CHAPTER -I

1. INTRODUCTION

The gamma subdivision proteobacteria *Salmonella enterica*, with its various serovars, has considerable importance with respect to infectious disease, public health and basic microbiology research (Noordewier and Brown, 2002). Typhoid fever caused by infection with *Salmonella enterica* serovar Typhi (*S.* Typhi hereafter) causes an estimated 21.6 million cases annually, with 220 000 deaths and remains a major health problem in developing countries (Bhan *et al.*, 2005; Crump *et al.*, 2004; Parry *et al.*, 2002). *Salmonella enterica* serovar Paratyphi A (*S.* Paratyphi A hereafter) causes additional 5·4 million illnesses and is emerging cause of enteric fever in south Asian countries. Enteric fever (typhoid and paratyphoid fever) has a very high social and economic impact because of the hospitalization of patients with acute disease, severe complications and loss of income attributable to the duration of the clinical illness (Punjabi, 1998). Enteric fever caused by *S.* Typhi and *S.* Paratyphi A is the most common clinical diagnosis among febrile patients presenting to hospital in Nepal (Maskey *et al.*, 2008; Pokhrel *et al.*, 2006; Shirakawa *et al.*, 2006; Malla *et al.*, 2005; Murdoch *et al*., 2004).

Although the first successful definition of typhoid fever was given by William Jenner in the mid-1800s, treatment was unavailable until the discovery of chloramphenical in 1948 (Woodward *et al.*, 1948). Traditional drugs such as chloramphenicol, ampicillin and co-trimoxazole were most effectively used as first-line drugs for the treatment of enteric fever (Bhutta, 2006; Bhan *et al.*, 2005). However, during the late 1980s and early 1990s the occurrence of multidrug-resistant (MDR) *S.* Typhi and *S.* Paratyphi A strains resistant to chloramphenicol, ampicillin and co-trimoxazole, led to the use of fluoroquinolones (FQs), in particularly ciprofloxacin, and third generation cephalosporins for the treatment of enteric fever (Bhutta, 2006; Bhan *et al.*, 2005; Threlfall *et al.*, 1992).

The widespread use of fluoroquinolones led to increased rates of *Salmonella enterica* strains with reduced susceptibility to fluoroquinolones that were being reported more frequently, particularly in Europe, Asia, and Africa (Molbak *et al.*, 2002; Hakanen *et al.*, 2001; Threlfall *et al.*, 1999, 2001). Although the development of high-level fluoroquinolone resistance in *Salmonella enterica* remains rare, strains with elevated minimum inhibitory concentration (MIC) values associated with point mutations in the quinolone resistance-determining region (QRDR) and/or active efflux (Guerra *et al.*, 2003; Ling *et al.*, 2003; Nakaya *et al.*, 2003; Giraud *et al.*, 2000) are common. It has been demonstrated that single *gyrA* mutations mediate resistance to nalidixic acid and increase the MIC to broader-spectrum fluoroquinolones and a further mutation in gyrA with or without in *parC* leads to strains highly resistant to fluoroquinolones (Renuka *et al.*, 2004; Ling *et al.*, 2003; Nakaya *et al.*, 2003; Heurtin *et al.*, 1999). It has also been observed that fluoroquinolone treatment, especially with short courses or low-dose regimens, for strains with elevated fluoroquinolone MIC values, although susceptible using current Clinical and Laboratory Standards Institute (CLSI, formerly the National Committee for Clinical Laboratory Standards [NCCLS]) breakpoint criteria, have led elevated rates of clinical failure (Bhan *et al.*, 2005; Nkemngu *et al.*, 2005; Rupali *et al.*, 2004; Kristiansen *et al.*, 2003; Piddock *et al.*, 1993).

Nepal faced a series of enteric fever epidemics over the last decade (Maskey *et al.*, 2008; Lewis *et al.*, 2005) with changing resistance patterns (Malla and Dumre, 2008). The first report of MDR *S.* Typhi in Nepal was published in 1991 (Watson and Pettibone, 1991). In the following years, with the introduction of fluoroquinolones in the treatment, nalidixic acid-resistant strains associated with reduced susceptibility to fluoroquinolones have been continuously reported from Nepal and trend of resistance is increasing (Maskey *et al.*, 2008; Shirakawa *et al.*, 2006). Subsequently, occasional isolation of highly FQ-resistant and third generation cephalosporins resistant *S.* Paratyphi A and *S.* Typhi strains has also been reported from Nepal (Chau *et al.*, 2007; Pokherel *et al.*, 2006) and other developing

countries such as India and Bangladesh (Ahmed *et al.*, 2006; Saha *et al.*, 2006; Renuka *et al.*, 2005).

Reduced susceptibility to fluoroquinolones not being detected by disc diffusion tests even strains are still considered susceptible according to the CLSI interpretive criteria led to discussions regarding whether the current fluoroquinolones breakpoint criteria used for *Salmonella* spp. remain appropriate (Aarestrup *et al.*, 2003; Crump *et al.*, 2003). The use of nalidixic acid disc diffusion test has been recommended by CLSI (CLSI, 2005) to screen reduced susceptibility to fluoroquinolones as later is well correlated to the high-level resistance to nalidixic acid, which has been studied and reported by several investigators (Albayrak *et al.*, 2004; Oteo *et al.*, 2000; Hakanen *et al.*, 1999).

Although fluoroquinolones, if susceptible, may still be useful for the treatment, clinicians should be aware of the possibility of treatment failures of infections with *S.* Typhi and *S.* Paratyphi A strains with reduced fluoroquinolone susceptibility which may not be eradicated by fluoroquinolone treatment (Crump *et al.*, 2003; Threlfall *et al.*, 2001). So, it is important to monitor the increase in the MIC of fluoroquinolones in *S.* Typhi and the emerging strains of *S.* Paratyphi A, which is worrying but not surprising, given that ciprofloxacin and ofloxacin are the most widely used antibiotic for the treatment of enteric fever in developing countries like Nepal, where it is available from pharmacies without prescription by physicians (Woods *et al.*, 2006).

The changing antimicrobial resistance pattern, new approaches to treatment, and control strategies in *Salmonella* infections are the emerging issues. Community and hospital-based studies in different Asian and African countries show changing antimicrobial susceptibility pattern in *S.* Typhi and Paratyphi A with decline in multidrug resistance in most areas, high incidence of reduced fluoroquinolone susceptibility, and increasing report of isolates with full fluoroquinolone resistance and resistance to third generation cephalosporins. Continual surveillance of resistance levels is critical for clinicians to guide appropriate therapy based

on the existing resistance trend and for policy makers to implement control strategies. This study aimed to evaluate antimicrobial resistance pattern along with fluoroquinolone susceptibility of *S.* Typhi and emerging strain of *S.* Paratyphi A which are the major cause of enteric fever in Nepal.

CHAPTER-II

2. OBJECTIVES

2.1 General objective

Determination of antibiotic susceptibility pattern of *Salmonella* isolated from enteric fever patients.

2.2 Specific objectives

- To isolate and identify *Salmonella enterica* serovars causing enteric fever from blood specimens.
- To determine the antimicrobial susceptibility pattern of *Salmonella* isolates.
- To determine the Minimum Inhibitory Concentration (MIC) of fluoroquinolones in *Salmonella* isolates.
- To compare the MIC and disc diffusion test result in ciprofloxacin and ofloxacin with that of nalidixic acid.
- To analyse the validity of nalidixic acid screeing test to determine reduced susceptibility to fluoroquinolones.

CHAPTER-III

3. LITERATURE REVIEW

3.1. Enteric fever

3.1.1 Global burden

Although enteric fever is a global health problem, its real impact is difficult to estimate because the clinical picture is confused with those of many other febrile infections. Additionally, the disease is underestimated because microbiologic facilities often are unavailable and epidemiologic studies with active surveillance are difficult and expensive to perform in most areas of developing countries (WHO, 2003).

Typhoid fever is estimated to have caused 21·6 million illnesses and 216 500 deaths per year globally in 2000 (Crump *et al.*, 2004). The incidence of typhoid fever was high (>100 cases per 100 000 population per year) in south-central Asia, southeast Asia, and southern Africa, medium (10–100 cases per 100 000) in the rest of Asia, Africa, Latin America, and Oceania, except for Australia and New Zealand, and low in the other parts of the world (<10 cases per 100 000) (Bhan *et al.*, 2005). However, the estimates have been biased because study populations is focused in data from only a few countries having high incidence, with only one study providing data from Africa (Bhan *et al.*, 2005).

Where typhoid is endemic, most cases are children aged 5–19 years and young adults. Recent population based studies from India, Indonesia, and Vietnam suggest that in some settings typhoid fever is also common in 1–5 year-old children (Lin *et al.*, 2001; Lin *et al.*, 2000; Sinha *et al.*, 1999; Simanjuntak *et al.*, 1991). Data from hospital-based studies in Bangladesh and Thailand (Saha *et al.*, 2001; Thisyakorn *et al.*, 1987) also support these findings. Surveillance data from the USA indicate that the proportion of cases of typhoid is constant over the first 25 years of life (Crump *et al.*, 2004). Typhoid fever is more common in urban than in rural areas, but rural area in Nepal and Vietnam found a high disease burden (Lin *et al.*, 2001; Lin *et al.*, 2000; Acharya *et al.*, 1987). Most cases in developed countries arise in travelers returning from endemic countries, but domestically acquired cases are also reported (Ackers *et al.*, 2000).

Paratyphoid fever is estimated to have caused an additional 5·4 million illnesses in 2000 (Crump *et al.*, 2004). This number is based on an estimated one case of paratyphoid fever for every four cases of typhoid fever. Studies from China, Pakistan, India and Nepal suggest that in some settings and times, paratyphoid fever caused by *S.* paratyphi A can contribute up to half of all the enteric fever cases (WHO 2003; Sood *et al.*, 1999; Shlim *et al.*, 1995).

