gene amplicon was cloned into plasmid pCR 2.1- TOPO (Invitrogen). *E. coli* VU1 was to act as an internal control to monitor DNA extraction and amplification efficiency in all PCR reactions using primers pHV-1 forward and reverse and a specific probe (van Doornum et al., 2003). PCR amplicons for all target sequences were produced by monoplex conventional PCR using the primer sequences outlined below in **section 2.5.9.5**. *E. coli* TOPO10 cells (Invitrogen) were transformed with purified plasmid DNA containing target DNA sequence and PCR amplicons were sequenced (Applied Biosystems) to ensure accurate amplification. Purified plasmid DNA was used as template in all subsequent experiments, which utilized a standard curve.

2.5.9.5 Primers and PCR conditions

Primers and probes for the detection of *S.* Typhi and *S.* Paratyphi A were designed using the Primer Express Software (Applied Biosystems) and manufactured by Sigma-Proligo (Singapore). Primers and probe sequences were as shown in **Table 2.2**. PCR reactions were performed in 25 µl reaction volumes consisting of 5 mM $MgCl_2$, 0.2 mM each deoxynucleotide triphosphate, 1 U of Hot start Taq DNA polymerase (Qiagen) and 1-5 ng of template DNA. Final reaction concentrations of the three primer and probe sets for internal control, *S.* Typhi and *S.* Paratyphi A were 0.4 µM of each primer and 0.15 µM of each probe. PCR was performed on a Bio-Rad Chromo 4 real-time PCR system and fluorescence was released via the TaqMan 5' to 3' exonuclease activity. All PCRs were cycled under the following conditions; 15 min at 95°C and 45 cycles of 30 sec at 95°C, 30 sec at 60°C and 30 sec at 72°C.

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

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

Primers and probes used previously for the detection of *Shigella* spp. (Thiem et al., 2004) were used for this study designed. Primers and TaqMan probe sequences targeting the *ipaH* and *virB* genes were as shown in **Table 2.3**. PCR reactions were performed in 25 µl reaction volumes consisting of 2 mM $MgCl_2$, 100 nM each deoxynucleotide triphosphate, 200nM primers (both ipaH-U1 and ipaH-L1), 40nM fluorogenic probe, ipaH-P1 and 11 U of Hot start Taq DNA polymerase (Qiagen) and 1-5 ng of DNA template. PCR was performed on a LightCycler 480 II (Roche) system. All PCRs were cycled under the following conditions; 15 min at 95°C and 45 cycles of 30 sec at 95°C, 30 sec at 60°C for 1 minute. The assay result was considered to be positive when Cp value was less than 40.

Table 2.3: Oligonucleotide primers and probe used in the PCR reaction for the detection of *Shigella* spp

Primer and Probe sequence
ipaH-F-U1 5'CCT TTT CCG CGT TCC TTG A 3'
ipaH-R-L1 5' CGG AAT CCG GAG GTA TTG C 3'
ipaH-probe- 6-Cyan500-CGC CTT TCC GAT ACC GTC TCT GCA-BHQ1
virB-F764 GATTCTTGATCGCGTACATTCG
virB-R873 ACACTCCATTCTGGTAATAAAGTTTCC
virB-Probe788 Fam-TTTTGGTTGACGAAGGTTAAATCTTGGATCCTTTTA-BHQ1

For the detection of *Vibrio* spp internal transcribed spacer (ITS) region primers listed in **Table 2.4** were used. Conventional PCR was performed for the detection of *Vibrio* species in the metagenomic samples. PCR was cycled under the following conditions: 30 cycles of 95°C for 5 minutes, 94°C for 20 seconds, 60°C for 20 seconds and 72°C for 2 minutes. The amplification product was observed on a gel documentation system GelDOx (Biorad)

Table 2.4: Internal transcribed spacer (ITS) region primer sequences used for the detection of *Vibrio* species

Organism	**Primer sequences**
*V. cholerae*ITSF	-TTAAGCSTTTTCRCTGAGAATG
*V. cholerae*ITSR	-AGTCACTTAACCATACAACCCG

2.5.9.6 Real time PCR quantification

Plasmid DNA with cloned target DNA sequences (*S.* Typhi and *S.* Paratyphi A) was purified and concentrations (µg/ml) were calculated by a NanoDrop spectrophotometer (Thermo-Scientific). Concentrations were converted to copy number using the formula; mol/g x molecules/mol=molecules/g, via a DNA copy number calculator http://www.uri.edu/research/gsc/resources/cndna.html. Plasmid solutions were diluted in 10-fold serial dilutions ranging from 10^0 to 10^5 plasmid copies per µl. Serially diluted plasmid DNA was mixed in increasing (*S.* Typhi target) and decreasing (*S.* Paratyphi A target) concentrations and subjected to a real-time PCR amplification. Standard curves for *S.* Typhi and *S.* Paratyphi A copy number were constructed by plotting the C*t* value against the plasmid DNA copy number.

2.5.9.7 PCR product purification

PCR products were purified using QIA quick PCR purification kit following manufacturer's instructions (QIAGEN, GmbH Germany).

2.5.9.8 Cloning of DNA sequence from PCR amplifications

To ensure that amplifications were specific for *S.* Typhi and *S.* Paratyphi A amplified PCR products were cloned into a plasmid vector using TOPO TA Cloning kit (Invitrogen) and DNA sequenced.

For the preparation of competent cells a colony of lab strain *E.oli* DH5α was grown in 5ml of Luria Bertani (LB) broth. Two microlitres of the *E. coli* DH5α cultured broth was

transferred to a 100ml flask containing LB broth that had been pre-warmed at 37ºC, and then incubated with shaking at 37ºC for 3 hours. The culture was incubated on ice and aliquoted into two 50ml tubes. The cells were then harvested by centrifuging at 4,000 rpm (Hettich Zentrifugen, EBA 21,Germany) for 15 minutes at 4ºC. The bacterial pellet was subsequently washed with 10ml of ice-cold 10% glycerol and then centrifuged at 4,000 rpm (Hettich Zentrifugen, EBA 21,Germany) for 10 minutes at 4ºC. The bacterial pellet was washed another 5 times with 5 ml of 10% ice-cold glycerol with centrifugation as previously mentioned. The tubes were always kept on ice and centrifugation was performed at 4ºC. Once washed, the pellet was re-suspended in 100 µl of 10% glycerol and aliquoted in 2 microfuge tubes, each contained 50µl of competent cells. The competent cells were subsequently stored at -80ºC until required.

For ligation, a Topo reaction was prepared in a 1.5ml microfuge tube by mixing 1 µl Topo vector, 1 µl salt solution and 4µl of the PCR amplicon. The mixture was gently mixed and incubated at ambient temperature for 15 minutes. For transformation, 100µl of thawed competent cells were added to the TOPO mixture and gently mixed. The mixture was then heat shocked by placing the tube in a water bath equilibrated to a temperature of 42°C for 45 to 50 seconds. 150µl of ambient temperature S.O.C. media was added to the mixture and incubated at 37°C for 1 hour and then centrifuged at 3,000rpm for 2 minutes. The supernatant was discarded and the pellet was gently mixed and spread (~30µl) on prepared LB agar plates containing galactose and ampicillin. The plates were then incubated at 37°C overnight. After incubation plates were observed for the growth of blue and white colonies. Individual white colonies were picked and suspended in 20µl of LB broth with 50µl/ml of ampicillin. To the PCR premixes, 5µl of bacterial suspension were added and PCR amplification cycles were performed specifically using the M13 primers **(Table 2.5)**

and primers specific for *S.* Typhi and *S.* Paratyphi A as before.

Table 2.5: Table describing the sequence of the M13 primers

Primer	Sequence
M13 Forward (-20)	5′-GTAAAACGACGGCCAG-3′
M13 Reverse	5′-CAGGAAACAGCTATGAC-3′

2.5.9.9 M13 PCR amplification for sequencing

Table 2.6: Premix quantities for M13 PCR

Reagent	Final concentration	Volume µl
$MgCl_2$ (25mM)	1.25mM	2.5
10X buffer	1X	5
dNTPs	0.1mM	0.5
Primers F/R (100ng/ µl)	1ng/ µl	0.5 each
Hot start Taq	0.5 U	0.2

The PCR amplification premix for the M13 specific PCR was prepared as shown in **Table 2.6**. PCR for M13 was performed in a volume of 50 µl, consisting of 5 µl of 10 X buffer, 2.5 µl of MgCl2 25 mM, 0.5 µl of dNTPs, 0.5 µl of each forward and backward primer, 0.2 µl of Hot start Taq (5U/µl), 5µl of template DNA and made up to volume with distilled water.

PCR amplification with the M13 primers was performed using the following program

i. 100°C for 5 minutes

ii. 95°C for 15 minutes

iii. 30 cycles of 95°C for 15 seconds, 55°C for 30 seconds and 72°C for 1 minute

iv. 72°C for 10 minutes

PCR amplicons were visualized on 1% agarose gel containing ethidium bromide. PCR amplicons were purified using the qiaquick PCR purification kit (Qiagen) and the amount of DNA was then measured using a nanodrop spectrophotometer.

2.5.9.10 DNA Sequencing

PCR premixes were mixed separately for forward and backward sequences on ice as shown in Table 2.5.

Table 2.7: PCR premixes for each sequence for the purpose of DNA sequencing

Ingredient	Quantity
Big dye:	1 µl
5X Buffer:	2 µl
Primer:	0.5 µl
Water:	5.5 µl
DNA:	1 µl
Total volume	**10 µl**

The PCR for sequencing was then carried out using the following program:

i. 96°C for 1 minute

ii. 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 4 minutes.

DNA sequence alignment was conducted using the Align X programme of the Vector NTI Suit 7 Software on the 3130XL ABI sequencer (Applied Biosytems, USA).

2.6 Methods specifically related to Chapter 6: Matched case control study for enteric fever within LSMC.

2.6.1 Ethical approval informed consent process

This study was approved by the institutional Ethics Boards of Patan Hospital (PH) and The Nepal Health Research Council (NHRC). All enrollees were required to provide written informed consent **(Appendix 9.5)** as described in **Section 2.1.1.**

2.6.2 Cases for case control study

A case of enteric fever was defined as a patient from whom a *S.* Typhi or *S.* Paratyphi organism was cultured from their blood on arrival in the clinic at the outpatients department according to the methodologies previously described in **section 2.2**. Cases were all attendees of the outpatients department or emergency department at Patan Hospital between April 2011and October 2011 and were all enrolled in one of three consecutive randomized controlled trials for the treatment of uncomplicated enteric fever as previously described.

2.6.3 Controls for case control study

Controls were used to try and identify risk factors as in comparison to the cases within the same population. Controls were ± 5 years of age to the case, of the same gender as the

case, and had not had fever, gastrointestinal disturbances or history of enteric fever for the past month. All controls were enrolled within 2 weeks of the case having been enrolled. For enrollment of controls, in instances where the cases lived in individual houses, the house to the right of the "case-household" was selected. In case of fever 30 days preceding the interview in the household or refusal to participate, the house on alternating sides of the initially selected household was approached.

For enrollment of controls, in instances where the cases lived in multi-storey buildings, the household above the "case-household" was approached. In case of fever 30 days preceding the interview in the household or refusal to participate, the storey above or below the initially selected household was approached.

2.6.4 Household definition

A household for the case control study was defined as the number of people sharing a kitchen. Multiple household infections were defined as more than one culture positive (*S.* Typhi or *S.* Paratyphi A) infection in a single household.

2.6.5 Questionnaires

I interviewed all individuals providing written informed consent forms who were eligible to be cases with a standardized questionnaire **(Appendix 9.6)**. Cases were interviewed on the day of enrollment while controls were interviewed within 2 weeks of the positive culture of the cases.

3 Burden and characteristics of enteric fever at Patan Hospital

3.1 Abstract

Enteric fever caused by *S.* Typhi and *S.* Paratyphi A is a major public health concern within the urban population of Kathmandu. Demographic patterns of enteric fever suggest that the disease is limited to locations with poor sanitation that permit the continued transmission of the infecting organisms. Efficacious and inexpensive vaccines are available for *S.* Typhi, yet are not commonly deployed to control the disease. A lack of vaccination is partly due to an uncertainty of the disease burden arising from a paucity of epidemiologic information in key endemic locations. I sought to investigate some of the epidemiological patterns of enteric fever in LSMC. Data from 3,898 cases of blood culture confirmed enteric fever from Patan Hospital in LSMC between June 2005 and May 2009 was collected and analyzed to estimate incidence and seasonal patterns. Demographic data was available for a subset of these patients (n=527) that were resident in LSMC and who were enrolled in the clinical trials. I found that there is an ongoing considerable burden of enteric fever caused by *S.* Typhi (2,672; 68.5%) and *S.* Paratyphi A (1,226; 31.5%) at Patan Hospital over the four year period investigated, which correlated with seasonal fluctuations in rainfall. It was found that local population density was not related to incidence and there was a focus of infection to the east of LSMC study area. With available data from patients resident in LSMC, the median age of those with *S.* Typhi (16 years) was significantly less than those with *S.* Paratyphi A (20 years) and that males aged between 15 to 25 years were disproportionately infected. The results provide a snapshot into the epidemiologic patterns of enteric fever in LSMC. The uneven distribution of enteric fever patients within the population suggests local variation in risk factors, such as

contaminated drinking water. These findings are important for basic understanding of some of the epidemiological features of enteric fever in this location.

3.2 Introduction

Very few countries with endemic and epidemic enteric fever have implemented typhoid immunization programs, despite the global incidence and current WHO recommendations to employ vaccines based on knowledge of the local epidemiological situation (DeRoeck et al., 2007, WHO, 2008b, WHO, 2008a). Therefore, for an effective control program, vaccination or otherwise, in locations such as Kathmandu, it is essential to understand the local distribution and burden of enteric fever. Knowing the proportions of the disease burden caused by *S.* Typhi and *S.* Paratyphi A is also significant for intervention (Steele, 2008) as these serovars may have subtle differences in their modes of transmission and may necessitate differing control measures (Sur et al., 2007, Vollaard, 2004). *S.* Paratyphi A is a particular challenge as there is currently no licensed vaccine, and a vaccine study in Kolkata, India demonstrated that whilst the Vi vaccine was highly protective for *S.* Typhi infections, there was no cross protection for *S* Paratyphi A infections (Cook et al., 2009, Sur et al., 2009a). There is a paucity of data regarding *S.* Typhi and *S.* Paratyphi A infections originating from Nepal and in particular Kathmandu, which is known to have a significant incidence of enteric fever (Karkey et al., 2008).

Previous work at Patan Hospital in LSMC, an urban district of Kathmandu includes a retrospective and a prospective clinical assessment of enteric fever and a series of randomized controlled treatment trials (Maskey et al., 2008, Maskey et al., 2006 , Pandit et al., 2007, Arjyal et al., 2009). The purpose of the work presented in this chapter was to calculate the burden of enteric fever attributable to *S.* Typhi and *S.* Paratyphi A at Patan Hospital in Kathmandu and estimate incidence in the population residing in LSMC. To

address this aim, data were obtained from two sources; retrospective data from culture confirmed enteric fever patients between June 2005 and May 2009 were extracted from the diagnostic microbiology database at Patan Hospital and prospective demographic data were obtained from patients enrolled in three clinical trials conducted over the same period **(Section 2.1.3)**. Here I present the epidemiological characteristics and the etiology of enteric fever in patients presenting to Patan Hospital in LSMC, Kathmandu between 2005 and 2009.

3.3 Results

3.3.1 Enteric fever at Patan Hospital: June 2005 to May 2009

The number of enteric fever patients presenting to Patan Hospital by month, the number of outpatient attendees and the number of blood cultures performed between June 2005 and May 2009 are shown in **Figure3.1**.During this period, there were 54,536 blood cultures performed at Patan Hospital, of which 3,898 (7.15%) were positive for invasive salmonellae. Of the 3,898 invasive salmonellae identified, 2,672 (68.5%) were *S.* Typhi and 1,226 (31.5%) were *S.* Paratyphi A. *S.* Paratyphi B and *S.* Paratyphi C were not observed. Over this four-year period there was an overall decline in the number of enteric fever cases; despite a slight increase from 2007 to 2008. This decline was not related to the number of blood cultures performed (14,295 in 2005 and 13,869 in 2008) or the number of patients attending the outpatients departments (relative risk=0.82 per year, 95% CI 0.75 to 0.90, $p<0.001$) **(Figure 3.1)** indicating that the observed decline in the number of enteric fever patients during this period is probably not an artefact of overall healthcare-seeking behaviour. Some of the reasons could be, to some extent, the impact of the randomized control trials and better diagnosis and treatment regimens that ensure patients take

complete antimicrobial courses.

The overall decline in enteric fever appears to be dominated by an annual decrease in *S.* Typhi cases (728 in 2006, 605 in 2007, and 511 in 2009). The increase in enteric fever from 2007 to 2008 (813 in 2007 and 857 in 2008) was due to an increase in the number of *S.* Paratyphi A cases (192 in 2007, 324 in 2008) **(Figure 3.1)**. From October 2008 to April 2009 the number of *S.* Paratyphi A cases was almost equivalent to the number of *S.* Typhi cases. Annual peaks in enteric fever cases correlated with the peak attendance in the outpatients department, suggesting seasonal variation of febrile disease in this location

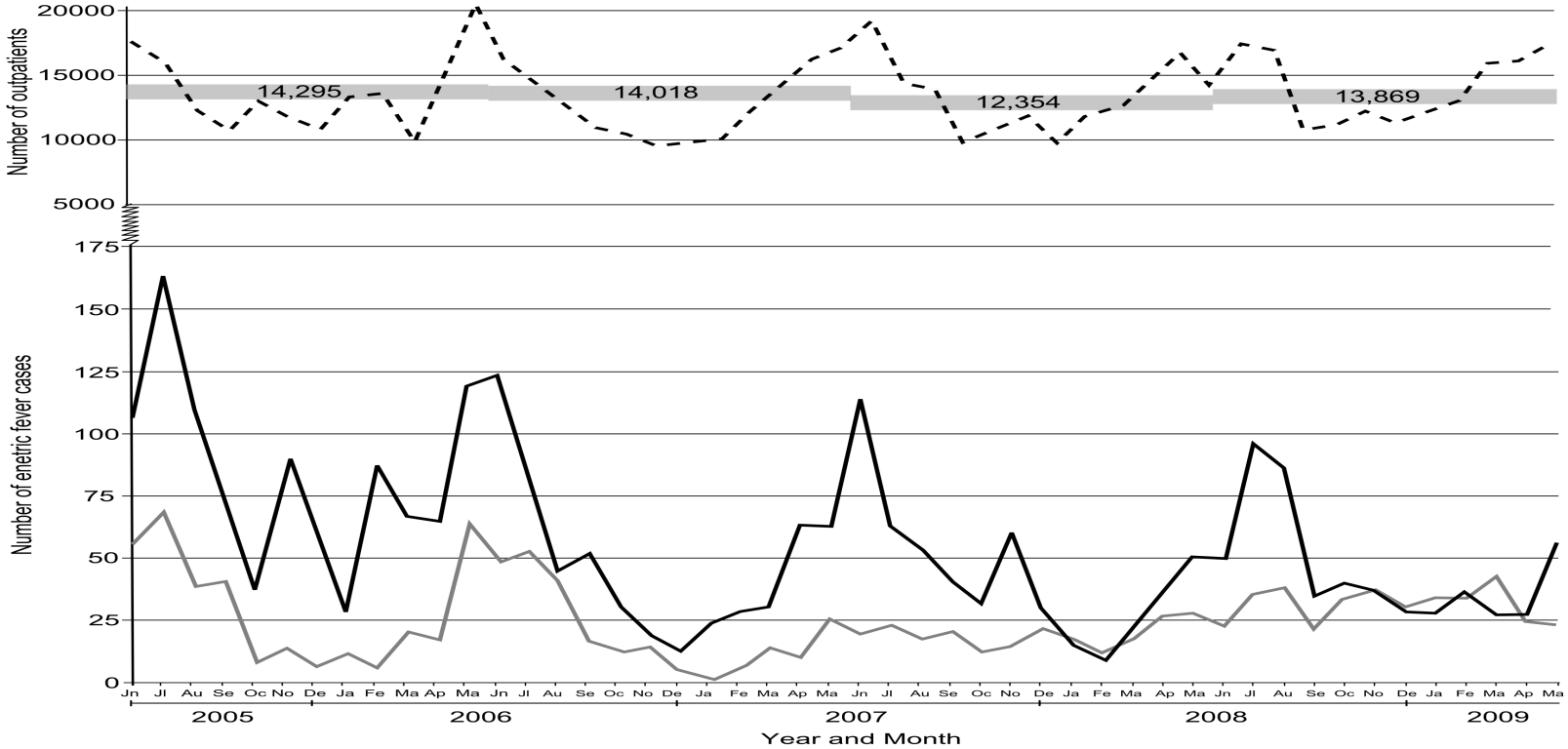


Figure 3.1: Enteric fever case burden in patients attending Patan Hospital, 2005-2009.

Plot showing the number of culture positive enteric fever cases at Patan Hospital per month between June 2005 and May 2009 (solid black line represents *S.* Typhi and solid grey line represents *S.* Paratyphi A). The broken black line shows the number of patients attending the outpatient department per month over the same period, with numbers in grey boxes equating to the annual number of blood cultures performed

3.4 The seasonal distribution of enteric fever cases

Figure 3.2 shows the average number of blood culture confirmed cases of *S.* Typhi and *S.* Paratyphi A per month at Patan Hospital over the four year period of study (monthly data were combined and divided by the four years of the study).

There was a seasonal signal in the monthly frequency of enteric fever cases, which correlated with the average volume of rainfall per month over the same time period. The peak of the wet season in Kathmandu occurs in July, which corresponds with a peak in both *S.* Typhi (average number of cases in July 100) and *S.* Paratyphi A cases (average number of cases in July 45) at Patan Hospital. The rainfall subsides after August and between October and April an average of less than 56 *S.* Typhi cases and 24 *S.* Paratyphi A cases were observed per month. The average seasonal distribution was comparable for the *S.* Typhi and *S.* Paratyphi A implying a parallel relationship between enteric fever of both aetiological agents to seasonal variation in rainfall (p=0.91; χ^2 test, 11 df).

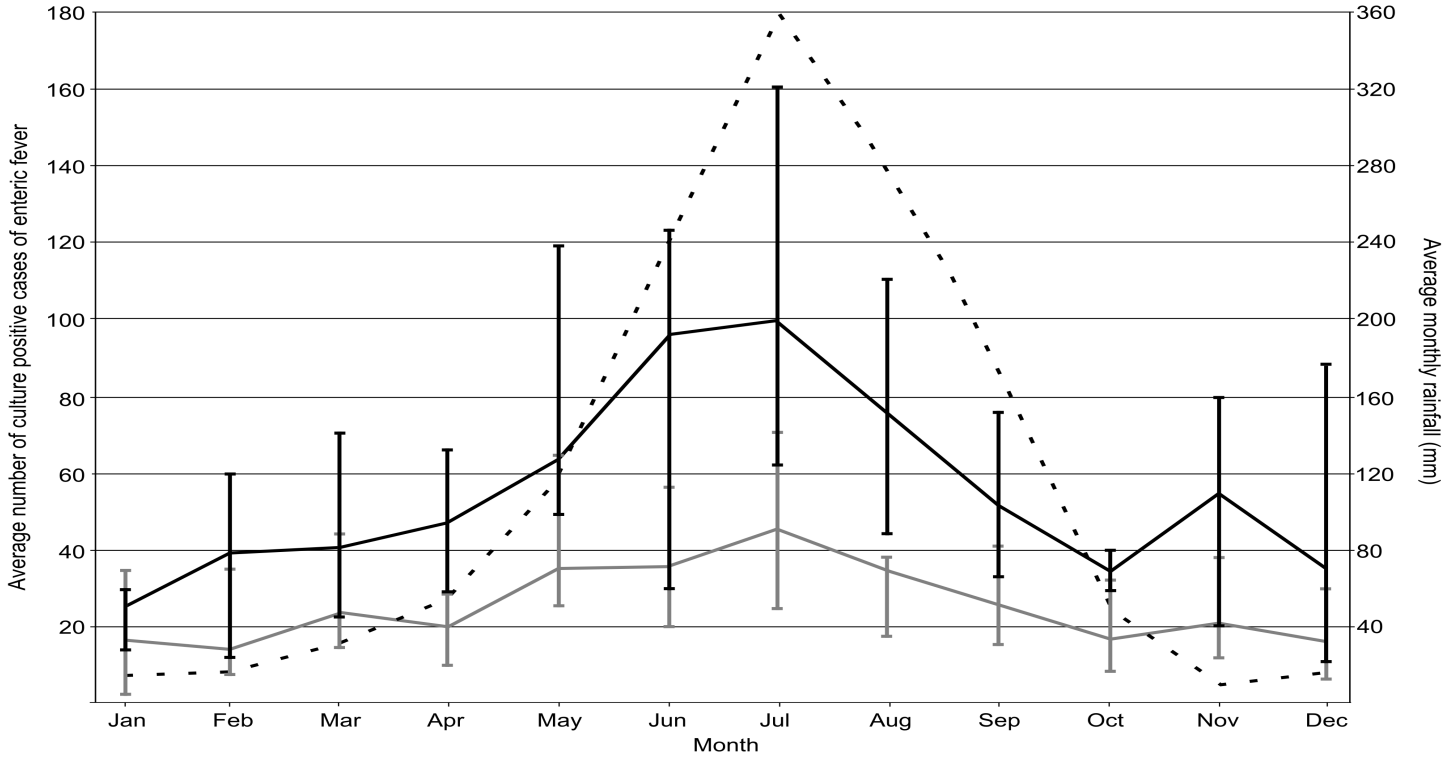


Figure 3.2: The seasonal distribution of enteric fever patients at Patan Hospital.

Plot of the seasonal distribution of enteric fever patients at Patan Hospital in Kathmandu. The average number of *S.* Typhi cases (black line) and *S.* Paratyphi A cases (grey line) for each month of the year was calculated over the period June 2005-May 2009. Vertical lines represent the range over the four-year period for each month. The average monthly rainfall (mm) is shown by the broken line and corresponds with the secondary y-axis.

3.4.1 The incidence of enteric fever within LSMC

During the period June 2005 to May 2009, we enrolled 527 patients that had a positive blood culture for either *S.* Typhi or *S.* Paratyphi A in clinical studies at Patan Hospital. These enrollees constituted 13.5% (527/3,898) of all the blood culture positive enteric fever patients at Patan Hospital over the same period. The remainders of enteric fever patients were not enrolled in clinical studies, as enrollment was dependent on a range of criteria and additional factors such as time of admission to the hospital, admission during periods when clinical studies were not running, and residence outside LSMC.

All 527 enrolled patients were resident in the 28.5 km^2 that constitute the 22 wards of LSMC. These data were categorized by ward and using population figures for LSMC wards as a denominator, the average annual incidence of *S.* Typhi, *S.* Paratyphi A and overall within LSMC was calculated over this four year period and is shown in **Table 3.1**.

The three wards with the highest incidence of enteric fever during the study period, wards 9, 22 and 11 had an incidence of 1.66, 1.53 and 1.24 per 1,000 residents per year, respectively. These three wards are neighbouring, located to the east of the city and are bordered by the Bagmati River **(Figure 3.3)**. Ward 22 had both the highest incidence of *S.* Typhi and *S.* Paratyphi A and ward 1 had the lowest **(Figure 3.3)**. Despite enteric fever following a human-to-human transmission route, there was no correlation between population density and enteric fever incidence in the 22 wards (p=0.857, r=0.037, Spearman's ρ). There was also no association between ward level incidence in this group of patients and the average distance of patients' residence to Patan Hospital (p=0.1706, r=-0.292757, Spearman's ρ). *S.* Typhi was predominant over *S.* Paratyphi A in all but two

wards and the ratio of *S.* Typhi to *S.* Paratyphi A ranged from 1:1 to 5.67:1. The average incidence of enteric fever in LSMC was calculated to be 3.78 cases per 1,000 households per year over the period 2005-2009. Similar to the population incidence of enteric fever, wards 9, 22 and 11 also had the highest household incidences.

Table 3.1: The average annual incidence of enteric fever cases in LSMC by ward of residence

Ward	Population[a]	Population/ ha[a]	*S.* Typhi		*S.* Paratyphi A		Total		Ratio[c]	Distance (km) [d]	Incidence/ 1,000
			Number	Incidence[b]	Number	Incidence[b]	Number	Incidence[b]			Households/ year
1	7,096	171.32	2	0.07	2	0.07	4	0.14	1.00	2.17	0.60
2	10,459	80.61	11	0.26	4	0.10	15	0.36	2.75	2.20	1.65
3	10,637	71.86	23	0.54	9	0.21	31	0.75	2.56	1.55	3.38
4	10,971	60.72	37	0.84	14	0.32	51	1.16	2.64	1.66	5.05
5	6,573	93.18	15	0.57	5	0.19	20	0.76	3.00	0.30	3.58
6	6,352	249.49	14	0.55	6	0.24	20	0.79	2.33	0.63	3.83
7	6,408	268.79	6	0.24	3	0.12	9	0.35	2.00	1.07	1.73
8	7,355	165.80	21	0.72	4	0.14	25	0.85	5.25	1.04	4.45
9	8,135	105.35	44	1.35	10	0.31	54	1.66	4.40	1.15	7.93
10	5,430	66.95	12	0.55	8	0.37	20	0.92	1.50	1.75	4.10
11	4,238	338.50	17	1.00	4	0.24	21	1.24	4.25	0.92	6.73
12	5,677	430.40	10	0.44	8	0.35	18	0.79	1.25	0.43	3.98
13	6,553	68.79	11	0.42	5	0.19	16	0.61	2.20	1.14	2.85
14	11,530	62.45	15	0.33	7	0.15	22	0.48	2.14	1.44	2.20
15	11,352	46.66	27	0.60	9	0.20	36	0.79	3.00	1.26	3.35
16	5,294	540.20	12	0.57	4	0.19	16	0.76	3.00	1.00	4.05
17	6,693	118.08	17	0.64	3	0.11	20	0.75	5.67	0.94	3.33
18	6,915	539.39	11	0.40	3	0.11	14	0.51	3.67	0.66	2.73
19	6,048	345.21	12	0.50	12	0.50	24	0.99	1.00	0.42	4.75
20	6,519	328.58	17	0.65	7	0.27	24	0.92	2.43	0.66	4.15
21	4,249	452.99	11	0.65	3	0.18	14	0.82	3.67	0.83	3.88
22	8,513	184.66	34	1.00	18	0.53	52	1.53	1.89	1.16	6.88
Total/ Average	**162,997**	**217.73**	**379**	**0.59**	**148**	**0.23**	**527**	**0.82**	**2.80**	**1.11**	**3.78**

[a] Data from the 2001 Nepal census (Nepal, 2002); [b] Incidence calculated as cases per 1,000 population per year; [c] Ratio of *S.* Typhi to *S.* Paratyphi A per ward; [d] Average distance from Patan Hospital to patients' location of residence

Figure 3.3: The geographical distribution of enteric fever cases in LSMC.

Maps depicting the average annual incidence of enteric fever per 1,000 population in the 22 wards (numbered) that constitute the Lalitpur Sub-Metropolitan City (LSMC), based on enteric fever cases enrolled in clinical trials at Patan Hospital between June 2005 and May 2009 for (a) *S.* Typhi and (b) *S.* Paratyphi A. Patan Hospital is located in ward 20 and is highlighted. Ward population figures are depicted in **Table 3.1**

3.4.2 Age and gender distribution of enteric fever patients

The distribution of all the 527 enrolled enteric fever cases by age and gender are shown in **Figure 3.4**. Of all the enteric fever cases 64% were male (male:female, 1.76:1). The number of male cases of enteric fever exceeded female cases across almost all age groups and, in particular, among young adults aged between 15 and 30 years. The median age of male cases (18 years) was higher than that of female cases (15 years), [$p< 0.003$ (t-test)]. The median age of *S.* Typhi cases (16 years) was significantly lower than that of *S.* Paratyphi A cases (20 years), [$p<0.0001$ (t-test)]. In both males and females *S.* Typhi was the predominant aetiological agent across all age groups, which is consistent with our observation that *S.* Typhi is more prevalent than *S.* Paratyphi A in this location. Notably, among children under the age of 10 years, the ratio of *S.* Typhi to *S.* Paratyphi A exceeded 8:1, then declined with age **(Figure 3.4).**

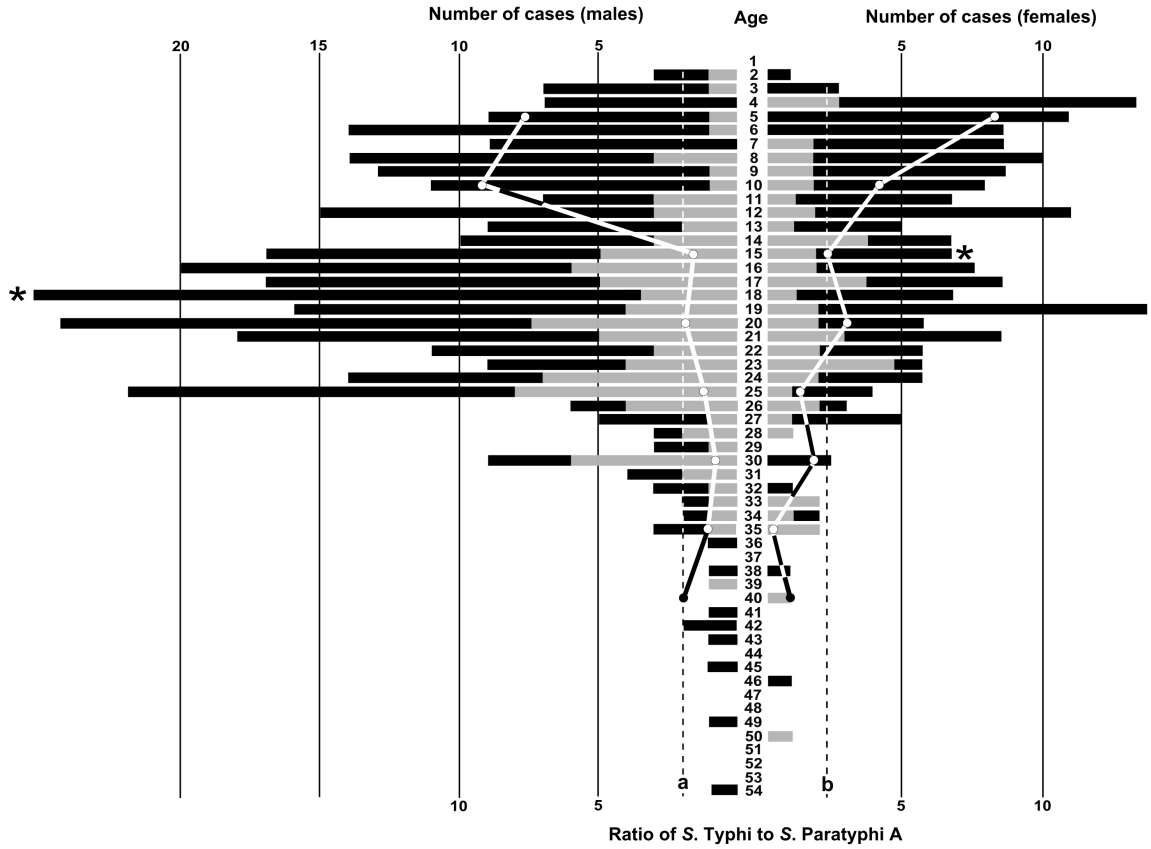


Figure 3.4: The age distribution of a subset of enteric fever cases of Patan Hospital, LSMC.

A double-sided bar chart showing the age distribution (central scale; y-axis) of blood culture positive enteric fever cases enrolled in clinical trials at Patan Hospital in LSMC between June 2005 and May 2009 (n=527). Bar sizes correspond to the number of patients (upper x-axis) of each sex (left: male and right: female). Cases that were culture positive for *S.* Typhi are shown in dark shading, those that were culture positive *S.* Paratyphi A are shown in light shading. The ratio of the culture positive *S.* Typhi to *S.* Paratyphi A cases over the period of investigation in each five year age group for males and females are shown by the solid lines with solid circles (lower x-axis). The overall ratio of *S.* Typhi to *S.* Paratyphi A was 2.69:1 in males (shown by the black broken line a) and 2.73:1 in females (shown by the black broken line b). An asterisk shows the median age of the male and female patients.