3.1.2 Transmission and risk factor

S. Typhi and *S.* Paratyphi A are exclusively human pathogens and principally transmitted by the fecal-oral route. The pathogen can survive for days in groundwater, pondwater, or seawater, and for months in contaminated eggs (Cho *et al.*, 1999; Wait Sobsey, 1981; Elsarnagawy, 1978). The infection is transmitted by ingestion of food or water that has been contaminated with feces either from acutely infected persons, persistent excretors, or from chronic asymptomatic carriers. Established risk factors are contaminated water supply, eating ice cream, flavoured iced drinks or food from street vendors, and raw fruit and vegetables grown in fields fertilised with sewage (Swaddiwudhipong and Kanlayanaphotporn, 2001; Mermin *et al.*, 1999; Luby *et al.*, 1998; Black *et al.*, 1985; Levine

et al., 1982). Other reported risk factors include a history of contact with other patients before illness, poor personal hygiene, poor housing, and past evidence of infection with *Helicobacter pylori* (Bhan *et al.*, 2002; Gasem *et al.*, 2001; Luxemburger *et al.*, 2001). Factors within the household (eg, poor personal hygiene and housing) might be more important risk factors for typhoid fever, whereas factors outside the household (eg, food from street vendors, flooding) are more important for transmission of paratyphoid fever (Vollaard *et al.*, 2004). A possible reason proposed for this difference is the higher infective dose necessary for paratyphoid, which is more likely to be present in food from street vendors as waterborne transmission of *S.* Typhi usually, involves small inocula, whereas foodborne transmission is associated with large inocula (WHO, 2003). In developed countries, on the other hand, typhoid is transmitted when chronic carriers contaminate food as a consequence of unsatisfactory food-related hygiene practices. Most cases of typhoid fever in developed countries (*eg*, the United States) are related to foreign travel.

Although chronic typhoid carriers are important for survival of the pathogen, they are less important as a direct source of infection in endemic areas than contaminated water or food (WHO, 2003; Levine *et al.*, 1982). Involvement of host genetic factors has also been implicated in the pathogenesis of typhoid fever. Work on typhoid fever patients in Vietnam has suggested an important role of HLA-linked genes in governing susceptibility or resistance to this infection (Dharmana *et al.*, 2002; Dunstan *et al.*, 2001). The evidence for an association between typhoid and infection with the human immunodeficiency virus (HIV) is conflicting (Gotuzzo *et al.*, 1991), whereas there is a large increase in the incidence of non-typhi *Salmonella* bacteremia in HIV infection.

3.1.3 Enteric fever in Nepal and south Asia

Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and Vietnam are home to approximately 80% of the world's typhoid fever cases (Chau *et al.*, 2007). In a prospective population-based surveillance in five Asian countries, the annual typhoid incidence (per

1000 000 person years) varied from 24.2 and 29.3 in sites in Viet Nam and China, respectively, to 180.3 in the site in Indonesia; and to 412.9 and 493.5 in sites in Pakistan and India, respectively (Ochiai *et al*., 2008).

Enteric fever is the most commonly identified cause of febrile illness among adults in Nepal (Maskey *et al.*, 2008; Pokhrel *et al.*, 2006; Malla *et al.*, 2005; Murdoch *et al.*, 2004). *S.* Typhi and *S.* Paratyphi A have been reported as the most common culture isolates from patients with febrile illnesses. In addition to *S.* Typhi, *S.* Paratyphi A has emerged as an important cause of enteric fever in South Asia during the last decade (Ochiai *et al.*, 2005; Rodrigues *et al.*, 2003; Sood *et al.*, 1999; Kapil *et al.*, 1997). A largest ever-reported outbreak with MDR *S.* Typhi from a contaminated drinking water supply in a small Nepali town has been described (Lewis *et al*., 2005). The poor microbiological quality of the urban water supply is major risk factor and indicates possibility of fatal outbreaks of enteric fever in Nepal (Bhatta *et al*., 2007). Established risk factors like food from street vendors, raw fruit and vegetables grown in fields fertilised with sewage, poor personal hygiene etc. also play major role in enteric fever transmission in Nepal.

3.2 The causative agent

Typhoid fever is a prolonged febrile illnes caused by a systemic infection with *S.* Typhi and the closely related organisms *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C. cause Paratyphoid fever. Other serotype of *Salmonella* such as *S.* Enteritidis may rarely cause enteric fever (WHO, 2003).

3.2.1 Classification of *Salmonella* serotype

The complicated nomenclature system of *Salmonella* has long been a subject of discussion (Su and Chiu, 2007). In 2005, *Salmonella enterica* finally gained official approval as the type species of the genus *Salmonella*. The genus *Salmonella* also contains the species

Salmonella bongori in addition to a new species, *Salmonella subterranean*, which was recognized in 2005. Unlike other bacterial genera, *Salmonella* are differentiated by serotyping. Presently, new serotypes (serovars) are still being discovered each year, adding to the complexity of this large bacterial population.

Le Minor and Popoff suggested that two species should be recognized: *Salmonella bongori* and *Salmonella enterica*. *S. enterica* included six subspecies, of which subspecies I contained all the pathogens of warm-blooded animals. *S.* Typhi is a serotype within subspecies I: *Salmonella enterica* subspecies I serovar Typhi. The International Judicial Commission however rejected this proposal because the name was not well known to clinicians.

The antigenic classification system of various *Salmonella* serovars used today has accumulated from many years of studies on antibody interactions with surface antigens of *Salmonella* organisms established by Kauffman and White almost a century ago. The identification of serotypes in this scheme depends on the presence of the O (somatic) and H (flagellar) antigens. The antigenic formulae of recognized *Salmonella* serotypes consists of 3 parts, describing the somatic O antigen, the phase-1 H antigen and the phase-2 H antigen in order separated by colons, and components of each part separated by commas. The WHO Collaborating Centre for Reference and Research on *Salmonella* at the Pasteur Institute, Paris, France is responsible for updating the scheme. Every year newly recognized serovars are reported in the journal Research in Microbiology. In the latest report published in 2004, there were a total of 2,541 serovars in the genus *Salmonella* (Table3.1).

Table 3.1: Current *Salmonella* nomenclature (Popoff *et al.*, 2004)

Genus (Capitalized, italic	Species (Italic)	Subspecies(Italic)	Serotypes (capitalized, not italic)	No of serotypes
Salmonella	*enterica*	*enterica* (or subspecies I)	Choleraesuis, Enteritidis,	1504

		arizonae (or subspecies II) *salamae* (or subspecies IIIa) *diarizonae*(or subspecies IIIb) *houtanae* (or subspecies IV) *indica* (or subspecies VI)	Typhi, Paratyphi, 9,46:z:z39 43:z29:- 6,7:l,v:1,5,7 21:m,t:- 59:z36:-	502 95 33 72 13
	bongori	subspecies V	13,22:z39:-	22
	subterranea	Under review		

3.2.2 Morphology and biochemical characteristics

Salmonellae are the organisms that confirm to the definition of the *Enterobacteriaceae* with respect to morphology, cultural and biochemical characteristics (Old and Threlfall, 1998). Salmonellae grow aerobically or anaerobically over a wide temperature range from 7 to 48˚C, at pH 4-8 and water activities above 0.93, using simple carbon compound as a source of carbon and energy and a wide range of nitrogenous sources. Although non-motile strains may be isolated from clinical cases, most salmonellae (with the exception of Gallinarum-Pullorum) are motile with peritrichous flagella and 80% of the fimbriate strains possess type-1 fimbriae associated with mannose sensitive adhesive properties. Some serotypes of salmonellae, particularly Paratyphi B, produce mucoid colonies best developed at low temperature, low humidity and high osmolarity.

Most salmonellae ferment glucose, mannitol, and sorbitol with the production of acid and gas; rarely ferment sucrose or adonitol; do not ferment salicin and do not form indole. Gas is not formed by Typhi, biotype Gallinarum or by occasional strains of other serotypes; maltose is not fermented by biotype Pullorum strains; and Typhisuis does not ferment mannitol. Occasional strains that form acid from lactose, sucrose or raffinose, produce

indole, or split urea have been reported. The positive methyl red reaction and negative reaction to the Voges-Proskauer tests is constant (Old and Threlfall, 1998).

Most salmonellae grow with citrate as the sole source of carbon though exceptions include Typhi, Paratyphi A, Typhisuis, Sendai and biotype Pullorum. Most salmonellae also give a positive reaction for H_2S in TSI agar; Typhi produce little or no H_2S; strains of Paratyphi A, Choleraesuis, Typhisuis, Sendai, Abortusequi and Berta do not produce H_2S. Nearly all salmonellae produce arginine dihydrolase and lysine and ornithine decarboxylases but not glutamic acid decarboxylase. Notable exceptions are Typhi and biotype Gallinarum which fail to decarboxylase ornithine, and Paratyphi A, which doesnot attack lysine (Old and Threlfall, 1998). The common biochemical characteristics of the salmonellae used in differentiation of genus, species, subspecies and some serotypes are shown in table 3.2 and table 3.3 (Old and Threlfall, 1998).

Table 3.2: Biochemical differentiation of species and subspecies of *salmonella*

Reaction	Subspecies of *S. enterica*						*S. bongori*
	I	II	III	IV	VI	VII	
ONPG	-	-	+	+	-	d	+
Gelatinase	-	+	+	+	+	+	-
Galacturonate	-	+	-	+	+	+	+
Growth on KCN	-	-	-	-	+	-	+
Malonate	-	+	+	+	-	-	-
Dulcitol	+	+	-	-	-	d	+
Mucate	+	+	+	d	-	+	+
d-Tartate	+	-	-	-	-	-	-
y-Glutamultransferase	+	+	-	+	+	+	+
b-Glucuronidase	d	d	-	+	-	d	-
Salicin	-	-	-	-	+	-	-

Sorbitol	+	+	+	+	+	-	+

Source: Old and Threlfall, 1998.
Note: Subspecies I *enterica*, II *salamae*, III *arizonae*, IV *diarizonae*, VI *houtenae*, VII *indica*; +, >90% of strain positive; -, >90% of strain negative; d, some strains positive other negative

Table 3.3: Biochemical reactions of some *Salmonella* serotypes of subspecies *enterica*

Reactions	Most serotypes	Typhi	Paratyphi A	Choleraesuis	Gallinarum	Pullorum
Gas	+	-	+	+	-	+
Citrate	+	-	-	d	+	-
H_2S	+	w	-	-	-	-
Lysine	+	+	-	+	+	+
Ornithine	+	-	+	+	-	+
Motility	+	+	+	+	-	-

Source: Old and Threlfall, 1998.
Note: +, >90% of strain positive; -, >90% of strain negative; d, delayed positive; w, weak positive

3.2.3 Antigenic characteristics

Common antigenic structures and antigenic variations that occur in salmonellae are as follows.

a) O antigens: These somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide layer of the bacterial cell wall. The somatic O antigens are represented by Arabic numerals. They are hydrophilic and enable the bacteria to form stable, homogenous suspensions in saline (0.85% NaCl) solution (Old, 1996).

b) H antigens: These antigens represent determinant groups on the flagellar protein. They are heat-labile and alcohol-labile but are well preserved in 0.04-0.2% formaldehyde. Heating at temperature above 60°C detaches the flagella from the bacteria. In many salmonellae i.e. other than serotypes of subspecies IIIa, IV and V, the production of flagellar antigens is

diphasic, each strain varying spontaneously and reversibly between two phases (phase I and phase II) with different sets of H antigens. In phase I, the bacteria from flagella with one or more antigens form a set of over 70 antigen designated by the small letters of the alphabet 'a to z', a series that is complete except for 'j' then z_1, z_2, z_3, etc. In phase 2, the bacterial form flagella with one or more antigens from a mainly different set of antigens.

c) Other surface antigen

These include Vi antigen, the slime (mucus) or M antigen and the fimbrial (F) antigens. Such antigens may cause difficulty in the serological identification of bacteria either by masking the agglutination by O antibodies or by causing non specific cross-reaction due to their presence in unrelated bacteria (Old, 1996).

- Vi antigen: This is a heat-labile surface polysaccharide of β(1-4) linked N-acetyl-D-galactosaminuronic acid, the C_3 groups of which are variably acetylated and forms the covering layer outside their cell wall in almost all recently isolated strains of *S.* Typhi. Vi rich strains which produce more opaque colonies when maintained by subculture on conventional media are rapidly replaced by spontaneously originating Vi deficient mutans (V→W variation) (Old, 1996).
- M antigen: This loose extra-cellular polysaccharide slime consisting of colanic acid occurs in a serologically similar form in various unrelated enterobacteria including serotypes of *Salmonella* and many strains of *Escherichia coli* and resembles the Vi antigen in preventing agglutination by O antiserum (Old, 1996).
- Fimbrial antigen: The antigenic determinants of the type -1 fimbriae of salmonellae are unrelated to those of most other *Enterobacteriaceae*. Duguid and Campbell described five type-1 fimbrial antigens in 1969 (Old, 1990).
- R antigen: In S→R mutation, the O antigens are lost and new 'R' antigens are revealed at the bacterial surface. The R antigens are the same in the R variants from

different *Salmonella* serotypes though different from the R antigens of other enterobacteria (Old, 1996).

d) Antigenic variation: Variation in the O-antigen and H antigen occurs commonly in *Salmonella*. The O antigens are liable to be changed in character by form variation and lysogenic conversion and to be lost from the bacteria in S→R mutation (Old, 1996). Rough mutant strains are not agglutinated by homologous O antiserum (Old and Threlfall, 1998). Form variation is a spontaneous reversible variation in the amount of one of the O antigens (Old, 1996). The specificities of O antigens may be modified in the process of lysogenic conversion by phages which genetically determine subtle change in the chemistry of the repeating unit of polysaccharide.

In H antigen variation, non-motile (Mot ¯) strains of salmonellae may occasionally be found with flagella that are antigenically and morphologically like those of motile parent strains. Flagellate salmonellae sometimes give rise to non-flagellate H- strains or fail to rotate the flagella due to defects in the mechanism of energy transduction. This kind of change observed in both laboratory and field strains and originally described as the OH→H variation results from defects in *fla, flb* genes. Besides this, most serotypes of *Salmonella* of subspecies other than IIIa, IV and V express alternately 2 kinds of H flagellar specificity (phase I and phase II) showing diphesic variation.

3.2.4 Organization of genome

Because *Salmonella* is one of the closest relatives of *Escherichia coli*, much is known about the genetics, physiology and biochemistry. The recent tandem publication of the complete genome sequences of *S.* Typhi (Parkhill J *et al.*, 2001) and *S.* Typhimurium (McClelland *et al.*, 2001) is an important milestone in microbial genomics. In addition to a greater biological understanding, the availability of genomic sequences for *Salmonella* strains can be expected to greatly facilitate specific health subjects, including diagnosis, pathogenesis,

treatment and vaccination. More *Salmonella* genomic sequences are in the pipeline, including *S.* Dublin, Paratyphi A, and at least two other strains of *S.* Typhimurium, DT104 and SL1344, that differ in their pathogenicity.

S. Typhi has several unique features, the genetic basis of many of which is known as a result of early genetic studies and the recent sequencing of the whole genome. The complete genome sequence was determined for a multidrug-resistant strain of *S.* Typhi (CT18), which was isolated in 1993 from a child with typhoid fever in the Vietnam (Parkhill, *et al.*, 2001). The CT18 genome harbors 4,809,037 base pairs with an estimated 4599 coding sequences. The genomes of *S.* Typhi CT18, *S.* Typhimurium LT2, and *Escherichia coli* are essentially collinear, despite the fact that *E. coli* and *S. enterica* diverged about 100 million years ago (Parry *et al.*, 2002).

Unlike *E. coli, S.* Typhi has several large insertions in its genome, termed *Salmonella* pathogenicity islands (SPIs) that are thought to be recent horizontal acquisitions and these gene clusters unique to *S.* Typhi play important role in adaptations to particular environments or may contribute to pathogenicity. In addition, there are multiple insertions of many smaller gene blocks and individual genes scattered in the genome that may potentially be involved in pathogenicity. A striking feature of the *S.* Typhi genome is the presence of 204 pseudogenes, more than half of which are inactivated by the introduction of a single frame-shift or stop codon, suggesting that they are of recent origin. A substantial number are predicted to be involved in housekeeping functions or in virulence or host interactions. This apparent inactivation of genes responsible for host interactions may explain why *S.* Typhi, unlike other *Salmonella* serotypes, is restricted to one host (i.e., humans) and suggests that *S.* Typhi may have passed through a recent evolutionary bottleneck.

S. Typhi CT18 harbors two plasmids. The larger conjugative plasmid, pHCM1, is 218 kb in length and shares approximately 168 kb of DNA with the plasmid R27 which is an *Inc* H1 plasmid first isolated in the 1960s from *S. enterica,* that is closely related to the

chloramphenicol-resistance plasmids detected in *S.* Typhi in the 1970s with more than 99% sequence identity (Sherburne *et al.*, 2000; Taylor *et al.*, 1985). The pHCM1 plasmid encodes resistance to chloramphenicol (*catI*), ampicillin (TEM-1, *Bla)*, trimethoprim (*dhfr1b*), sulfonamides (*sulII*), and streptomycin (*strAB*). The smaller plasmid, pHCM2, is 106.5 kb in length and is phenotypically cryptic, but it has striking homology with the pMT1 virulence-associated plasmid of *Yersinia pestis.*

3.2.5 Typing and molecular characterization

Differentiation and classification into subtypes of the organism isolated from difference sources at different times and in different geographical region is of epidemiological importance as the pathogens involved in the outbreak of infectious disease often have a common origin and are clonally related (Old and Threlfall, 1998). A range of phenotypic methods of typing (serotyping, phage typing, and biotyping) and molecular typing methods based on plasmid and chromosomal DNA are currently used to differentiate and subtype isolates.

3.2.5.1 Serotyping

The *Salmonella* serotyping scheme, also called the Kauffmann-White scheme, is based on the combination of antigenic properties of the phase 1 and phase 2 flagellar proteins (flagella H1 and H2 antigens) and the cell-surface lipopolysaccharide (somatic O antigen). Flagella H1 and H2 are encoded by the *fliC* and *fljB* genes, respectively. The genes involved in the biosynthesis of O antigen are generally clustered at a specific locus of the chromosome as an O antigen gene cluster. Each distinctive combination of O (somatic), H1 and H2 (flagella) antigens is given formal recognition as a serovar. Those strains of the same serovar

combination (antigen formula) belonging to different subspecies are treated as separate serovars. There are 2541 serovars recognized in *Salmonella* presently (Popoff and Minor, 2001; Popoff *et al.*, 2004).

3.2.5 .2 Phage typing

Based on the underlying principle of host specificity of bacteriophages several phage typing schemes have been developed for some of the salmonellae serotypes of clinical or epidemiological importance. The first phage typing scheme, Vi phage typing, based on the principle of phage adaptation was that developed for the differentiation of Typhi. The method of Vi phage typing standardized in 1947 is now the internationally accepted methods for differentiation of Typhi and the scheme is used in specialized WHO-approved reference centres world wide. The types most widespread and abundant through the world are E1 and A, followed by B2, C1, D1 and F1. Most of the MDR strains of *S*. Typhi belong to Vi phage type E1. In addition to Typhi, phage typing schemes are also used in Paratyphi B, Typhimurium, Hadar, Enteritidis and Virchow.

3.2.5.3 Biotyping

Subdividing common *Salmonella* serotypes according to their biochemical characteristics is sometimes of value in epidemiological investigations. The biotyping method is useful in studying the epidemiology of infection with Typhimurium. Biotyping is also helpful in dividing strains of phage types. Biotyping scheme have also been successfully applied to the epidemiology of other salmonellae serotype: Agona, Livingstone, Crichton, Montevideo and Paratyphi B.

3.2.5.4 Molecular typing methods

A range of molecular typing methods based on characterization of the genotype of the the organism by analysis of plasmid and chromosomal DNA has now been developed for typing

Salmonella. Molecular typing methods based on the charactertization of plasmid DNA include plasmid profile typing, plasmid fingerprinting and identification of plasmid mediated virulence genes. Chromosomolly based methods used for *Salmonella* are ribotyping, random cloned chromosomal sequence typing, insertion sequence (IS) 200 typing, pulse field gel electropohoresis (PFGE), and polymerase chain reaction (PCR) based method such as random amplified polymorphic DNA typing (RAPD), enterobacterial repetitive intergenic consensus typing (ERIC-PCR) and repetitive extragenic pallindromic element typing (REP-PCR) (Old and Threlfall 1998).