3.5 Discussion

In this chapter, I have described some of the basic epidemiologic characteristics of enteric fever within LSMC. It was observed that there was a considerable burden of *S.* Typhi and *S.* Paratyphi A infections at Patan Hospital, which fluctuates with seasonal variation in rainfall. From available data on patients enrolled in clinical trials, a conservative average of annual enteric fever incidence in LSMC over a four-year period and the age distribution and spatial variation in disease incidence, by ward, was investigated. The significance of these findings is that this area of Kathmandu would be suitable for programmatic use of typhoid vaccines for controlling the disease as suggested by the WHO (WHO, 2008a) and as demonstrated in studies conducted in South Asia (Khan et al., 2006, Sur et al., 2009b).

These findings, however, have limitations as they are based upon available data from those people with enteric fever receiving a blood culture that present to a single health care facility in LSMC. Additionally, the age distribution, incidence and locality data are taken from a subset of patients entering clinical trials. Therefore, this data presents a snapshot of some of the epidemiological features of enteric fever in this location, and it is recognized that the subset of patients on whom the analysis was based, might not be fully representative of the enteric fever patient population of LSMC as a whole. However, in the absence of prospective population based surveillance data, these findings from a large dataset provide insight into the patterns of enteric fever within an urban population and will lead to more detailed epidemiological studies within this population.

It was observed that the proportion of *S.* Typhi to *S.* Paratyphi A cases fluctuated over the four year study period. There was a sequential annual decrease in *S.* Typhi and an increase

in *S.* Paratyphi A from June 2005 to May 2009, with an overall decrease in total enteric fever cases over the four year period. Previous studies have reported that an increasing fraction of the enteric fever cases in this population were caused by *S.* Paratyphi A between 1999 and 2003 (Maskey et al., 2008), and it appears that this trend is continuing. Variation in the proportion of *S.* Typhi to *S.* Paratyphi A at the ward level and by patient age was also observed. These observations may be explained by differing transmission factors between these two pathogens, as well as difference in the overall disease burden attributable to these two serovars. *S.* Paratyphi A incidence was lower than *S.* Typhi, suggesting that exposure to *S.* Paratyphi A is lower than to *S.* Typhi. It would, therefore, be expected that the average age of patients infected with *S.* Paratyphi A infections to be higher, since lower exposure implies a longer time to acquired immunity; and this is consistent with what was observed in the study. Previous studies have reported independent risk factors for transmission of *S.* Typhi and *S.* Paratyphi A within the same area (Vollaard, 2004), and the data from this study which shows differences in the ward level distribution of the two serovars are consistent with this. The observed decrease in *S.* Typhi at Patan Hospital over the four-year period may be due to changing health care seeking behaviour or increasing immunity within a highly exposed population (Pulickal et al., 2009).

Enteric fever is considered to be a particular public health issue in children (Lin et al., 2000). In this study, although a substantial number of enteric fever cases in children were observed, at the same time, a disproportionate number of enteric fever cases in young male adults among the subset of patients enrolled in clinical trials. Although robust conclusions cannot be drawn regarding age and gender distribution of enteric fever patients in LSMC, some observations warrant consideration. The majority of the cases were males aged

between 16 and 30 years. Kathmandu has a large predominantly male transient workforce, i.e. those coming from other areas of Nepal for employment. A demographic study of the Nepalese population in 2007 found that 37% of households had at least one person that had travelled away from the household for employment within Nepal at some time in the previous 12 months (Ministry of Health and Population (MOHP) et al., 2007). This observation is intriguing and if these data are representative, the disproportionate number of infected males may be attributable to increased risk of exposure (related to behaviour and/ or living conditions) or lack of immunity due to a lack of previous exposure.

Some variation in the spatial distribution of the enrolled enteric fever patients, with a major focus of infections in the east of the city, was observed. However, there was no direct relationship between population density or the distance from the hospital and the incidence of enteric fever cases in individual wards, suggesting the local environment plays an important role in transmission.

The minimum estimate of average annual incidence of enteric fever in LSMC was calculated to be 0.82 per 1,000 residents per year. This frequency makes LSMC an area of medium incidence according to the guidelines outlined by Crump *et.al.*(Crump et al., 2004) and equivalent to the (*S.* Typhi) incidence calculated from population surveillance in Jakarta, Indonesia (Ochiai et al., 2008). The calculation in this study is based on those with culture confirmed enteric fever enrolled in clinical studies attending one health care facility. Therefore, the figures are likely to be highly conservative and on estimation (based on the proportion of total outpatient attendees living in LSMC) it may be at least five times greater than this figure. This makes LSMC an area of high enteric fever incidence and enteric fever is likely to place considerable burden on the health care system

in this study area.

We propose that environmental factors play a significant role in the transmission of the infecting organisms, and this is supported by the association of incidence with rainfall, an association that has been observed to a lesser extent in Viet Nam (Lin et al., 2000).

3.6 Conclusion

This study shows that enteric fever caused by *S.* Typhi is largely predominant among young adolescents in this study population with the majority of the focus on children. Therefore, school age children are an obvious target group for a vaccination program. However, further investigation is also required of the high case burden we observed in young adult males, to determine whether particular risk group such as transient workers should be targeted in prevention and control activities. Young adult males were relatively more likely to be infected by *S.* Paratyphi A for which there is no vaccine, an *S.* Paratyphi A vaccine would be a valuable tool to prevent infection and control transmission.

The median age of enteric fever may be higher in LSMC than in other locations and we suggest that tackling the disease in school-age children and the transient male population may be key to controlling the ongoing transmission of enteric fever in this densely populated area. The uneven spatial distribution of enteric fever incidence within the Patan Hospital catchment population suggests local variation in risk factors, and control measures should be implemented throughout the LSMC area. In the absence of prospective population-based surveillance, these findings from a large clinical dataset provide significant information on the distribution of enteric fever within an urban population.

4 Geospatial mapping of enteric fever

4.1 Abstract

Having observed an uneven spatial distribution of enteric fever incidence within the study area, to improve the understanding of typhoid transmission, a longitudinal spatial case-control study for enteric fever was conducted, combining SNP genotyping and GPS case localization. I hypothesized that enteric fever cases are clustered and that the infecting organisms are transmitted indirectly through the environment, particularly in the water supply. If the hypothesis was true, it was expected that genotyping of the pathogens from this population would reveal a pattern of transmission or at least a semi-random distribution. During a period of four years we GPS located 584 households that had enteric fever, I subdivided them into *S.* Typhi and *S.* Paratyphi and investigated the distribution compared to an afebrile control population resident in the same area, I additionally genotyped all the *S.* Typhi isolates. Here, I observed extensive clustering of enteric fever, which as in chapter 3, occurred independently of population size and density. *S.* Typhi haplotyping identified multiple genotypes circulating randomly throughout the population and even within individual residences, furthermore, sequencing revealed local microevolution of a dominant clonal group. The data produced here did provide evidence for direct intrinsic transmission, yet I surmised that the local environment, potentially through contamination of the water supply is key in sustaining transmission. Consistent with this conclusion, a spatial association between enteric fever with public water sources and low elevation was calculated. These findings have profound implications for enteric fever-control strategies, and this approach could be applied to other study diseases caused by other emerging pathogens.

4.2 Introduction

S. Typhi and *S.* Paratyphi A have adapted to be restricted to a single niche through a variety of genetic mechanisms such as acquisition of virulence factors and serial gene inactivation (Holt et al., 2009, Parkhill et al., 2001a, Pickard et al., 2003). The inability of these pathogens to interact with other hosts appears to have resulted in them becoming genetically isolated and dependent on asymptomatic carriage for long-term survival and continued transmission within the human population. These pathogens are transmitted faeco-orally and in addition to being shed acutely during an infection, an estimated 5% of the cases may progress to become long term asymptomatic carriers as both pathogens can colonise and survive for prolonged periods within the gall bladder (Holt et al., 2008a, Khatri et al., 2009, Levine et al., 1982). A classic example of a typhoid carrier is the infamous case of Typhoid Mary, a New York cook (Marr, 1999). Typhoid carriers are capable of unknowingly excreting the causative pathogen into the environment indefinitely, thereby representing individual reservoirs of infection that may, potentially, maintain the local pathogen population (Nath et al., 2010, Ristori C et al., 1982).

Enteric fever transmission is thought to be through direct contact with individuals shedding the bacteria, either during acute infection or during asymptomatic carriage. Additionally, as enteric fever is associated with poor sanitation and is transmitted through a faeco-oral route, indirect transmission via the consumption of contaminated food or water evidently plays an important role (Swaddiwudhipong and Kanlayanaphotporn, 2001). However, the general understanding of enteric fever transmission patterns is not well understood as current theories are based on observations of declining incidence following improvements in general sanitation (Tulchinsky et al., 2000, Wolleswinkel-van den Bosch JH, 1997), or through risk factors identified during epidemiological studies

(Luxemburger et al., 2001, Sur et al., 2007, Vollaard, 2004). Previous epidemiological studies concerning endemic enteric fever have not included a thorough molecular examination of the pathogens or a spatio-temporal investigation of infection sites in a single setting. Therefore, potential transmission events contributing to endemic disease have never been elucidated, and the relative influence of indirect and direct transmission, or the relationship between *S.* Typhi and *S.* Paratyphi A in endemic urban areas are not understood. The fact that both *S.* Typhi and *S.* Paratyphi A are monophyletic clades within the bacterial species *S. enterica* could partially explain the general lack of comprehensive pathogen characterization in enteric fever studies. These two genetically distinct pathogens since entering the human population approximately 50,000 years ago (Kidgell et al., 2002) has undergone evolutionary convergence to cause indistinguishable diseases (Didelot et al., 2006, Holt et al., 2009, Roumagnac et al., 2006), frequently occurring together in the same locations (Karkey et al., 2010). Being recent clones, both pathogens exhibit very little genetic variations, thus the current gold standard for bacterial genotyping, MLST, has insufficient resolution for distinguishing within populations of these pathogens (Kidgell et al., 2002). Alternative methods such as PFGE could be used to discriminate between some isolates, but does not generate robust phylogenetic information that could be applicable for the study of global evolution or local transmission patterns (Baker et al., 2010b). However, there have been recent developments using SNP genotyping to precisely define the global *S.* Typhi population in the form of a highly parsimonius phylogenetic tree (Holt et al., 2008a, Roumagnac et al., 2006). The use of such high-resolution genotyping allows for precise and consistent distinguishing among circulating *S.* Typhi within localized human populations, thereby allowing the diversity of the organism to be precisely defined within any given temporal or geographical boundary (Baker et al., 2008, Holt et al., 2010, Holt et al., 2011, Kariuki et al., 2010).

In an attempt to better understand the dynamics of enteric fever transmission in LSMC, I combined bacterial isolation, SNP- based genotyping and GPS case localization. I identified the genetic diversity and the corresponding spatio-temporal distribution of *S.* Typhi found within the study population, and have provided novel insights into the transmission of enteric fever in this urban setting. The methodology used in this study offers the potential for the design of rational and efficient intervention strategies against enteric fever and other bacterial infections with similar limited genetic diversity.

4.3 Results

4.3.1 Geographical clustering of typhoid fever

Over a period of four years, from June 2005 to May 2009, handheld GPS devices were used to locate the residences of 584 culture confirmed typhoid fever patients of which 431 (73.8%) were infected with *S.* Typhi and 153 (26.2%) were infected with *S.* Paratyphi A. All patients were tracked after having attended the outpatient department at Patan Hospital and were resident within our study catchment area **(Figure 4.1)**.

Figure 4.1: Map of LSMC depicting its 22 wards generated from Google Earth Maps.

Each ward is demarcated with white lines and labeled (white balloons). Patan Hospital is situated in Ward 20 (yellow balloon). The River Bagmati (blue line) separates LSMC from the city of Kathmandu to the North (blue line).

The temporal typhoid distribution is shown in **Figure 4.2 a**, which can be seen to follow an annual seasonal trend with the majority of infections (420 out of 584, i.e. 71.9%) occurring during the monsoon season, between June and September. The mapping data revealed that enteric fever, caused by both *S.* Typhi and *S.* Paratyphi A, was heavily clustered to the northeast of the study area (**Figure 4.2b**).

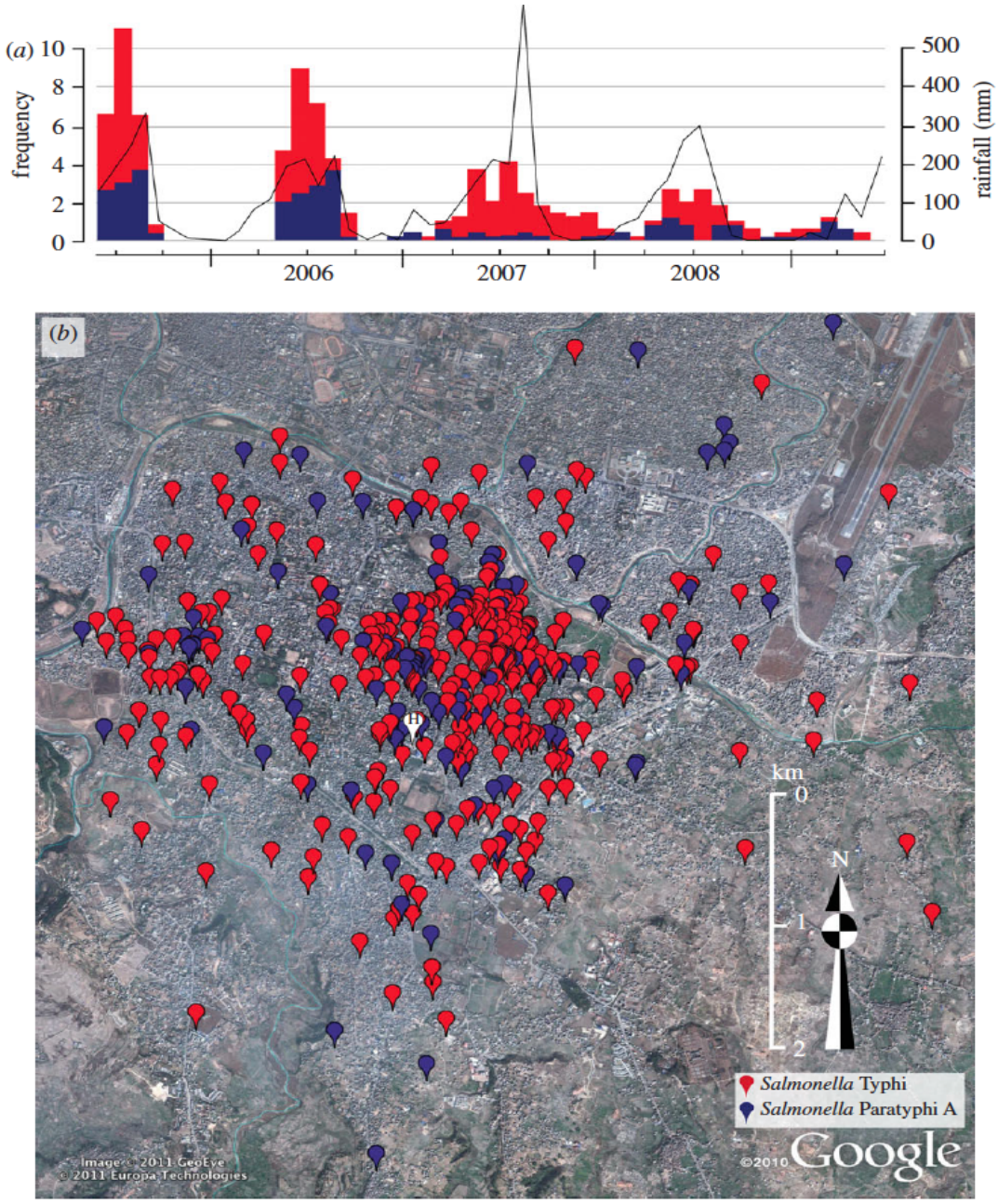


Figure 4.2: The temporal and spatial distribution of typhoid infections.

(a) A histogram showing the monthly temporal distribution of *S.* Typhi (red) and *S.* Paratyphi A (blue), and the corresponding monthly rainfall over the 4 year study period. (b) Google Earth map, of the study site showing locations of the residences of 584 culture confirmed enteric fever patients: 431 *S.* Typhi (red) and 153 *S.* Paratyphi A (blue). The site of patient enrollment (Patan Hospital) is marked as H.

To assess the scale and significance of enteric fever clustering, a spatial model was fitted, using the residences of 2,048 afebrile outpatients, resident within the study catchment area, to control for population density and hospital referral patterns (Chetwynd et al., 2001b)(**Figure 4.3**). The dispersal of enteric fever cases was found to be non random, as both *S.* Typhi and *S.* Paratyphi A cases demonstrated extensive spatial clustering (up to approximately 4.4 km for *S.* Typhi and 1.7 km for *S.* Paratyphi A), in comparison to the controls.

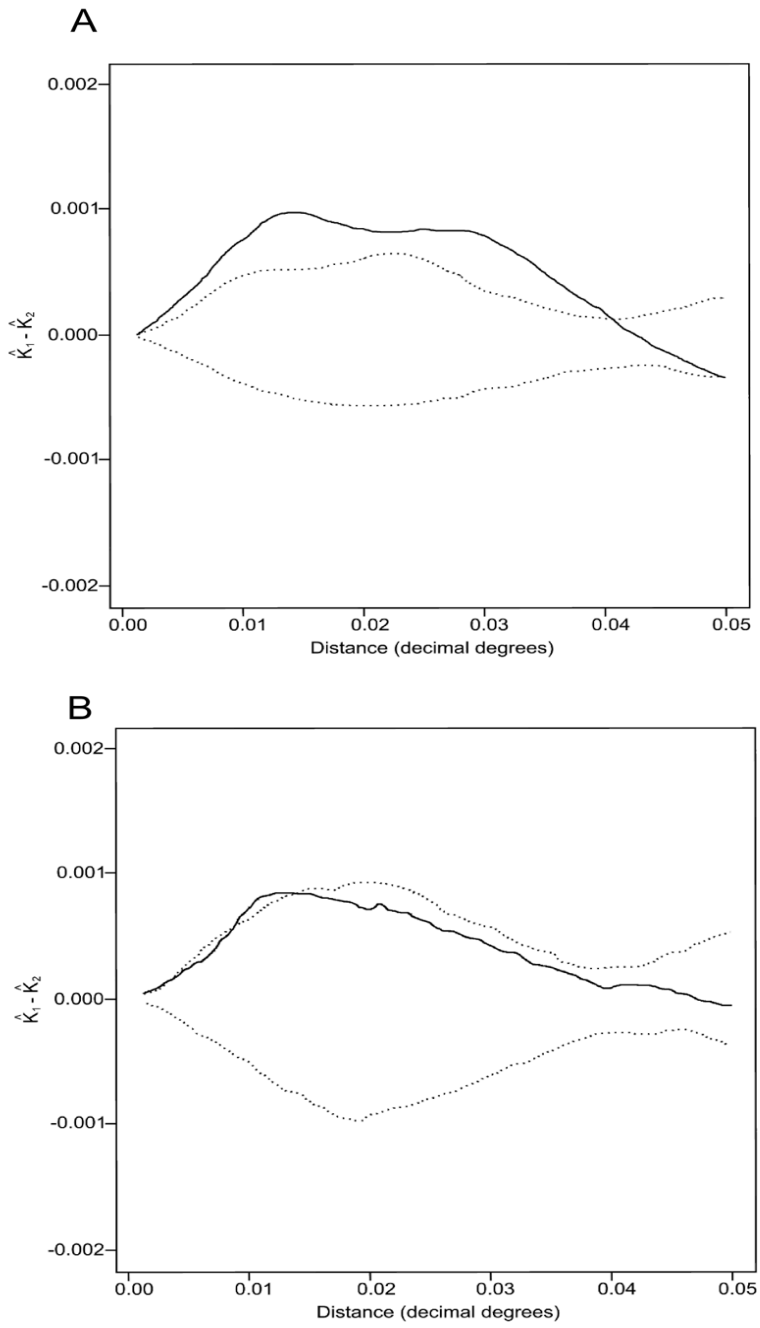


Figure 4.3: Bivariate K- functions for enteric fever infections.

Difference in K- function values for *S.* Typhi (A) and *S.* Paratyphi A (B) cases (K1) and controls (K2) (solid lines indicate K1-K2). Broken lines show an envelope of the minima and maxima of 199 simulations for K1-K2 under conditions of spatial randomness. Distance is defined in decimal degrees; 0.01 decimal degrees equals approximately 1.11

Furthermore, when the spatial distribution was compared with the date of bacterial isolation, spatio-temporal case clustering was evident throughout the study period (p=0.002; Mantel test) consistent with small outbreaks occurring during each monsoon season (**Figure 4.2a)**.

To identify specific enteric fever infection hotspots, a model to infer the spatial risk for *S.* Typhi and *S.* Paratyphi A infections was constructed which corrected for the effects of population density and hospital referral pattern with the afebrile controls (Webster et al., 2006). Despite *S.* Paratyphi A being more diffusely distributed than *S.* Typhi, the areas of highest and lowest spatial risk were comparable for both pathogens and demonstrated considerable commonality, yet *S.* Paratyphi A infections were more diffuse than *S.* Typhi infections and appeared to be associated with downstream river flow. The focal point for enteric fever infections was located towards the north of the study area and formed an elongated cluster following the route of the Bagmati River **(Figure 4.4)**.

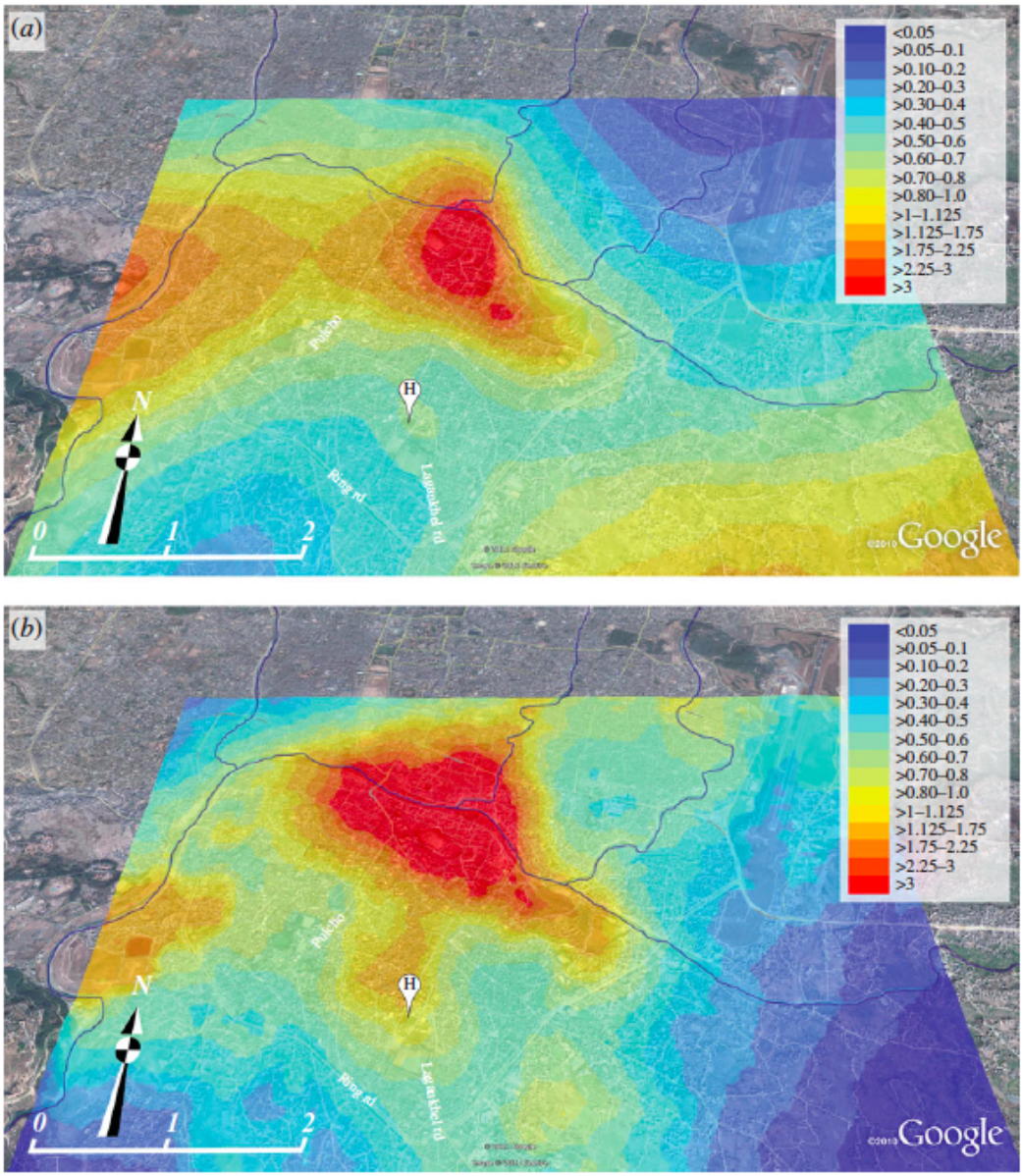


Figure 4.4: The variable spatial risk of enteric infections.

Elevated Google Earth map of the study terrain with heat map overlays showing the predicted spatial odds for (a) *S.* Typhi and (b) *S.* Paratyphi A infections compared with controls, as calculated by spatial risk modelling. Spatial odds for typhoid infections are scaled from low (blue) to high (red) as shown by the key. The site of patient enrolment is marked H; the lower-left scale represents distance in kilometres and the route of the Bagmati River is highlighted (flow: east to west).

4.3.2 Genotyping of circulating *S.* Typhi

SNP based genotyping has been shown to be a powerful approach to discriminate within *S.* Typhi populations, providing information on phylogenetic lineage and related phenotypes, such as antimicrobial resistance (Baker et al., 2008, Holt et al., 2011). Re-sequencing of highly related local genotypes renders possible the ability to define microevolution in real time by identifying SNP accumulation **(Figure 4.5)**. In this study the Sequenom platform was used to genotype DNA extracted from 387 local *S.* Typhi (89.8%) with 73 previously identified informative SNP loci (Baker et al., 2008). This initial typing identified 14 genetically distinct *S.* Typhi clades circulating in this district during the study period **(Figure 4.5 a)**. However, 68% of the isolates (n=259) were of haplotype H58, which has been shown to be expanding globally and associated with resistance to multiple antimicrobials (Kariuki et al., 2010, Roumagnac et al., 2006).

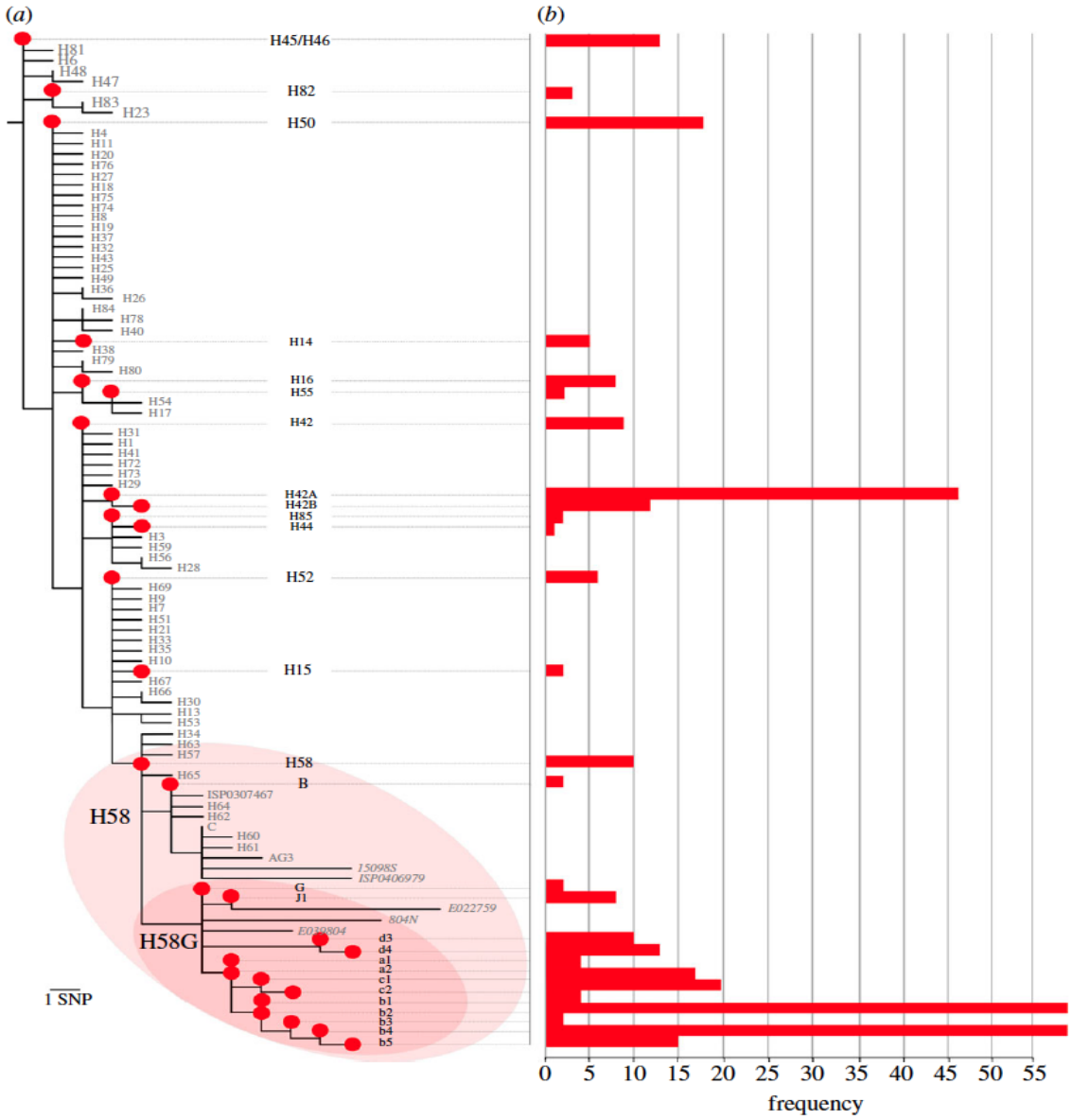


Figure 4.5: Phylogenetic tree and frequencies of *S.* Typhi genotypes.

(a) Phylogenetic tree showing the haplotype distribution of 387 *S.* Typhi strains isolated in the study area between June 2005 and May 2009. Red circles and black text indicate genotypes that were detected among the study isolates; grey text indicates genotypes that were defined by assayed loci, but not detected among the study isolates (i.e. would have been detected if present). The H58 and H58G clonal groups are highlighted. **(b)** Horizontal bar plot indicating the frequency of isolation of each *S.* Typhi genotype over the 4-year study period, according to the scale denoted on the X-axis.

As H58 represents a highly clonal group, an additional 38 SNPs that had been identified earlier were exploited to re-analyse the 259 H58 isolates (Kariuki et al., 2010). It was observed that 237 of the H58 *S.* Typhi (92%) belonged to a single subgroup that has previously been named H58G (Holt et al., 2010). It was hypothesized that the H58G subgroup had undergone expansion during the period of the study, leading to the prediction that this dominant group would have accumulated further mutations during its persistence within the local human population, which could be useful to further differentiate within the clonal group. Consequently, 40 H58G isolates were selected for Illumina whole genome sequencing and SNP discovery. Using the criterion that a SNP was present in at least two of the 40 H58G isolates, 13 novel SNPs were identified, and all H58G isolates were screened with these additional SNPs **(Figure 4.6)**.

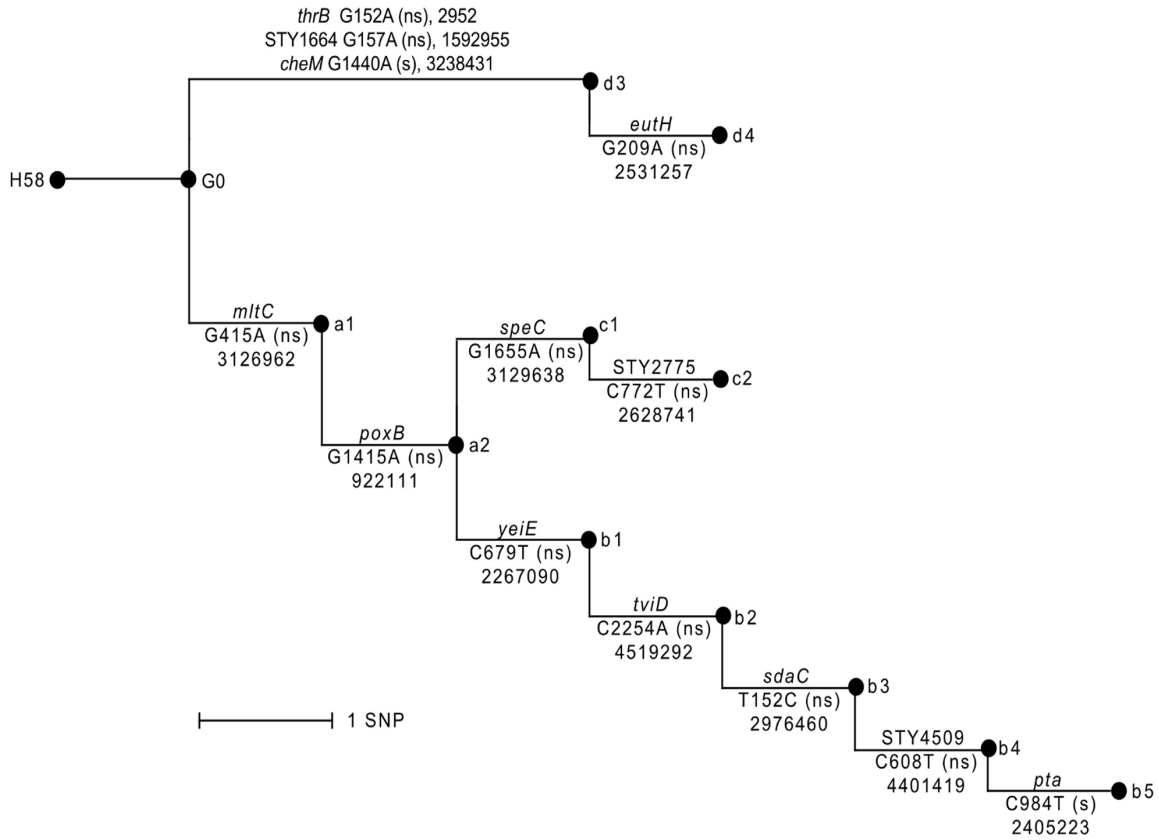


Figure 4.6: SNPs defining *S.* Typhi H58G microevolution

Minimum spanning tree describing the SNPs that define *S.* Typhi H58G microevolution found in the study. Nodes (black circles) correspond to haplotypes observed among the *S.* Typhi strains, branches are labeled with the gene name in which the SNP occurs (above the branch), the precise mutation (ancestral allele; nucleotide coordinate within the gene; derived allele), the effect of the mutation on the encoded protein (s: synonymous SNP, i.e. no change; ns: non-synonymous SNP, i.e. change in the encoded amino acid), and the coordinate of the SNP in the Typhi CT18 genome (EMBL: AL513382)

The resulting phylogeny revealed the expansion of three lineages from H58G, the distribution and designations of which are outlined in **Figures 4.5a and 4.6**. There were annual fluctuations in the proportions of the various genotypes detected, with some genotypes detected annually through the period of the investigation, yet this variability had no obvious pattern **(Figure 4.7)**.