3.3 The disease

After ingestion in food or water, typhoid organisms pass through the pylorus and reach the small intestine. The infectious dose is between 1000 and 1 million organisms given orally (Hornick *et al.*, 1970) but the inoculum size and the type of vehicle in which the organisms are ingested greatly influence both the attack rate and the incubation period. It is believed that typhoid bacilli reach the bloodstream principally by lymph drainage from mesenteric nodes, after which they enter the thoracic duct and then the general circulation.

As a result of this silent primary bacteraemia the pathogen reaches an intracellular haven within 24 hours after ingestion throughout the organs of the reticuloendothelial system (spleen, liver, bone marrow, etc.), where it resides during the incubation period, usually of 8 to 14 days. The incubation period in a particular individual depends on the quantity of inoculum, i.e. it decreases as the quantity of inoculum increases, and on host factors. Incubation periods ranging from 3 days to more than 60 days have been reported. During an acute infection, *S.* Typhi multiplies in mononuclear phagocytic cells before being released into the bloodstream. Clinical illness is accompanied by a fairly sustained but low level of secondary bacteraemia.

3.3.1 Pathogenesis

The current understanding of the natural history and pathogenesis of *S.* Typhi infection comes from observing the disease in humans, volunteer studies and animal models, the best-characterized animal model of typhoid fever being the murine model using *Salmonella* Typhimurium. In the small intestine, the bacteria adhere to mucosal cells and then invade the mucosa. The M cells, specialized epithelial cells overlying peyer's patches, are probably the site of the internalization of *S.* Typhi and its transport to the underlying lymphoid tissue. Briefly, ingested *S.* Typhi enters the small intestine and, via the M cells of the peyer's patches, migrates into the mesenteric lymph nodes where multiplication occurs. Bacteria are released into the bloodstream where they disseminate widely (transient primary bacteraemia).

Because epithelial cells are not inherently phagocytic, *Salmonella* have evolved a sophisticated repertoire of virulence factors that allow them to penetrate the intestinal epithelium, from which they may disseminate systemically to colonize the liver and spleen. Ingested bacteria colonize the intestinal epithelium by triggering their own phagocytosis, using a sophisticated array of effector proteins that are injected into the host cell cytoplasm through a type III secretion system (T3SS). The synergistic action of these secreted effectors leads to a dramatic reorganization of the host actin cytoskeleton, resulting in vigorous membrane protrusion (membrane ruffling) and the engulfment of attached bacteria (Ly and Casanova 2007). The *Salmonella* genome contains several clusters of genes, referred to as SPIs that encode virulence factors. In general, SPI-1 is thought to contain genes required for bacterial entry (Galán, 1996), while SPI-2 is necessary for intracellularsurvival (Shea *et al*., 1996). Both SPI-1 and SPI-2 encode T3SS, a sort of molecular syringe consisting of more than 20 proteins that spans both inner and outer bacterial membranes and inserts into the plasma membrane of the host (Galán and Wolf-Watz, 2006). This sophisticated apparatus,

which is evolutionarily conserved among many Gram-negative pathogens, mediates the translocation of bacterial virulence factors into the host cell cytosol.

Salmonella are able to survive and multiply within the mononuclear phagocytic cells of the lymphoid follicles, liver, and spleen (House *et al.*, 2001). At a critical point that is probably determined by the number of bacteria, their virulence, and the host response, bacteria are released from this sequestered intracellular habitat into the bloodstream. The re-entry of bacteria into the blood (secondary bacteraemia) marks the onset of clinical disease. Organisms are then removed from blood via the liver. *S.* Typhi infection of the gall bladder can lead to reinfection of the intestinal tract, causing inflammation, ulceration and necrosis (Everest *et al.*, 2001). Haemorrhage from ulcers during the third week of illness or perforation of the peyer's patches occurs in <5% of patients and can cause generalized peritonitis and septicaemia, the commonest cause of death in typhoid fever (Bitar *et al.*, 1995; Butler *et al.*, 1985). Even though *S.* Typhi produces a potent endotoxin, mortality from treated typhoid fever is less than 1%. Studies have shown increased levels of circulating proinflammatory and antiinflammatory cytokines in patients with typhoid and a reduced capacity of whole blood to produce inflammatory cytokines in patients with severe disease (Bulter *et al.*, 1993; Keuter *et al.*, 1994; Bhutta *et al.*, 1997).

Survival within macrophages appears to be crucial for the virulence of *S.* Typhimurium in murine models and is important for typhoid fever caused by *S.* Typhi in humans. Intracellular survival of *S.* Typhimurium has been shown to be attributable to various factors, including resistance to reactive oxygen metabolites (Ishibashi and Arai, 1989), inhibition of phagosome-lysosome fusion (Ishibashi and Arai, 1990; Buchmeier *et al.*, 1990), acidification of phagolysosomes (Aranda *et al.*, 1992), and resistance to bactericidal peptides such as defensin (Fields *et al.*, 1989).

Vi-negative strains of *S.* Typhi are less infectious and less virulent than Vi-positive strains (Hornick *et al.*, 1970). Achlorhydria as a result of aging, previous gastrectomy, or treatment

with histamine, H2-receptor antagonists, proton-pump inhibitors, or large amounts of antacids lowers the infective dose. *S.* Typhi must survive the gastric acid barrier to reach the small intestine, and a low gastric pH is an important defense mechanism.

Typhoid induces systemic and local humoral and cellular immune responses, but these confer incomplete protection against relapse and reinfection. The interaction of host immunologic mediators and bacterial factors in infected tissue may contribute to the necrosis of peyer's patches in severe disease (Everest *et al.*, 2001). Major-histocompatibility-complex class II and class III alleles have been shown to be associated with susceptibility to typhoid fever and disease resistance in Vietnam (Dunstan *et al.*, 2001). Polymorphisms in the genes encoding the natural-resistance–associated macrophage protein were not associated with resistance to typhoid, in contrast to the importance of this allele in the murine model.

3.3.2 Sign and symptoms

The clinical presentation of typhoid fever is very variable, ranging from a mild illness with low-grade fever, malaise, and slight dry cough to a severe clinical picture with abdominal discomfort, marked toxaemia and associated complications involving many systems. In endemic regions, diagnosis can be missed because of non-specific features like diarrhoea and vomiting, or predominant respiratory symptoms. Because of this variable profile, the disease has to be differentiated from tuberculosis, brucellosis, sepsis due to other bacterial pathogens, infectious mononucleosis, anicteric hepatitis, and, infrequently, from leukaemia and lymphoma. An average case of acute non-complicated typhoid fever has an incubation period of 10–14 days and is usually associated with prolonged low-grade fever, dull frontal headache, malaise, myalgia, a dry bronchitic cough, anorexia, and nausea. The fever might rise progressively in a stepwise manner to become persistent and high grade by the second week of illness.

Continuous high-grade fever can continue for up to 4 weeks if left untreated, followed by a return to normal temperature. Malaise and lethargy can continue for a couple of months later. Although not present consistently, relative bradycardia at the peak of high fever is an indicator of typhoid fever. Coated tongue, alteration of bowel habits varying from constipation in adults to diarrhoea in children, tender abdomen, hepatomegaly, and splenomegaly are often present (Pary *et al.*, 2002; Vinh *et al.*, 1996). Small erythematous maculopapular lesions (rose spots) are seen on the back, arms, and legs in up to a quarter of cases late in the first week of fever, particularly in fair-skinned people. Rhonchi and scattered crepts might be heard on chest auscultation. Liver involvement is common with elevated concentrations of serum bilirubin and alanine transferase; in endemic areas, typhoid fever should be a differential diagnosis for a patient with fever and jaundice (Shetty *et al.*, 1999).

Neonatal typhoid fever resulting from vertical transmission during late pregnancy is a rare but often life-threatening illness. It usually begins during 3 days of delivery with fever, vomiting, diarrhoea, and abdominal distension. Hepatomegaly, jaundice and seizures can occur sometimes. In children younger than 5 years, typhoid fever can be milder and can mimic a viral syndrome, the rate of severe complications being lower than at later ages (Sinha *et al.*, 1999; Chiu *et al.*, 1999). Without therapy, the illness may last for 3–4 weeks and case-fatality rates may exceed 10%. With appropriate treatment, clinical symptoms subside within a few days, fever recedes within 5 days, and mortality is reduced to approximately 1%. Overall, about 10–15% of patients develop severe disease. Factors affecting severity include duration of illness before the initiation of appropriate therapy, the choice of antimicrobial treatment, age, the previous exposure or vaccination history, the virulence of the bacterial strain, the quantity of inoculum ingested, and host factors such as HLA type, AIDS or other immune suppression and whether the individual was taking other medications such as H2 blockers or antacids to diminish gastric acid.

Clinical features of paratyphoid fever are similar to those of typhoid fever but are usually milder with a shorter incubation period. *S.* Paratyphi A or Paratyphi B can manifest with jaundice, thrombosis (Rajgopal *et al.*, 2002; Mohanty *et al.*, 2003) and systemic infections (Lee *et al.*, 2000). *S.* Paratyphi B might occasionally have an onset similar to non-specific *Salmonella* gastroenteritis. Gastrointestinal symptoms are usually not present with *S.* Paratyphi C but there have been cases with systemic complications such as septicaemia and arthritis (Lang *et al.*, 1992). A relapse rate of 8% has been reported with *S.* Paratyphi A (Goh, 1981).

3.3.3 Complications

Depending on the clinical setting and the quality of available medical care, up to 10% of typhoid patients may develop serious complications. The commonest complications are gastrointestinal bleeding, intestinal perforation and typhoid encephalopathy (WHO, 2003; Parry *et al.*, 2002). Gastrointestinal bleeding occurs in 10–20% of cases due to erosion of the peyer's patch into an intestinal vessel and is usually restricted to either occult blood in stool or malaena (Hosoglu *et al.*, 2004; Lee *at al.*, 2004) and intestinal perforation occurs in 1–3% of cases (Van Basten *et al.*, 1960) in hospital; the commonest site is the ileum. The symptoms and signs of intestinal perforation and peritonitis sometimes follow, accompanied by a sudden rise in pulse rate, hypotension, marked abdominal tenderness, rebound tenderness and guarding, and subsequent abdominal rigidity.

Intermittent confusion, insomnia, and dizziness are reported in 3–10% of cases and these symptoms are associated with high case fatality. Some patients manifest neuropsychiatric symptoms such as picking at bedclothes or imaginary objects, which are described as muttering delirium or coma vigil but deep coma is rare. Typhoid meningitis, encephalomyelitis, Guillain-Barré syndrome, and cranial or peripheral neuritis have been reported from different regions with incidence of 2–40%. Convulsions occasionally arise in young children (Bulter *et al.*, 1991). Severe complications such as disseminated

intravascular coagulation or haemorrhages can lead rapidly to death. Severe pneumonia is more frequent in children than adults. The other rare complications reported include hepatic, splenic, and bone-marrow granulomas, splenic and liver abscesses, pleural effusion, multiple organ dysfunction syndrome, haematophagocytic syndrome, pseudotumour cerebri, haemolytic uraemic syndrome, glomerulonephritis or pylonephritis, endocarditis, and pericarditis (Chaudhry *et al.*,2003; Mert *et al.*, 2004; Snyder *et al.*, 2004; Albaqali *et al.*, 2003; Balasubramanian *et al.*, 2003). Arrhythmia or cardiogenic shock can be a manifestation of toxic myocarditis with fatty infiltration of the heart.

With the introduction of early and appropriate antibiotic therapy, the average case fatality rates for typhoid are less than 1%; however, mortality as high as 30–50% has been reported from Papua New Guinea and Indonesia for severe typhoid fever (Punjabi *et al.*, 1988; Rogerson *et al.*, 1991). In 552 culture-positive patients with typhoid fever in hospital in Bangladesh, the overall case-fatality rate was 4·3%, with the highest rates for those younger than 1 year (11%) and for adults 31 years or older (10%)(Bulter *et al.*, 1991).

3.3.4 Relapse

Relapse can occur in 5–10% of cases after 2–3 weeks of resolution of the initial fever, and is characterized by a less severe but otherwise typical illness. Relapse can happen without a history of therapeutic intervention but more often it follows antibiotic treatment (Gotuzzo *et al.*, 1987; Smith *et al.*, 1994; Yew *et al.*, 1991) and is presented by the return of fever soon after the completion of antibiotic treatment usually after an afebrile period of 1–2 weeks. The incidence off relapse after treatment with fluoroquinolones (1·5%) or broad-spectrum cephalosporins (5%) is lower than that after treatment with chloramphenicol, trimethoprim-sulfamethoxazole, and ampicillin. and most relapses are caused by the same *S.* Typhi strain with the same antibiotic susceptibility patterns as the initial episode excluding some individuals who may become reinfected with distinct and possibly newly acquired isolates (Wein *et al.*, 1999).

3.3.5 Carrier stage

An individual is considered a chronic carrier if he or she is asymptomatic and continues to have positive stool or rectal swab cultures for *S.* Typhi a year following recovery from acute illness. Between 1% and 5% of patients with acute typhoid infection have been reported to become chronic carriers of the infection in the gall bladder, depending on age, sex and treatment regimen. The propensity to become a carrier follows the epidemiology of gall bladder disease, increasing with age and being greater in females than in males. The rate of carriage is slightly higher among female patients, patients older than 50 years, and patients with cholelithiasis or schistosomiasis. The propensity to become a chronic carrier may have changed with the present availability and selection of antibiotics as well as with the antibiotic resistance of the prevalent strains (WHO, 2003).

3.3.6 Treatment, prevention and control

3.3.6.1 Treatment

More than 90% of patients are managed at home with oral antimicrobials, bedrest, and close medical follow up but patients with persistent vomiting, severe diarrhoea, or abdominal distension need admission to hospital and parenteral antibiotic therapy (WHO, 2003). Important considerations for treatment are the prevention of severe complications and death, prompt resolution of clinical disease, and eradication the organism from the body to prevent relapses and faecal carriage. Initial choice of antibiotic depends on the sensitivity patterns of *S.* Typhi and Paratyphi isolates.

Where the bacterium is still fully susceptible to traditonal first-line drugs (chloramphenicol, amoxicillin or trimethoprim-sulfamethoxazole), these remain appropriate for the treatment of typhoid fever. They are inexpensive, widely available, and rarely associated with side effects (WHO, 2003). Fluoroquinolones (ciprofloxacin, ofloxacin, and pefloxacin) are the

most effective drugs for treatment of typhoid fever caused by isolates that are not quinolone resistant. Chloramphenicol, the traditional first-line drug of choice, is less effective than fluoroquinolones due mainly to the persistence of the organism in bone marrow, even for treatment of patients with fully susceptible isolates. Azithromycin and cefixime are regarded as acceptable therapy for quinolone-resistant typhoid and paratyphoid fever. Combinations of antimicrobials are being assessed to provide more affordable options for treatment of quinolone-resistant typhoid fever. Supportive treatment includes maintenance of hydration, appropriate nutrition, and antipyretics.

Patients with intestinal haemorrhage need intensive care, monitoring, and blood transfusion when blood loss is substantial. Patients with intestinal perforation should be resuscitated with fluids or blood and should undergo early surgical intervention within 6 hrs to prevent death. Relapses should be treated in the same way as initial infections. Most carriers without gallstones can be cured by a long course of antimicrobials. In patients with gallstones, cholecystectomy along with antibiotic therapy might be required.

3.3.6.2 Prevention and control

The key preventive strategies are safe water, safe food, personal hygiene, and appropriate sanitation (WHO, 2003). The importance of prevention has greatly increased with the emergence of antibiotic resistance. However, provision of safe water and appropriate sanitation are expensive and are usually linked with economic development. Carriers should be excluded from any activities involving food preparation and serving, as should be people recovering from typhoid and paratyphoid fever.

Vaccination is an additional effective tool for prevention of typhoid fever. Vaccination is useful for prevention of typhoid in travellers from developed countries to typhoid endemic countries, in preventing and controlling epidemics, as well for children in endemic settings aged 2–19 years (WHO, 2003). Travellers to typhoid endemic countries, especially those

visiting for 2 weeks or more and those visiting friends and relatives, should be targeted for vaccination (Steinberg *et al.*, 2004). The old parenteral whole-cell typhoid-paratyphoid A and B vaccine was effective against both typhoid and paratyphoid fevers but has been largely discontinued because of strong side-effects (Engels *et al.*, 1998). Two currently licensed vaccines for typhoid fever are Vi polysaccharide based vaccine and whole-cell live attenuated bacteria, but there is no licenced vaccine for paratyphoid fever (WHO, 2003). A new Vi-conjugate vaccine is highly effective in children younger than 5 years but it has not been tested in infants. Vaccines are under development based on outer membrane proteins known as porins and new live oral vaccines (eg CVD 908-htrA and Ty2 candidate vaccines) (Tacket *et al.*, 2004; Singh *et al.*, 1999). A new vaccine against *S.* Paratyphi A composed of surface-O-specific polysaccharide conjugated with tetanus toxoid has proved safe and immunogenic (Konadu *et al.*, 2002).

In developing countries, provision of safe drinking water, effective sewage disposal, and hygienic food preparation play important role in reducing the number of cases (Ivanoff, 1995). Mass immunization has been used successfully in some areas. Identification and treatment of chronic carriers is also important. Besides this, the widespread emergence of multidrug-resistant strains, dramatic increase in the strains with reduced susceptibility to fluoroquinolones and sporadic reports of full resistance to fluoroquinolones and resistance third-generation cephalosporins are serious considerations.

3.4 Drug resistance

3.4.1 Use of antibiotics in treatment of enteric fever

Although the first successful definition of typhoid fever was given by William Jenner in the mid-1800s, treatment was unavailable until the discovery of chloramphenical in 1948. In 1948, chloramphenicol became the standard antibiotic for treating typhoid (Woodward *et*

al., 1948). Besides chloramphenicol, other traditional drugs such as ampicillin and co-trimoxazole were most effectively used as first-line drugs for the treatment of typhoid cases (Bhutta, 2006; Bhan *et al.*, 2005). However, during the late 1980s and early 1990s the occurrence of MDR *S.* Typhi strains, i.e. resistant to chloramphenicol, ampicillin and co-trimoxazole, led to the widespread use of fluoroquinolones (FQs), in particularly ciprofloxacin and third generation cephalosporins for the treatment of typhoid fever (Bhutta, 2006; Bhan *et al.*, 2005; Threlfall *et al.*, 1992).

3.4.2 Evolution of drug resistance

Although resistance emerged within two years after its introduction and a few sporadic isolates of chloramphenicol resistant Typhi have been reported before 1970, the first epidemic caused by a chloramphenicol resistant strains occurred in Mexico in 1972 (olarate and Galindo 1973) followed by a second outbreak in India (Panikar and Vilma 1972). After 1972 chloramphenicol-resistant typhoid fever became a major problem when outbreaks occurred in Mexico, India, Vietnam, Thailand, Korea, and Peru. Chloramphenicol resistance was associated with high-molecular-weight, self-transferable, *Inc*HI plasmids. Other drugs such as trimethoprim, sulfamethoxazole, and ampicillin were then extensively used as first-line treatment of chloramphenicol resistant typhoid fever.

Toward the end of the 1980s and the 1990s, *S.* Typhi developed resistance simultaneously to all the drugs that were then used as first-line treatment (chloramphenicol, trimethoprim, sulfamethoxazole, and ampicillin) (Mirza *et al.*, 1996). Outbreaks of infections with these strains occurred in India (Threlfall *et al.*, 1992; Shanahan *et al.*, 1998), Pakistan (Bhutta *et al.*, 1996), Bangladesh (Hermans *et al.*, 1996), Vietnam (Hoa *et al.*,1998), the Middle East and Africa (Karauki *et al.*, 2000). These MDR strains carried the 100,000-to-120,000-kD *Inc* HI plasmids that encoded the resistance genes. Spread results from the clonal dissemination of individual MDR *S.* Typhi strains or from transfer of the plasmid to multiple *S.* Typhi strains (Mirza *et al.*, 2000; Thong *et al.*, 2000; Connerton *et al.*, 2000).