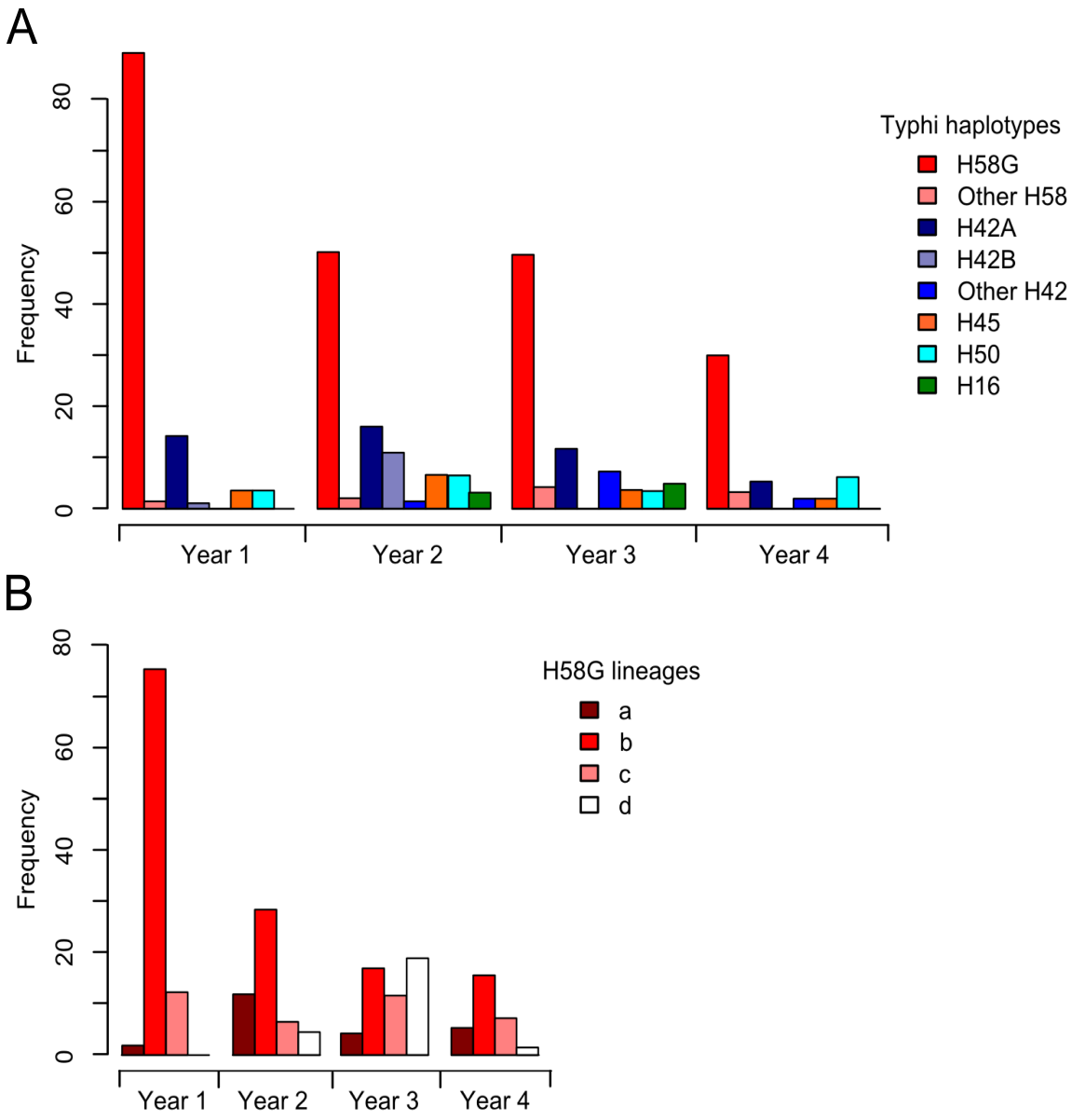


Figure 4.7: The annual distribution of *S.* Typhi genotypes

Histograms showing: (A) the annual distribution of Typhi haplotypes and (B) the annual distribution of Typhi H58G lineages over the four-year study period that was initiated in June 2005

4.3.3 *S.* Typhi genotype distribution within enteric fever clusters

The spatial distribution of the 28 different *S.* Typhi genotypes was investigated by combining the genotype information with the GPS location data **(Figure 4.8)**. I hypothesized that a succession of single source infections would be temporally and spatially related, and would be comprised of an individual genotype. On a micro-level, a 1km^2 cluster of *S.* Typhi cases located in the northwest region of the study area, which met the criteria for the hypothesis, was identified **(Figure 4.8 b)**. Within this cluster, 28 of 39 (71.2%) isolates in year 1 of the study were of identical genotype H58G-b4. In subsequent years, the number of *S.* Typhi cases in this area decreased with an approximate 7 to 13 cases per year (p= 0.03; χ^2-test), as did the contribution of *S.* Typhi H58G-b4 (2 to 6 cases per year; p<0.0001; χ^2-test), suggesting an isolated single genotype outbreak in year 1 of the study. However, this small genotype outbreak was an exception as no other genotype clustering was observed. Despite the spatial and temporal case clustering, the distribution of *S.* Typhi genotypes was random **(Figure 4.8)**. Case clusters occurring over limited time periods were habitually composed of multiple genotypes and there was no overall evidence of spatial (p= 0.61; Mantel test) or temporal (p=1; Mantel test) genotype clustering. These data suggest an overwhelming contribution of indirect transmission of individual organisms.

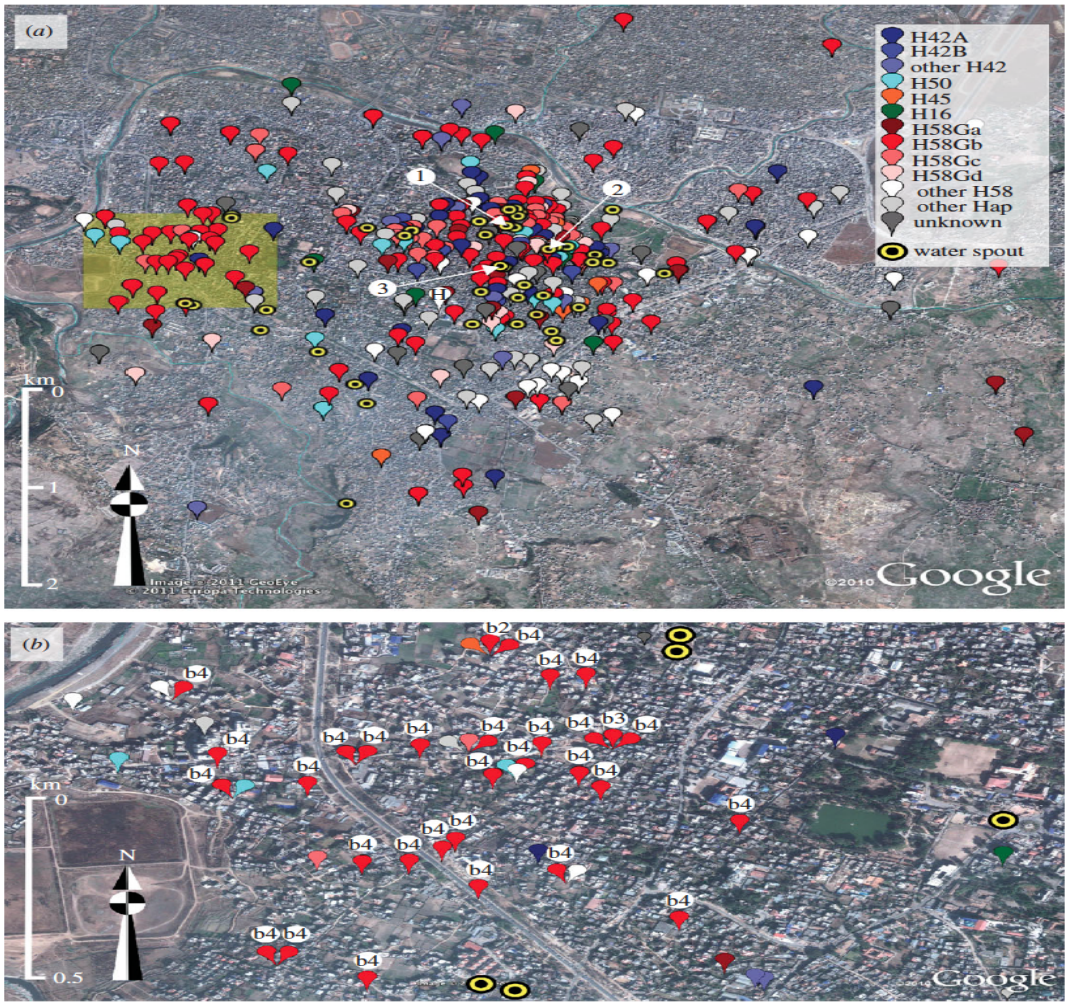


Figure 4.8: The spatial distribution of *S.* Typhi genotypes

Google Earth maps of the study site showing locations of the residences of culture confirmed *S.* Typhi infections, categorized by *S.* Typhi genotype (defined in figure 4.5) and the 42 functional waterspouts including the three water-sampling sites (labeled 1, 2 and 3) according to the legend provided. (a) All 431 culture-confirmed *S.* Typhi infections from the 4-year study period. The site of patient enrollment is marked H. (b) Culture-confirmed *S.* Typhi infections occurring in a concentrated 1km^2 cluster to the west of the hospital (shaded as in (a)). The subgroups of the H58Gb are shown by the lower-case letters and numerals associated with red markers.

Waterborne transmission of enteric fever has been demonstrated in several epidemic settings (Lewis et al., 2005 , Mermin et al., 1999), and I had previously hypothesized that the local water supply could play an important role in the transmission of enteric fever in the study catchment area (Karkey et al., 2010). To explore further this hypothesis, the location of 42 functional water stone spouts within the study catchment were mapped using GPS devices (Pradhananga, 1997). With this GPS data the spatial risk model was used to assess the effects of water spout proximity and elevation on enteric fever risk. Proximity to a water stone spout was significantly associated with the risk of enteric fever caused by both *S.* Typhi (OR 0.48, 95% CI (0.37, 0.62), $p< 0.0001$) and *S.* Paratyphi A (OR 0.60, 95% CI (0.40, 0.88), $p=0.009$) **(Figure 4.9)**. Lower elevation was also significantly associated with enteric fever risk, as the mean elevation of *S.* Typhi and *S.* Paratyphi A patient residences were respectively 3.27 and 3.78m lower than those of afebrile controls (OR 0.83, 95% CI (0.77, 0.90), $p<0.0001$ for *S.* Typhi; OR 0.83, 95% CI (0.75, 0.93), $p=0.001$ for *S.* Paratyphi A) **(Figure 4.10)**.

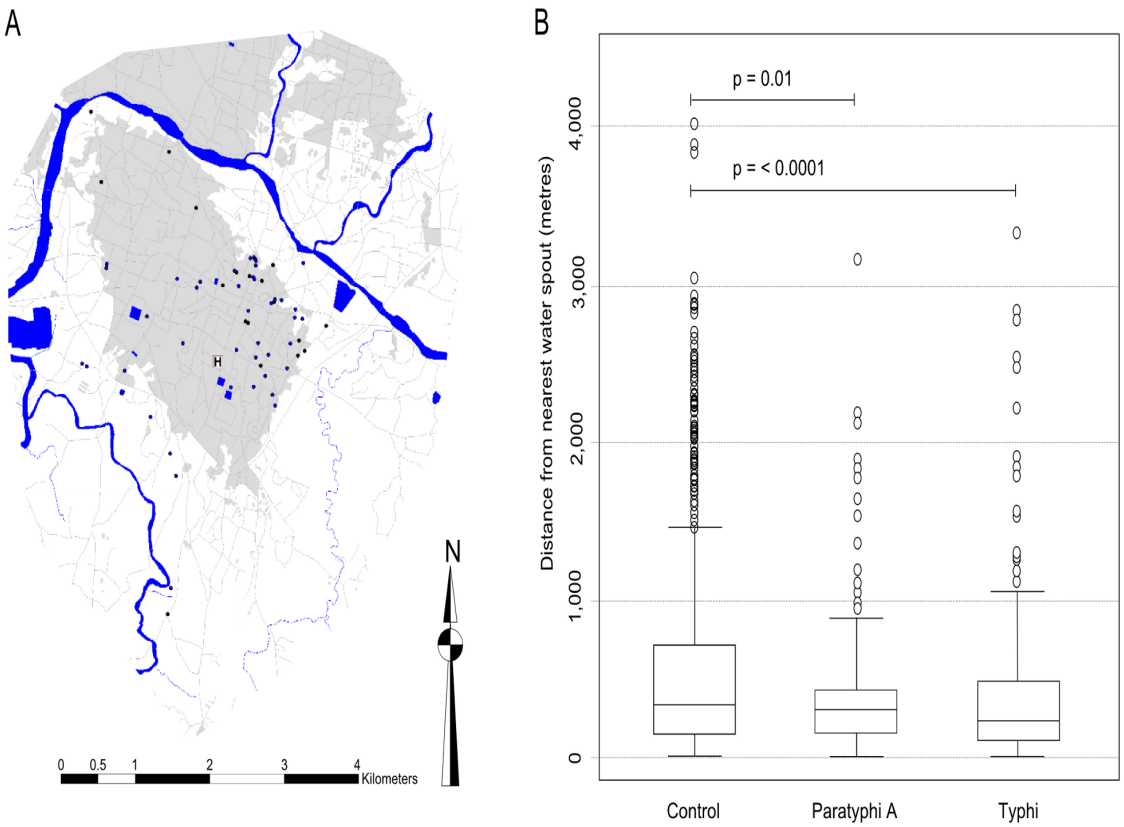


Figure 4.9: The spatial distribution and relative spatial risk of water spout proximity to enteric fever infections.

(A) Map showing the spatial distribution of residents with *S.* Typhi and *S.* Paratyphi A infections (black spots) and their relative risk with respect to controls over the same geographical area. Built up areas and thoroughfares are shown in grey and the route of the Bagmati River (flow: east to west) and major bodies of water are shown in blue. The location of Patan Hospital is depicted by the symbol H, and the positions of the functional water stone spouts are highlighted by the blue points.

(B) Box plots comparing distance from water stone spouts of *S.* Typhi and *S.* Paratyphi A infection to that of controls. A line and a corresponding p value highlight statistical significance between groups (2-tailed t test).

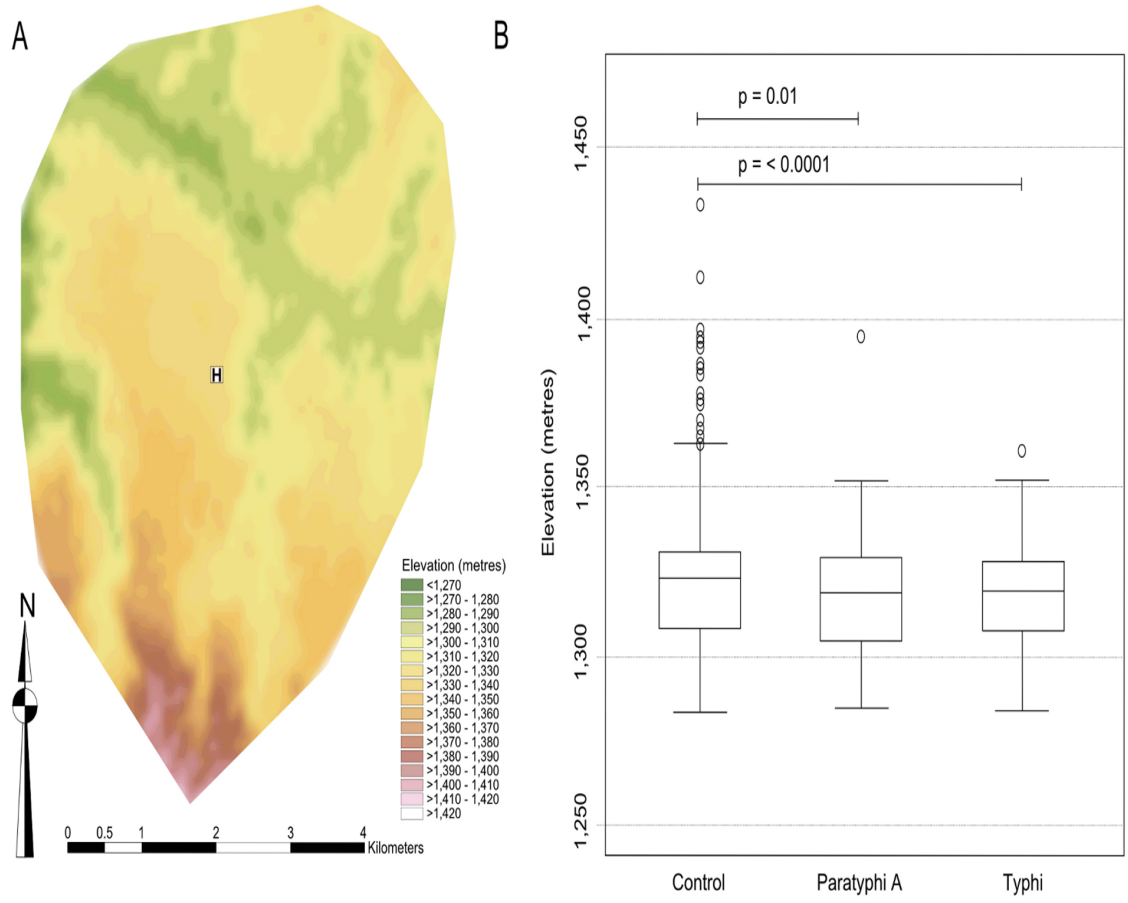


Figure 4.10: The spatial distribution and relative spatial risk of elevation to enteric fever infections.

(A) Heat maps showing the predicted spatial odds for enteric fever infections compared with controls, as calculated by spatial risk modeling. Spatial odds are scaled from low (green) to high (red) as shown by the key. The site of patient enrollment, Patan Hospital, is marked with an H.

(B) A box plot comparing elevation of households of cases to those of controls. A line and a corresponding p value highlight statistical significance between groups (2-tailed t test).

4.3.4 Intra-household typhoid transmission

Over the 4-year study period, multiple residences within the study catchment area had more than one case of enteric fever, which allowed for the exploration of potential transmission routes in finer detail. 37 residences (43%) had both *S.* Typhi and *S.* Paratyphi A infections, and 59 had multiple *S.* Typhi infections. Genotype data were available for more than one *S.* Typhi in 55 of these households. Of these 55, 44 (80%) households had infections caused by identical *S.* Typhi genotype only. The observed number of intra-household *S.* Typhi pairs of identical genotype was greater than that expected by chance (p= 0.027; randomization test), providing evidence of direct transmission within the individual residences. For the paired *S.* Typhi isolates from the same residence within a week, 44% (7 of 16) shared the same genotype, consistent with direct intrinsic transmission during an acute infection. However, multiple genotypes within individual residences were more commonly observed **(Figure 4.11)**. Given multiple cases of enteric fever infections in a single residence, the odds of the infections being caused by distinct organisms as opposed to an identical genotype were in excess of 3:1.

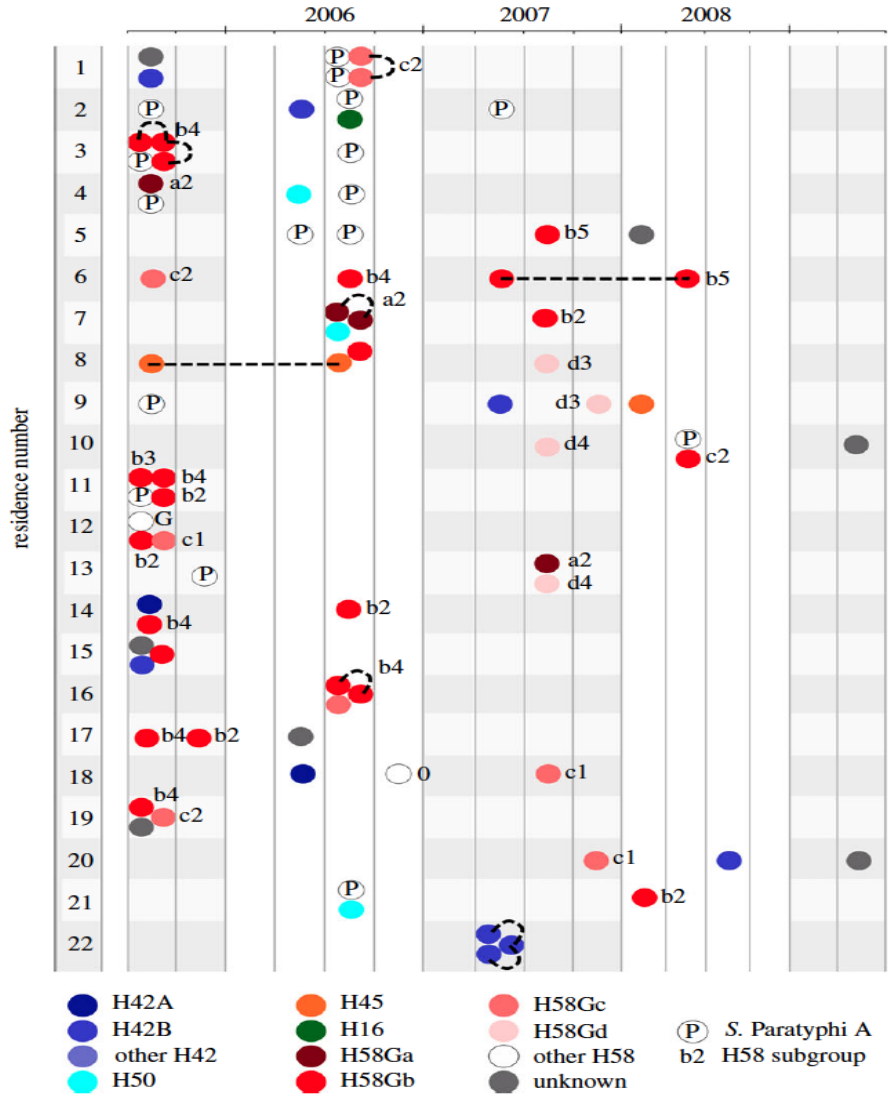


Figure 4.11: Intra-residence typhoid infections. The figure depicts the 22 residences (vertical axis) with three or more culture confirmed typhoid infections over the period of the investigation (horizontal axis). Each individual infection is shown by coloured circles, which are grouped into three-month periods. Colours indicate the *Salmonella* serotype (the letter P indicates an *S.* Paratyphi A infection) or *S.* Typhi genotype associated with each infection, according to the legend provided (as defined in **Figure 4.4**). Lower case letters and numerals associated with red circles refer to the individual H58G subgroup (defined in **Figure 4.5**). Broken black lines link isolates of the same genotype within a single residence.

4.4 Discussion

Study of the local ecology and transmission patterns of bacterial pathogens with low diversity had until recently been hindered due to the inability of distinguishing such pathogens. However, technological advancement in molecular biology and the resolution now permitted by high-throughput sequencing and SNP analysis has facilitated an enhanced understanding of the global population of *S.* Typhi and other bacterial pathogens with limited genetic diversity (Harris et al., 2010b, Morelli et al., 2010, Roumagnac et al., 2006). In this study, for the first time, elements of classical epidemiology have been combined with high throughput sequence analysis and GPS based spatial analyses to longitudinally study the local distribution and infer the transmission of a human restricted bacterial pathogen in an endemic region.

The application of these methods has increased the understanding of enteric fever transmission in a densely populated and highly endemic urban area. The methods applied were able to show a widespread diversity in the spatial risk of typhoid in the study area. In particular, extensive clustering of enteric fever cases in specific locations was observed, the main regions of which were comparable for both *S.* Typhi and *S.* Paratyphi A. However, it was observed that *S.* Paratyphi A was more diffuse than *S.* Typhi infections, and was strongly associated with proximity to the Bagmati River. These data suggest that, both pathogens exhibit a similar spatial risk and transmission pattern, with some evidence that *S.* Paratyphi A infection risk spreads downstream from the main focal point. It appears that *S.* Typhi infections are associated with a limited number of specific locations, whereas *S.* Paratyphi A may possess an enhanced ability to disseminate, potentially by contaminating ground water via the Bagmati River. Previous studies have observed enteric fever case clustering in other

urban settings, which have been associated to population density, overcrowding and hygiene practices (Sinha et al., 1999b, Sur et al., 2007). Here, spatial clustering of enteric fever was unrelated to the local population density. This fact, for a human restricted pathogen is counterintuitive, and again predicts strong local variability in risk factors for infection. The transmission of enteric fever is associated with poor sanitation, contaminated water and contact with an acute case or carrier (Kelly-Hope et al., 2007, Lin et al., 2000, Vollaard, 2004, Farooqi et al., 2009). These data strongly suggest that indirect transmission is dominant within our study setting, which is substantiated by the association of enteric fever cases with low elevation and water stone spout proximity. A study linking transmission through isolation of *S.* Typhi from water supplies during an enteric fever epidemic in Chile provides a comparable argument (Fica et al., 1996). I propose that people living near water stone spouts are of greater dependence on these for household water, and enteric fever incidence is likely to be associated with faecal contamination of ground water during the monsoon, particularly in areas with low elevation. The two variables, elevation and water spout proximity, are likely to be interconnected, as the water is gravity dependent and the water spouts are more common in low lying areas.

I additionally described 28 specific *S.* Typhi genotypes circulating in varying annual proportions within the study area over the 4-years study period. This degree of genetic diversity was sufficient to allow for further investigation of potential spatial and temporal relationships between the circulating *S.* Typhi. A plausible theory would be that proximal cases occurring as a result of direct transmission from a single source, such as a chronic or acute shedder, would be indistinguishable by SNP typing. However, with the exception of one

localized area, there was no temporal or spatial association of individual genotypes and no evidence of local clonal replacement. In fact, the overall pattern of strain distribution was one of erratic, random genotype fluctuation rather than localized genotype clustering. This suggests of an exposure to a wide variety of genotypes, rather than direct transmission of the same organism in close proximity. Evidence of direct transmission would be through the isolation of identical genotypes within individual households at a greater rate than would be expected by chance. However, it was observed that even with individual households, multiple cases were more frequently caused by a variety of genotypes, suggesting potential acquisition from outside the residence. One drawback of these findings is that this study presumes that individuals are infected exclusively with one bacterial strain, rather than co-infection with multiple strains. Conversely, owing to SNP discovery bias, it is more likely that SNP typing underestimates diversity within a dwelling, as it is possible for two isolates to be identical at all assayed loci despite being genetically distinct at unassayed loci (Pearson et al., 2004).

Previous data from Patan Hospital has demonstrated the presence of *S.* Typhi and *S.* Paratyphi A carriers within the local community (Khatri et al., 2009), but their precise role in transmission remains unclear. Data from this spatio-temporal genotyping are not consistent with such carriers playing a prevailing role in direct transmission. Rather, I suggest that in this urban endemic enteric fever environment, asymptomatic transient carriers are essential for sustaining a diverse *S.* Typhi population from year to year, providing individual isolated reservoirs for the long term persistence of a wide variety of genotypes, rather than facilitating direct transmission.

4.5 Conclusion

Within urban settings such as LSMC, improvements in infrastructure are fundamental to the control and elimination of enteric fever. Poor water quality, unsanitary conditions and the presence of carriers are likely to facilitate the continued persistence of the organism in the community long after the limited window of immunity induced by the current polysaccharide vaccine (Fraser et al., 2007, Sur et al., 2009b). For enteric fever to be controlled in the long term in such endemic settings, the integration of infrastructural improvements alongside other control measures such as accurate diagnosis, appropriate treatment and vaccinations is necessary. Improvements in water sanitation would be consistent with the historical elimination of enteric fever from many countries where intervention strategies have invariably been coupled with economic development and improvements in infrastructure.

High-resolution SNP-based genotyping and local GPS location data have been combined for the first time for a bacterial pathogen such as *S.* Typhi. This work provides a better understanding of the ecology and transmission of enteric fever pathogens in an endemic setting. It represents an integrated unique approach to molecular epidemiology that may be directly applied to other diseases caused by other monophyletic or emerging bacterial pathogens.

5 Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

5.1 Abstract

Various epidemiological studies have implicated food and water in the transmission of enteric fever. To investigate the extent of faecal contamination of water and street food, various food and water samples from within the study area were analysed. Weekly water samples were collected for the period of a year, when permitted by seasonal flow, from three different types of sources in10 locations situated in areas of LSMC known to suffer high burdens of enteric fever. Similarly over a period of 12 weeks, 48 food samples were analysed the majority of which was found to be contaminated with faecal coliforms. In the food samples consistent faecal contamination ranging from 7.9 – $2x10^3$ cfu/ml was observed with spiced raw meat and dumpling sauce being the most contaminated food products. Water samples were also faecally contaminated consistently with contamination ranging from 3.3 - $1.4x10^6$cfu/ml^{-1}. Among the sources analysed, water from traditional stone spouts was found to have the highest level of contamination and municipal supplied water the least. Although *S.* Typhi and *S.* Paratyphi A were not isolated through conventional microbiological culture methods from either food or water, using a pathogen specific real-time PCR assay the presence of DNA sequences specific for *S.* Typhi and *S.* Paratyphi A was detected in a majority of the water samples from all 10 locations. The findings show significant faecal contamination of water sources around areas of high enteric fever burden, suggesting that it is probably a major risk factor for infection.

5.2 Introduction

Water sources are often focal points of communities within Kathmandu and are generally used for drinking, washing, cooking and recreation. Majority of people living within the referral area of Patan Hospital do not have access to piped water within the home and rely on gathering water from the traditional local water stone spouts or from sunken wells. The historic nature of the waterways system feeding the stone spouts from local mountain springs implies a high risk of drinking water mixing with sewage as a consequence of poor maintenance and increasing population. In the late 1800s, a piped water system was introduced to the Valley, however due to limited resources and weak management over time, maintenance of the piped water and sewage systems has been poor, making water vulnerable to environmental contamination. The principle resource base of the municipal water supply in the Kathmandu Valley is the Bagmati River, which provides 92% of the supply during the wet season and 60% during the dry season (CBS, 1998). Unfortunately, intensive urbanisation has had a significant impact in deteriorating the surface water and hence the ground water quality along the Bagmati River (Kannel et al., 2008).

Faecal contamination of urban water supplies in Nepal has been reported previously. In 2002, an outbreak of *S.* Typhi infecting 5,936 people in Bharatpur (150km west of Kathmandu) was traced to the municipal water supply (Lewis et al., 2005). Additionally, a study from the Terai region of southern Nepal demonstrated that coliforms were present in 61% (61/ 100) of the provided water samples (Atreya et al., 2006). In an urban setting, Bhatta *et.al.,* isolated and identified multiple drug resistant *S.* Typhi and *S.* Paratyphi A in the drinking water supply of

14% (42/300) of their samples (Bhatta et al., 2007). These data support the hypothesis of this study that most of the transmission of enteric fever within LSMC occurs through contaminated water. Transmission through water supply is also evidenced by the seasonal variation in disease incidence observed in the previous analysis (Chapter 3), where an association with the average monthly rainfall and a peak in the seasonal temperatures saw an obvious increase in cases between June and August with a maximum in July **(Figure 3.2)**. Seasonal variation in enteric fever has been highlighted before and tends to peak with highest temperatures as in Karachi, Pakistan (Siddiqui et al., 2006) and with rainfall as in the Mekong Delta area of southern Viet Nam (Kelly-Hope et al., 2007). The theory for an increased incidence following seasonal change is that during the monsoon the ground water becomes saturated and the intermingling of faecal matter and the water supply occurs more frequently. To investigate this theory, water was collected for a year from different sources in 10 endemic locations and their quality was assessed.

In addition to water, food has also been implicated as a potential route of transmission for enteric fever in several epidemiologic studies (Luby et al., 1998, Bhan et al., 2002, Black et al., 1985, Velema et al., 1997). Urban population growth has stimulated a rise in the number of street food vendors worldwide, and the migration from rural to urban centres has created a daily need among many working people to eat outside the home (Winarno and Allain, 1991). In Kathmandu, a large number of vendors, approximately 30,000, are earning their livelihoods on the street with a quarter of this population vending food items. Internal conflict and socio-economic hardships have pushed families into the Valley for employment and safety. These

people, in the absence of formal education and skills for well-paid jobs within the private and public sector, have started working in the informal sector as street vendors. Though the income from this livelihood is not much, the investment required is low so people find this a feasible method of earning a livelihood (Timalsina, 2007). Additionally, as the urban population in Kathmandu Valley increases, the size of living spaces has dramatically reduced, with people living in cramped rooms without a place to cook. Food in eateries and restaurants is expensive and unaffordable on a daily basis as in comparison to street food, which is cheaper and more readily available. Due to these circumstantial conditions the demand for and supply of street food has increased rapidly over the past few years. Unfortunately with the increase of supply, the quality of the food has been compromised, as it is an informal industry with no food inspection or surveillance rules or regulations in place. Thus, there is an increased risk of exposing the population to contaminated food due to these poor hygiene standards. In regard to enteric fever, this problem could be exacerbated with the potential role of food handlers who could be carriers of the pathogen.

Here I present the microbiological quality of water and street food from various sources available within our study catchment area in LSMC and discuss its probable relation to disease incidence.

5.3 Results

5.3.1 Levels of water contamination

A total of 431 water samples from various sources were analysed using the MPN method, over the study period, consistent faecal contamination ranging from 3.3 to $1.4x10^6$cfu $ml^{-1}$was observed. Among the 3 different water sources tested, faecal contamination was found to be highest in the water from stone spouts while the municipal supplied water was found to be the least contaminated (**Figure 5.1**) (one-way ANOVA, $p<0.001$).

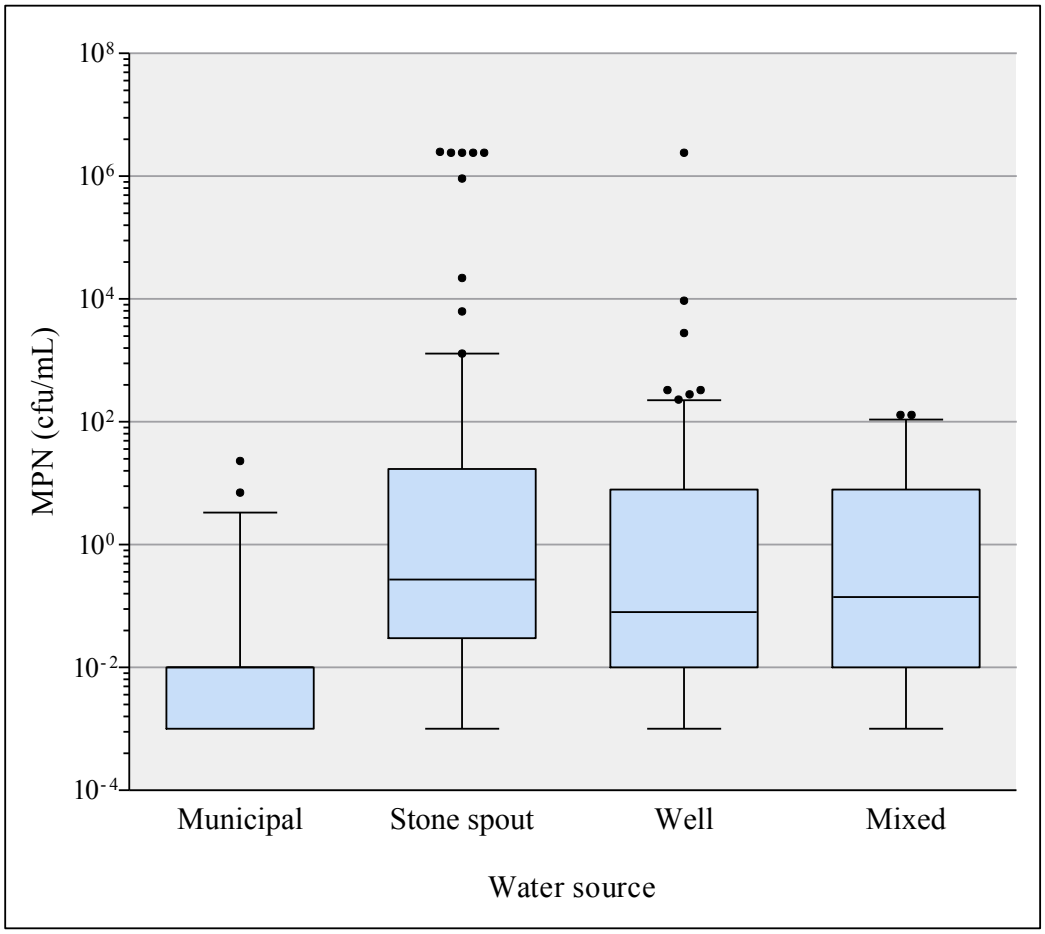


Figure 5.1: A box plot showing varying levels of faecal contamination among the sources that were tested. The most probable number test was performed on a total of 431 samples from 5 water stone spouts, 3 wells and 1 municipal supply. One of the sources was a mixed sample of well and municipal supplied water. Each of the boxes in the figure represent a source with the lower limit of the box representing the sample minimum value and the upper limit of the box representing the sample maximum value. The black lines within the boxes indicate the median values while the whiskers protruding from the box indicate the lower quartile (lower whisker) and the upper quartile (upper whisker). The dots indicate values that are outliers.