A longitudinal investigation of the molecular evolution of multidrug resistance in nontyphoid *Salmonella* demonstrated that progressive acquisition and accumulation of plasmid-mediated resistance determinants through the exchange of plasmids or other mobile elements between *Salmonella* and/or other members of the family *Enterobacteriaceae* might have led to multidrug resistance. In addition to the generally recognized selective pressure from antimicrobial use in food animals, *Salmonella* could acquire drug resistance plasmids from other enteric pathogens in the intestinal tract of individual patients. Horizontal gene transfer facilitates the adaptation of microorganisms to a new set of environmental conditions such as resistance to antibiotics. As an example, the drug trimethoprim blocks DNA synthesis by inhibiting dihydrofolate reductase (Dhfr). Trimethoprim resistance in *S.* Typhi CT18 has been linked to a mutant version of the gene encoding Dhfr, *dhfr*1b, located on the large plasmid pHCM1, which also has other antibiotic resistance loci including *sul*II (sulfonamide), *cat*I (chloramphenicol), *bla* (TEM-1 β-lactamase; ampicillin) and *str*AB (streptomycin) (Noordewier and Brown, 2002).

After 1990 fluoroquinolones, particularly ciprofloxacin, and third generation cephalosporins were used in the treatment of MDR typhoid cases. Emergence of *S.* Typhi strains with reduced susceptibility to fluoroquinolones in the following year become a major problem in Asia (Brown *et al.*, 1996; Threlfall *et al.*, 1999; Wein at al., 1997). The emergence of resistance to multiple antimicrobial agents in *S.* Typhi and *S.* Paratyphi A has been a major problem in Asia in the past 15 years. MDR isolates with resistance to chloramphenicol, ampicillin, and cotrimoxazole, and with reduced susceptibility to fluoroquinolones, have caused epidemics and become endemic in the Indian Subcontinent, Southeast Asia, China, and some countries of central Asia (Parry, 2003). Multidrug-resistant strains of *S.* Typhi were still common in many areas of Asia, although in some areas strains that are fully susceptible to all first-line antibiotics have reemerged (Sood *et al.*, 1998).

3.4.3 Generation of fluoroquinolone resistance

Resistance to fluoroquinolones typically occurs by point mutations in the genes encoding the bacterial DNA gyrase and topoisomerase IV enzymes, which are the target for these agents, or in the genes controlling the transport of the drug into the bacterial cell. The newly identified plasmid-mediated mechanism of quinolone resistance has not been detected in *S.* Typhi or *S.* Paratyphi A.

As found in other bacterial species, acquired quinolone resistance in *Salmonella* is usually due to point mutations in the genes encoding DNA gyrase (*gyrA, gyrB*), or DNA topoisomerase IV (*parC, parE*) (Su *et al.*, 2004; Chiu *et al.*, 2002), leading to modifications in the target. Some "hot-spots" in a region called the quinolone resistance-determining region (QRDR) found in both DNA gyrase and topoisomerase IV are particularly prone to mutation. The most commonly described mutation sites are codon 83 (serine to phenylalanine, tyrosine, or alanine) and codon 87 (aspartic acid to glycine, asparagine, or tyrosine) in *gyrA* (Giraud 2000). Other frequently reported mutation sites include: codon 464 (serine to phenylalanine) in *gyrB* and codon 57 (threonine to serine), codon 80 (serine to isoleucine or arginine), and codon 84 (glutamic acid to lysine) in *parC*. Besides these, mutations in *parE* have also been reported at various codons (Eaves *et al.*, 2004).

Furthermore, two global regulatory systems, *marRAB* and *soxRS*, have been associated with multiple antimicrobial resistance in *Salmonella* and other members of *Enterobacteriaceae* (Cloeckaert and Chaslus-Dancla, 2001)). The *marRAB* and *soxRS* genes code for proteins that up-regulate and down-regulate the expression the AcrAB-TolC efflux system. MarA and SoxS also up-regulate the production of *micF*, an antisense RNA, which is responsible for the reduced synthesis of a major porin (OmpF), resulting in decreased outer membrane permeability and resistance to fluoroquinolones and other antibiotics (Cloeckaert and Chaslus-Dancla, 2001).

3.4.3.1 Reduced susceptibility to fluoroquinolones and treatment failure

Use of ciprofloxacin as the drug of choice for routine outpatient treatment has resulted in the rapid emergence of *S.* Typhi strains with reduced susceptibility to ciprofloxacin which were being reported particularly in many Asian countries (Parry, 2002). Fluoroquinolone resistance is characterized by a step-wise process. A single mutation in any of the mutation sites (*gyr*A or *gyr*B) may result in resistance to nalidixic acid but only a slightly reduced susceptibility to fluoroquinolones (MIC < 4 µg/mL) (Saha *et al.*, 2006; Wain *et al.*, 1997).

Common single mutations in the *gyrA* gene can produce a ten-fold increase in the fluoroquinolone MIC compared with the wild type (Mermin *et al.*, 1999). The MIC of such isolates is still within the susceptible range according to current guidelines, and are reported as susceptible when using disc susceptibility tests. However, the reduction in susceptibility results in a poor clinical response to treatment and translates into an increased chance for clinical failure (Bhan *et al.*, 2005; Nkemngu *et al.*, 2005; Rupali *et al.*, 2004; Kristiansen *et al.*, 2003; Mermin *et al.*, 1999; Piddock *et al.*, 1993) when infection with these isolates is treated with fluoroquinolones.

3.4.3.2 Generation of full fluoroquinolones resistance

Complete fluoroquinolone resistance in the *Enterobacteriaceae* usually results when double or more mutations are present concurrently within the QRDRs of the DNA gyrase and topoisomerase IV genes (Cloekaert *et al.*, 2001; Hooper *et al.*, 2001). In *Salmonella*, the most common residues associated with mutation leading to quinolone resistance have been Ser-83 and Asp-87 in the *gyrA* gene, either alone or together (Hirose *et al.*, 2002; Piddock *et al.*, 1998; Brown *et al.*, 1996; Wain *et al.*, 1997). However, together with the target gene mutations, concomitant over-expression of AcrAB-TolC efflux systems is also required to achieve high-level fluoroquinolone resistance (MIC ≥ 32 µg/mL).

Recent reports of infections because of strains of *S.* Typhi and *S.* Paratyphi A with high-level resistance to fluoroquinolones are particularly worrying (Nair *et al.*, 2006; Adachi *et*

al., 2005). On the Indian subcontinent, there are sporadic reports of *S.* Typhi isolates fully resistant to ciprofloxacin, although the numbers are low. Molecular characterization of *S.* Typhi and *S.* Paratyphi A with fluoroquinolone resistance has been described previously (Gaind *et al.*, 2006; Adachi *et al.*, 2005). Generally, a single *gyrA* mutation (Ser-83 to Phe or Ser-83 to Tyr) was associated with reduced susceptibility to ciprofloxacin (MICs 0.125–1µg/mL); an additional mutation in *parC* (Ser-80 to Ile, Ser-80 to Arg, Asp-69 to Glu or Gly-78 to Asp) was accompanied by an increase in ciprofloxacin MIC (≥ 0.5 µg/mL). Three mutations conferred ciprofloxacin resistance: two in *gyrA* (Ser-83 to Phe and Asp-87 to Asn or Asp-87 to Gly) and one in *parC*. Moreover, transferable plasmid carrying a class 1 integron (*dfrA15/aadA1*) that confers resistance to co-trimoxazole and tetracycline was also characterized from ciprofloxacin resistant strains (Gaind *et al.*, 2006).

3.4.4 ESBL production and resistance to third generation cephalosporins

Extended-spectrum cephalosporins are used for treatment of invasive infections caused by multidrug- resistant *Salmonella* and strains that are resistant to fluoroquinolones. There have been sporadic reports of high-level resistance to ceftriaxone *S.* Typhi and *S.* Paratyphi A, although these strains are very rare. Resistance to these antimicrobial agents, however, has been on the rise among several *Salmonella* serotypes. A variety of Ambler class A and class C beta lactamases have been described in different serotypes of *Salmonella* spp. (Parry 2003). Szych *et al.* in 2001 reported 0.3% occurrence of ESBL *S. enterica* in Poland. Pokhrel *et al.* in 2006 has reported ESBL production by *S.* Paratyphi A in Nepal.

The major mechanism for this resistance is through the production of specific enzymes to hydrolyze the associated extended-spectrum cephalosporins. The ceftriaxone resistance was attributed to the presence of a plasmid-mediated *blaCMY-2* and *blaCTX-M-3* gene, which were located on a transposon like DNA element consisting of a specific *tnpAbla CMY-2-blc-sugE* structure. Plasmid borne resistance to ceftriaxone can be transmitted among bacterial

organisms of the same or different species, resulting in widespread resistance that may result in untreatable typhoid fever.

3.5 Laboratory diagnosis of enteric fever

Clinical diagnosis of enteric fever is not specific because the presenting signs and symptoms are diverse and similar to those of other common febrile illnesses, such as malaria and dengue fever. A specific diagnosis requires access to a competent laboratory that can process blood cultures. The definitive diagnosis depends on the isolation of *S.* Typhi from blood, bone marrow or a specific anatomical lesion. The presence of characteristic clinical symptoms or the detection of a specific antibody response is suggestive of enteric fever but not definitive.

3.5.1 Microbiological procedures

Confirmation of typhoid or paratyphoid fever requires isolation of *S.* Typhi or *S.* Paratyphi, respectively, from blood, bone marrow, stool, or duodenal fluid. Cultures from skin above rose spots, buffy coats, and blood clots treated with streptokinase have been used (Gilman *et al.*, 1975; Vallenas *et al.*, 1985., Hoffman *et al.*, 1986; Rubin *et al.*, 1990). Counts of bacteria in patients with acute typhoid fever indicate a median concentration of 1 bacterium per milliliter of blood (about 66 percent of which are inside phagocytic cells) and about 10 bacteria per mL of bone marrow (Wain *et al.*, 1991).