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

Faecal contamination of water sources was observed to have a significant association with the seasons, increasing particularly during the monsoons (Spearman's $\rho = 0.236$, $p<0.001$). The maximum water contamination was observed in the monsoon months of June, July and August, with a peak in average contamination level in July (**Figure 5.2**).

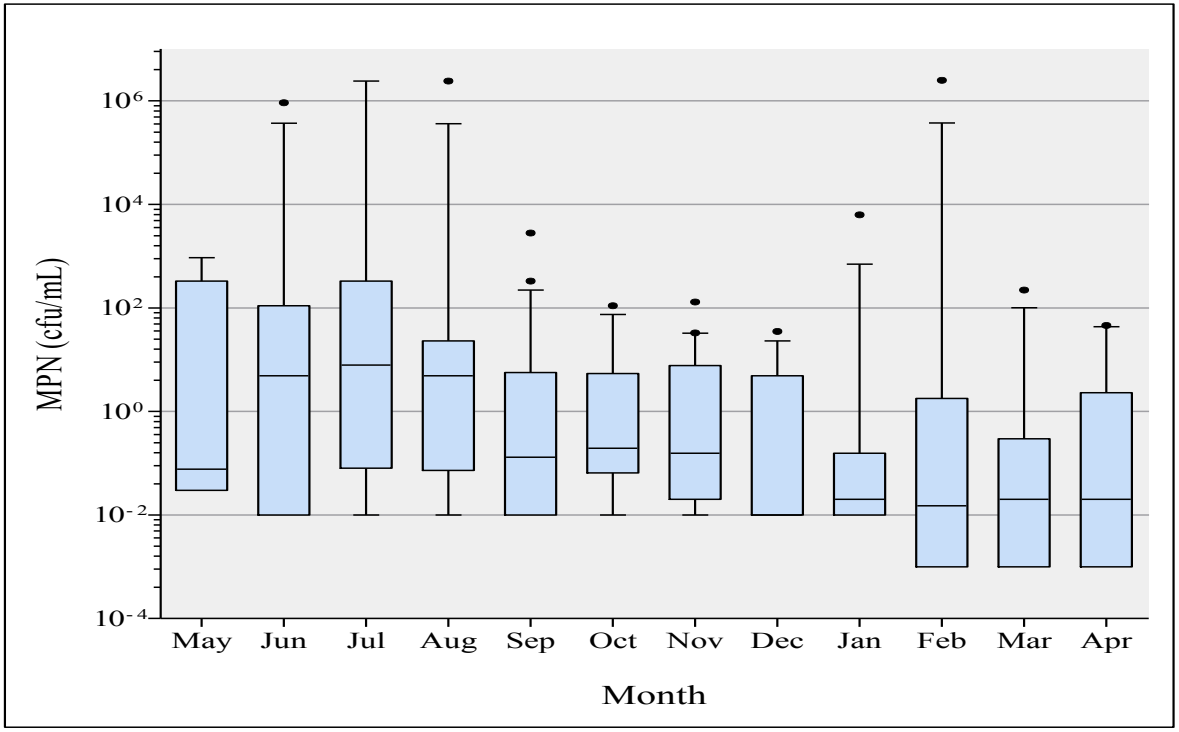


Figure 5.2: A box plot showing fluctuations of faecal contamination over 12 months beginning in May 2009.

Peak contamination is observed in July and August. Each of the boxes in the figure represent a month of the year with the lower limit of the box representing the minimum amount of contamination noted in that month and the upper limit of the box representing the maximum contamination. The black lines within the boxes indicate the median values while the whiskers protruding from the box indicate the lower quartile (lower whisker) and the upper quartile (upper whisker). The dots indicate values that are outliers.

At the same time, the maximum volume of rainfall was observed in the month of July (353.44mm) and August (522.025 mm) **(Figure 5.3)**. An increase in faecal contamination was also observed with significant increase in the levels of nitrates (Spearman's ρ 0.367, $p<0.001$), nitrites (Spearman's ρ 0.276, $p< 0.001$) and chloride (Spearman's ρ 0.444, $p< 0.001$) in the water. However, the level of pH, and levels of the chemical elements such as iron, ammonia, and arsenic remained constant for all water sources though out the period of analysis. Levels of iron and arsenic were found to be within acceptable limits according to the recommended WHO guidelines for potable water.

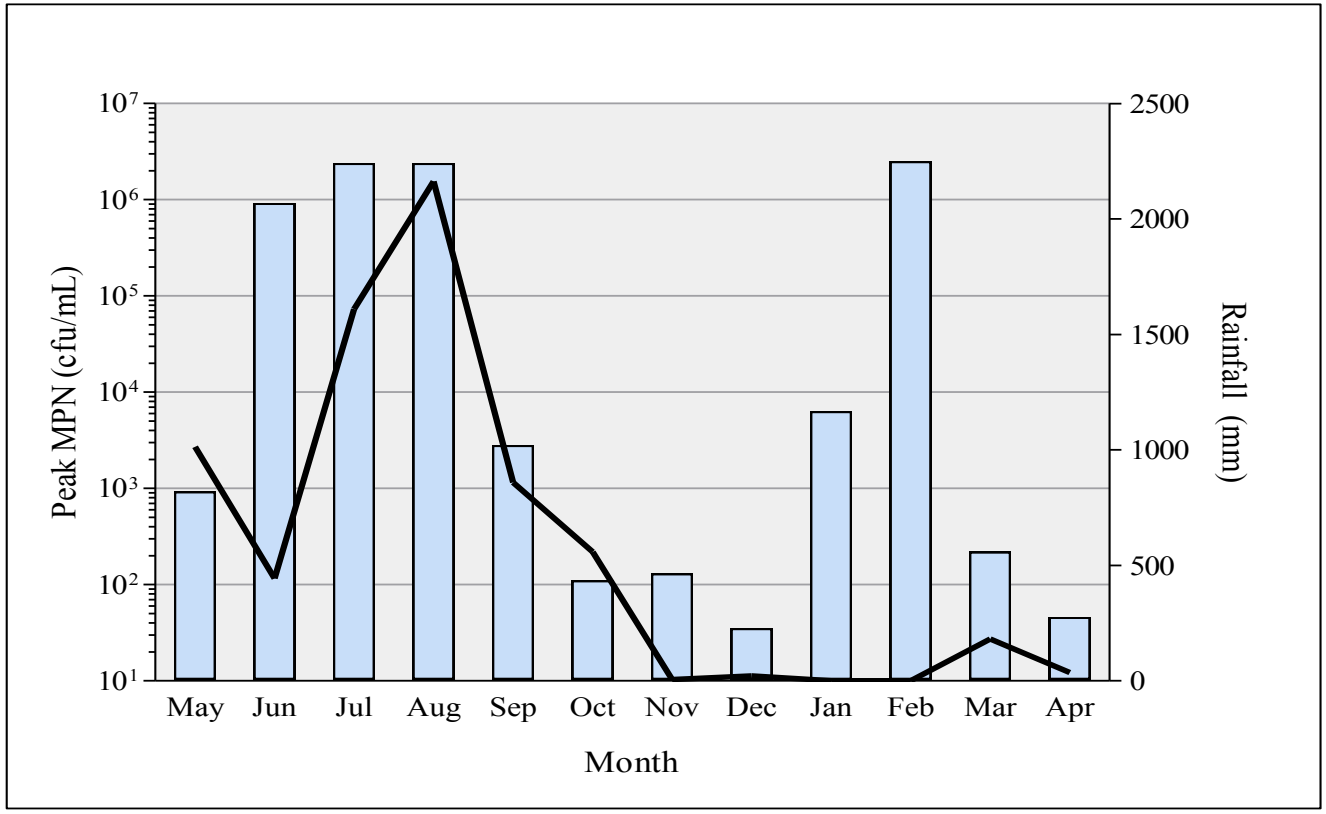


Figure 5.3: Graph showing peak water contamination coinciding with peak rainfall.

Each bar represents the peak log10 MPN values for the various sources over a year, beginning in May 2009. The line plots the average rainfall of each month. The months of June, July and August had the maximum water contamination and also the maximum rainfall.

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

A wide spectrum of microorganisms was isolated from the water samples over the period of a year. A complete list of the isolated microorganisms is shown in **Table 5.1**.

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

Table 5.1: List of *Enterobacteriaceae* isolated from water sources over the period of a year(May 2009 to April 2010)

BACTERIAL SPECIES	BACTERIAL GENUS
Achromobacter spp	
[b,c]*Acinetobacter* spp	
[b,c]*Aeromonas* spp	*caviae, hydrophila, salmonicida, sobria*
Alcaligenes spp	
Bordetella spp	
Chromomonas spp	*violaceum*
Chryseomonas spp	*luteola*
Erwinia spp	
Escherichia spp	[a]*coli,* [a]*coli 1,* [a]*coli 2, fergusonii, hermanii, vulneris*
[b]*Flavobacterium* spp	*indologenes, oryzihabitans, meningosepticum*
Hafnia spp	*alvei 1, alvei 2*
[b]*Klebsiella* spp	*oxytoca, planticola, pneumoniae ozaenae, pneumoniae pneumoniae, terrigena, ascorbata, cryocrescens*
Leclercia spp	*adecarboxylata*
[c]*Moraxella* spp	
Morganella spp	*morganii*
Pasteurella spp	
Plesiomonas spp	*shigelloides*
Proteus spp	*mirabilis, vulgaris*
Providencia spp	
[a,b,c]*Pseudomonas* spp	*alcalifaciens, stuartii, rettgeri, fluorescens,* [b]*aeruginosa, cepacia, paucimobilis,* [a]*pseudomallei, putida*

BACTERIAL SPECIES	BACTERIAL GENUS
Rahnella spp	*aquatilis*
[a]*Salmonella* spp	
[b]*Serratia* spp	*ficaria, fonticola, marcescens, plymuthica*
Shewanella spp	*putrefaciens*
[a]*Shigella* spp	*boydii C2, dysenteriae 01, flexneri, sonnei D*
Sphingobacterium spp	*paucimobilis*
Tatumella spp	*ptyseos*
[a]*Vibrio* spp	*alginolyticus,* [a]*cholerae,* [a]*cholerae 01, fluvialis, metschnikovii, mimicus*
Weeksella spp	*virosa*
[c]*Xanthomonas* spp	*maltophila*
Yersinia spp	*intermedia, rucker*

[a] Pathogens classified as having high health risks; [b] Pathogens classified as opportunistic;

[c] Pathogens that have little evidence of being transmitted through water (WHO, 2008c)

Of specific significance was the isolation of *Vibrio cholera*e in week 19 (September 20 to 26th), week 21 (October 4th to 10th), week 41 (February 21 to 27th) and week 42 (February 28th to March 6th); 6 isolates of various serotypes of *Shigella* species such as *boydii C2* (Week 10: July 19th to 25th), *flexneri* (Week 13: August 9th to 15th), *sonnei D* (Weeks 13 and 16: August 9th to 15th and August 30th to September 5th) and *dysenteriae 01* (Week 20: September 27th to October 3rd); and 3 isolates of *Salmonella* species, none of which were either Typhi or Paratyphi A, in week 14 (August 16th to 22nd), week 29 (November 29th to December 5th) and week 50 (April 25th to May 1st). All of these pathogens were isolated from stone spout and well waters. Despite the considerable amount of faecal contamination indicated by the most probable number, I was unable to isolate any *S.* Typhi or *S.* Paratyphi A through selective culture techniques. To further investigate possible reasons behind the failure to isolate any *S.* Typhi or *S.* Paratyphi A, bottled sterile water was spiked with varying loads of *S.* Typhi and cultured as described in **Section 2.9.4**. Though *S.* Typhi was subsequently isolated from these spiked samples, the recovery of these pathogens was not proportional to the initial bacterial load. From the inoculum with cell densities of $1x10^8$, $1x10^7$, $1x10^6$cfu/ml, only 8, 5, and 6 colony-forming units respectively were recovered. In samples with lower cell densities of $1x10^5$, $1x10^4$ and $1x10^3$cfu/ml, the pathogen was not recovered.

However, using a real time PCR assay developed for identification of *S.* Typhi and *S.* Paratyphi A in biological specimens, the presence of DNA sequences specific for *S.* Typhi and *S.* Paratyphi A were detected in all the water sources from all locations. Out of the 413 metagenomic DNA samples from filter surfaces, 333 (80.6%) were positive for *S.* Typhi and

301 (72.8%) were positive for *S.* Paratyphi A and 266 (64.4%) were positive for both *S.* Typhi and *S.* Paratyphi A. To further ensure that the PCR amplicons were *S.* Typhi and *S.* Paratyphi A, the PCR amplicons were further cloned, sequenced and compared to identified unique sequences of *S.* Typhi or *S.* Paratyphi A that had been previously identified as discussed in **Section 2.9.7.7** through**2.9.7.10**. Our sequence comparison confirmed the presence of DNA sequences specific for the aetiologic agents of enteric fever **(Figure 5.4** and **5.5)**.

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

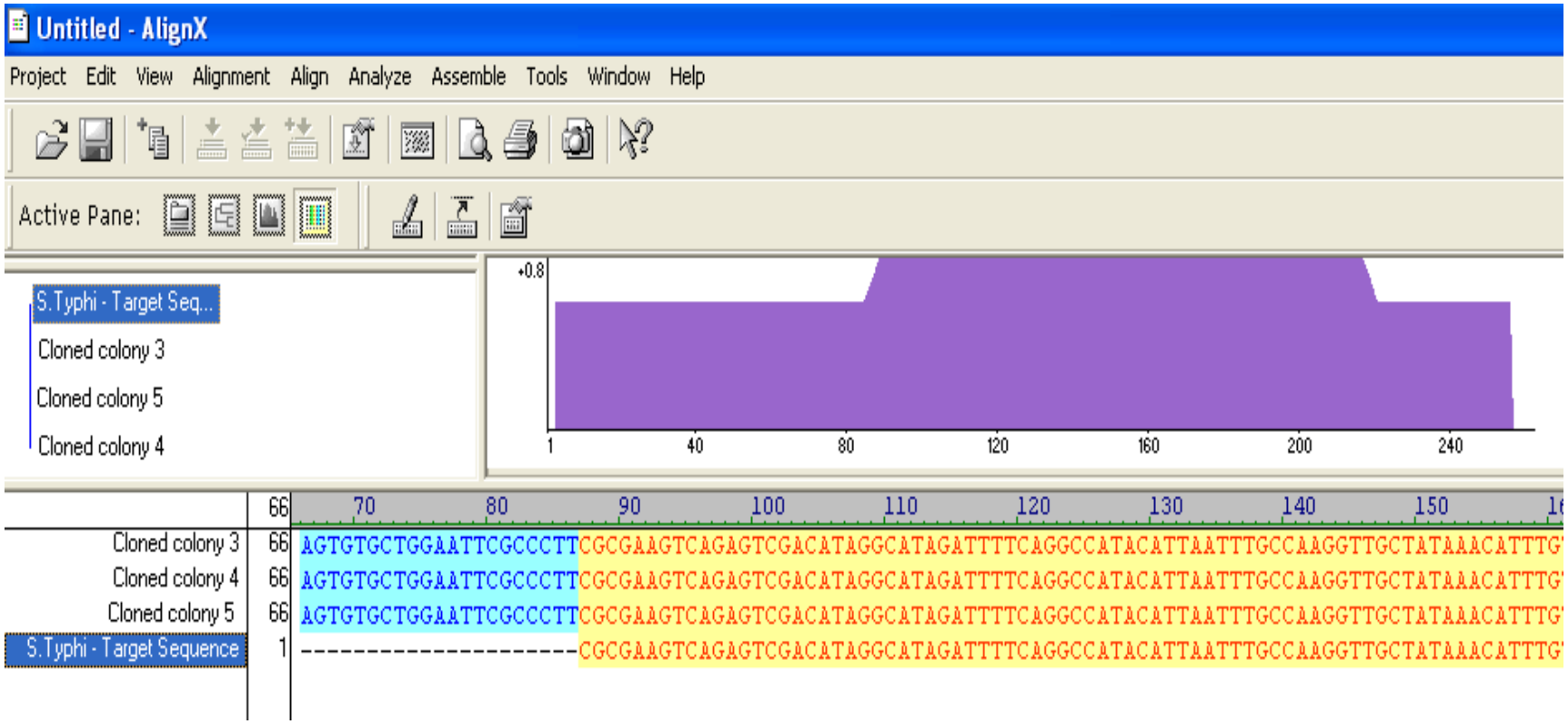


Figure 5.4: DNA sequence alignment of PCR amplicons against known sequence of *S.* Typhi using the Align X programme of the Vector NTI. In the bottom window, the first three rows show DNA sequences of cloned colonies believed to be those of *S.* Typhi. The bottom most sequence is the target sequence that is known to be unique to *S.* Typhi. The yellow highlights show that the sequences are exact matches. Thus the PCR amplicons detected were DNA sequences of *S.* Typhi.

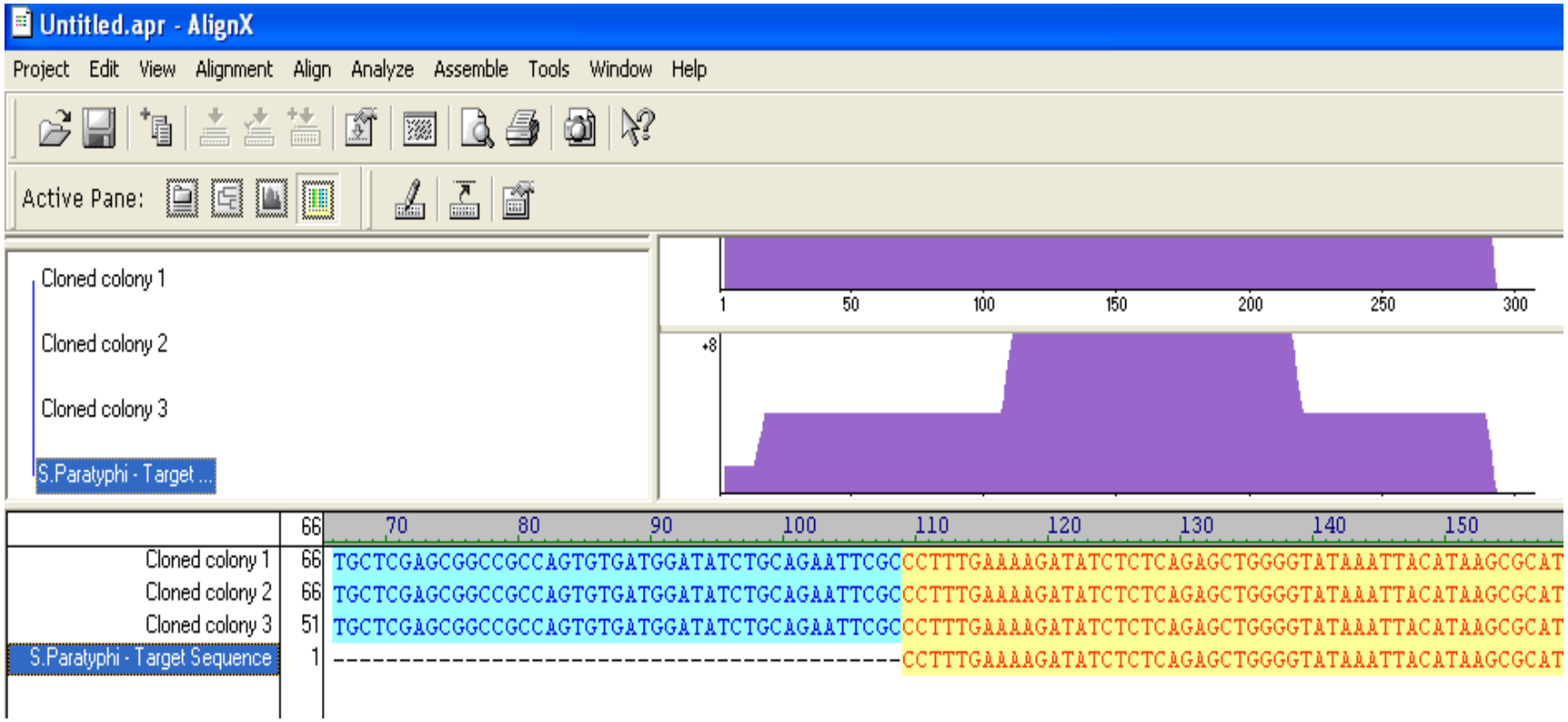


Figure 5.5: DNA sequence alignment of PCR amplicons against known sequence of *S.* Paratyphi A using the Align X program of the Vector NTI.In the bottom window, the first three rows show DNA sequences of cloned colonies believed to be those of *S.* Paratyphi. The bottom most sequence is the target sequence that is known to be unique to *S.* Paratyphi. The yellow highlights show that the sequences are exact matches. Thus the PCR amplicons detected were DNA sequences of *S.* Paratyphi.

The PCR amplicons were quantified by running a standard curve with each batch of PCR and when quantified, the DNA copies per reaction were higher for *S.* Typhi than for *S.* Paratyphi A. However, the number of copies per reaction fluctuated, ranging between $1.4x10^{1} – 2.6x10^{7}$ copies for *S.* Typhi and $1.6x10^{-1} – 8.4x10^{3}$ copies for *S.* Paratyphi A. There was evidence of a positive relationship between rainfall and presence of *S.* Typhi ($p<0.001$, Spearman's $\rho=0.345$). This association was also observed for *S.* Paratyphi A ($p=0.0136$, Spearman's $\rho=0.148$) **(Figure 5.6)**.

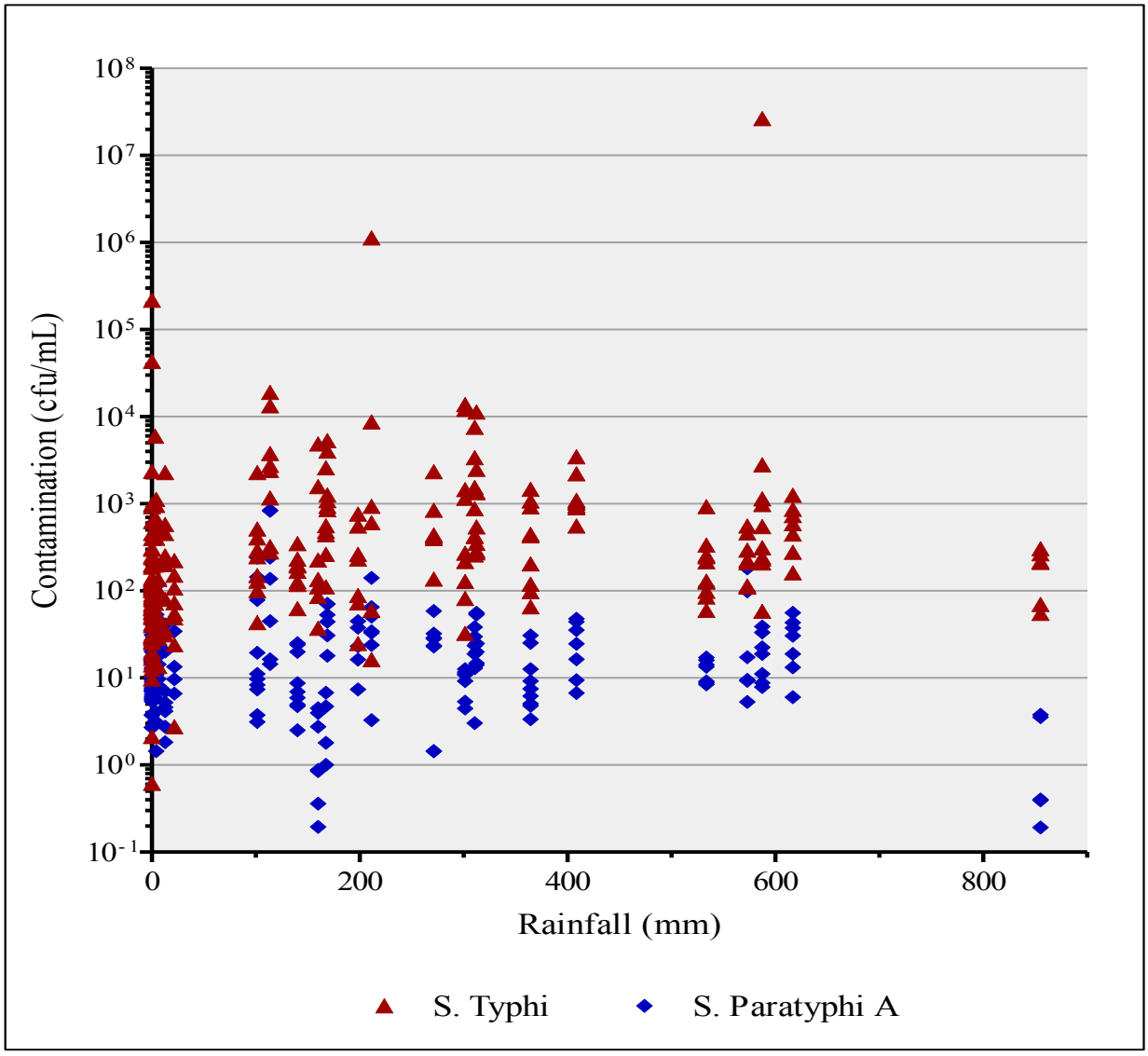


Figure 5.6: Relationship between *S.* Typhi and *S.* Paratyphi A contamination as a function of rainfall

5.3.2 Levels of food contamination

A total of 48 food samples from various food vendors were analysed over a period of 12 weeks (February to May 2010). MPN analysis exhibited consistent faecal contamination ranging from $<1x10^{-2}$- $2.4x10^{7}$cfu/ml. A local dish consisting of shredded raw buffalo meat (Kachila) was found to have the highest faecal contamination with a bacterial load of $2.4x10^{7}$cfu/ml. This was followed by a sweet confection high in milk content (Peda), which had an average bacterial load of $1.6x10^{7}$ cfu/ml. Rice flour patties with various kinds of stuffing (Wa) were also found to have high levels of faecal contamination among all food items analysed. Rice patties with meat stuffing were found to constitute a higher level of faecal contamination ($7.5x10^{3}$ cfu/ml) as in comparison to those without stuffing ($1.9x10^{3}$cfu/ml). Dumplings (momo), chatpat, samosa and mango drinks were found to be free of faecal contamination (MPN less than1 cfu/ml) (**Figure 5.7**).

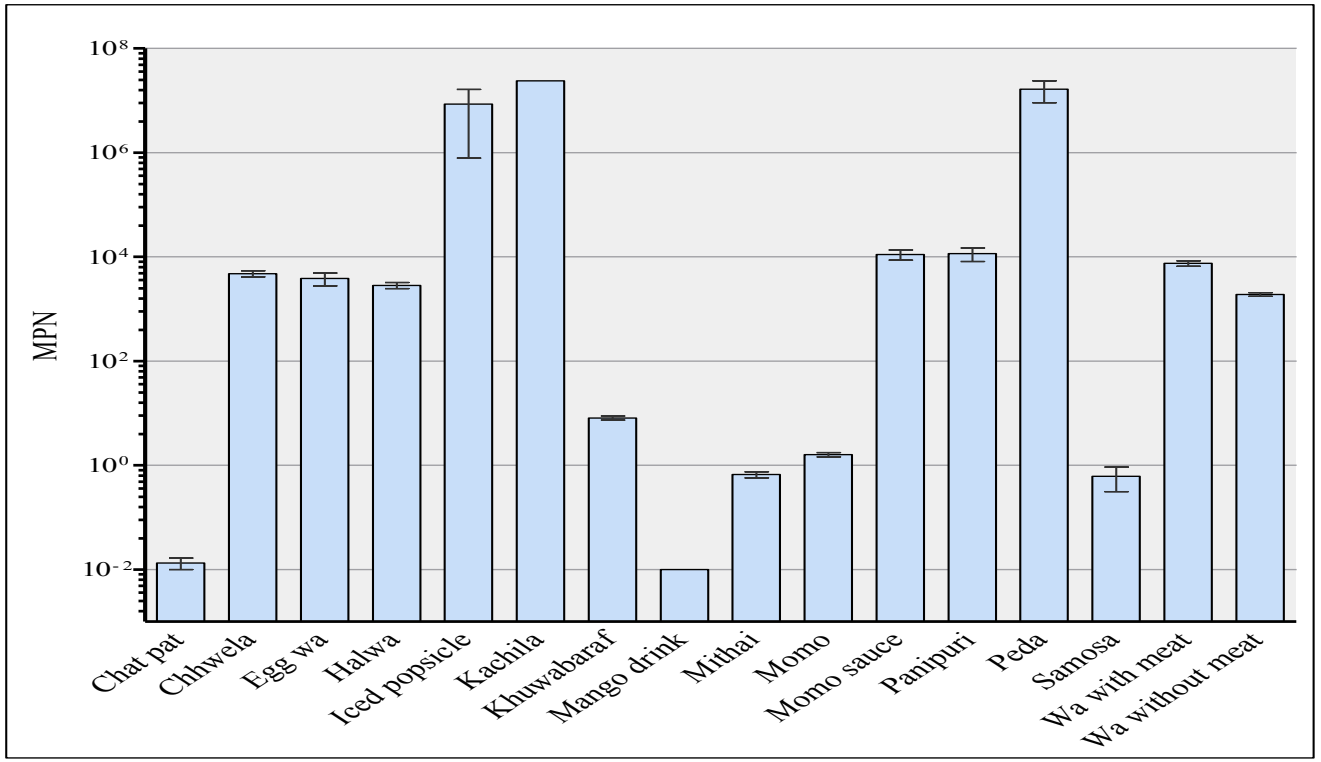


Figure 5.7: Bar graph showing levels of faecal contamination in various street foods that were analysed

Kachila had the highest mean level of contamination ($2.7x10^7$cfu/ml) followed by Peda ($1.6x10^7$cfu/ml). Varieties of Wa were also contaminated with meat stuffed ones beingthe most contaminated ($7.5x10^7$cfu/ml) and those without stuffing the least contaminated ($1.9x10^3$cfu/ml). Momo, chatpat, mango drink, samosa and mithai were found to be the least contaminated ($<1x10^{-2}$cfu/ml).

While species of *Enterobacter* were isolated from a majority of the food items, alarmingly, *Shigella*spp, was isolated from the meat stuffing of rice flour patties. From the dish consisting of raw meat, a host of pathogenic bacteria were isolated including *Proteus vulgaris, Eshcerichia coli,* and *Plesiomonas shigelloides.* Even though the MPN for samosa was less than $1x10^{-2}$ cfu/ml, *Enterobacter* spp. and *Leclercia adecarboxylata* were isolated. A wide variety of pathogens were isolated and identified from the food items and a complete list of *Enterobacteriaceae* isolated from various food samples is listed in **Table 5.2**.

Table 5.2: List of *Enterobacteriaceae* isolated from the various street foods analysed between February and May 2010

ANALYSED FOOD	ORGANISMS ISOLATED
Barfi	*Klebsiella oxytoca, Klebsiella ornitholytica, Serratia odorifera*
Chatpat	NO GROWTH
Chhwella	*Kluyvera spp, Enterobacter sakazakii, Enterobacter aerogenes, Enterobacter agglomerans, Enterobacter cloacae, Leclercia adecarboxylata*
Halwa	*Enterobacter sakazakii, Enterobacter cloacae*
Iced popsicle	*Enterobacter sakazakii, Enterobacter cloacae, Klebsiella oxytoca, Klebsiella ornitholytica, Serratia odorifera*
Kachila	*Citrobacter freundii, Proteus vulgaris, Kluyvera spp., Plesiomonas shigelloides, Moellerella wisconsensis, Escherichia coli 1 and 2, Klebsiella pneumoniae*
Khuwabaraf	*Escherichia coli 1 and 2, Citrobacter freundii*
Mango drink	NO GROWTH
Momo	NO GROWTH
Momo sauce	*Enterobacter cloacae, Enterobacter intermedius, Escherichia coli*
Pani puri	*Enterobacter sakazakii, cloacae, Escherichia coli*

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

ANALYSED FOOD	ORGANISMS ISOLATED
Peda	*Klebsiella oxytoca, Klebsiella ornitholytica, Serratia odorifera, Enterobacter sakazakii*
Samosa	*Leclercia adecarboxylata, Enterobacter agglomerans, Enterobacter cloacae*
Wa with egg	*Chromobacterium violaceum, Pseudomonas aeruginosa, Pseudomonas fluorescens, Pseudomonas putida, Enterobacter aerogenes, Enterobacter gregoirae*
Wa with meat	*Shigella* spp, *Acinetobacter* spp, *Tatumella ptyseos*, *Enterobacter sakazakii, Enterobacter cloacae, Enterobacter amnigenus*
Wa without meat	*Enterobacter sakazakii, Enterobacter cloacae*

5. Water quality among various water sources and food quality among various street vendors within Lalitpur Sub Metropolitan City (LSMC)

From the 48 samples, the most frequently identified species were *Enterobacter* spp (44%), *Klebsiella* spp, *Escherichia coli* (7%), *Pseudomonas* spp (6%) and *Serratia odorifera* (6%) **(Figure 5.8)**. Among the *Enterobacter* species the most commonly isolated were *Enterobacter cloacae* and *Enterobacter sakazakii*; for the *Klebsiella* species the most commonly identified were *Klebsiella oxytoca* and *Klebsiella ornitholytica*. Of all the samples 9 samples that included dumpligs, chatpat and the mango drink had no bacterial growth.

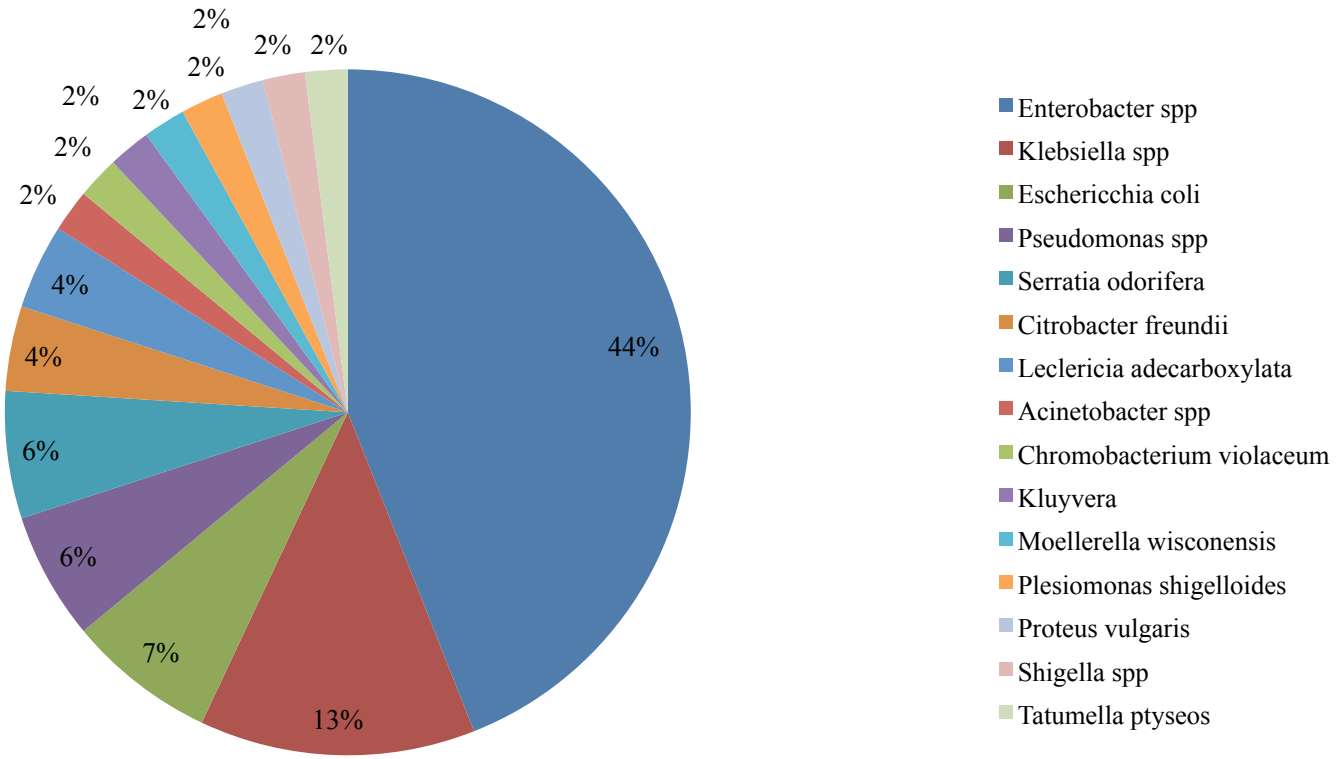


Figure 5.8: Pie chart showing the percent of each bacterial species identified from the various food samples that was isolated. *Enterobacter* species was the most commonly identified pathogen from food (21/48) followed by *Klebsiella* species (7/48 samples) and *Eshcerichia coli* (4/48 samples).

5.4 Discussion

In this chapter, the microbiological quality of water from various sources and food sold by street vendors from within LSMC has been described.

Considerable faecal contamination was observed among the various analysed water sources. Enteric pathogens of significant clinical relevance such as *Salmonella* spp, *Shigella* spp, and *Vibrio* spp were isolated through conventional microbiological methods, and DNA amplifications specific for *S.* Typhi and *S.* Paratyphi A were also detected throughout the year. Faecal contamination was seen to fluctuate with the seasonal variation in rainfall although *S.* Typhi and *S.* Paratyphi A DNA was detected in all the water sources at all times of the year, with varying bacterial loads. The burden was greatest in the wetter monsoon months due presumably to increased mixing of water sources and sewage. Though there was evidence of an association between seasonality and water samples testing positive for enteric fever pathogens during the monsoon season, it is difficult to draw conclusions regarding seasonal contamination as the water stone spouts generally produce flowing water when the aquifers are filled as a result of rainwater replenishment. It is clear from these data that the population within LSMC would greatly benefit from access to clean water and adequate sanitation to control not only enteric but also diseases caused by other waterborne pathogens as mandated by the WHO (WHO, 2008c), and in observational studies where decline in disease incidence was noted following improvements in sanitation (Tulchinsky et al., 2000, Wolleswinkel-van den Bosch JH, 1997).

Increasing demand by a growing population has placed pressure on the water quality within Kathmandu Valley. Infectious diseases caused by pathogenic bacteria, viruses and parasites are the most common and widespread health risk associated with drinking water and the array of pathogens that can be transmitted through contaminated water is diverse. Opportunistic pathogens such as *Flavobacterium, Acinetobater, Klebsiella, Serratia and Aeromonas,* which are capable of causing diseases of the skin and mucous membrane of the eye, ear, nose and throat, are naturally present in the environment but cause disease in the immunologically challenged individuals and are of concern when repeatedly isolated from the same water sources. However, the perpetual presence of high health risk pathogens such as *Shigella* spp, *V. cholerae, E. coli* and *Salmonella* spp, (WHO, 2008c) in the water samples is of serious public health concern. Alarmingly, I was able to isolate a number of the pathogens classified as medium and high health risk pathogens by the WHO **(Table 5.1)** from all the water sources analysed. These results indicate water pollution at levels that pose a serious threat to human health. Unplanned urbanisation and inadequate sewage facilities have accelerated the discharge of untreated domestic liquid waste into the river and as a result, degradation of both surface and groundwater levels have been reported (MOPE, 2000). Due to the massive increase of the urban population within the Valley over the last decade resulting from civil unrest, groundwater has been exploited for many purposes and has led to a decline in the water level, aggravating the fact that the recharge area for groundwater is shrinking at an alarming rate (Kannel et al., 2008, MOPE, 2001). The total sustainable level of groundwater withdrawal per day is 26.3 million litres (Stanley et al., 1994) but currently 800 million litres of groundwater are being extracted daily according to the Kathmandu Valley Water Supply Management Board (KVWSMB). The impact of

such water withdrawal from the groundwater reservoir is the infiltration of polluted stream and river water(Kannel et al., 2008, SMEC, 1992). The Bagmati River, which is the main river of the Valley is a repository for the capital's untreated sewage, solid waste and industrial effluent, and has been shown to be highly polluted (Kannel et al., 2008). The wells and the stone spouts all draw on groundwater, and thus the seepage of polluted river water continuously exposes the population to contaminated water. The presence of pathogenic bacteria in the municipal supply could be due to a variety of reasons such as inadequate chlorination and infiltration of sewage due to faulty connections or leakage points.

My inability to directly culture *S.* Typhi and *S.* Paratyphi A, despite molecular evidence of their presence, requires further investigations. Recovery of only a few pathogens by comparison to the huge inocula spiked into sterile conditions, an environment where they did not have to fight for micronutrients with other micrflora, suggests that for some reason these microorganisms are highly elusive in water. These pathogens are known to be notoriously furtive in the environment, and culturing salmonellae from water sources have been reported rarely and only in the context of single source outbreaks (Lewis et al., 2005 , Mermin et al., 1999). As previously proposed, the reason for their elusiveness could be either due to the organism residing in a quiescent form and not responding after exposure to rich microbiological media (Roszak and Colwell, 1987), or, could be the result of extensive faecal contamination and they were simply overwhelmed by other less fastidious organisms.

As *S.* Typhi and *S* Paratyphi A were not cultured from water, and various studies have

implicated food as a risk factor for enteric fever (Bhan et al., 2002, Black et al., 1985, Luby et al., 1998, Velema et al., 1997), popular street food available from ambulatory street food vendors within LSMC were analysed. Consistent faecal contamination was observed in the majority of food products, particularly spiced raw meat and the meat stuffing of rice flour patties. This was consistent with studies where contamination of meat products within Kathmandu has been reported (Joshi et al., 2003, Maharjan et al., 2006) with *S.* Typhi and *S.* Paratyphi A. However, dumplings with meat stuffing were free of contamination, which could be due to the fact that dumplings are steamed prior to serving. However, eating dumplings still poses a health hazard as the sauce served with the dumplings was found to have considerable contamination. The mango drink and the chatpat were also found to be free of contamination. While the mango drink was in a tetra pack and hence the results not surprising, the chatpat being free of contamination was unexpected as they are generally made unhygienically and kept uncovered in street stalls.

However, no definite conclusion regarding the safety of the street foods can be drawn from this study, as the number of samples and period of analysis needs to be larger. As the safety of food is affected by several factors, from the quality of the raw materials used to the food handling and storage practices, appropriate measures need to be taken to reduce the risk of contamination. Further analysis should be performed at every point in the production process to identify the contamination points and to reduce health hazards.

5.5 Conclusion

This study supports previous findings regarding the poor water and food quality within the urban areas of Kathmandu Valley. However, further investigation is also required to look at other possible routes of transmission. For a disease like enteric fever, which has a faecal-oral mode of transmission, water is just one vehicle of transmission in addition to contaminated food and asymptomatic carriers.

Identification of possible routes of transmission for enteric fever within Kathmandu Valley will aid in the development of a rational control strategy. With accurate identification, resources should be allocated toward interventions known to be effective such as chlorination of drinking water, construction of proper water distribution and sewage networks, implementing regulations on extraction of groundwater, regulating food sold by vendors, identification of carriers within or outside affected households, vaccination campaigns and hygiene education.

6 Matched case-control study evaluating typhoid and paratyphoid fever within Lalitpur Sub-Metropolitan City

6.1 Abstract

Enteric fever was a significant cause of morbidity and mortality in the overcrowded and unsanitary conditions of European and North American cities for centuries. With improvements in public health infrastructures, such as the provision of reliable clean water supplies and sewage systems, these places witnessed a dramatic decrease in the incidence of enteric fever over time. Today the vast majority of the disease burden is found in developing countries where poor sanitary conditions remain and facilitate transmission, particularly among the impoverished population in urban centres such as LSMC. The central study question for this epidemiological study was to examine why, in an endemic region when everyone is constantly exposed to various risk factors for enteric fever, do some people develop the disease while others do not. To evaluate risk factors within this region, a case control study matched on age, sex and area of residence was conducted for 6 months. A total of 103 cases of blood culture confirmed enteric fever and 294 matched neighborhood controls were enrolled within a two-week window of case diagnosis. The overall median age of enteric fever cases was 18 years (IQR 10-22.5 years) with more males being infected. Though major risk factors for either typhoid or paratyphoid fever were not observed, associations between water from stone spouts to typhoid fever and eating street food to paratyphoid fever were recorded. Additionally, several important behavioural characteristics were found to be protective in this population.

6.2 Introduction

With an annual estimated 22 million cases and 200,000 deaths from typhoid fever globally in addition to 5.5 million cases of paratyphoid fever, enteric fever is a major public health concern (Crump et al., 2004). In comparison to paratyphoid fever, typhoid fever has historically been thought to be more common, have a more severe clinical course and result in more frequent and severe sequelae (Sur et al., 2007). However, more recent studies have suggested that infections caused by *S.* Paratyphi A are now more frequent in endemic areas and that infections caused by *S.* Typhi and *S.* Paratyphi A present with indistinguishable clinical syndromes (Maskey et al., 2006 , Ochiai et al., 2005, Vollaard et al., 2005). Enteric fever is endemic in Nepal and remains the most common clinical and blood culture-confirmed diagnosis among patients with febrile illnesses (Maskey et al., 2008).

Several epidemiologic studies have identified the risk factors for enteric fever, implicating both water and food as important routes of transmission (Bhan et al., 2002, Black et al., 1985, Gasem et al., 2001, King et al., 1989, Luby et al., 1998, Mermin et al., 1999, Swaddiwudhipong and Kanlayanaphotporn, 2001, Velema et al., 1997, Vollaard, 2004, Bhunia et al., 2009). This could be due to the fact that salmonellae are capable of surviving in water for prolonged periods of time and can also multiply readily in food (Christie, 1988, Wait and Sobsey, 2001). To date no epidemiological investigations have been conducted to examine risk factors for enteric fever in Kathmandu. Accurate identification of risk factors and probable routes of transmission of enteric fever are necessary, particularly in developing countries, for the development of rational control strategies. Once risk factors are identified,

steps can be made to control those that contribute significantly to disease burden. It is thought that paratyphoid fever requires a higher infectious dose than typhoid for clinical disease, which suggests that these two serovars may have different main routes of infection (Vollaard, 2004). Therefore, investigating differences between the modes of transmission of both diseases has also become increasingly relevant.

There have been recent reports of a proportional increase in the incidence of paratyphoid fever in China and India, a phenomenon which has also been observed in Kathmandu **(Figure 3.1)** (Karkey et al., 2010, Maskey et al., 2006 , Ochiai et al., 2005, Padmapriya et al., 2003, Sood et al., 1999a, Tankhiwale et al., 2003). However, whether this is due to incomplete epidemiological data in endemic countries or is a consequence of the downward trend in the incidence of typhoid fever compared to an absolute increase in the incidence of paratyphoid fever is still not clear (Pang et al., 1998, Saha et al., 2002, Vollaard, 2004). Clarifications of the nature of this increase in paratyphoid cases could help to refocus public health measures such as implementation of the typhoid vaccines, parenteral Vi and oral Ty21a, which do not protect against paratyphoid fever (Arya and Sharma, 1995, Ochiai et al., 2008).

In this study a hospital based case control study was conducted within an endemic area of LSMC. Cases with enteric fever were identified prospectively through passive surveillance and were compared with community controls matched for age, sex and ward to help identify potential risks of enteric fever infections through examining differences in hygienic practices, eating habits and environmental and household

characteristics in addition to gathering information to potentially elucidate important transmission routes.

Cases and controls were enrolled in the study as described in **section 2.10.**

6.3 Results

6.3.1 Cases versus controls

6.3.1.1 Baseline characteristics of cases and controls

A total of 229 febrile patients were approached for enrollment into the study. Of those approached, 12 individuals were excluded as 8 individuals lived outside the study area and 4 denied consent. Consent from a total of 217 patients with fever, defined as being more than 37.5°C and lasting for 3 or more days were included. Almost half (48%, 103/217) of these febrile patients had a positive blood culture for either *S.* Typhi or *S.* Paratyphi A. Of the blood culture positive patients, 36% (37/103) were female and 64% (66/103) were male. Among the 103 blood culture positive cases, 47.5% (49/103) were identified as *S.* Typhi and 52.4% (54/103) were identified as *S.* Paratyphi A. The overall median age for blood culture confirmed enrollees was 18 years (IQR 10-22.5years) with females having a median age of 22 years (IQR 13-24 years) and males a median age of 18 years (IQR 9-21 years). A majority of the cases and controls were students who had come from rural areas into the Valley. While the number of illiterate cases and controls were few, the majority of cases had a pre-primary to primary level of education.

6.3.1.2 Clinical Presentation

Most of the clinical features between typhoid and paratyphoid were indistinguishable. However, anorexia, (p= 0.047,χ^2 test), abdominal pain (p=0.005,χ^2 test) and diarrhoea (p=0.038, χ^2 test) were more common among typhoid cases than paratyphoid cases.

6.3.1.3 Drug resistance

Antimicrobial sensitivity and MIC determination was conducted on all the isolated pathogens **(Appendix 9.7)**. Of all the isolates from the cases, 96% (97/101) were resistant to nalidixic acid. In addition, 92% (43/47) of the *S.* Typhi isolates and all of the *S.* Paratyphi A isolates were resistant to nalidixic acid. Antimicrobial profiling could not be performed on two isolates of *S.* Typhi as they were unable to be sub-cultured. A total of 62% (63/101) of the isolates were sensitive to ciprofloxacin and the remainder (38/101) displayed intermediate resistance. All isolates were sensitive to ofloxacin. Among the *S.* Paratyphi A isolates, 40% (21/53) were sensitive to ciprofloxacin while 60% (32/53) displayed intermediate resistance. None of the isolates from the cases were MDR (resistant to chloramphenicol, ampicillin, and trimethoprim).

6.3.1.4 Univariate analysis

A univariate analysis of potential risk factors for enteric fever cases compared to matched community controls exhibited several variables that were seen to be significantly associated with enteric fever infection **(Table 6.1)**. These included having had a typhoid contact in the previous eight weeks, using a metal cover for water

storage containers, being aware of enteric fever, using extra water from wells, wiping hands after washing, routinely eating pani puri, eating sliced fruit from street vendors, the number of people using a latrine, and having eaten street food in the two weeks preceding day of interview.

Table 6.1: Risk factors for enteric fever, n (%) or median (IQR), univariate and multivariate matched regression analyses on data from 100 case-control pairs, including a-priori risk factor of flooding

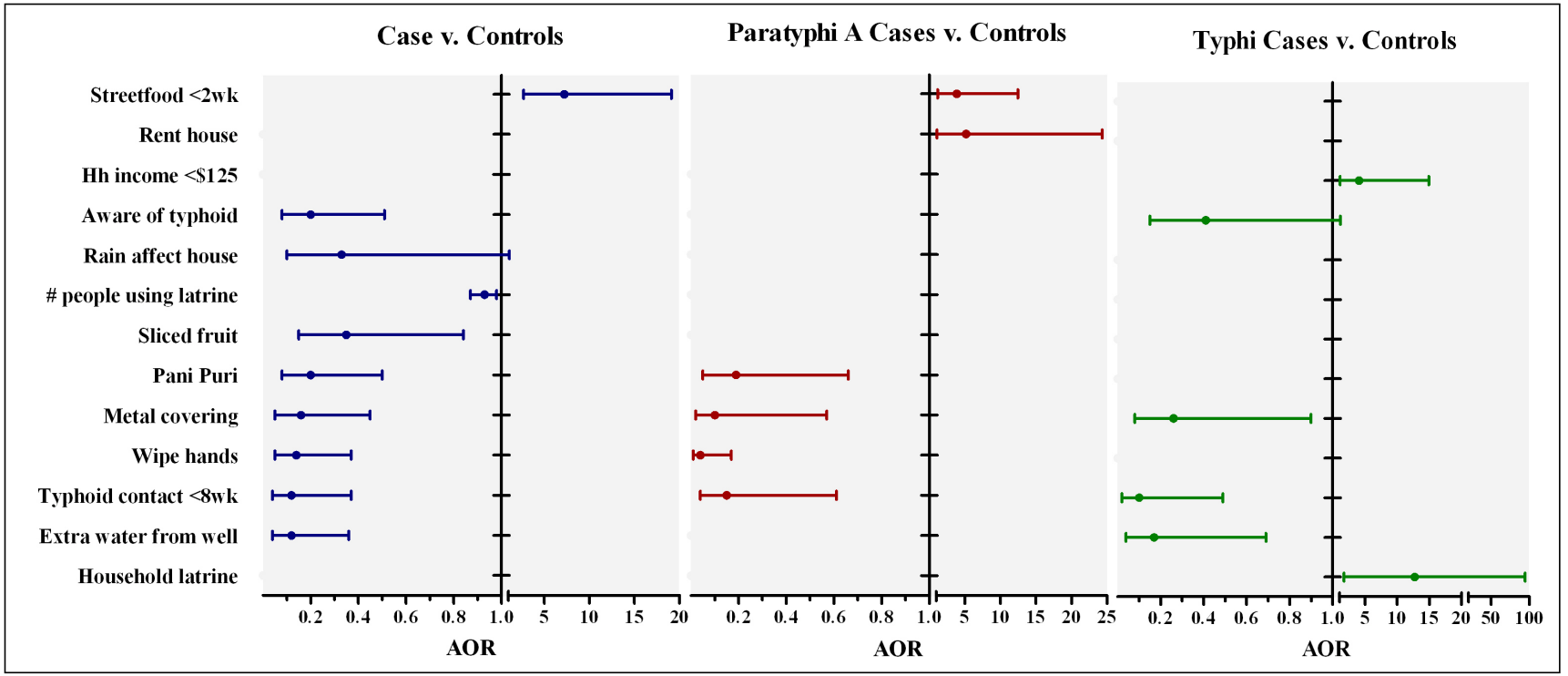
Variable	Cases n=103	Controls n=294	MOR	95%CI	p	AOR	95%CI	p
Aware of enteric fever	29 (28.2)	159 (54.1)	0.26	0.15-0.44	**<0.001**	0.20	0.08-0.51	**0.001**
Enteric fever contact in last 8 weeks	11 (10.7)	115 (39.1)	0.12	0.05-0.27	**<0.001**	0.12	0.04-0.37	**<0.001**
Extra water from well	45 (43.7)	190 (64.6)	0.27	0.14-0.50	**<0.001**	0.12	0.04-0.36	**<0.001**
Rain affect house during monsoon?	17 (16.5)	42 (14.3)	1.31	0.63-2.70	0.469	0.33	0.10-1.11	0.073
Metal covering of water storage	14 (13.6)	113 (38.4)	0.17	0.08-0.37	**<0.001**	0.16	0.05-0.45	**0.001**
Wipe hands after washing*	54 (52.4)	222 (76.6)	0.30	0.18-0.50	**<0.001**	0.14	0.05-0.37	**<0.001**
Number of people using latrine	8 (6-12)	10 (6-20)	0.95	0.92-0.98	**0.005**	0.93	0.87-0.98	**0.011**
Eaten pani puri	34 (33)	143 (48.6)	0.41	0.23-0.73	**0.003**	0.20	0.08-0.50	**0.001**
Eaten street-food in the last 2 weeks	46 (44.7)	102 (34.7)	1.76	1.04-2.97	**0.034**	7.20	2.71-19.14	**<0.001**
Eaten sliced street fruit	22 (21.4)	95 (32.3)	0.53	0.30-0.94	**0.030**	0.35	0.15-0.84	**0.019**

*data from 290 controls; **Boldface** indicates $p \leq 0.05$; MOR: Matched Odds Ratio; AOR: Adjusted Odds Ratio

6.3.1.5 Multivariate analysis:

After controlling for factors significant in the univariate analysis as well as *a priori* confounders **(Table 6.1)** several variables remained independently and significantly associated with typhoid fever infection **(Figure 6.1)**. All of the factors found to be significantly associated with infection in the univariate analysis were also found to be significant in the multivariate analysis. Again, these include having had a typhoid contact in the last eight weeks, using extra water from wells, wiping hands after washing, using a metal cover for water storage containers, being aware of enteric fever, eating pani puri regularly, living in a house affected by rain during the monsoon months, eating sliced fruit from street vendors on a normal basis, the number of people using a latrine, and having eaten street food in the last two weeks.

Figure 6.1: Adjusted Odds Ratios (AOR) among the three analysed groups. The variables on the Y-axis remained independently and significantly associated with enteric fever infection after controlling for factors significant in the univariate analysis as well as *a priori* confounders. The central black line marks the point where AOR=1. All variables with confidence limits less than one (left of the central bar) have protective effects while variables with confidence limits greater than one (right from the central bar) are considered as risk factors.



6.3.1.5.1 Awareness of enteric fever

Being aware of enteric fever, including knowledge of transmission, was seen to have a protective effect (Adjusted odds ratio [AOR] 0.20; 95% Confidence interval [CI] 0.08-0.51). Among the cases, 28% (29/103) were aware of enteric fever as in comparison to 54% (159/294) of the controls.

6.3.1.5.2 Exposure to enteric fever

Having had an exposure to an individual with enteric fever in the past eight weeks was strongly protective against the development of enteric fever (AOR 0.04; 95% CI 0.08-0.51). Among our cases, only 11% (11/103) had been exposed to an enteric fever patient in the past eight weeks compared to 39% (115/294) of the controls.

6.3.1.5.3 Water sources and storage

Although the effects of using water from various sources was not seen to have any effect on the risk of contracting enteric fever after controlling for confounding, using water from a well when regular water source was insufficient was seen to be significantly associated (AOR 0.12, 95%CI 0.04-0.36) with protection against enteric fever. A total of 44% (45/103) of the cases obtained extra water from a well compared to 64% (190/294) of the controls. Similarly, having a metal cover for water storage containers was found to have a significant protective effect against contracting enteric fever (AOR0.16, 95%CI 0.05-0.45). Among the cases, 14% (14/103) had metal coverings for water storage containers compared to 38% (113/294) of the controls.

6.3.1.5.4 Flooding

Living in a house that was affected by rain during the monsoon months was included in the regression model, as previous studies had found it to be an important risk factor for enteric fever (Gasem et al., 2001, Kumar and Harada, 2002). Although it was not seen to have a significant association (AOR 0.33; 95%CI 0.10-1.11) with contracting enteric fever, it remained in the backward stepwise logistic regression model due to a significant likelihood ratio test result ($p \leq 0.05$).

6.3.1.5.5 Sanitary Behaviour

Wiping hands with a towel after washing was seen to have a significant protective effect (AOR 0.14, 95% CI 0.05-0.37) against contracting enteric fever. This habit was reported among 52% (54/103) of the cases compared to 77% (222/290) of the controls who practiced the habit.

6.3.1.5.6 Latrine use

The number of people using a latrine was seen to have a slight effect (AOR 0.93, 95% CI 0.87-0.98) on the risk of infection with enteric fever. The median number of people using the same latrine among the cases was 8 (IQR 6-12) while among the controls it was slightly higher at10 (IQR 6-20).

6.3.1.5.7 Food

When comparing cases to controls, eating street food in the two weeks prior to

questioning was seen to be a significant risk factor (AOR 7.20; 95%CI 2.71-19.14) for infection with enteric fever. Almost half (46/103) of the cases had taken a meal from street vendors in the two weeks prior to becoming symptomatic with clinical features of enteric fever compared to only 35% (102/294) of the controls.

On the contrary, reporting eating pani puri (AOR 0.20, 95%CI 0.08-0.50) or sliced fruit (AOR 0.35, 95% CI 0.15-0.84) regularly (although not necessarily in the last two weeks) from street vendors were found to have significant protective effects. While 33% (34/103) of cases and 49% (143/294) of the controls said they often consumed pani puri from a street vendor, 21% (22/103) of the cases and 32% (95/294) of the controls reported routinely eating sliced fruit from a street vendor.

6.3.2 *S.* Typhi versus matched controls

Among individuals identified with *S.* Typhi septicemia, 40.8% (20/49) were female with a median age of 22 years (IQR 16-25years) and 59% (29/49) were male with a median age of 16 years (IQR 9-21years). There were 49 total *S.* Typhi patients and 136 controls matched for sex, age and ward.

6.3.2.1 Univariate analysis

Univariate analysis of potential risk factors comparing typhoid cases with community controls exhibited several variables that were seen to be significantly associated with enteric fever infection **(Table 6.2)** including using water from stone spouts and storing water after collection.

Table 6.2: Risk factors for *S.* Typhi, n (%) or median (IQR). Univariate and multivariate matched regression analyses on data from 49 case-control pairs showing all variables entered into a backward stepwise model (p(r)=0.10) and those selected by the likelihood ratio test to produce adjusted odds ratios

Variable	*S.* Typhi n=49	Controls n=136	MOR	95%CI	p	AOR	95%CI	p
Typhoid								
Aware of enteric fever	13 (26.5)	68 (50.0)	0.26	0.12-0.57	**0.001**	0.41	0.15-1.12	0.083
Previous episode	8 (16.3)	37 (27.2)	0.35	0.13-0.97	**0.043**			
Household enteric fever in last 8 weeks	3 (6.1)	41 (30.1)	0.14	0.04-0.49	**0.002**			
Enteric fever contact in last 8 weeks	4 (8.2)	57 (41.9)	0.10	0.03-0.33	**<0.001**	0.10	0.02-0.49	**0.004**
Socioeconomic factors								
Household income <10000 NPR	16 (32.7)	27 (19.9)	2.62	1.12-6.09	**0.026**	4.05	1.10-14.91	**0.035**
Own a motorbike	8 (16.3)	44 (32.4)	0.37	0.14-0.95	**0.039**			
Household size	13 (7-25)	12 (5-25)	1.00	0.98-1.04	0.674			
Water sources								
Any water from municipal supply	26 (53.1)	92 (67.6)	0.30	0.11-0.79	**0.015**			
Any water from stone spouts	27 (55.1)	56 (41.2)	3.70	1.23-11.15	**0.020**			
Any water from covered well	19 (38.8)	79 (58.1)	0.36	0.16-0.83	**0.017**			
Drinking water from municipal water	19 (38.8)	73 (53.7)	0.32	0.12-0.85	**0.022**			
Drinking water from stone spout	18 (36.7)	32 (23.5)	5.05	1.29-19.85	**0.020**			

Variable	*S.* Typhi n=49	Controls n=136	MOR	95%CI	p	AOR	95%CI	p
Extra water from stone spout	23 (46.9)	44 (32.4)	2.61	1.04-6.56	**0.041**			
Extra water from well	17 (34.7)	94 (69.1)	0.11	0.04-0.33	**<0.001**	0.17	0.04-0.69	**0.013**
General use water from stone spout	9 (18.4)	8 (5.9)	4.62	1.11-19.31	**0.036**			
Hand-washing water from stone spout	12 (24.5)	10 (7.4)	6.84	1.79-26.18	**0.005**			
Water for bath from stone spout	9 (18.4)	8 (5.9)	6.02	1.15-31.49	**0.033**			
Water shortage affect daily life?	30 (61.2)	106 (77.9)	0.42	0.19-0.94	**0.034**			
Do you use ice cubes?	4 (8.2)	10 (7.4)	0.86	0.22-3.32	0.823			
Water storage								
Water stored after collected*	42 (87.5)	94 (69.6)	3.09	1.18-8.12	**0.022**			
Wide mouth of storage container	6 (12.2)	36 (26.5)	0.31	0.11-0.85	**0.024**			
Metal covering of water storage	7 (14.3)	54 (39.7)	0.22	0.08-0.59	**0.003**	0.26	0.08-0.90	**0.033**
Sanitation behavior								
Wipe hands after washing	25 (51.0)	96 (70.6)	0.43	0.22-0.85	**0.016**			
Wash with soap after using toilet*	45 (93.8)	114 (84.4)	5.57	1.10-28.28	**0.038**			
Latrine								
Community	2 (4.1)	28 (20.6)	8.52	1.81-40.05	0.007	12.71	1.71-94.49	0.013
Household	46 (93.9)	108 (79.4)	1.00	-	-	1.00	-	-
Number of people using latrine	9 (5-12)	10 (6-17)	0.95	0.91-0.99	**0.049**			

*data on 48 cases and 135 controls for both water storage and wash with soap; **Boldface** indicates $p \leq 0.05$; Matched Odds Ratio (MOR), Adjusted Odds Ratio (AOR)

6.3.2.1.1 Water sources

The use of water for any purpose from stone spouts was seen as a significant risk factor (Matched odds ration [MOR] 3.70, 95% CI 1.23-11.15) for developing typhoid fever. Over half (27/49) of the cases used water from stone spouts for drinking and all relevant general use as compared to 41% (56/136) of controls.

Storing water after collection was also found to be a significant risk factor (MOR 3.09, 95% CI 1.18-8.12) for typhoid fever. A full 88% (42/48) of the cases stored water after collection compared to 70% (94/135) of the controls.

6.3.2.1.2 Hygiene

Although almost all cases and controls reported washing their hands after toilet use, washing hands with soap was observed to be a significant risk factor (MOR 5.57, 95% CI 1.10-28.28). Among the typhoid fever cases 94% (45/48) washed their hands with soap rather than just water compared to 84% (114/135) of the cases.

6.3.2.2 Multivariate analysis

After controlling for factors significant in the univariate analysis as well as *a priori* confounders **(Table 6.2)** several variables remained independently and significantly associated with typhoid fever infection **(Figure 6.2)**. These factors included using a community latrine, having had exposure to an individual with enteric fever in the past eight weeks, using extra water from a well, using a metal cover for water storage

containers, being aware of enteric fever, and having a monthly household income of less than US $125.

6.3.2.2.1 Exposure to enteric fever

Having had an exposure to an individual with enteric fever in the past eight weeks was seen to be strongly protective against infection with *S.* Typhi (AOR 0.10, 95% CI 0.02-0.49). Among our cases, only 8% (4/49) had been exposed to an enteric fever patient in the past eight weeks compared to 42% (57/136) of the controls.

6.3.2.2.2 Socioeconomic factors

Having a low household income (less than US $125) was found to be a significant and independently associated risk factor (AOR 4.05; 95% CI 1.10-14.91), reported in 33% (16/49) of the typhoid cases and 20% (27/136) of the controls.

6.3.2.2.3 Water sources

Although the effects of using water from various sources, particularly from stone spouts, were not seen to have any effect on the risk of contracting enteric fever after controlling for potential confounders, using water from a well when regular water source was insufficient was protective (AOR 0.17, 95% CI 0.004-0.69) from contracting enteric fever as 35% (17/49) of the cases obtained extra water from a well compared to 69% (94/136) of the cases. Similarly, having a metal cover for water storage containers was found to be significantly protective (AOR 0.26, 95%CI 0.08-

0.90). A total of only 14% (7/49) of the cases had metal coverings for water storage containers compared to 40% (54/136) of the controls.

6.3.2.2.4 Latrine

The use of household latrines appeared to be a significant risk factor for infection with *S.* Typhi compared to the use of latrines located in the community (AOR 12.71, 95% CI1.71-94.49). A total of 94% (46/49) of typhoid cases used household latrines compared to 79% (108/136) of the controls.

6.3.3 *S.* Paratyphi versus matched controls

Among the individuals identified with *S.* Paratyphi A septicaemia, 31.5% (17/54) were female with a median age of 20 years (IQR 12-24years) and 66.7% (36/54) were male with a median age of 18 years (IQR 10-21years). There were 54 *S.* Paratyphi A patients and 158 age, sex and ward matched controls.

6.3.3.1 Univariate analysis

Univariate analysis of probable risk factors comparing paratyphoid cases with community controls exhibited several variables that were found to be significantly associated with paratyphoid fever infection **(Table 6.3)**. Among the significantly associated risk factors were living in a neighbourhood affected by flooding, having eaten street food in the last two weeks, having recently moved into Kathmandu, having low monthly house-hold income, and renting a house.

Table 6.3:Risk factors for *S.* Paratyphi A, n (%) or median (IQR). Univariate and multivariate matched regression analyses on data from 49 case-control pairs showing all variables entered into a backward stepwise model (p(r)=0.10) and those selected by the likelihood ratio test to produce adjusted odds ratios

Variable	***S.* Paratyphi A** n=54	**Controls** n=158	**MOR**	**95%CI**	**p**	**AOR**	**95%CI**	**p**
Typhoid								
Aware of typhoid	16 (29.6)	91 (57.6)	0.26	0.12-0.54	**<0.001**			
Household enteric fever in last 8 weeks	6 (11.1)	43 (27.2)	0.22	0.07-0.67	**0.008**			
Enteric fever contact in last 8 weeks	7 (13.0)	58 (36.7)	0.15	0.05-0.45	**0.001**	0.15	0.04-0.61	**0.008**
Socioeconomic factors								
Household income <10000 NPR*	19 (35.2)	31 (20.0)	2.74	1.28-5.86	**0.009**			
Duration of stay in Kathmandu < 2 years*	23 (46.9)	47 (31.3)	2.27	1.02-5.02	**0.044**			
Rent house*	40 (74.1)	86 (55.5)	3.66	1.54-8.74	**0.003**	5.20	1.11-24.27	**0.036**
Water								
Water shortage affect daily life?	29 (53.7)	119 (75.3)	0.29	0.14-0.62	**0.001**			
Flooding affect house or neighborhood?	13 (24.1)	30 (19.0)	1.56	0.61-4.00	**0.350**			

Variable	*S.* Paratyphi A n=54	Controls n=158	MOR	95%CI	p	AOR	95%CI	p
Water storage								
Wide mouth of storage container	9 (16.7)	46 (29.1)	0.40	0.16-0.99	**0.047**			
Metal covering of water storage	7 (13.0)	59 (37.3)	0.14	0.05-0.41	**<0.001**	0.10	0.02-0.57	**0.009**
Sanitation behavior								
Wipe hands after washing*	29 (53.7)	126 (81.8)	0.18	0.08-0.40	**<0.001**	0.04	0.01-0.17	**<0.001**
Food								
Sliced fruit	10 (18.5)	52 (32.9)	0.41	0.18-0.94	**0.035**			
Eat pani puri	17 (31.5)	76 (48.1)	0.36	0.15-0.82	**0.016**	0.19	0.05-0.66	**0.009**
Eaten streetfood in the last 2 weeks	22 (40.7)	50 (31.6)	1.79	0.84-3.82	0.131	3.89	1.22-12.47	**0.020**
Latrine								
Community	5 (9.3)	37 (23.4)	6.10	1.68-22.07	0.006			
Household	49 (90.7)	118 (74.7)	1.00	-	-			
Number of people using latrine	8 (6-12)	9 (6-20)	0.94	0.89-0.99	**0.047**			

*data from 155 controls (household income) 49 cases & 150 controls (duration of stay in Kathmandu), 155 controls (rent house) and 154 controls (wipe hands); **Boldface** indicates $p \leq 0.05$

6.3.3.1.1 Socioeconomic factors

Having recently moved into Kathmandu was seen to be a significant (MOR 2.27, 95% CI 1.02-5.02) risk factor for contracting paratyphoid fever. Almost half, i.e. 47% (23/49) of the cases had been in Kathmandu for a significantly shorter period of time compared to only 31% (47/150) of the cases.

6.3.3.2 Multivariate analysis

After controlling for factors significant in the univariate analysis as well as *a priori* confounders **(Table 6.3)** several variables remained independently and significantly associated with typhoid fever infection **(Figure 6.2)**. These include wiping hands after washing, using a metal cover for water storage containers, having had an exposure to enteric fever in the last eight weeks, eating pani puri regularly, having eaten street food within two weeks preceding the questionnaire, and renting a house.

6.3.3.2.1 Exposure to enteric fever

Having had an exposure to an individual with enteric fever in the past eight weeks was seen to have a significant protective effect (AOR 0.10, 95% CI 0.02-0.49) against paratyphoid fever. Among our cases 11% (6/54) had been exposed to an enteric fever patient in the past eight weeks compared to 27% (43/158) of the controls.

6.3.3.2.2 Socioeconomic factors

Renting a house was seen to be a significant risk factor as well (AOR 5.20, 95% CI 1.11-24.27) for contracting paratyphoid fever as74% (40/54) of the cases reported renting as opposed to only 27% (43/155) of the controls.