3.5.1.1 Blood and bone marrow culture

Blood culture is the mainstay of the diagnosis as more than 80% of patients with typhoid fever have the causative organism in their blood. Although ox bile medium (Oxgall) is

recommended for enteric fever pathogens (*S.* Typhi and *S.* Paratyphi), only these pathogens can be grown on it. In a general diagnostic laboratory, therefore, where other pathogens are suspected, a general blood culture medium should be used. A failure to isolate the organism may be caused by several factors: (i) the limitations of laboratory media (ii) the presence of antibiotics (iii) the volume of the specimen cultured or (iv) the time of collection. Patients with a history of fever for 7 to 10 days were more likely than others to have a positive blood culture and sensitivity decreases with increasing duration of fever (Hoffman *et al.*, 1986). Failure to maintain ambient temperatures of 15–40ºC during specimen transportation, inappropriate laboratory methods, and antimicrobials compromise the yield.

Bone marrow aspirate culture, the gold standard for the diagnosis of typhoid fever, is positive in 80–95% of enteric fever patients (Farooqui *et al.*, 1991) It is particularly valuable for lengthy illness and for patients who have been previously treated (Gasem *et al.*, 1995), who have a long history of illness and for whom there has been a negative blood culture with the recommended volume of blood.

3.5.1.2 Culture of stool, urine and other sample

Duodenal aspirate culture has also proved highly satisfactory as a diagnostic test but has not found widespread acceptance because of poor tolerance of duodenal aspiration, particularly in children. Stool isolation of *S.* Typhi alone is insufficient for diagnosis and only marginally improves diagnosis by blood culture. However, it is confirmatory for carrier detection.

3.5.1.3 Isolation and identification

Any growth in culture of specimens from suspected enteric fever must be subjected for identification of causative agent. Conventional biochemical tests helps identification of the pathogen. Conformation of the *Salmonella enterica* involved in enteric fever is performed by serotyping with specific antisera.

3.5.1.3.1 Conventional biochemical test

Several biochemical reactions can be used for the identifiocation of *Salmonella* into genus, species and subspecies level (table 3.3). Besides these, some of the biochemical reactions (table 3.4) are found only in particular serotypes and are used in differentiation of serotypes.

3.5.1.3.2 Serotyping

Salmonellae can be characterized by their somatic and flagellar antigens, the latter existing in some serotypes in phases 1 and 2. Strains of *S.* Typhi and *S.* Paratyphi C may possess Vi antigen that render the strains non-agglutinable in O antisera. The O antigen is usually determined by means of the slide agglutination test with group-specific antiserum followed by agglutination with factor antiserum. Growth from non-selective agar or Kliger's iron agar can be used for the determination of O antigen.The antigenic compositions of the *Salmonella* that cause enteric fever are shown in Table 3.5.

Table 3.4 Antigenic formulae of serotype of *Salmonella* causing enteric fever

Serotype	Serogroup	O antigens and Vi	H antigens	
			Phase 1	Phase 2
Typhi	9	9, 12, Vi	d	-
Paratyphi A	2	1, 2, 12	a	(1, 5)
Paratyphi B	4	1, 4, 5, 12	b	1, 2
Paratyphi C	7	6, 7, Vi	c	1,5

Source: Old, 1996

3.5.2 Serological tests

3.5.2.1 Widal agglutination test

The Widal test identifies the agglutinating antibodies against the O (somatic) and H (flagellar) *S.* Typhi and *S.* Paratyphi antigens, which appear respectively on days 6-8 and 10-12 after the onset of the disease. The test is usually performed on an acute serum. A convalescent serum should preferably also be collected so that paired titrations can be performed but the later, however, often difficult in practice. The sensitivity, specificity, and predictive values reported from different centres vary because of sharing of O and H antigens and cross-reacting epitopes with other *Enterobacteriaceae* (Olopoenia and King 2000). The Widal test result can be negative in up to 30% of culture-proven cases of typhoid fever. The high numbers of false positive and false-negative Widal test results limit its clinical usefulness. To make a diagnosis, results from a single acute sample should be interpreted against the appropriate local cut-off values or there should be a fourfold rise in the antibody titres between convalescent and acute sera (Pang and Puthucheary 1983; Parry *et al.*, 1999). It is therefore important to establish the antibody level in the normal population in a particular locality in order to determine a threshold above which the antibody titre is considered significant. This is particularly important if, as usually happens, a single acute sample is available for testing

Despite these limitations, the test may be useful, particularly in areas that cannot afford the more expensive diagnostic methods. In developed countries, the use of Widal agglutination as a laboratory tool to aid in the diagnosis of typhoid fever during the acute phase of the illness, has largely been abandoned, as the need for such a test is minimal, especially in view of the low prevalence of typhoid fever. The Widal test appears to be the only laboratory means employed in the diagnosis of typhoid fever among suspected patients in some resource poor area of developing countries.

3.5.2.2 New diagnostic tests

There is a need for a quick and reliable diagnostic test for typhoid fever as an alternative to the Widal test. Recent advances include the IDL Tubex® test marketed by a Swedish company, which reportedly can detect IgM O9 antibodies from patient's serum within a few minutes. Another rapid serological test, Typhidot®, developed in Malaysia for the detection of specific IgM and IgG antibodies against a 50 kd antigen of *S.* Typhi, takes three hours to perform. A newer version of the test, Typhidot-M®, was recently developed to detect specific IgM antibodies only. The dipstick test, developed in the Netherlands, is based on the binding of *S.* Typhi-specific IgM antibodies in samples to *S.* Typhi lipopolysaccharide (LPS) antigen and the staining of bound antibodies by an anti-human IgM antibody conjugated to colloidal dye particles.

Serological tests based on agglutination of Vi antigens have 70–80% sensitivity and up to 95% specificity in identifying carriers of *S.* Typhi (Lanata *et al.*, 1983). The understanding of genome sequence of *S* Typhi might now result in identification of specific antibodies to fimbrial and other antigens. Urinary Vi antigen detection by ELISA within the first febrile week shows promise, but its positivity in brucellosis patients is an obstacle to further development (Fadeel *et al.*, 2004).

3.5.3 Molecular methods

Isolation of *S.* Typhi from culture takes 2 to 7 days and this delay might prove to be fatal especially in young children. Widal test helpful in making a presumptive diagnosis in non-endemic areas is still controversial in endemic areas (Levine *et al.*, 1978) and false positivity have been reported in certain non-typhoid infections. In view of this, DNA-based diagnosis of enteric fever has been attempted in different laboratories (Way *et al.*, 1993). Use of DNA probe specific to Vi antigen of *S.* Typhi (Rubin *et al.*, 1985, 1988, 1990) and polymerase

chain reaction (PCR) amplification of a specific fragment corresponding to flagellar gene (*H1-d*) (Hashimoto *et al.*, 1995) has been used to detect organisms in patients' blood with typhoid fever. PCR approach has been found to discriminate typhoid from other febrile conditions including paratyphoid and culture negative cases (Song *et al.*, 1993). DNA probes and PCR-based tests to detect flagellar genes (Hirose *et al.*, 2002) are not routinely useful in developing countries, but they are of value in surveillance and research.

3.5.4 Diagnosis of enteric fever in Nepal

Clinical diagnosis of enteric fever is not specific because the presenting signs and symptoms are diverse and similar to those of other common febrile illnesses, such as malaria and dengue fever that are also prevalent in Nepal. A specific diagnosis of typhoid requires access to a competent laboratory that can process blood cultures; such laboratories are uncommon in resource-poor regions of developing countries including Nepal (Ochiai *et al.*, 2008). In some well-equipped laboratories of Nepal, both the culture and Widal tests are done simultaneously whereas other laboratories, where the culture facility is not available, only the Widal test is done and in some part diagnosis is solely based on clinical background.

3.6 Antimicrobial susceptibility testing

Antimicrobial susceptibility testing is crucial for the guidance of clinical management of enteric fever. Ampicillin, chloramphenicol, sulfonamide, trimethoprim, streptomycin and tetracycline are the conventional drug used in the treatment of enteric fever, although isolates from many parts of the world are now MDR being usually resistance to all these first line drugs. Alternative drugs that are used for treatment include fluoroquinolones (e.g. ciprofloxacin), third-generation cephalosporins (e.g. ceftriaxone, cefotaxime), a monobactum beta-lactam (aztreonam) and a macrolide (azithromycin). Even though

resistance to the first two has been noted they nevertheless remain useful. Reduced susceptibility to fluoroquinolones that cannot be appropriately intreprated according to CLSI guidelines is important problem in antimicrobial susceptibility testing.

3.6.1 Disc diffusion methods

In vitro susceptibility testing usually involves disc diffusion following standard guidelines. The agents that are currently being used for treatment and the desire to determine the prevalence of MDR strains dictate the choice of antimicrobial agents for the test. It is therefore recommended that susceptibility tests be performed against the following antimicrobial agents: a fluoroquinolone, a third-generation cephalosporin and any other drug currently used for treatment, nalidixic acid (for determining reduced susceptibility to fluoroquinolones because of the possibility of false in vitro susceptibility against the fluoroquinolone used for treatment), and the previous first-line antimicrobials to which the strains could be resistant (chloramphenicol, ampicillin, trimethoprim/sulfamethoxazole, streptomycin and tetracycline) (WHO, 2003). Azithromycin disc test is also recommended (Andrews, 2008) but results should be interpreted with caution as break-point recommendations for azithromycin against *S.* Typhi are still not clear and patients may respond satisfactorily to azithromycin even if isolates are intermediate according to current guidelines.

Based on the antimicrobial susceptibility testing, the isolates can be broadly classified as sensitive to first-line antimicrobials, multidrug resistant but nalidixic-acid susceptible, only nalidixic acid resistant, and multidrug resistant with nalidixic-acid resistant (MDR-NAR).