6.3.3.2.3 Water

Having a metal cover for the water storage containers found to be significantly protective against paratyphoid fever (AOR 0.10, 95%CI 0.02-0.57) with only13% (7/54) of cases reporting metal coverings for water storage containers compared to 37% (59/154) of the controls.

6.3.3.2.4 Hygiene

Wiping hands with a towel after washing had a strongly protective effect (AOR 0.04, 95% CI 0.01-0.17) as 54% (29/54) of the cases reported wiping their hands after washing compared to 82% (126/158) of the controls.

6.3.3.2.5 Food

Having eaten street food two weeks prior to the date of interview was observed to be a significant risk factor for paratyphoid fever (AOR 3.89, 95% CI 1.22-12.47) **(Table 6.3)**. Compared to 41% (22/54) of the cases who had street food in the previous two weeks, only 32% (50/158) of controls had done so. However, eating pani puri regularly was found to have

a significant protective effect (AOR 0.19, 95% CI 0.05-0.66) against paratyphoid fever 32% (17/54) of the paratyphoid cases were observed to have consumed pani puri compared to 48% (76/158) of the controls.

6.4 Discussion

Historical surveillance data suggest that enteric fever rates decreased in parallel with the introduction of treatment of water supplies, pasteurization of dairy products, and exclusion of human faeces from food production (Crump et al., 2004). In areas of endemicity, where various risk factors and transmission routes have been identified (Black et al., 1985, Gasem et al., 2001, Luby et al., 1998, Luxemburger et al., 2001

, Parry et al., 2002), deciphering the interactions of various risk factors amongst themselves and with the population could lead to the identification of groups most at risk. This process would help in rationalising control strategies on a priority basis. In order to better understand these subtle interactions, a matched case control study was conducted to elucidate the finer dynamics of enteric fever within LSMC. The study was designed to investigate why some individuals within this endemic area develop enteric fever while others fail to do so. We matched on age, sex and area of residence in order to evaluate finer differences between enteric fever cases and disease free controls. Age and sex have been shown to be strongly associated with risk of typhoid fever and were thus matched to reduce any confounding effects they may have been introduced into our data. From previous works conducted at Patan Hospital, location of residence have also been found to heavily influence risk of enteric fever

and was therefore chosen to be a matched variable in an effort to closely examine any behavioural or environmental differences between the cases and controls. **(Chapter 3, section 3.3)**.

Due to the study design and level of endemicity of enteric fever in this population we were unable to identify major risk factors for enteric fever and certain variables that are known to be risk factors, such as having had a recent contact with typhoid, were observed to have a protective effect against enteric fever. Our matched design has allowed us, however, to identify several important and subtle behavioural factors that are protective against enteric fever that may provide important prevention strategies for this heavily exposed population.

For typhoid fever, having had prior contact with an enteric fever patient was counter-intuitively observed to have a protective effect. This may be due to the matched study design as controls were selected from neighbouring households and upon the recommendation of the case in order to efficiently locate age and sex matched individuals. Therefore many of the controls would have been aware of the typhoid case (although this information was not divulged by the CMAs) as it was likely to be a friend or acquaintance. Additionally, within an endemic area, where almost every household has a case, especially during the peak monsoon season when the majority of cases are observed and were enrolled, most people in the study population would have been exposed to enteric fever cases within a period of eight weeks. So whether physically having contact with a case of typhoid is a particularly risky behavior is questionable, more likely this finding is a result of our control selection.

The observation that use of a household latrine in comparison to a community latrine is a risk factor for *S.* Typhi is interesting. It could be possible that a constant and heavy exposure to contaminated fecal matter from a large number of individuals acts as an immune boosting mechanism and is protective in a community latrine setting. Sharing a toilet with only members of one household would decrease exposure to various pathogens and could feasibly lead to increased susceptibility to infection and thus make it riskier. Alternatively, the observed protective effect may be an artefact of our study design or control selection procedures.

Awareness of enteric fever, using well water when general supply was insufficient and having a metal covering for water containers were also observed to have a protective effect. An awareness of enteric fever was defined as knowledge of the signs and symptoms of the disease, transmission, and precautions to observe to avoid being infected. This knowledge was found to be protective against infection, which provides evidence for the use of an effective education-based prevention strategy for LSMC.

The noted protective effects of several other factors are less clear, however. For example, the strong protective effect of having a metal covering for water storage is likely explained by other uncollected information. The storage container that was covered was defined as the container in which drinking water was stored. From our previous experience with this

population, we know that some households store water in permanent kitchenware such as filters and pots while other households simply use either glass or plastic bottles. We believe that households that filter their water prior to drinking are likely to have a covering made either of steel, aluminum, or copper. These containers with metal covers are often heavy, permanent and filled through the action of physically bringing water from a source to the container and filtering it. Normally water is filtered by passing it through ceramic or clay candles whose small pore size filter dirt and bacteria out of water. Containers with plastic covers, however, are likely to be plastic bottles with narrow mouths, making them both portable and difficult to clean properly. It is unlikely that water put into these plastic containers is ever filtered prior to use and the water probably comes from several different sources as they are able to be re-filled at any time. So therefore it is probable that it is not the physical covering itself but the act of filtering water prior to consumption that is protective against infection with pathogens of enteric fever.

Risk from particular water sources proved difficult to evaluate from this study for two reasons. First, the majority of the population used water from a variety of different sources for most activities so the combination of mixed sources at varying proportions and inaccuracy of patient recall makes it challenging to identify specific sources as risky or protective. Secondly, our controls were matched for ward of residence and thus both cases and controls within a particular neighborhood were likely to report using the same water sources due to proximity. Ward matching therefore limits our ability to evaluate any substantial differences in water sources of cases and controls in the matched analysis.

Taking these considerations into account however, some interesting water-related observations arose nonetheless. For example, in the initial univariate analysis the use of water from stone spouts for any purpose was observed to be a major risk factor for contraction of *S.* Typhi **(Table 6.2)**. However, this effect disappeared in the multivariate analysis and was confounded by other factors present in the model. This was an unexpected observation as high levels of contamination and presence of *S.* Typhi and *S.* Paratyphi A DNA in the water from stone spouts had already been observed in our study (Chapter 5). Similarly, residences within closer proximity to stone spouts were observed to have higher cases of enteric fever reported (Chapter 4). Therefore it is likely that stone spout water is still a strong risk factor for contracting *S.* Typhi (and potentially *S.* Paratyphi A) but our study design prohibits these effects from remaining significant in the analysis.

The study population has extremely limited access to piped municipal supply, particularly during the dry season, and only a limited number of houses have dug wells. The majority of the households are dependent on water from stone spouts, and when water from this source is insufficient the next option is well water. This study was conducted during the rainy season so most people had sufficient water supply at the time of interview. However, cases reported collecting extra water from stone spouts more often than controls (although this did not remain significant in the adjusted analysis), and controls were significantly more likely to report obtaining extra water from wells. This could be due to the fact that well water may be less contaminated than that of the stone spout or perhaps it could also be explained by

uncollected behavioural differences relating to well water collection (ie distance from house or taste preference).

For paratyphoid fever, two factors were found to strongly associate with risk of infection: renting a house and eating street food in the two weeks preceding illness. Having had contact with an enteric fever patient in the previous eight weeks, using a metal cover for water storage containers, wiping hands after washing and eating pani puri regularly were observed to have a protective effect.

Eating street food has been implicated in previous studies as a risk factor for paratyphoid fever (Vollaard, 2004) and supports our findings. Corroborating this, we were able to isolate *Enterobacteriaceae* from various street foods, suggesting that the observation of street food as a risk factor is strong and not confounded by any other factors. However, routinely eating pani puri, a very popular street food, was reported significantly more frequently in controls. Although initially counterintuitive, more frequent consumption of such a heavily contaminated food product would lead to a constant exposure and thus potential boosting of the immune system against enteric fever. Due to the living conditions and high documented prevalence of enteric fever in LSMC, it is probable that most of the population has had a previous enteric fever infection and, thus, noting a protective effect of a contaminated food is reasonable.

Renting a house was also seen as a risk factor for paratyphoid fever and could imply a recent

migration into the Kathmandu Valley. The median age of the overall cases was 18 years with an age range of 10 to 22.5 years, and more males were observed than females. This is the average age when individuals migrate to the capital city from the surrounding rural areas in search of work or for higher education. In a patriarchal society such as Nepal, it is generally males who migrate more often than women for education and employment. Recent movement of an immunologically naïve individual into an endemic area would lead these individuals to be highly susceptible to infection, particularly in such an endemic setting.

Enteric fever is generally a disease of the young within the LSMC population and this has also been observed in Bangladesh, India and Pakistan (Brooks et al., 2005, Sinha et al., 1999a, KHAN et al., 2012). An increased incidence of enteric fever at a younger age has been attributed to immunological factors as have a naïve immune system, rather than to environmental factors (Butler et al., 1991). One of the main reasons that we excluded children under the age of 5 years was because this age group has different behavioural and immunological interactions relevant to enteric fever in comparison to older children and young adults. However, our finding of a median age of 18 years supports our assumption that constant and frequent exposure could lead to a natural development in immunological resistance to *S.* Typhi and *S.* Paratyphi for those native to an endemic area. Repeated exposure to the pathogen in such an endemic settings as children grow or as naïve individuals acclimatise, may result in lower incidence, though the mechanism of protection is poorly understood (KHAN et al., 2012). Studies have shown that in endemic regions, the incidence of enteric fever in pre-school children aged less than 5 years of age approximates to the

incidence among that for older school aged children (Ochiai et al., 2008 , Sinha et al., 1999a). This coupled with the movement of transient workers or students into Kathmandu could explain the median age (18 years) of our cases.

Comparing risk factors between typhoid and paratyphoid infection yielded some interesting findings. It seems as though risk or protection from typhoid is more heavily dependent on water or sanitation related factors, whereas food and socioeconomic factors play a larger role in risk of paratyphoid fever. The relationship between contaminated water and typhoid fever has been previously observed in epidemic outbreaks (Lewis et al., 2005 , Mermin et al., 1999) as has been various food items from street vendors (Black et al., 1985, Luby et al., 1998). Studies have also suggested that transmission routes for typhoid and paratyphoid fever are distinctly different (Sur et al., 2007, Vollaard, 2004) which was also observed in our study.

There were notable limitations within this study. Firstly, this population is heavily exposed to typhoid related research activities due to several recent clinical trials conducted in LSMC. This close contact with the community has provided many advantages, particularly in allowing the CMAs to integrate into the community and develop the trust of the population. Yet as a result the general population has a high level of awareness of typhoid and related risks, which may have biased our results.

Additionally, matching our controls for ward of residence heavily influenced the analysis and results of this study. We may have inadvertently overmatched our controls, which altered the

interpretation of the results as many of the individual strata were completely concordant and therefore didn't contribute to the overall estimate of effect. So although we did not identify many of the known risk factors for either *S.* Typhi or *S.* Paratyphi A infection (flooding, crowding, water sources) we were able to identify protective behavioral differences between our cases and controls which still provides valuable information.

6.5 Conclusion

This study supports our previous findings that enteric fever is highly endemic within this study population and the case burden is among young adult males. The results estimate an increased risk of enteric fever transmission for immunologically naïve individuals, i.e. those who have not been exposed to salmonellae before and young children. Risk or protection from typhoid appears to be more heavily dependent on water or sanitation related factors, whereas food and socioeconomic factors appear to play a larger role in risk of paratyphoid fever.As this population is highly endemic and constantly exposed, control and prevention through simple behavioral changes would provide a palatable and feasible method of reducing overall disease burden in LSMC. This is particularly appealing considering the low likelihood of any dramatic improvements in sanitation infrastructure of Kathmandu in the near future.

7 General Discussion

Even-though enteric fever has been all but eradicated in developed countries it remains a significant problem in many developing countries particularly in South and South-East Asia (Crump and Mintz, 2010, Ochiai et al., 2008). The most concerning issue for enteric fever today is that it has been almost forgotten by the international health community. It has been assumed that the omnipresence of enteric fever in the developing world has led to it being invisible (Maurice, 2012). In accordance with these observations, my work in Kathmandu observed a high incidence of enteric fever within the study population. However, though there have been short term immediate interventions through better diagnosis and treatment at Patan Hospital through the randomized control trials for a subset of the population within LSMC, no other immediate interventions, either in the short term, or in the long term to reduce the burden within this population has been devised. Antimicrobials are the standard treatment for enteric fever but the capacity of the pathogen to acquire resistance and spread rapidly threatens to condemn any antimicrobial control strategies to failure (Parry et al., 2002). These issues need to be addressed and enteric fever needs to go back onto the agendas of international and national health policy makers. The Coalition against Typhoid (CaT) has been established at the Sabin Vaccine Institute in Washington, D.C., U.S.A and endeavours to bring together epidemiologists, clinicians, vaccine industry representatives and mathematical modelers to tackle this issue. It is too early to tell or to forecast if such a group of non-political individuals can achieve this goal.

Despite WHO recommendations (WHO, 2008b), and the availability of two licensed vaccines against typhoid fever, i.e. the injectable Vi polysaccharide and the oral attenuated strain Ty21,

for over two decades, their use has not been widely disseminated in public health programs in countries endemic for enteric fever (Acosta CJ, 2004). This is in part related to uncertainties about the disease burden due to inadequate diagnostic tools which makes it difficult to estimate mortality rates due to enteric fever in any given area (Baker et al., 2010a, Maurice, 2012). The best enteric fever incidence assessment is based on available, sparse surveillance data that has been extrapolated from limited studies (Crump et al., 2004). These figures could therefore be imprecise and the matter is further aggravated by the lack of accurate diagnostics available for enteric fever (Baker et al., 2010a, Baker et al., 2010b). In this study the majority of the disease burden in addition to being among young children, was observed also among young adult males. Though this high case burden requires further investigation, targeting this particular at risk group in prevention activities such as vaccination programmes seems to be important. Mathematical modeling of the disease epidemiology in conjunction with improved diagnostics could play a key role in decreasing the incidence of enteric fever by informing governments of endemic countries such as Nepal of the magnitude of enteric fever burden within their population and assess the most feasible, cost effective means of reducing the disease burden.

The development of reliable and cheap diagnostics for enteric fever would undoubtedly benefit the long-term disease control and treatment. Reassessment of enteric fever diagnostics and considerations to the potentials and limitations of different approaches is now more optimistic than ever due to the significant advances that have been made towards the understanding of the biology and genomics of both, *S.* Typhi and *S.* Paratyphi A (Holt et al., 2008a, McClelland et al., 2004, Parkhill J et al., 2001, Roumagnac et al., 2006). Enteric fever diagnostics represent a paradigm of how technology must be driven by the human and

microbiological realities of the natural infection, as the infection has a unique molecular pathogenesis with a specific host response (Parry et al., 2011a). There are a wide range of potential clinical specimens and possible technological approaches, but methods that are practically applicable have to be conceived. Hence, the development of any new diagnostics has to address both the low count of the bacteria in various sterile sites and the cross-reactive nature of any potential antigens (Baker et al., 2010a, Parry et al., 2011a).

Apart from unreliable diagnostics and paucity in epidemiological understanding, the low uptake of the licensed vaccines are also due to a few shortcomings of the vaccines as these vaccines are effective in only 65-70% of the recipients (Acosta et al., 2005, Dipika Sur, 2009, Engels et al., 1998, Fraser et al., 2007, Guzman et al., 2006), the protection they afford is of relatively short duration (Guzman et al., 2006, Kietel WA, 1994), they have little or no efficacy in children under the age of two (Guzman et al., 2006, Mai et al., 2003, Plotkin SA, 1995), their role against Vi negative strains of *S.* Typhi is doubtful(Baker et al., 2005, Wain et al., 2005) and are ineffective against *S.* Paratyphi A(Stephen et al., 1996). A new generation conjugate vaccine that is linked to a protein polysaccharide providing strong, long lasting protection in all age groups and promising cross protection against *S.* Paratyphi holds some promise but this vaccine is still in development(Maurice, 2012). Additionally, carriers of the pathogens of enteric fever are believed to play a significant role in the transmission of the disease. While vaccination with available vaccines prevents infection and contributes to the reduction of incidence of chronic carriers, vaccination does not reduce carriage. Thus, enteric fever endemic regions such as LSMC should ensure surveillance for carriage and treat them appropriately as this would complement significantly mass vaccination campaigns in reducing the burden of disease (Khan et al., 2010).

Formidable obstacles exist for the reduction of enteric fever. While provision of safe water and basic sanitation for populations are long term solutions to reduce the incidence of the disease, this requires massive financial investments, political will and behavioural change, which are difficult goals to attain for a developing country such as Nepal which still faces economic, political and social instability. While vaccination costs limit the use of vaccines in developing countries various studies have shown that immunisation programs using the Vi vaccine are extremely cost effective in areas of high disease burden (Cook et al., 2008). Therefore, vaccines should be considered as an effective short to medium term intervention for the prevention and control of enteric fever.

The results of this thesis indicate a high prevalence of enteric fever within LSMC with the population being constantly exposed to a various risk factors including heavily contaminated water and street food. The data from this thesis strongly suggests that indirect transmission is dominant in this setting. Thus, in settings such as Kathmandu valley, improvements in infrastructure are fundamental to the control and elimination of enteric fever. Though this study was unable to accurately pin-point definite risk factors within the study population contaminated water and poor sanitary conditions along with the presence of carriers are likely to facilitate the continued persistence of the organisms in the community long after the limited window of immunity induced by the current vaccine (Fraser et al., 2007, Sur et al., 2009a).

To further aid in control and prevention measures one of the things that I would like to further investigate is the identification of risk factors within this population. I would like to optimise and condense the questionnaire that was used for this study and continue conducting the study for the period of a year. As this study has identified the high faecal contamination of water

used by this population, I would like to look into intervention methods that is user friendly and cost effective. One of the things that I have been interested in is looking at filtration methods, in particular sand filters that have been engineered by local companies through the NGO, Enpho-Nepal. These sand filters can be made locally, are relatively cheap (35 USD), and because the water is filtered through a natural process, the change in water taste that people complain about when water is boiled or put through candle filters could be overcome and I believe that we could get the population to drink safer water.

Due to time and logistic restrictions, I was only briefly able to look at food contamination among street vendors within this population. I would very much like to further analyse food contamination levels for the period of a year among food sold by street vendors.

It is hoped that the results of this study and our continued investigations will help in the planning and integration of infrastructural improvements alongside other control measures such as accurate diagnosis, appropriate therapy and principally vaccination programs for the long-term control of enteric fever within this population.

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9 Appendices

9.1 Genotyped *S.* Typhi isolates

Table 9.1: *S.* Typhi genotypes and corresponding isolate data from this study

Strain	Patient	Patient age	Date of	Cases in	Sequence	Genoty	Elevation
DM0	F	2	05/06/2005	1	NA	H42A	1314
DM0	F	21	05/06/2005	2	32.1	H58G-	1312
DM0	M	22	05/06/2005	1	NA	H58G-	1288
DM0	F	10	09/06/2005	6	NA	unk	1326
DM0	F	12	13/06/2005	5	NA	H58G-	1313
DM0	M	12	10/06/2005	2	NA	H42A	1314
DM0	F	27	10/06/2005	1	NA	H58G-	1319
DM0	M	28	15/06/2005	1	NA	H58G-	1319
DM0	M	18	16/06/2005	1	NA	H58G-	1310
DM0	F	46	16/06/2005	1	NA	H58G-	1311
DM0	F	24	25/06/2005	3	NA	H58G-	1302
DM0	F	5	17/06/2005	1	NA	H58G-	1302
DM0	F	13	20/06/2005	2	16.8	H58G-	1302
DM0	M	5	21/06/2005	2	NA	H58G-	1327
DM0	M	20	24/06/2005	2	NA	H58G-	1294
DM0	F	8	24/06/2005	1	NA	H58G-	1304
DM0	F	16	26/06/2005	1	NA	H50	1324
DM0	F	19	26/06/2005	1	NA	H42A	1313
DM0	M	15	26/06/2005	1	NA	unk	1304
DM0	M	11	28/06/2005	2	NA	H58G-	1333
DM0	M	16	28/06/2005	1	NA	H58G-	1320
DM0	F	6	29/06/2005	2	NA	H58G-	1334
DM0	F	25	29/06/2005	2	NA	H58G-	1334
DM0	M	17	01/07/2005	1	NA	H45	1302
DM0	F	18	01/07/2005	3	NA	H42A	1325
DM0	M	18	01/07/2005	1	NA	H58G-	1308
DM0	M	6	01/07/2005	2	NA	H58G-	1330
DM0	M	8	02/07/2005	3	NA	H58G-	1302
DM0	F	22	02/07/2005	2	NA	H42A	1330
DM0	F	27	03/07/2005	2	NA	unk	1320
DM0	F	17	03/07/2005	1	NA	H58G-	1316
DM1	F	24	04/07/2005	1	18.9	H58G-	1304
DM1	M	9	05/07/2005	2	NA	H58G-	1320
DM1	M	4	05/07/2005	2	22.1	H58G-	1310
DM1	M	19	05/07/2005	1	NA	H58G-	1335
DM1	M	12	05/07/2005	1	NA	H42A	1327
DM1	M	5	05/07/2005	1	NA	H58G-	1320

DM110	M	18	06/07/2005	1	NA	unk	1321
DM121	M	36	08/07/2005	1	NA	H58G-b2	1323
DM123	M	25	09/07/2005	2	NA	H58G-b4	1284
DM138	F	17	12/07/2005	2	NA	H58G-b4	1310
DM141	M	17	12/07/2005	1	NA	H58G-b2	1318
DM144	M	7	13/07/2005	1	NA	H58	1325

DM145	M	19	13/07/2005	2	NA	H45	1322
DM147	F	17	13/07/2005	1	NA	H58G-a2	1320
DM149	F	9	14/07/2005	2	NA	H58G-b4	1296
DM155	M	3	15/07/2005	2	NA	H58G-a2	1290
DM156	M	22	15/07/2005	4	NA	unk	1311
DM157	F	14	16/07/2005	5	NA	H58G-b4	1319
DM159	M	9	18/07/2005	2	NA	H58G-b2	1288
DM161	F	30	16/07/2005	1	NA	H58G-b4	1319
DM166	F	18	17/07/2005	1	NA	H58G-b2	1297
DM168	M	17	17/07/2005	2	NA	H58G-b4	1330
DM169	F	4	17/07/2005	2	NA	H58G-b4	1332
DM171	M	8	17/07/2005	1	NA	H58G-b2	1322
DM172	M	25	17/07/2005	5	NA	H58G-b3	1331
DM173	F	12	17/07/2005	1	NA	H58G-b3	1331
DM173	F	19	17/07/2005	4	NA	H58G-c2	1301
DM174	F	13	17/07/2005	4	NA	H58G-b4	1308
DM176	M	18	18/07/2005	2	NA	H58G-b4	1314
DM179	M	9	19/07/2005	1	NA	H42A	1320
DM180	M	25	19/07/2005	1	13.5	H58G-b4	1326
DM181	M	20	19/07/2005	1	NA	H50	1324
DM189	M	21	21/07/2005	2	NA	H58G-b4	1301
DM197	M	5	23/07/2005	1	NA	H58G-b4	1321
DM199	M	19	24/07/2005	3	NA	H58G-b2	1297
DM202	M	20	24/07/2005	2	NA	H58B	1296
DM203	M	8	24/07/2005	1	NA	H58G-b4	1314
DM206	M	35	25/07/2005	1	NA	H58G-b2	1326
DM207	M	16	25/07/2005	2	NA	H58G-b4	1295
DM213	M	6	26/07/2005	4	NA	H58G-b2	1330
DM215	M	20	27/07/2005	1	NA	H58G-b2	1337
DM216	M	26	28/07/2005	1	NA	H42A	1329
DM217	M	31	28/07/2005	1	NA	H42A	1316
DM218	M	15	28/07/2005	2	NA	H58G-b2	1323
DM221	F	19	28/07/2005	1	NA	H42A	1313
DM223	M	21	29/07/2005	1	NA	H58G-b2	1312
DM224	F	16	29/07/2005	2	NA	H58G-c1	1310
DM226	F	9	30/07/2005	1	NA	H58G-b2	1322

DM230	M	7	31/07/2005	1	NA	H50	1315
DM231	M	18	31/07/2005	1	NA	H58G-b2	1318
DM237	M	25	01/08/2005	2	NA	H58G-b2	1297
DM239	M	19	01/08/2005	1	NA	H58B	1336
DM240	M	14	01/08/2005	1	NA	H58G-b4	1326
DM242	F	4	12/08/2005	4	NA	H42A	1297
DM243	M	45	02/08/2005	3	NA	H58G-b2	1329
DM249	F	24	02/08/2005	1	7.1	H58G-b4	1311
DM255	M	6	04/08/2005	1	NA	H58G-b3	1323
DM257	M	15	04/08/2005	1	NA	H58G-c2	1337
DM258	F	9	04/08/2005	2	NA	H42A	1326
DM262	M	24	05/08/2005	6	NA	unk	1304
DM266	M	8	07/08/2005	3	NA	H58G-c2	1304
DM267	F	12	07/08/2005	1	NA	H58G-b2	1314
DM268	M	13	07/08/2005	3	NA	H58G-c2	1313
DM282	M	13	10/08/2005	1	NA	H58G-b2	1322
DM283	M	25	10/08/2005	2	NA	H58G-b2	1317
DM288	M	20	11/08/2005	1	NA	H58G-c2	1319
DM290	M	20	11/08/2005	1	NA	H45	1328
DM298	M	9	14/08/2005	2	NA	H58G-b2	1310
DM299	M	7	14/08/2005	2	NA	H58G-b2	1310
DM301	F	22	16/08/2005	3	NA	H58G-c1	1296
DM302	F	10	16/08/2005	2	NA	H58G-b4	1311
DM304	F	17	16/08/2005	2	NA	H58G-b4	1303
DM306	F	21	18/08/2005	1	NA	H58G-b2	1296
DM307	F	18	18/08/2005	3	NA	unk	1324
DM309	F	6	18/08/2005	1	NA	H58G-b4	1324
DM312	M	8	19/08/2005	1	NA	H42B	1323
DM314	M	15	19/08/2005	1	NA	H58G-b4	1304
DM316	F	20	20/08/2005	3	19.3	H58G-0	1296
DM317	M	10	21/08/2005	1	NA	H58G-b4	1311
DM330	F	8	25/08/2005	1	NA	H58G-b4	1304
DM331	M	20	25/08/2005	3	NA	H58G-b4	1311
DM340	M	18	28/08/2005	2	NA	H42A	1299
DM345	M	16	29/08/2005	3	12.7	H58G-c2	1325
DM348	M	18	29/08/2005	4	16.5	H58G-b4	1310
DM349	M	4	30/08/2005	2	8.2	H58G-b2	1315
DM353	F	3	30/08/2005	2	7.5	H58G-b5	1325
DM368	M	8	02/09/2005	1	NA	H58G-b4	1325
DM373	F	13	04/09/2005	1	15.6	H58G-b4	1299
DM377	M	21	05/09/2005	3	NA	H58G-b2	1304
DM383	M	6	06/09/2005	1	NA	H42A	1327
DM386	M	10	07/09/2005	1	NA	H52	1319

ED001	M	14	07/05/2006	3	NA	unk	1304

ED004	F	10	11/05/2006	1	NA	H42B	1326
ED006	M	8	14/05/2006	1	NA	H14	1338
ED007	F	12	14/05/2006	1	NA	H58G-J1	1329
ED008	M	10	14/05/2006	1	NA	unk	1330
ED012	M	22	15/05/2006	5	NA	H42B	1293
ED020	F	4	18/05/2006	1	NA	H58G-b2	1326
ED022	M	3	18/05/2006	1	NA	H42B	1330
ED024	F	6	18/05/2006	1	NA	H14	1331
ED028	M	21	21/05/2006	2	NA	H82	1312
ED030	M	9	22/05/2006	1	NA	H50	1290

ED031	M	17	22/05/2006	1	NA	H58	1343
ED032	F	5	22/05/2006	2	NA	H45	1322
ED033	F	35	22/05/2006	4	NA	H82	1326
ED038	F	7	23/05/2006	1	NA	H42B	1322
ED041	F	8	23/05/2006	1	NA	unk	1335
ED042	M	27	23/05/2006	1	NA	unk	1334
ED046	M	4	24/05/2006	1	NA	H14	1304
ED047	F	18	26/05/2006	1	NA	H82	1336
ED049	M	20	26/05/2006	1	NA	H58G-b2	1305
ED050	M	12	26/05/2006	1	NA	H58G-a1	1330
ED052	F	11	28/05/2006	1	NA	unk	1332
ED059	M	16	29/05/2006	3	NA	H42A	1327
ED063	M	38	31/05/2006	2	NA	unk	1309
ED069	M	22	05/06/2006	1	NA	H58G-a2	1323
ED072	F	9	05/06/2006	1	NA	H58G-d3	1292
ED076	F	20	06/06/2006	1	NA	H14	1304
ED082	M	8	07/06/2006	1	NA	H42B	1361
ED083	F	13	07/06/2006	1	NA	H42B	1329
ED088	F	7	08/06/2006	1	NA	unk	1333
ED089	M	9	08/06/2006	1	NA	H58G-J1	1330
ED090	F	26	08/06/2006	2	NA	H58G-b2	1326
ED091	M	18	09/06/2006	1	NA	H42B	1323
ED092	F	4	09/06/2006	1	NA	H58G-b4	1303
ED093	F	30	09/06/2006	1	NA	H58G-b4	1337
ED094	F	32	09/06/2006	2	NA	H14	1284
ED097	M	13	09/06/2006	1	NA	H58G-b2	1305
ED098	M	17	10/06/2006	1	NA	H42A	1301
ED102	M	12	12/06/2006	1	NA	H42B	1323
ED104	M	23	13/06/2006	2	NA	H58G-b2	1299
ED106	M	13	12/06/2006	2	NA	H42B	1294
ED108	M	19	13/06/2006	2	NA	H58G-J1	1333
ED110	M	5	13/06/2006	1	NA	H42B	1307

ED111	F	10	14/06/2006	1	NA	H58G-J1	1320
ED114	F	11	14/06/2006	1	NA	H58G-a1	1327
ED117	F	4	14/06/2006	1	NA	H58G-b2	1312
ED119	F	15	15/06/2006	1	NA	H58G-d3	1327
ED126	F	14	16/06/2006	3	404.1	H58G-b2	1302
ED130	F	25	17/06/2006	1	NA	H42A	1324
ED134	M	16	18/06/2006	2	NA	H42A	1335
ED138	M	12	19/06/2006	2	NA	H58G-J1	1333
ED139	M	17	19/06/2006	1	NA	H42A	1335
ED140	M	3	19/06/2006	1	NA	H58G-J1	1308
ED141	M	9	19/06/2006	1	NA	H42A	1303
ED142	M	20	19/06/2006	1	NA	H58G-J1	1305
ED143	F	10	20/06/2006	1	NA	H52	1315
ED145	M	13	22/06/2006	1	NA	H58G-a1	1331
ED147	F	4	22/06/2006	1	NA	H42A	1311
ED148	M	30	22/06/2006	1	NA	H58G-b2	1323
ED149	M	27	22/06/2006	2	NA	H45	1313
ED150	F	4	23/06/2006	1	NA	H58G-b4	1327
ED152	F	4	23/06/2006	3	NA	H58G-c2	1327
ED156	F	15	23/06/2006	3	NA	H58G-b2	1301
ED158	M	11	24/06/2006	3	NA	H58G-b4	1327
ED169	F	19	27/06/2006	1	NA	unk	1341
ED171	M	35	28/06/2006	1	NA	H42A	1343
ED174	F	8	29/06/2006	1	41.9	H58G-b2	1305
ED176	F	9	30/06/2006	1	NA	H58G-a2	1327
ED177	F	38	30/06/2006	1	NA	H50	1316
ED181	M	8	03/07/2006	1	NA	H58G-a2	1331
ED188	M	8	04/07/2006	1	9.9	H58G-b2	1330
ED190	M	3	04/07/2006	1	NA	H58G-b2	1302
ED195	M	20	05/07/2006	1	NA	H45	1352
ED197	F	16	06/07/2006	2	NA	H58G-b2	1315
ED203	M	4	06/07/2006	1	NA	H16	1305
ED206	F	6	07/07/2006	1	NA	H42A	1338
ED214	F	9	09/07/2006	4	NA	H58G-a2	1314
ED215	M	19	09/07/2006	1	NA	H58G-a2	1325
ED216	F	20	09/07/2006	1	NA	H58G-b2	1293
ED219	F	16	11/07/2006	4	NA	H50	1314
ED220	M	15	11/07/2006	1	NA	H58G-a2	1301
ED223	M	20	12/07/2006	1	NA	H58G-a2	1314
ED224	M	12	12/07/2006	4	NA	H58G-b2	1315
ED226	M	18	13/07/2006	1	NA	H16	1335
ED227	M	20	13/07/2006	1	NA	H42A	1328
ED231	F	3	16/07/2006	2	NA	unk	1319

ED232	M	29	16/07/2006	1	NA	H58G-c2	1312
ED233	M	20	16/07/2006	2	NA	H42A	1313
ED234	F	19	16/07/2006	1	NA	H55	1332
ED236	M	21	17/07/2006	5	NA	H55	1293
ED237	M	14	17/07/2006	1	NA	H85	1325
ED240	F	4	18/07/2006	2	NA	H58G-d3	1308
ED243	F	12	19/07/2006	2	NA	H42A	1323
ED247	F	18	20/07/2006	2	NA	H58G-c2	1344
ED248	F	16	20/07/2006	2	NA	H58G-a2	1309
ED249	M	7	21/07/2006	1	23.3	H58G-a2	1335
ED254	F	14	22/07/2006	4	NA	H58G-b4	1328
ED260	F	7	23/07/2006	1	NA	unk	1295
ED265	M	18	25/07/2006	1	NA	H58G-c2	1326
ED269	F	7	25/07/2006	6	NA	H58G-b2	1331
ED282	F	9	28/07/2006	1	NA	H42A	1309
ED285	M	3	28/07/2006	2	NA	H58G-J1	1333
ED292	F	11	30/07/2006	2	NA	H58G-b4	1325
ED294	M	21	30/07/2006	1	NA	H58G-b2	1303
ED295	F	5	30/07/2006	1	NA	H50	1322
ED296	M	42	30/07/2006	4	NA	H16	1331
ED297	M	17	30/07/2006	1	NA	H58G-c2	1329
ED302	F	34	01/08/2006	4	NA	H45	1328
ED304	M	25	01/08/2006	3	NA	H50	1328
ED307	F	15	03/08/2006	1	NA	H58G-b2	1294
ED325	M	6	08/08/2006	1	NA	H42B	1303
ED329	M	18	09/08/2006	2	NA	H58G-b1	1319
ED341	M	12	16/08/2006	1	8.6	H58G-b2	1300
ED343	M	3	17/08/2006	2	NA	H58G-b2	1319
ED349	M	6	18/08/2006	1	NA	H58G-b2	1349
ED350	M	18	18/08/2006	1	NA	H42A	1327
ED351	M	10	18/08/2006	2	NA	unk	1317
ED353	M	19	20/08/2006	1	32.3	H58G-d3	1327
ED359	F	27	22/08/2006	6	NA	H58G-c2	1326
ED373	F	15	27/08/2006	2	NA	H42A	1328
ED384	M	26	01/09/2006	2	NA	H50	1343
ED390	F	16	04/09/2006	1	NA	H45	1317
ED393	F	12	05/09/2006	2	NA	H45	1349
ED399	M	7	11/09/2006	2	NA	H42A	1323
ED400	M	10	12/09/2006	1	4.1	H58G-b2	1295
ED401	M	25	13/09/2006	1	NA	H42	1312
ED402	M	23	14/09/2006	3	NA	H58	1327
ED405	M	14	12/12/2006	1	30.3	H58G-unk	1325
ED425	M	19	22/02/2007	1	6.7	H58G-b2	1333

ED429	M	6	26/02/2007	1	NA	H42	1323
ED434	F	18	08/03/2007	1	NA	H16	1319
ED439	F	5	11/03/2007	1	14.1	H58	1304
ED440	M	23	13/03/2007	1	NA	H58G-b2	1325
ED448	F	10	23/03/2007	1	NA	H42A	1330
ED455	M	7	30/03/2007	3	NA	H42A	1340
ED464	F	4	09/04/2007	1	5.4	H58G-unk	1321
ED471	M	6	16/04/2007	1	NA	H58G-b2	1329
ED473	M	25	16/04/2007	1	15.8	H58G-b5	1326
ED478	M	20	18/04/2007	1	NA	H44	1296
ED479	M	6	22/04/2007	1	NA	H16	1292
ED482	M	9	29/04/2007	2	NA	H58G-b5	1325
ED483	F	7	29/04/2007	4	NA	H16	1298
ED484	F	12	30/04/2007	1	NA	H52	1305
ED492	M	16	06/05/2007	1	NA	H16	1290
ED497	M	10	11/05/2007	2	NA	H58G-0	1296
ED498	M	54	11/05/2007	2	NA	H58	1327
ED500	F	6	13/05/2007	3	NA	H42A	1340
ED504	M	18	17/05/2007	1	NA	H42A	1324
ED506	M	8	18/05/2007	1	NA	H58G-c1	1321
ED508	M	9	20/05/2007	2	NA	H42A	1335
ED509	M	16	20/05/2007	1	NA	H50	1325
ED512	M	30	21/05/2007	1	16.3	H58G-c1	1329
ED513	F	21	22/05/2007	1	NA	H58G-unk	1328
ED514	M	19	23/05/2007	4	NA	H42A	1323
ED516	F	5	24/05/2007	1	NA	H42A	1340
ED517	F	6	24/05/2007	3	NA	H58G-c2	1327
ED518	M	12	24/05/2007	1	NA	H58G-d3	1321
ED519	M	7	24/05/2007	1	NA	H45	1314
ED530	F	11	07/06/2007	1	NA	H16	1296
ED533	F	5	02/06/2007	1	NA	H85	1307
ED538	F	5	15/06/2007	1	22	H58G-d4	1313
ED540	M	6	19/06/2007	2	NA	H42A	1320
ED541	F	19	17/06/2007	1	NA	H58G-unk	1325
ED544	F	11	18/06/2007	1	NA	H58G-unk	1310
ED548	M	29	21/06/2007	1	NA	H58G-c1	1328
ED549	F	5	24/06/2007	2	NA	H58G-c1	1329
ED550	M	11	24/06/2007	1	NA	H58G-d4	1308
ED552	M	22	24/06/2007	2	NA	H58G-c1	1328
ED555	M	30	29/06/2007	4	12.7	H58G-d3	1328
ED557	F	16	29/06/2007	4	NA	H58G-b2	1314
ED558	M	17	01/07/2007	2	NA	H58G-b5	1323
ED560	F	25	01/07/2007	4	NA	H45	1297

ED561	F	4	02/07/2007	1	NA	H58G-unk	1327
ED564	M	10	05/07/2007	1	14.9	H58G-a2	1298
ED567	M	16	05/07/2007	3	NA	H58G-d4	1313
ED568	M	7	06/07/2007	3	NA	H58G-c1	1327
ED569	M	21	08/07/2007	2	NA	H58G-c1	1326
ED570	F	3	08/07/2007	1	NA	H50	1319
ED571	M	23	08/07/2007	1	NA	H42A	1321
ED574	M	2	10/07/2007	2	NA	H58G-c1	1329
ED577	M	12	10/07/2007	1	NA	H58G-d3	1325
ED581	F	15	12/07/2007	1	NA	H52	1301
ED585	M	8	15/07/2007	1	NA	H58G-unk	1329
ED587	M	3	15/07/2007	2	NA	H58G-d3	1322
ED589	F	19	15/07/2007	2	NA	H58G-unk	1323
ED590	M	9	16/07/2007	1	NA	H58G-d4	1313
ED593	M	24	16/07/2007	1	30.5	H58	1318
ED603	M	16	20/07/2007	1	failed	H58G-b2	1323
ED607	F	12	25/07/2007	1	NA	H58G-b4	1303
ED611	M	13	27/07/2007	2	NA	H58G-a2	1325
ED612	M	4	29/07/2007	1	NA	H58G-c1	1309
ED614	M	9	31/07/2007	1	NA	H58G-d4	1312
ED619	F	8	05/08/2007	1	14	H58G-b4	1316
ED621	M	9	06/08/2007	1	NA	H58G-unk	1297
ED622	M	25	06/08/2007	2	NA	H58G-b5	1297
ED623	M	27	06/08/2007	2	NA	H58G-b2	1321
ED627	M	6	17/08/2007	4	NA	H58G-d4	1318
ED628	M	18	20/08/2007	2	NA	H58G-d4	1291
ED629	M	6	18/08/2007	1	NA	H58G-b5	1294
ED630	F	8	22/08/2007	1	NA	H58G-d3	1337
ED633	F	19	24/08/2007	1	NA	H58G-b5	1346
ED634	M	18	26/08/2007	1	9.9	H58G-d4	1302
ED637	M	16	29/08/2007	1	NA	H58G-unk	1321
ED638	F	18	30/08/2007	3	NA	H58G-d4	1298
ED639	M	4	31/08/2007	1	NA	H58G-c1	1320
ED643	F	5	02/09/2007	2	NA	H42A	1303
ED645	M	34	05/09/2007	1	NA	H58G-b5	1315
ED648	F	5	13/09/2007	1	NA	H42	1346
ED652	M	32	18/09/2007	1	NA	H42A	1325
ED659	M	14	27/09/2007	2	11.1	H58G-unk	1284
ED661	M	19	28/09/2007	3	NA	H50	1329
ED662	M	9	28/09/2007	1	NA	H58G-c1	1318
ED665	M	21	02/10/2007	2	NA	H58G-d4	1326
ED666	M	5	02/10/2007	1	NA	H58G-d4	1337
ED669	M	42	02/10/2007	1	14.3	H58G-d4	1313

ED670	M	20	14/10/2007	4	NA	H58G-d3	1323
ED672	M	24	26/10/2007	1	NA	H42	1326
ED673	F	19	30/10/2007	1	NA	H42	1328
ED678	M	22	02/11/2007	1	NA	unk	1326
ED679	M	6	05/11/2007	2	NA	H58G-b1	1345
ED682	F	5	07/11/2007	1	17.4	H58G-b5	1327
ED685	F	8	19/11/2007	1	NA	H58	1341
ED688	M	10	25/11/2007	1	NA	H58G-unk	1309
ED692	F	27	29/11/2007	2	NA	unk	1345
ED693	M	21	30/11/2007	2	NA	H42	1328
ED694	M	7	02/12/2007	1	NA	H58G-b2	1328
ED695	M	13	02/12/2007	3	20.7	H58G-b1	1302
ED697	M	18	07/12/2007	1	NA	H58G-a2	1313
ED698	M	25	04/12/2007	1	NA	H42	1328
ED707	M	17	26/12/2007	4	NA	H45	1323
ED714	F	6	06/01/2008	1	NA	H42A	1336
ED729	M	16	14/03/2008	1	NA	H42	1323
ED737	M	17	25/03/2008	2	NA	H52	1326
ED740	M	17	04/04/2008	2	NA	H42	1324
ED747	F	19	15/04/2008	1	NA	H58	1296
ED748	M	22	14/04/2008	1	NA	H52	1346
ED750	F	7	17/04/2008	1	NA	H58G-a2	1299
ED753	M	25	21/04/2008	1	NA	unk	1325
ED754	F	6	22/04/2008	1	NA	H42A	1321
ED757	F	21	24/04/2008	1	13.6	H58G-b2	1314
ED758	M	18	25/04/2008	2	NA	H58G-c1	1320
ED760	M	19	08/01/2008	4	NA	H58G-c2	1312
ED762	M	17	28/04/2008	1	NA	H58G-b5	1320
ED763	M	31	28/04/2008	1	NA	H58G-a2	1315
ED763	F	5	28/04/2008	2	NA	H58G-a2	1315
ED765	M	5	01/05/2008	1	NA	unk	1327
ED767	F	22	06/05/2008	1	NA	H58G-b4	1313
ED772	M	25	11/05/2008	1	NA	H58G-b5	1302
ED774	F	5	11/05/2008	1	5.9	H58G-b2	1327
ED775	M	12	12/05/2008	1	NA	H58G-b2	1330
ED777	M	18	15/05/2008	1	NA	H58G-b1	1301
ED786	F	21	20/05/2008	1	NA	H58G-b5	1325
ED787	F	8	21/05/2008	1	NA	H58G-b5	1315
ED791	M	21	20/05/2008	4	NA	H15	1293
ED793	M	27	23/05/2008	4	NA	H58G-c2	1318
ED794	M	14	23/05/2008	1	NA	H58G-b5	1333
ED795	F	22	23/05/2008	2	NA	H42A	1303
ED806	M	32	04/06/2008	1	NA	H42A	1313

ED808	M	33	14/06/2008	1	NA	H58G-c2	1308
ED809	M	15	09/06/2008	2	NA	H15	1315
ED811	F	30	10/06/2008	1	NA	H42A	1322
ED821	M	20	20/06/2008	1	NA	H50	1314
ED822	M	23	19/06/2008	1	NA	H45	1312
ED824	M	21	22/06/2008	1	NA	unk	1333
ED825	F	8	23/06/2008	3	NA	H42A	1318
ED826	M	14	27/06/2008	1	failed	H58G-c2	1313
ED829	M	18	30/06/2008	1	NA	H58G-b4	1313
ED831	F	6	04/07/2008	1	NA	unk	1333
ED833	F	21	15/07/2008	1	NA	H58G-d4	1295
ED835	M	5	09/07/2008	1	19	H58G-b4	1327
ED838	M	12	13/07/2008	1	NA	H58G-b4	1321
ED841	M	19	15/07/2008	2	NA	H58G-b5	1326
ED846	M	15	21/07/2008	1	NA	H50	1310
ED850	M	15	23/07/2008	2	NA	H58G-unk	1302
ED851	F	12	23/07/2008	2	NA	H58G-b2	1329
TY003	F	7	28/07/2008	1	NA	H58G-b4	1313
TY004	M	15	29/06/2008	2	NA	H50	1296
TY013	M	4	05/08/2008	1	NA	H58	1313
TY014	M	17	05/07/2008	1	NA	H58G-c2	1311
TY019	F	19	08/08/2008	1	NA	H58G-b4	1300
TY020	F	20	10/08/2008	1	NA	H50	1329
TY021	M	5	10/08/2008	1	NA	H50	1292
TY022	M	10	11/08/2008	1	NA	H50	1297
TY026	F	17	19/08/2008	1	NA	H58	1285
TY028	F	7	01/09/2008	1	NA	H58G-a2	1328
TY029	M	25	01/09/2008	1	NA	H58G-c1	1302
TY031	M	11	29/08/2008	1	NA	H58G-a1	1283
TY034	M	15	11/09/2008	1	NA	H58G-unk	1334
TY042	M	43	23/09/2008	1	NA	H58G-a2	1305
TY045	M	7	25/09/2008	1	NA	unk	1309
TY046	F	23	25/09/2008	1	NA	unk	1319
TY052	M	41	04/11/2008	1	NA	unk	1330
TY061	M	16	30/11/2008	1	NA	unk	1296
TY066	F	11	09/12/2008	1	NA	unk	1299
TY073	F	24	16/12/2008	3	NA	unk	1318
TY080	M	21	09/01/2009	1	NA	unk	1303
TY082	M	10	11/01/2009	4	NA	unk	1318
TY084	M	25	30/01/2009	1	NA	unk	1297
TY086	M	12	28/01/2009	1	NA	unk	1312
TY088	M	18	22/02/2009	1	NA	unk	1311
TY095	M	16	19/02/2009	1	NA	unk	1311

TY096	M	15	20/02/2009	1	NA	unk	1288
TY099	F	19	24/02/2009	1	NA	unk	1329
TY102	M	18	03/02/2009	1	NA	unk	1317
TY105	M	22	09/03/2009	1	NA	unk	1297
TY107	M	21	11/03/2009	1	NA	unk	1305
TY116	M	16	26/03/2009	1	NA	unk	1329
TY126	M	15	01/05/2009	1	NA	unk	1319
TY127	M	20	03/05/2009	1	NA	unk	1317

a: Total number of culture positive typhoid cases (*S*. Typhi and *S*. Paratyphi A) in the residence; b: Average read depth for the 40 strains sequenced in Index-tagged Illumina libraries; c: unk: unknown d, Elevation of residence as extracted from digital elevation model

9.2 The 113 *S.* Typhi chromosomal loci assayed in 387 *S.* Typhi isolates using the Sequenom iPLEX Gold assay

Position in *S.* Typhi CT18	Name	Ancestral	Derived	Source (reference)
6317	BiP1	C	T	P. Roumagnac *et al.* (17)
955273	BiP10	G	A	P. Roumagnac *et al.* (17)
2271858	BiP102	G	A	P. Roumagnac *et al.* (17)
2463900	BiP103	G	A	P. Roumagnac *et al.* (17)
4391261	BiP104	C	T	P. Roumagnac *et al.* (17)
2221217	BiP106	G	A	P. Roumagnac *et al.* (17)
2412815	BiP107	T	C	P. Roumagnac *et al.* (17)
2947124	BiP108	G	A	P. Roumagnac *et al.* (17)
4218089	BiP109	C	T	P. Roumagnac *et al.* (17)
958002	BiP11	G	A	P. Roumagnac *et al.* (17)
1076027	BiP12	C	T	P. Roumagnac *et al.* (17)
1089517	BiP13	T	C	P. Roumagnac *et al.* (17)
1240637	BiP14	G	A	P. Roumagnac *et al.* (17)
1285129	BiP15	C	T	P. Roumagnac *et al.* (17)
1509712	BiP16	C	T	P. Roumagnac *et al.* (17)
1619556	BiP17	C	T	P. Roumagnac *et al.* (17)
1641434	BiP18	T	C	P. Roumagnac *et al.* (17)
6261	BiP2	C	T	P. Roumagnac *et al.* (17)
1812993	BiP20	A	G	P. Roumagnac *et al.* (17)
1838206	BiP23	C	T	P. Roumagnac *et al.* (17)
1943523	BiP24	C	T	P. Roumagnac *et al.* (17)
2048732	BiP25	G	A	P. Roumagnac *et al.* (17)
2048739	BiP26	T	G	P. Roumagnac *et al.* (17)
2108140	BiP27	C	T	P. Roumagnac *et al.* (17)
2108322	BiP28	G	A	P. Roumagnac *et al.* (17)
2221079	BiP29	C	T	P. Roumagnac *et al.* (17)
138680	BiP3	C	T	P. Roumagnac *et al.* (17)
2220955	BiP30	G	A	P. Roumagnac *et al.* (17)
2348902	BiP33	C	T	P. Roumagnac *et al.* (17)
2413077	BiP34	A	G	P. Roumagnac *et al.* (17)
2456099	BiP35	C	T	P. Roumagnac *et al.* (17)
2464185	BiP36	A	G	P. Roumagnac *et al.* (17)
2463810	BiP37	C	A	P. Roumagnac *et al.* (17)
2463831	BiP38	G	A	P. Roumagnac *et al.* (17)
180029	BiP4	C	T	P. Roumagnac *et al.* (17)
2544044	BiP40	G	A	P. Roumagnac *et al.* (17)
2643935	BiP41	G	A	P. Roumagnac *et al.* (17)
2697121	BiP42	G	A	P. Roumagnac *et al.* (17)
2825733	BiP43	G	A	P. Roumagnac *et al.* (17)
2847564	BiP44	G	A	P. Roumagnac *et al.* (17)

2902990	BiP45	G	A	P. Roumagnac *et al.* (17)
2947118	BiP47	G	A	P. Roumagnac *et al.* (17)
3062270	BiP48	C	T	P. Roumagnac *et al.* (17)
3287913	BiP49	C	T	P. Roumagnac *et al.* (17)
3339598	BiP51	C	T	P. Roumagnac *et al.* (17)
3469152	BiP54	T	C	P. Roumagnac *et al.* (17)
3476189	BiP56	C	T	P. Roumagnac *et al.* (17)
3731468	BiP57	G	A	P. Roumagnac *et al.* (17)
3731453	BiP58	C	T	P. Roumagnac *et al.* (17)
3806041	BiP59	G	A	P. Roumagnac *et al.* (17)
332645	BiP6	G	A	P. Roumagnac *et al.* (17)
4286222	BiP60	C	T	P. Roumagnac *et al.* (17)
4391077	BiP61	C	T	P. Roumagnac *et al.* (17)
4429843	BiP62	G	A	P. Roumagnac *et al.* (17)
4443365	BiP63	G	A	P. Roumagnac *et al.* (17)
4651341	BiP65	T	G	P. Roumagnac *et al.* (17)
2757499	BiP67	T	G	P. Roumagnac *et al.* (17)
546949	BiP68	T	C	P. Roumagnac *et al.* (17)
343912	BiP7	C	T	P. Roumagnac *et al.* (17)
3245128	BiP76	G	A	P. Roumagnac *et al.* (17)
3415355	BiP77	C	T	P. Roumagnac *et al.* (17)
4422904	BiP79	C	T	P. Roumagnac *et al.* (17)
580916	BiP8	T	C	P. Roumagnac *et al.* (17)
1808734	BiP81	G	A	P. Roumagnac *et al.* (17)
138729	BiP89	C	A	P. Roumagnac *et al.* (17)
332683	BiP90	G	A	P. Roumagnac *et al.* (17)
830308	BiP91	C	T	P. Roumagnac *et al.* (17)
976842	BiP92	G	A	P. Roumagnac *et al.* (17)
1813023	BiP93	G	A	P. Roumagnac *et al.* (17)
2221033	BiP94	C	T	P. Roumagnac *et al.* (17)
2221015	BiP95	A	G	P. Roumagnac *et al.* (17)
2220992	BiP96	C	A	P. Roumagnac *et al.* (17)
2221084	BiP99	A	G	P. Roumagnac *et al.* (17)
1057075	H42a	C	T	K.E. Holt *et al.* (20)
1161982	H42b	C	T	K.E. Holt *et al.* (20)
229722	H58.1	G	A	K.E. Holt *et al.* (20)
241789	H58.2	A	G	K.E. Holt *et al.* (20)
522174	H58.3	C	T	K.E. Holt *et al.* (20)
789538	H58.4	C	T	K.E. Holt *et al.* (20)
991913	H58.5	G	A	K.E. Holt *et al.* (20)
1193220	H58.6	T	C	K.E. Holt *et al.* (20)
1534019	H58.7	G	A	K.E. Holt *et al.* (20)
1579791	H58.8	C	T	K.E. Holt *et al.* (20)
1750900	H58.9	G	A	K.E. Holt *et al.* (20)
2003906	H58.10	G	A	K.E. Holt *et al.* (20)
2014424	H58.11	G	A	K.E. Holt *et al.* (20)
2418082	H58.12	G	A	K.E. Holt *et al.* (20)

2499777	H58.13	G	A	K.E. Holt *et al.* (20)
2843437	H58.14	A	G	K.E. Holt *et al.* (20)
2915916	H58.15	G	A	K.E. Holt *et al.* (20)
2915943	H58.16	C	A	K.E. Holt *et al.* (20)
2916079	H58.17	C	T	K.E. Holt *et al.* (20)
3061270	H58.18	C	T	K.E. Holt *et al.* (20)
3180740	H58.19	G	A	K.E. Holt *et al.* (20)
3196458	H58.20	T	C	K.E. Holt *et al.* (20)
3202099	H58.21	C	T	K.E. Holt *et al.* (20)
3259213	H58.22	C	T	K.E. Holt *et al.* (20)
3514815	H58.23	C	T	K.E. Holt *et al.* (20)
3694947	H58.24	C	G	K.E. Holt *et al.* (20)
3784470	H58.25	G	T	K.E. Holt *et al.* (20)
3788492	H58.26	C	T	K.E. Holt *et al.* (20)
3810322	H58.27	C	T	K.E. Holt *et al.* (20)
3826375	H58.28	C	T	K.E. Holt *et al.* (20)
4297511	H58.29	C	A	K.E. Holt *et al.* (20)
4379937	H58.30	G	A	K.E. Holt *et al.* (20)
4387832	H58.31	C	T	K.E. Holt *et al.* (20)
4546085	H58.32	C	T	K.E. Holt *et al.* (20)
4546132	H58.33	G	A	K.E. Holt *et al.* (20)
4581043	H58.34	T	A	K.E. Holt *et al.* (20)
4610837	H58.35	C	T	K.E. Holt *et al.* (20)
4653894	H58.36	C	T	K.E. Holt *et al.* (20)
4798685	H58.37	C	T	K.E. Holt *et al.* (20)
35555	H58.38	G	A	K.E. Holt *et al.* (20)
3126962	H58.39	G	A	This work
922111	H58.40	G	A	This work
2267090	H58.41	C	T	This work
4519292	H58.42	C	A	This work
2976460	H58.43	T	C	This work
4401419	H58.44	C	T	This work
2405223	H58.45	C	T	This work
3129638	H58.46	G	A	This work
2628741	H58.47	C	T	This work
2952	H58.48	G	T	This work
1592955	H58.49	G	A	This work
3238431	H58.50	G	A	This work
2531257	H58.51	G	A	This work

9.3 Analysis of physical parameter of water

9.3.1 pH

The pH metric method was applied to determine the pH levels of the various water samples.

pH measurement is the determination of the activity of the hydrogen ions by potentiometric measurement using a standard hydrogen electrode and a reference electrode.

Procedure

The pH meter was stabilized before measuring pH for at least 10 minutes. It was then calibrated with standard buffer solutions (pH 4, pH 7 and pH 9 buffer tablets). This should be done at least once a day. The electrode was washed with distilled water, wiped gently with tissue paper and immersed into the beaker containing sample water. The pH value was noted and read by the pH meter.After measuring the pH of the sample the cell was washed, wiped and immersed into the beaker containing saturated potassium chloride (KCl) solution.

9.3.2 Temperature

Temperature of the water samples was determined by using a digital thermometer. Probe of the thermometer was immersed into the water sample and the observed value was recorded in the analysis book.

9.3.3 Conductivity

The conductivity meter method was used to measure the conductivity of the water samples.

Procedure

The conductivity meter was allowed to stabilize before measuring the conductance for at least 15 minutes. The electrode was washed several times with distilled water and wiped with tissue paper. The electrode was dipped into the beaker containing sample water and the conductance read by the conductometer was noted.

9.3.4 Colour

Colour was estimated through the UV spectrophotometric method.The absorbance given by the sample at 270nm was compared with the absorbance produced by the solution of the potassium chloroplatinate. The intensity of the colour was expressed in chloroplatinate colour unit.

Procedure

Stock colour standard was prepared by dissolving 1.2460gm of potassium chloroplatinate (K_2PtCl_6) and 1.00gm of crystallized cobaltous chloride ($CoCl_2.6H_2O$) in distilled water. To the solution 100ml concentrated hydrochloric acid was added and diluted with distilled water to 1000ml. This stock standard had the colour of 500units.

Working colour standards of 5, 10, 15, 20 colour units were prepared by taking 1, 2, 3 and 4 ml stock solution and adjusting their volume to 100ml with distilled water. These standards were protected against evaporation and contamination when not in use.

Adequate amount of sample was filtered through the membrane filter paper. Absorbance of standards and samples were read at 270nm using distilled water as reference.From the observed absorbency the amount of colour in the sample was calculated the by comparing it

with the absorbency produced by standard colour standards.

9.3.5 Turbidity

Turbidity of water samples was calculated through the nephlometric method.Reference for this method was taken from 2130 B, APHA - AWWA - WPCF 1989, 17th Edition

Turbidity in water is caused by the suspended matter, such as, clay; silt, finely divided organic and inorganic matters, soluble colored organic compounds, plankton and other microscopic organisms. The nephelometric method is based on a comparison of the light intensity of the scattered light under certain conditions with the intensity of the scattered light by the reference suspension solution under the same condition.

Turbidity of free water was assumed to be filtered distilled water.

Stock turbidity standards were prepared. The first solution was prepared by dissolving 1.00 gm hydrazine sulphate $(NH_2)_2.H_2SO_4$ in distilled water. The volume was then adjusted 100 ml with water. The second solution was prepared by dissolving 10.00 gm hexamethylenetetramine $(CH_2)_6N_4$ in distilled water and diluting it to 100 ml in a volumetric flask.

In a 100 ml volumetric flask, 5.0 ml of solution 1 and 5.00 ml of solution 2 was mixed. It was then allowed to stand for 24 hrs at 25 ± 3^oC. The solution was then diluted to 100 with turbidity free water. This solution would give a turbidity of 400 NTU.

Standard turbidity solution was prepared by diluting 5 and 10.00ml stock turbidity suspension in 100 ml of turbidity free water, which would give a turbidity of 20 NTU and 40 NTU

respectively.

Procedure

Allow the nephelometer to stabilise. The turbidity free water was poured into the turbidity tube and a zero value was set for the blank by rotating the zero knob. Then the standard of 40 NTU was poured into the turbidity tube and the reading was adjusted at 40 with the help of 100-adjustment knob. The 20 NTU standard was introduced into the turbidity tube and the turbidity was read in the nephelometer. It was read be exactly(or could be very close to 20). The sample was then introduced into the tube and the value of turbidity displayed by nephelometer was noted.

9.4 Chemical Parameters

9.4.1 Total Hardness as CaCO3

The EDTA titrimetric method was used for analysis. Reference for this method was taken from 2340 C, APHA - AWWA - WPCF 1989, 17th Edition

Hardness is mainly caused by the calcium and magnesium ions present in the given water. Beside that hardness is also caused by other polyvalent cations. Hardness in water sample is determined by version titration method.

Reagents

Standard calcium solution was prepared. For this, 1.0000gm anhydrous calcium carbonate ($CaCO_3$) powder was weighed and put in a 500ml conical flask. A funnel was placed in the flask (1 + 1) hydrochloric acid solution was slowly added until all the calcium carbonate ($CaCO_3$) had dissolved. To the solution 200ml distilled water was added and boiled for a few minutes to expel carbon dioxide (CO_2). It was allowed to cool, and a few drops of methyl red indicatorwas added. The colour of the solution was adjusted to the intermediate orange colour by adding 3N ammonia solution or (1 + 1) hydrochloric acid solution, as required. Into a 1000ml volumetric flask the solution was quantitatively transferred and diluted to 1000ml with distilled water. (1ml = 1.00mg $CaCO_3$).

0.01M EDTA solution was prepared. Ethylenediamine tetra acetic acid di-sodium salt, $[CH_2.N(CH_2.COOH)CH_2.COONa]_2.2H_2O$ was dried at 80°C for one hour and allowed to cool. In 1000ml distilled water3.7225gms of EDTA was dissolved. The solution was kept in a polythene bottle. The EDTA was standardised with standard calcium solution.

Buffer solution (pH = 10 ± 0.1) was prepared by dissolving 16.9gm ammonium chloride (NH_4Cl) in 143ml concentrated ammonia solution to which was added1.25 g of magnesium salt of EDTA that was then diluted to 250ml with distilled water.

Hydroxylamine hydrochloride solution (3 %) was prepared by dissolving3g hydroxylamine hydrochloride ($H_3NO.HCl$) in 100ml distilled water.

EBT indicator was prepared by dissolving 0.5gm sodium salt of 1 - (1 - hydroxy - 2 - naphthylazo) - 5 - nitro - 2 - naphthol - 4 - sulfonic acid i.e., erichrome balck T; No. 203 in the Colour Index dye in 100ml triethanolamine, N $(CH_2.CH_2.OH)_3$.

Procedure

Into a 250ml conical flask 50ml of the sample was pipetted. To the sample, 1 ml of 3% hydroxylamine hydrochloride solution, 2 ml buffer solution, and 2 drops of EBT indicator was added. The solution was then titrated against standard 0.01M EDTA solution till the wine red colour changed to blue, the end point (A). The blank was run with 50 ml distilled water with the same procedure (X).

Calculation:

Total hardness as $CaCO_3$, (mg/l) = (A - X) M * 2000

Where, M = Molarity of EDTA solution; A = Volume of standard 0.01M EDTA consumed by

the sample (ml); X = Volume of standard 0.01M EDTA consumed by the blank (ml).

9.4.2 Total Alkalinity as CaCO3

To calculate the total alkalinity of the water samples the pH metric titration method was used. The summation of phenolphthalein alkalinity and the methyl orange alkalinity gives the total alkalinity. Alternately the total alkalinity can be determined by titrating the sample against the standard acid to the methyl orange end point.

Reagents

Methyl orange indicator was prepared by dissolving 0.05gm of methyl orange indicator powder to 100ml of ethanol. Standard sulphuric acid (0.1N) was prepared by adding 2.8ml concentrated sulphuric acid (H_2SO_4) to distilled water. The volume was adjusted up to 1000ml with distilled water. This gives the sulphuric acid solution of approximately 0.1N strength. This acid solution was standardised against 0.1N sodium carbonate solution.Standard sulphuric acid solution (0.02N)was prepared by pipetting 100ml 0.1N sulphuric acid solution in a 500ml capacity volumetric flask. Volume was adjusted up to the mark with the addition of water and well shaken.Standard sodium carbonate solution (0.1N) was prepared by drying 6 m of anhydrous sodium carbonate at 250^{o}C for 4 hours. Approximately 5.000gm of the dried standard was weighed and dissolved in distilled water to make a volume of a litre.

Procedure

50ml sample was pipetted into a 250ml conical flask and 1-2 drop of methyl orange indicator was added. Titration with 0.02N standard sulphuric acid was done until the yellow colour changes to pink (up to pH 4.5).

Calculation

Total alkalinity as $CaCO_3$, (mg/l) = X *N * 1000

Where, N = Strength of standard sulphuric acid consumed during titration; X = Volume of acid consumed during titration (ml).

9.4.3 Chloride

The method used for the analysis of chloride in water was the argentometric titration method. The reference used for this method was 4500 - Cl - B, APHA - AWWA - WPCF 1989, 17^{th}Edition

In a neutral or slightly alkaline condition, potassium chromate can indicate the point of the silver nitrate titration of chloride. Before the formation of red silver chromate, silver chloride is precipitated quantitatively.

Reagents

Potassium chromate indicator solution (5 %) was prepared by dissolving 50gm potassium chromate (K_2CrO_4) in a little amount of distilled water. Silver nitrate ($AgNO_3$) solution was added until a definite red precipitate was formed. It was then allowed to stand for 12 hours, filtered, and diluted to one litre with distilled water.1000 ml of standard silver nitrate titrant (0.0141N) was prepared by dissolving 2.395gm silver nitrate ($AgNO_3$) in distilled water. It was standardized against standard sodium chloride (NaCl) solution.Standard sodium chloride solution (0.0141N) was prepared by dissolving 824.0mg sodium chloride (dried at 140^oC) in one litre of distilled water.

Procedure

A total of 50ml sample was pipetted into a 250ml conical flask. The pH was adjusted to between 7 and10 with either sulphuric acid or sodium hydroxide. To it was added 1ml of 5% potassium chromate indicator solution. It was then titrated against standard 0.0141N silver nitrate solution to a pinkish yellow end point (a). A blank was run with 100ml distilled water with the same procedure as above (b).

Calculation

Chloride, (mg/l) = (a - b) * N * 35.45 * 1000 / V

Where, a = Volume of standard silver nitrate consumed by the sample (ml); b = Volume of standard silver nitrate consumed by the blank (ml); N = Strength of standard silver nitrate solution; V = Volume of sample taken for analysis (ml).

9.4.4 Ammonia

For the analysis of ammonia in the water samples the method of nesslerisation was use.The reference used for this method was 4500 - NH_3 C, APHA - AWWA - WPCF 1989, 17^{th} Edition

Ammonia ion reacts withthe Nessler reagent and the intensity of the developed yellow colour is measured at 400 - 425nm. If the concentration of ammonia in the sample is high then it develops reddish brown hues on addition of Nessler reagent. The intensity of the developed colour can be measured at 450 - 500nm. The measurement at 450-500 nm enables to measure

the concentration up to the range of 10mg/l without dilution.

However Nessler reagent reacts with other l ions such as calcium, magnesium, iron and sulphides producing precipitates of these ions. Therefore presence these ions interferes the determination of ammonia. Interference from these ions can be eliminated by treating sample with zinc sulphate solution in an alkaline condition prior to Nesslerization.

If sample contains sufficient level of interfering ions then treat the sample with zinc sulphate solution (10%). Take 100ml of sample and add 1.0 ml of zinc sulphate heptahydrate solution. Then after add 0.4-.06 ml of 6N sodium hydroxide solution to make pH 10.5. Shake well and let stand to settle down the heavy flock. Centrifuge or filter and determine the ammonia by Nesslerization method.

Reagents

Rochelle salt solution was prepared by dissolving 50gm potassium sodium tartrate tetrahydrate, $KNaC_4H_4O_6.4H_2O$, in 100ml of distilled water. Ammonia, which is usually present in salt form, was removed by boiling off 30ml of the solution. After allowing it to cool it was diluted to 100ml. Nessler reagent was prepared by dissolving 100gm of mercuric iodide (HgI_2) and 70gm of potassium iodide (KI) in a small volume of distilled water and this mixture was slowly added while stirring to a solution of 160gm sodium hydroxide dissolved in 500ml distilled water. The solution was then diluted to a litre. Stock ammonia solution was prepared by dissolving 3.819gm anhydrous NH_4Cl, dried at 100^oC in distilled water, and diluted to 1000ml in a volumetric flask. 1.0ml=1.00mg-N=1.22mg NH_3. The working standard solution was diluted with10 ml of the stock solution to 1000cc with distilled water. 1.0 ml of

this solution = 10.00 µg N = 12.2µg NH_3.

Procedure

A solution with 50ml of filtered sample or / diluted sample was taken in one volumetric flask and into another flask distilled water was taken. The water was run as a blank. One drop of Rochelle salt solution was added in each volumetric flask followed by the addition of one ml of Nessler reagent. The solution was shaken and allowed to stand for 5 - 10 minutes for complete colour development. The absorbance of each aliquot was read at 420nm taking distilled water as a reference solution.

Calibration Curve

In to 50 ml capacity volumetric flasks, 0.0, 1.0, 2.0 and 3.0 ml of the working ammonia solution was pipetted. Water was added to make a total volume of 50 ml. This blank and standards were treated as samples and the absorbance was read after 5-10 minutes at 420nm.

Calculation

A standard curve was constructed by plotting absorbance due to ammonia against NH_3 - N concentration of standard and the sample concentration from the standard curve wasobtainedby using the following formula:

NH_3 - N, (mg/l) = (µg from curve * dilution factor) / 50

9.4.5 Nitrate

For the determination of nitrate in the water samples the UV spectrophotometric screening method was used. The reference for this method was 4500-NO3 B, APHA-AWWA-WPCF 1989, 17th Edition.This method is applicable for those samples, which are least contaminated with organic matters. Nitrate exhibits absorbency at 220nm however organic impurities also interferes for the determination exhibiting the absorbency at the same wavelength. This problem however can be over come by measuring the absorbance at 275 nm. Organic impurities give absorbency at 275nm also.

Reagents

Stock nitrate solution (100ppm) was prepared and for this potassium nitrate was dried in an oven at 105^{o}C for 24 hours. Then 0.7218gm of the dried potassium nitrate was dissolved in distilled water and diluted to 1 litre in a volumetric flask. Working nitrate standards were prepared and for this a series of working standards of 0.5ppm, 1.0ppm and 1.5ppm NO_3 - N was prepared by the dilution method.

Procedure

The spectrophotometer was set to the wavelength of 275 nm. The absorbency was set to zero using distilled water.The absorbance of standards as well as samples was read.The wavelength was set at 220 nm and the absorbency was adjusted to zero with distilled water.The absorbencies exhibited by standards and samples were then read.

Calculation

For samples and standards, the absorbency obtained at 275nm was deduced bymultiplying the absorbency obtained at 220nm by two. A standard curve was constructed by plotting the subtracted absorbencies against the standard concentration of NO_3 - N. The absorbency of the sample was inserted into the calibration to obtain the nitrate level in the sample.

9.4.6 Nitrite

To calculate the level of nitrite in the water samples, spectrophotometric method was used.

NEDA solution is added to the sample diazozied with sulphanilamide solution to produce the pink colour. Intensity of the pink colour is measured at 540nm to estimate the nitrite level of the sample.

Reagents

4 Aminobenzene sulphonamide solution was prepared and for this 2gm of 4 aminobenzene sulphonamide, $C_6H_8N_2O_2S$ was dissolved in a mixture of 60ml hydrochloric acid and 80ml distilled water. A volume of 200ml was made with distilled water.

N - 1 Napthylethylene diammonium dichloride (0.1 %) was prepared by dissolve 0.2 gm N - 1 napthylethylene diammonium dichloride ($C_{10}H_7HNCH_2CH_2NH_2.2HCl$) in 200ml distilled water.

Stock nitrite solution (250ppm, NO_2 - N) was prepared and for this sodium nitrite ($NaNO_2$) was dried at 105^oC - 110^oC for four hours and cooled in a desiccator. In distilled water, 1.232

gm of the dried sodium nitrite was dissolved and the volume was adjusted to 1000ml in a volumetric flask with distilled water.

Working nitrite standard solution (1ppm, NO_2 - N) was prepared with the required strength of nitrite - N standard solution from the above stock standard solution by dilution method.

Procedure

Turbidity of the sample was removed by filtering the sample through membrane filter paper. Of the filtered sample, 10ml was pipettedinto a dry test tube.Nitrite standard of 1 ppm was separately pipetted in the amounts of 0.5ml, 1ml and 1.5ml into three test tubes. Water was added in each test tube containing standard solution to make a final volume of 10ml.Similarly 10ml of distilled water for blankwas pipetted. One mililitre of 4 - aminobenzene sulphonamide was added to each test tube, mixed and allowed to stand for 5 minutes. Further, 1ml N - 1 napthylethylene diammonium dichloride was added, mixed well and allowed to stand for 20 minutes. The absorbance was read at 540nm.

Calculation

Calibration curve was constructed from the absorbance (solution absorbance - blank absorbance) read off the standard sample. The absorbance of the sample was located on the calibration curve and micrograms of nitrite concentration in the sample was calculated.

Nitrite as NO_2 - N, (mg/l) = Concentration from the graph / 10 = Y

Nitrite as NO_2, (mg/l) = Y * 46 / 14

Where, Y = Concentration of nitrite - N as calculated

9.4.7 Trace elements and Heavy metals

For the estimation of iron in the water samples two separate but related methods was used. The first method used was the Atomic Absorption Spectrophotometric (AAS) method.

Metals in the water and wastewater samples can be determined by flame atomic absorption / emission method. Samples are aspirated in to the flame and the absorbency given is recorded to estimate the level of trace metals in the sample.

It is necessary to treat the sample properly prior to estimation of the trace metals levels on it. The pre-treatment may be either the concentration of the sample or the complete acid digestion to convert undissolved metals to the solution. The concentration or the reduction in volume can be carried out by the slow evaporation of the sample over hot plate in an acidic condition.

Procedure

Procedure of the Concentration:

Approximately 100-150 ml of the sample was taken in a 250 ml capacity acid washed beaker. To it 1.0 ml of concentrated nitric acid was added and evaporated slowly over the hot plate.After adequate reduction in volume, it was cooled and the interior of the beaker was washed with small amount of water. The content was then transferred in a

volumetric flask (10 ml) and the beaker was washed with a small portion of the water. Each washing was transferred to the same volumetric flask and finally the volume was adjusted up to mark with water.

Procedure of the Sample Digestion:

Approximately 50-100 ml of the well-mixed sample was taken in a clean beaker. To the sample 5.0 ml concentrated nitric acid and a few boiling chips was added. It was then heated over a hot plate and slow evaporation was performed until the lowest possible volume was obtained. Heating by the addition of concentrated nitric acid was performed until a light coloured clear solution was obtained. After digestion, the beaker was cooled and the interior walls were washed down with small amount of water and then solution was filtered if found to be necessary. The filtrate was transferred into the volumetric flask and after cooling down to room temperature adjusted to the required volume with water.

Determination of the Metal Concentration:

After the pre treatment of the sample, determination of the trace metal level was carried out by direct air-acetylene flame method. The detailed procedure is given in the instrument manual book. Both emission and absorption mode can be used depending upon the element. Also consult Manufacturer's specification and Cookbook of the Atomic Absorption Spectrophotometeter (AAS).

The second method used in the detection of trace elements and heavy metals is the Hydride Generation - AAS Technique

Elements such as arsenic (III), Selenium (IV), Lead (II), Tin (II) etc., have the property to form the corresponding hydride by reaction with nascent hydrogen. The so formed hydride can be easily driven out from the solution by a current of inert gas and carried into the flame.

Sodium borohydride has been proved to be an effective reducing agent in terms of the speed and efficiency of reduction that can quickly and efficiently converts all the arsenic into its hydride.

Reagents

Reagents that were used to estimate the level of arsenic by AAS - Hydride Generator method included concentrated hydrochloric acid (AR / GR), sodium borohydride (SD's LR), arsenious oxide (AR), sodium hydroxide (GR), and potassium iodide (AR).

Working Solutions

Various working solutions were prepared and used during arsenic analysis by this method.20% Hydrochloric acid solution in distilled water was prepared by adding100 ml of the concentrated hydrochloric acid to 400 ml of the distilled water to make 500 ml 20% hydrochloric acid solution.2% Sodium borohydride in 0.05N sodium hydroxide solution was prepared by dissolving 2 gm sodium borohydride in 100 ml 0.05 N sodium hydroxide solution. Arsenic (III) solution was prepared and for this 0.1320gm of arsenic trioxide AR grade powder was weighed and transferred into a 100ml beaker. Minimum quantity of 1N

sodium hydroxide solution was added and the powder was dissolved completely. Then the solution was acidified with hydrochloric acid and the volume was made up to 100ml with distilled water. This was given the arsenic solution of 1000ppm concentration.One ml of the stock solution was taken in 100 cc volumetric flask and the volume was adjusted up to mark with 100 ml in a volumetric flask with 20% hydrochloric that was given 10ppm arsenic solution. From 10ppm secondary stock solution, working arsenic solution of 200 ppb was prepared by appropriate dilution with 20% hydrochloric acid.Potassium iodide 10% in distilled water was prepared by dissolving 50 g of potassium iodide salt in 500 ml distilled water.

Procedure

Pentavelent arsenic if presented was converted into its trivalent form by the reduction with potassium iodide. The trivalent arsenic was then reacted with sodium borohydride to generated AsH_3 vapor. Vapor of AsH_3 was carried out into the quartz tube by the current of nitrogen gas. Arsenic hydride was heated in to the quartz tube to decompose it into the elemental arsenic. Elemental arsenic so formed inside the quartz tube absorbed light of specific wavelength to produce corresponding wavelength.

The AAS unit was set up with arsenic lamp and it was optimized to give the maximum absorbance at 193.7 ηm.The stainless steel stand for quartz tube was fixed on the air / acetylene burner head with the four screws and the quartz 'T' tube was also arranged in such a manner that minimum absorbance was observed by manipulating vertical, horizontal and angular positions of the burner head.Nitrogen gas input was given to the hydride generator stand arranged over the monochromator part of the AAS unit and the nitrogen gas flow was

set to 1 liter per minute in the rotameter on the hydride generator stand at 1 kg/cm^2.The rubber stopper cum septum on the reaction vessel was removed and 20ml of 20% hydrochloric acid and 1ml of potassium iodide was added into the reaction vessel and it was stoppered again.The three-way stopcock on the hydride generator stand was manipulated to get nitrogen gas flow through the reaction vessel.The air / acetylene flame was lit and its height was reduced to just around the lift off position by adjusting acetylene flow.The quartz tube was allowed to heat uniformly for 10 minutes.Absorbance reading was adjusted to read position.Then 2 ml of borohydride was injected into the reaction vessel through the septum and the peak absorbance was recorded.The 3 - way stopcock was manipulated, so that nitrogen gas was flowed through the reaction vessel. The contents of reaction vessel were drained out and flushed with distilled water and allowed it to drain out. 20 ml of 20% hydrochloric acid, 1ml of potassium iodide and then 1 ml or 2 ml or 3 ml of 0.2 ppm arsenic solution was added into the reaction vessel. The inlet was stopperred well and the 3 ways stopcock was manipulated to get nitrogen gas flow through the reaction vessel.The absorbance reading was adjusted to a null absorbance and then 2ml borohydride solution was injected into the reaction vessel.The peak absorbance value was recorded.The above-mentioned 10 steps were followed and the analysis was continued with sample solutions. After obtaining absorbencies of blank and standard solutions calibration graph was constructed and the insertion of the sample absorbencies was made to estimate the concentration of arsenic into the sample.

9.5 Consent form for the case control study

Case-Control Study No.:……..

Patan Hospital

Information on the study of infectious diseases (Enteric Fever)

Contact person: Abhilasha Karkey

Introduction to the study

You (or your child) are being asked to be in a research study on enteric fever (typhoid fever). In the past few years various studies have been conducted through Patan Hospital to further enhance our understanding of infectious diseases, particularly enteric fever within the population. In our experience we have found that some areas report more number of cases of enteric fever while some areas report lesser amounts. In this study we are trying to understand why this phenomenon happens and what the probable risk factors to enteric fever are.

What we will do in this study

If you wish to take part in this study we will first identify and locate your residence and then map your residence with the help of a global positioning system. For this our CMAs (community medical assistants) will come to your house within the next few days with a hand held GPS recording machine. Your house co-ordinates will then be mapped. For this we need you to give us your detailed residential address.

Second thing we will do is ask you a series of questions pertaining to your socio-economic status, demography, personal hygiene habits, kind of water you use, kind of food you use etcetra. Your answers will help us to understand better how infectious diseases (enteric fever) is spreading within your residential area. The questionnaire will take approximately 45 minutes to fill.

Confidentiality

All the information that we receive from you will be kept confidential. Your name will not appear anywhere in the data as we will identify you with a study code number.

Risks

As there are no drug interventions or invasive methods involved in this study you have absolutely no risks being involved in this study.

Refusal to participate

You may refuse to participate at any given point of the study. Your refusal will not in any way interfere with your ability to receive proper medical care or attention within Patan Hospital.

Questions

If you have any questions about this study please feel free to ask the person administering this questionnaire or the person named at the top of this document.

Consent

I have fully been informed of the undertakings of this study and agree to participate in this study.

Name of patient: _____Date: _____Signature:_____

Name of interviewer: _____Date: _____Signature:_____

9.6 Questionnaire for the case control study

Patan Hospital-Oxford University Clinical Research Unit, Nepal

Section 1- Registration

1.1	Study Registration number		
1.2	Date of interview (Western) DD/MM/YYYY		
1.3	Interview done with	☐ Respondent ☐ Parent of respondent ☐ Relative of respondent ☐ Friend	

Section 2 – Identification and Demographic data

For Cases and Controls

2.1	Participant status	☐ Case ☐ Concurrent case ☐ Control ☐ Concurrent control	
2.2	If control or concurrent case, write the corresponding case's study ID number		
2.3	Name		
2.4	Sex	☐ Male ☐ Female	
2.5.1	Date of Birth (Western) (DD/MM/YYYY)		
2.5.2	Date of Birth (Nepali; if Western unknown)(DD/MM/YYYY)		
2.6	Religious group	☐ Hindu ☐ Buddhism ☐ Muslim ☐ Christianity ☐ Others _____	

2.7.1a	**Permanent** Address: Area		
2.7.1b	Address: Ward No.		
2.7.1c	Address: Town/Village		
2.7.1d	Address: District		
2.7.2a	**Temporary** Address: Area		
2.7.2b	Address: Ward No.		
2.7.2c	Address: Town/Village		
2.7.2d	Address: District		
2.8.1	Telephone number: Home		
2.8.2	Telephone number: Cell		
2.9	GPS locations		
2.10	Which address is the current address?	☐ Temporary ☐ Permanent	
2.11	How long have you been living at the current address?	☐ Less than 1 month ☐ Less than 2 months ☐ Less than 6 months ☐ Less than 1 year ☐ Less than 2 years ☐ More than 2 years	
2.12	Do you go back to your hometown from time to time?	☐ Yes ☐ No	
2.13	When was the last time you went back to your hometown?	_____ months ago	
2.14	How long did you stay in your hometown?	_____days/ months/ years	

2.15	Have you spent more than one month out of the last year living outside Kathmandu?	☐ Yes ☐ No	
2.16	If yes, for how long?	_____ days/ months/ years	
2.17	Why are you in Kathmandu?	☐ Study ☐ Work ☐ Business ☐ Personal	
2.18	Daily Activity	☐ Student ☐ Work ☐ Housewife ☐ Unemployed	
2.19	Education level of the respondent	☐ Illiterate ☐ Never attended primary school but can read and write ☐ Primary school not finished ☐ Primary school finished ☐ Junior high school ☐ Senior high school ☐ Higher education	

2.20	Type of profession of the respondent	☐ Student ☐ Worker ☐ Civil service ☐ Private company ☐ Entrepreneur ☐ Free professions (Doctor, lawyer, Consultant) ☐ Housewife ☐ Unemployed	
2.21	Field of profession of the respondent	☐ Agriculture ☐ Industry and manual labour ☐ Public utility (Electricity, gas and water) ☐ Construction ☐ Trade and commerce (shopkeeper) ☐ Transport, Distribution and communication ☐ Commerce, Finance, Insurance, Leasing ☐ Civil Service ☐ Armed forces ☐ Domestic work ☐ Food preparation: cook, street vendor, restaurant keeper, Street vendor ☐ Manufacturing (carpet factory, metal works) ☐ Other	

2.22	Location of work/ school of respondent		
2.22.1a	Work Address: Area		
2.22.1b	Ward No.		
2.22.1c	Town/Village		
2.22.1d	District		
2.23	How long have you been studying/ working here?	☐0-6 months ☐Less than one year ☐Less than 2 years ☐ More than 2 years	

For Cases

2.24	Hospital number		
2.25	Laboratory number		

2.26	Microbiology result	☐ *S.* Typhi ☐ *S.* Paratyphi A ☐ *S.* Paratyphi B ☐ *S.* Paratyphi C ☐ Other Salmonellae ☐ No growth ☐ Other	

Section 3 – Questionnaire

3.1	Did you / the child have fever in the past three days?	☐Yes ☐No ☐Not sure		
3.2	If yes, what was the pattern of fever	☐Continuous ☐Intermittent ☐Remittent		
3.3	3.4	Did you/ the child have any of the following symptoms in the past week?	Fever at night Y/N Progressive fever Y/N Fever longer than 2 weeks Y/N Anorexia Y/N Nausea Y/N Abdominal pain Y/N Diarrhea Y/N Constipation Y/N Rectal blood loss Y/N Cough Y/N Headache Y/N Impaired consciousness Y/N Petechiae and rose spots: skin spots Y/N Dark colour of urine Y/N Rhinorrhea Y/N	

3.5	Do you/ the child have any existing chronic illness?	☐Yes ☐No ☐Not sure	
3.6	Do you know what typhoid fever is?	☐Yes ☐No ☐ Not sure	
3.7	Can you name 3 of the most prominent symptoms in order of severity?	☐ Yes ☐ No ☐ Choices _____	

3.8	Do you know where you catch it from?	Ill people Y/N/DK Food Y/N/DK Water Y/N/DK Air Y/N/DK Body fluids Y/N/DK Mosquito Y/N/DK Fly Y/N/DK Human faeces Y/N/DK Coughing Y/N/DK Hands Y/N/DK Animal faeces Y/N/DK Rats Y/N/DK Cockroach Y/N/DK Unsafe sex Y/N/DK	
3.9	Did you/ child have typhoid fever before?	☐ Yes ☐ No ☐ Not sure	

3.10	If yes, was it culture confirmed?	☐ Yes ☐ No ☐ Not sure	
3.11	Did anybody in the household have typhoid fever in the past 8 weeks?	☐Yes ☐No ☐Not sure	
3.12	Did you/ the child have contact with typhoid patient in past 8 weeks?	☐Yes ☐No ☐Not sure	
3.13	Did you/ the child visit a health care provider in the past one week?	☐Yes ☐No ☐Not sure	
3.14	Did you/ the child receive any medication in the past week for this episode of fever?	☐Yes ☐No ☐Not sure	
3.15	What type of medicine did you/ the child take?	☐ Don't know ☐ Antibiotic ☐ Antipyretic ☐ Others……..	

3.16	What antibiotics have you/ the child received for this episode of fever?	☐ Don't know ☐ Chloramphenicol ☐ Amoxicillin ☐ Cefixime ☐ Ciprofloxacin ☐ Ceftriaxone ☐ TMP-SMX ☐ Azithromycin ☐ Ofloxacin ☐ Other _____	
3.17	For how many days did you/ the child take the medication?	Days	
Socio economic conditions			
3.18	With whom do you live at the moment?	☐ Together with family ☐ With friends ☐ Alone ☐ Orphanage ☐ Hostel ☐ Others ……	
3.19	Who is the owner of the house	☐ Respondent ☐ Family ☐ Rent ☐ Others ……..	
3.20	What is the rent per month	Rupees …….	

3.21	Is your house affected by rainfall in the monsoons?	☐ Yes ☐ No ☐ Only some years with heavy rainfall	
3.22	How is your house affected during the rainfall?	☐ Mild flooding ☐ Complete flooding of the ground floor ☐ Others. Define	
3.23	Is your neighbourhood affected by rainfall in the monsoons?	☐ Yes ☐ No ☐ Only some years with heavy rainfall	
3.24	How is your neighbourhood affected during the rainfall?	☐ Mild flooding ☐ Severe flooding ☐ Others. Define	
3.25	Do you notice overflowing water pipes or sewage pipes in your neighbourhood during the monsoons?	☐ Yes ☐ No ☐ Have not noticed	
3.26	Is there an open garbage disposal point next to/ near your house?	☐ Yes ☐ No ☐ Have not noticed	
3.27	Do people or animals defecate on the streets in your neighbourhood?	☐ Yes ☐ No ☐ Have not noticed	

3.28	How many people live in your household?		
3.29	How many children are in your household?		
3.30	How many people sleep in one room?		
3.31	Do you have a radio?	Yes/No	
3.32	Do you have a TV?	Yes/No	
3.33	Do you have a bicycle?	Yes/ No	
3.34	Do you have a motorbike?	Yes/No	
3.35	Do you have a car?	Yes/No	
3.36	Do you have a fridge?	Yes/No	
3.37	Do you have a telephone?	Yes/No	
3.48	Do you have a mobile phone?	Yes/No	
3.39	How many family members are earning? (Any contribution counts)		

3.40	What is the total household monthly income?	☐ Less than 3000 NRs ☐ Less than 5000 NRs ☐ Less than 10000 NRS ☐ Less than 15000 NRs ☐ Less than 20000 NRs ☐ More than 20000 NRs	
3.41	How many people depend on the income?		
3.42	What is the average monthly expenditure of the household?	☐ Less than 3000 NRs ☐ Less than 5000 NRs ☐ Less than 10000 NRS ☐ Less than 15000 NRs ☐ Less than 20000 NRs ☐ More than 20000 NRs	
Water source			

3.43	What is the main source of drinking water at home?	☐ Jar (what is a jar?) ☐ Mineral (bottled) water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Private company water ☐ River water ☐ Other. Define_____	
3.44	Does your house have municipal supplied water?	☐ Yes ☐ No	
3.45	If yes, how many days a week do you get municipal supply?	☐ Once ☐ Twice ☐ Thrice ☐ Four times ☐ Don't know	
3.46	Do you store the municipal supplied water?	☐ Yes ☐ No	
3.47	If no municipal supply/ or if municipal supply is not enough, where do you get your water from?	☐ Stone spout ☐ Well ☐ Private water company ☐ River ☐ Rainfall	

3.48	Is water stored after collection from the source?	Yes/No	
3.49	Do you have a major storage area?	Yes/No	
3.50	If yes, what do you store it in	☐ Plastic storage tanks ☐ Concrete storage tanks ☐ Metal storage tanks ☐ Others……..	
3.51	Just before use, what do you store it in?	☐ Jerry can ☐ Gagri ☐ Surai ☐ Bottles ☐ Jars ☐ Buckets ☐ Kitchen utensils ☐ Others……..	
3.52	What is the mouth of the container like?	☐ Narrow ☐ Wide	
3.53	Is the stored water container covered?	☐ Yes ☐ No	

3.54	What is the material of the storage container?	☐ Clay ☐ Concrete ☐ Plastic ☐ Aluminum ☐ Copper ☐ Brass ☐ Others……..	
3.55	Do you know the location of your nearest water spout (name)	☐Yes ☐ No ☐ Name……	
3.56	Are major water storage areas cleaned?	☐Yes ☐ No ☐ Don't know	
3.57	If yes, how often?	☐ Monthly ☐ Twice a year ☐ Annually ☐ Never	
3.58	How do you treat the drinking water at home?	☐ Do not treat ☐ Boil ☐ Filter ☐ Chlorinate ☐ SODIS ☐ Others……	

3.59	What is the source of water for household chores (washing dishes, clothes etc)?	☐ Collected rain water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Private water company ☐ Others……..	
3.60	What is the source of water for washing your hands before eating?	☐ Collected rain water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Water bought from private company	
3.61	What is the source of water for washing your hands after defecation?	☐ Collected rain water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Water bought from private company	

3.62	What water do you use for bathing?	☐ Collected rain water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Water bought from private company	
3.63	What water do you drink at work/ school?	☐ Jar ☐ Mineral (bottled) water ☐ Stone Spout ☐ Well ☐ Hand pump ☐ Piped water supply ☐ Private company water ☐ River water ☐ Other. Define _____	
Personal Hygiene			
3.64	Does water shortage affect your daily activities (bathing, washing clothes etc)	Yes/No	

3.65	Do you wash your hands before eating?	☐ Always ☐ Often ☐ Sometimes ☐ Never	
3.66	If yes, how do you wash your hands?	☐ Water and soap ☐ Water only ☐ Ash	
3.67	Do you wipe your hands after washing?	Yes/No	
3.68	If yes, what do you wipe it on?	☐ Towel ☐ Clothes ☐ Other	
3.69	What is the type of latrine used by the household?	☐ No toilet present ☐ Household latrine ☐ Community latrine ☐ River ☐ Gutter ☐ Field	
3.70	If a latrine is used, where is it located?	☐ Indoor ☐ Outdoor	
3.71	If a latrine is used, how many people share it?		
3.72	Does it have water flushing system?	☐ Yes ☐ No	

3.73	Do you wash hands after defecation?	☐Yes ☐No	
3.74	What do you wash your hands with?	☐ Soap ☐ Only water ☐ Ash ☐ Others……..	
Eating habits			
3.75	Do you have a kitchen?	☐ Yes ☐ No	
3.76	How many people share the kitchen?		
3.77	Do you eat with your hands?	☐ Always ☐ Often (more than half the time) ☐ Seldom ☐Never	
3.78	Who prepares food in the household?	☐ No one, eat out ☐ Respondent ☐ Family member ☐Other household member ☐ Domestic help	
3.79	Do you wash your hands before preparing food?	☐ Always ☐ Often ☐ Never	

3.80	What do you wash your hands with?	☐ Soap ☐ Water ☐Do not wash	
3.81	What is the source of water for washing your hands before food preparation?	☐ Municipal supply tap water ☐ Private water company provided tap water ☐ Protected well ☐ Uncovered well ☐ Stone spout ☐ River water ☐ Collected rain water ☐ Stored water ☐ Others……..	
3.82	What is the source of water for washing your vegetables, fruits and kitchen utensils?	☐ Municipal supply tap water ☐ Private water company provided tap water ☐ Protected well ☐ Uncovered well ☐ Stone spout ☐ River water ☐ Collected rain water ☐ Stored water ☐ Others……..	

3.83	How often do you eat cooked food sold by street vendors?	☐ Never ☐ ____ times/day ☐ ___ times/week ☐ ___times/month ☐ Not sure	
3.84	How often do you eat sliced fruit sold by street vendors?	☐ Never ☐ ____ times/day ☐ ___ times/week ☐ ___times/month ☐ Not sure	
3.85	How often do you eat ice cream sold by street vendors?	☐ Never ☐ ____ times/day ☐ ___ times/week ☐ ___times/month ☐ Not sure	
3.86	How often do you eat milk or dairy products from street vendors?	☐ Never ☐ ____ times/day ☐ ___ times/week ☐ ___times/month ☐ Not sure	
3.87	Where do you get your milk or dairy products from?	☐ DDC ☐ ND ☐ Grocery shop ☐ Private farmer ☐ Not sure	

3.88	How often do you eat pani puri, chat sold by street vendors?	☐ Never ☐ ____ times/day ☐ ___ times/week ☐ ___times/month ☐ Not sure	
3.89	Did you recently (last two weeks) eat food sold by street vendors?	☐ Yes ☐ No ☐ Not Sure	
3.90	Did you recently (last two weeks) eat at a restaurant?	☐ Yes ☐ No ☐ Not sure	
3.91	Do you buy food at street vendors to eat at home?	☐ Yes ☐ No	
3.92	If yes, how often?	☐ Every day ☐ 3-4 times weekly ☐ Once a week ☐ Less than once a week	
3.93	Do you use ice cubes in your drinks?	☐ Yes ☐ No	
3.94	Where do you get your ice cubes from?	☐ From own fridge ☐ From the restaurant ☐ From the ice vendor	

3.95	How do you make your ice cubes?	☐ Mineral water ☐ Boiled water ☐ Untreated tap water ☐ Untreated other water	

Section 4 – Vaccination History

4.1	Have you ever received vaccines for typhoid?	☐ Yes ☐ No ☐ Not sure	
4.2	If yes, where did you/ the child receive the vaccine?	☐ School ☐ Hospital _____ ☐ Clinic _____ ☐ Other _____	
4.3	If yes, what was the date in which you/ the child received the vaccine (Western date dd/mm/yyyy)		
4.4	Did anyone else in the household receive typhoid vaccine?	☐ Yes ☐ No	
4.5	If yes, how many people received the vaccine?		

Section 5 – Administration

5.1	Was the interview complete?	☐ Yes ☐ No	
5.2	If no, what was / were the reason(s)?	☐ Refusal ☐ Time constraints ☐ Comprehension ☐ Lack of interests ☐ Other ……	

9.7 Antimicrobial sensitivity testing through disk diffusion and MIC determination through E test.

Study ID	Organism	E test						Disc diffusion					
		NA	C	CTX	GAT	CIP	OFX	NA	C	CTX	GAT	CIP	OFX
01	Salmonella Typhi	R	3	0.25	0.094	0.25	0.38	R	26	31	26	23	22
02	Salmonella Paratyphi A	R	4	0.125	0.38	0.25	1	R	25	32	25	25	22
03	Salmonella Typhi	R	3	0.125	0.94	0.125	0.38	R	30	34	30	28	27
04	Salmonella Typhi	R	3	0.125	0.094	0.19	0.38	R	27	31	28	26	27
05	Salmonella Typhi	R	3	0.19	0.094	0.19	0.38	R	26	34	29	27	27
06	Salmonella Paratyphi A	R	4	0.19	0.38	0.38	1	R	26	36	24	25	21
07	Salmonella Paratyphi A	R	4	0.19	0.38	0.25	1	R	26	33	26	26	23
08	Salmonella Typhi	R	2	0.094	0.094	0.19	0.38	R	30	36	28	22	25
09	Salmonella Paratyphi A	R	4	0.19	0.38	0.5	1	R	28	32	26	26	24
10	Salmonella Paratyphi A	R	3	0.125	0.125	0.25	0.75	R	31	33	26	26	24
11	Salmonella Typhi	R	2	0.125	0.064	0.25	0.25	R	32	30	28	25	25
12	Salmonella Typhi	1	2	0.064	0.006	0.004	0.016	30	33	32	35	36	34
13	Salmonella Typhi	R	3	0.19	0.094	0.19	0.38	R	29	37	31	28	27
14	Salmonella Paratyphi A	R	4	0.25	0.38	0.5	1.5	R	27	34	26	27	24
15	Salmonella Paratyphi A	R	6	0.19	0.25	0.38	1	R	30	32	26	25	20
16	Salmonella Typhi	1	2	0.064	0.006	0.004	0.023	32	30	39	37	40	38
17	Salmonella Paratyphi A	R	3	0.125	0.38	0.25	0.75	R	25	35	25	24	23
18	Salmonella Paratyphi A	R	3	0.094	0.25	0.19	0.75	R	26	35	25	25	22
19	Salmonella Paratyphi A	R	4	0.125	0.25	0.38	0.75	R	25	33	24	25	21
20	Salmonella Paratyphi A	R	3	0.19	0.25	0.5	1	R	27	33	24	26	22
21	Salmonella Paratyphi A	R	4	0.19	0.38	0.38	1	R	23	29	23	22	20
22	Salmonella Typhi	96	1.5	0.25	0.094	0.25	0.38	R	27	30	27	24	26
23	Salmonella Paratyphi A	R	3	0.19	0.28	0.38	1	R	24	30	26	25	22
24	Salmonella Paratyphi A	R	6	0.125	0.38	0.5	1	R	24	29	23	22	21
25	Salmonella Paratyphi A	R	4	0.25	0.38	0.5	1.5	R	23	28	22	21	19
26	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1.5	R	25	30	24	23	21

Study ID	Organism	E test						Disc diffusion					
27	Salmonella Typhi	R	3	0.19	0.125	0.38	0.38	R	26	28	27	25	24
28	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1	R	26	29	25	23	23
29	Salmonella Paratyphi A	R	3	0.19	0.25	0.38	1	R	25	30	24	24	22
30	Salmonella Paratyphi A	R	4	0.19	0.38	0.5	1	R	23	31	23	22	21
31	Salmonella Typhi	R	4	0.125	0.094	0.25	0.38	R	26	32	27	24	25
32	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1	R	22	31	23	22	20
33	Salmonella Paratyphi A	R	3	0.125	0.25	0.38	1	R	25	33	24	24	21
34	Salmonella Paratyphi A	R	3	0.19	0.25	0.38	1	R	23	30	25	24	21
35	Salmonella Paratyphi A												
36	Salmonella Paratyphi A												
37	Salmonella Paratyphi A	R	4	0.125	0.38	0.5	1	R	25	33	24	25	26
38	Salmonella Paratyphi A	R	3	0.125	0.25	0.38	1	R	23	31	24	23	20
39	Salmonella Typhi	R	3	0.19	0.094	0.25	0.38	R	27	34	31	27	27
40	Salmonella Typhi	R	2	0.19	0.125	0.38	0.38	R	26	33	26	23	24
41	Salmonella Typhi	128	3	0.094	0.064	0.125	0.25	R	30	36	29	27	25
42	Salmonella Paratyphi A	R	4	0.125	0.38	0.38	1	R	22	30	24	22	19
43	Salmonella Typhi	R	3	0.125	0.125	0.38	0.38	R	26	30	27	25	22
44	Salmonella Typhi	R	4	0.19	0.094	0.25	0.38	R	25	32	28	25	23
45	Salmonella Typhi	1	2	0.064	0.006	0.008	0.023	33	25	31	34	36	31
46	Salmonella Paratyphi A	R	4	0.125	0.38	0.5	1	R	22	31	23	22	18
47	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1	R	26	34	24	24	21
48	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1	R	24	30	23	22	20
49	Salmonella Typhi	96	3	0.25	0.094	0.25	0.38	R	28	31	27	24	24
50	Salmonella Typhi	64	3	0.064	0.064	0.125	0.25	R	25	30	26	25	24
51	Salmonella Typhi	128	3	0.19	0.094	0.38	0.38	R	28	33	27	25	24
52	Salmonella Paratyphi A	R	3	0.25	0.38	0.38	1	R	21	32	25	23	21
53	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	1	R	26	35	26	26	24
54	Salmonella Typhi	R	3	0.19	0.094	0.25	0.25	R	29	34	32	29	27
55	Salmonella Paratyphi A	R	4	0.19	0.38	0.25	1.5	R	25	37	26	25	22

56	Salmonella Paratyphi A	R	4	0.125	0.38	0.38	1	R	27	33	26	26	24
Study ID	Organism	E test						Disc diffusion					
57	Salmonella Paratyphi A	R	4	0.19	0.38	0.38	0.75	R	27	34	25	24	22
58	Salmonella Paratyphi A	R	3	0.19	0.38	0.38	0.75	R	26	36	25	26	22
59	Salmonella Typhi												
60	Salmonella Typhi	R	3	0.125	0.064	0.19	0.25	R	27	34	28	25	26
61	Salmonella Typhi	R	2	0.125	0.064	0.19	0.25	R	28	34	30	29	26
62	Salmonella Paratyphi A	R	3	0.125	0.25	0.25	1	R	26	27	24	25	21
63	Salmonella Paratyphi A	R	3	0.19	0.25	0.25	0.75	R	26	31	23	24	20
64	Salmonella Paratyphi A	R	3	0.125	0.38	0.25	0.75	R	24	32	24	24	22
65	Salmonella Typhi	R	2	0.125	0.094	0.125	0.19	R	28	34	30	26	27
66	Salmonella Paratyphi A												
67	Salmonella Typhi	96	1.5	0.19	0.094	0.19	0.25	R	27	35	28	25	26
68	Salmonella Paratyphi A												
69	Salmonella Typhi	96	1.5	0.125	0.047	0.125	0.19	R	29	32	29	26	25
70	Salmonella Typhi												
71	Salmonella Typhi												
72	Salmonella Paratyphi A	R	3	0.094	0.25	0.19	0.5	R	25	34	23	23	22
73	Salmonella Paratyphi A												
74	Salmonella Typhi	R	3	0.19	0.094	0.25	0.38	R	28	34	28	24	28
75	Salmonella Typhi	R	3	0.19	0.125	0.25	0.38	R	27	30	29	25	26
76	Salmonella Paratyphi A	R	3	0.125	0.25	0.38	0.75	R	24	38	29	27	23
77	Salmonella Paratyphi A	R	4	0.094	0.125	0.25	0.38	R	25	40	43	28	26
78	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	0.75	R	24	34	24	23	21
79	Salmonella Paratyphi A	R	6	0.25	0.38	0.5	0.75	R	24	32	23	24	21
80	Salmonella Typhi	R	3	0.19	0.125	0.19	0.38	R	27	31	28	24	25
81	Salmonella Typhi	R	2	0.19	0.064	0.19	0.25	R	28	30	27	24	23
82	Salmonella Paratyphi A	R	4	0.19	0.38	0.25	0.75	R	26	35	25	26	23
83	Salmonella Typhi	R	3	0.125	0.094	0.25	0.25	R	27	31	28	27	25
84	Salmonella Typhi												

85	Salmonella Paratyphi A	R	3	0.19	0.25	0.25	0.75	R	26	32	25	25	20
86	Salmonella Paratyphi A	R	4	0.19	0.38	0.5	0.75	R	24	31	24	25	22
Study ID	**Organism**	E test						Disc diffusion					
87	Salmonella Typhi	128	2	0.25	0.064	0.25	0.25	R	25	31	30	28	26
88	Salmonella Typhi	R	2	0.19	0.094	0.19	0.25	R	29	32	28	24	27
89	Salmonella Typhi	R	2	0.19	0.094	0.19	0.38	R	27	31	26	25	24
90	Salmonella Typhi	R	3	0.19	0.094	0.19	0.38	R	27	30	26	25	25
91	Salmonella Typhi												
92	Salmonella Typhi	R	2	0.125	0.094	0.19	0.25	R	27	31	26	24	26
93	Salmonella Typhi	R	3	0.19	0.094	0.19	0.25	R	25	30	26	24	25
94	Salmonella Typhi	R	3	0.19	0.064	0.19	0.25	R	25	31	28	24	24
95	Salmonella Typhi	R	2	0.125	0.064	0.19	0.25	R	21	30	25	23	24
96	Salmonella Paratyphi A	R	4	0.19	0.38	0.25	1	R	23	35	25	24	22
97	Salmonella Typhi	R	2	0.125	0.125	0.19	0.38	R	26	30	26	24	23
98	Salmonella Paratyphi A	R	4	0.19	0.38	0.38	1	R	26	32	21	23	19
99	Salmonella Typhi	128	2	0.094	0.094	0.094	0.25	R	30	35	31	29	29
100	Salmonella Paratyphi A	R	4	0.125	0.38	0.38	1	R	24	36	25	25	21
101	Salmonella Typhi	1.5	4	0.094	0.008	0.006	0.023	27	30	33	38	38	34
102	Salmonella Paratyphi A	R	4	0.19	0.25	0.38	0.75	R	25	31	27	24	23
103	Salmonella Typhi	1	3	0.094	0.016	0.004	0.125	31	27	34	33	40	34