3.6.2 Nalidixic acid screening tests

Common single mutations in the *gyrA* gene can produce a ten-fold increase in the fluoroquinolone MIC compared with the wild type. The MIC of such isolates is still within the susceptible range according to current guidelines, and they are reported as susceptible

when using disc sensitivity tests. However, the reduction in susceptibility translates into an increased chance for clinical failure when infection with these isolates is treated with fluoroquinolones. These strains usually are resistant to the first-generation quinolone, nalidixic acid, and this can be a useful screening test for the presence of such isolates (Hakanen *et al.*, 1999), although occasional isolates with reduced susceptibility to fluoroquinolones are nalidixic-acid susceptible (Hakanen *et al.*, 2001).

3.6.3 Determination of MIC

Minimim inhibitory concentrations (MICs) are considered gold standard for determining the susceptibility of organisms to antimicrobials and are therefore used to judge the performance of all other methods of susceptibility testing. MICs are used in diagnostic laboratories to confirm unusual resistance, to give a definitive answer when a borderline result is obtained by other methods of testing or when disc diffusion methods are not appropriate.

Determination of MICs, particularly of fluoroquinolones in *Salmonella* is important to detect the reduced susceptibility to these drugs that remains undectable by disc diffusion methods.

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials and equipments

Materials, equipments and reagents used in different stage of this study have been included in the appendix V1.

4.2 Methodology

4.2.1 Study site and study period

The study was carried out prospectively at National Public Health Laboratory (NPHL) from April through October 2008.

4.2.2 Study population

Study was conducted on clinically defined enteric fever patients who visited to NPHL requesting for blood culture and susceptibility testing**.**

4.2.3 Case definition

Cases included in the study were patient defined by physicians as probable case of enteric fever with fever (38°C and above) that has lasted for at least three days and showing clinical signs and symptoms of enteric fever.

4.2.4 Sample size and inclusion criteria

A total of 443 blood samples from patients following case definition of suspected enteric fever were included in the study. For the evaluation of fluoroquinolone susceptibility and validiation of nalidixic acid screening tests, isolated *Salmonella* serotypes which were preserved after confirmed identification were used. Samples with improper labeling, unsufficient blood volume, and inappropriate collection and transport were rejected.

4.2.5 Collection of blood sample

Blood samples were collected from patients visiting to NPHL following the case definition of enteric fever. Details on clinical history, age, sex etc. of the individual were recorded (appendix I). About 3ml of blood from children and 5ml of blood from adult was collected and aseptically inoculated into bile broth.

4.2.6 Isolation and identification of *Salmonella* serotype

4.2.6.1 Isolation

Causative agent of enteric fever was isolated by selective culture of blood in bile broth. Bile broth was incubated at 37˚C for upto 7 days, noted everyday for turbidity or any visual change, and subcultured on MacConkey agar. Any visual change on culture bottle and growth observed on subculture were recorded on worksheet (appendix II).

4.2.6.2 Biochemical tests

Isolates from bile broth on MacConkey agar were identified by standard conventional biochemical methods (appendix III).

4.2.6.3 Slide agglutination test

Serotyping by agglutination with specific antisera (Denka Seiken Co. Ltd., Tokyo, Japan) for O, H and Vi antigens was performed following manufacturer's instruction (appendix IV) to identify and confirm the serotype.

4.2.7 Preservation of isolates

After complete identification, isolates were preserved on tryptic soya broth with 25% glycerol at -70˚C.

4.2.8 Antimicrobial susceptibility testing

4.2.8.1 Disc diffusion method

Susceptibility to 8 antibiotics was performed by Kirby Bauer disc diffusion method (working protocol in appendix IV) with Mueller–Hinton agar using the guidelines and interpretive criteria of the CLSI. The antibiotic discs used were ampicillin (30µg), nalidixic acid (30µg), ofloxacin (5µg), ciprofloxacin (5µg), tetracycline (30µg), chloramphenicol (30µg), ceftriaxone (30µg), and trimethoprim–sulfamethoxazole (1.25/23.75µg). To ensure reproducibility, each tests and measurement were carried out twice.

4.2.8.2 Determination of MIC

MICs of ciprofloxacin, ofloxacin and nalidixic acid were determined by agar dilution method (Andrews, 2001) and the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2006) (working protocol in appendix IV). Briefly, a series of Mueller–Hinton agar plates, containing different concentration of antibiotics (0.25 to 512µg/mL for nalidixic acid, 0.0075 to 256µg/mL for ciprofloxacin and 0.0075 to 128µg/mL for ofloxacin) were prepared. Each plate was divided into a number of 25 sectors for spot inoculation. Using an inoculum of approximately 10^4 CFU per spot, 25 isolates (including two standard control strains at position 1 and 25) were inoculated at respective spot as prepared in worksheet. All tests were incubated at 37˚C for 18 h. The MIC was defined as the lowest concentration of antibiotic at which there was no visible growth. Hazy growth and one or two colonies on the spot were ignored. *Escherichia coli* ATCC 25922 and *Enterococcus faecalis* ATCC 29212 were used as the quality control strain. The breakpoints used were

those defined by the CLSI for *Enterobacteriaceae* (appendix VII). To ensure reproducibility, each tests and measurement were carried out twice.

4.3 Analysis of data

All the results were recorded in worksheet (appendix I to V) and entered into Microsoft Excel. Antimicrobial susceptibility test by disc diffusion and MIC values were analysed by using WHONET 5.4 software. Statistical analysis was done by using SPSS 11.5. Nalidixic acid validation test was determined by scatterplot analysis and determining sensitivity and specificity.

4.4 Quality control

4.4.1 Quality monitoring of the laboratory equipments, reagent and media

Laboratory equipment like incubator, hot air oven, autoclave, refrigerator etc. were regularly monitored for their performance and immediately corrected if any deviation occurred. The temperature of the incubator and refrigerator were monitored everyday.

Reagents and biochemical media were checked for manufacture and expiry date and proper storage. After preparation, each media and reagent were labelled with preparation date, expiry date and stored in proper conditions. Sterility testing and performance testing were carried out using standard control strains.

4.4.3 Quality control during isolation and identification

Blood culture broth that passed quality control for performance and sterility were used. During the identification, pure culture of the isolated colony of the organism was used. After

inoculation into different biochemical media, the inoculum was verified for pure culture and no contamination occurred during inoculation.

Suspension of the isolated colony from nonselected media was prepared on normal saline taken in a slide. Agglutination result should always be noted along with control to avoid false reporting due to auto- agglutination. Serotyping was performed strictly following the manufacturers' instruction.

4.4.5 Quality control during antimicrobial susceptibility testing

Muller Hinton agar and antibiotic disc were checked for each lot number, manufature date and expiry date and stored properly. Before use each antibiotics disc and Muller Hinton agar were monitored for their performance quality with *Escherichia coli* ATCC 25922 and *Staphylococcus aureus* 25923. For antimicrobial susceptibility testing, standard inoculum (matched with 0.5 Mc- farland solutions) was used.

4.4.6 Quality control during MIC determination

Antibiotic powders were checked for manufacture date, expiry date, potency and proper storage. Antibiotic stock solution of appropriate concentration was prepared and refrigerated at 4- 8˚C. Dilution range of antibiotic solution were prepared and mixed with autoclaved Muller Hinton agar (at 45˚C). Each series of antibiotic agar plate were inoculated with standard inoculum of test organism and standard control strains (*Escherichia coli* ATCC 25922 and *Staphylococcus aureus* 25923). Pre and post growth control plate without antibiotic were also included. The colony of the inoculum was verified by pour plate method. MIC value of the test organism was noted only if pre and post growth control plate showed growth, colony verification plate verified the inoculum used, and standard control strains showed appropriate MIC value. MIC values of isolates to antibiotics were measured twice to ensure reproducibility of results.

4.5 Limitation of the study

The relatively smaller size of the study group, lack of therapeutic response in patients with enteric fever caused by NARST infection are limitations of the present study. Besides this, our reliance upon a single blood culture and lack of susceptibility testing to higher generation fluoroquinolones (eg. gatifloxacin) and other alternative drugs (eg. azithromycin), and lacking of further molecular characterization of resistant isolates are also the limitations of the study.

CHAPTER-V

5. RESULTS

5.1 Cuture positivity

Out of the 443 enteric fever suspected febrile cases investigated during the study period, 305(68.84%) were male and 138(31.16%) were female. The patients under investigation were the age range of 1- 91 years (mean 26.37 years). The highest numbers of cases (256) were from the age group 19-45 years, followed by 130 cases from 5-18 years, 46 cases above 45 years and 11 cases below 5 years (table 5.1). The age group 19-45 comprised 57.78% of the all suspected cases. On blood culture, 41(9.26%) samples were culture positive (fig 5.1). Among male and female, 32(10.49%) and 9(6.52%) showed growth on blood culture, respectively. The difference in growth of organisms in male and female, however, is statistically insignificant ($P > 0.05$).

Although the overall growth rate was 9.26%, the percentage of growth in the age group 5-18 years was 20% followed by other (table 5.1). The mean age of enteric fever cases was 21.39 years. The age group 5-18 years consisted of 26(63.41%) enteric fever cases. The difference

in enteric fever cases in the age group 5-18 years in comparision to other age group is statistically significant (P < 0.001).

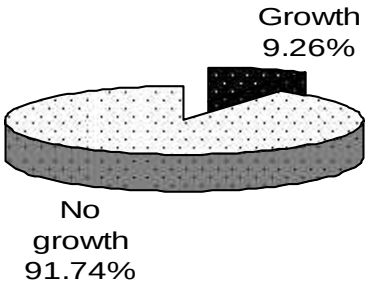


Fig 5.1: Percentage of growth on blood culture

Table 5.1: Distribution of the suspected cases and enteric fever in different age group

Age group (yrs)	Total number of suspected febrile cases		Number of growth (enteric fever cases)				Percentage of growth in different age group
	Male	Female	Male		Female		
			ST	SPA	ST	SPA	
<5	4	7	-	-	-	1	9.09
5-18	84	46	12	9	2	3	20.00
19-45	183	73	5	4	2	-	4.29
>45	34	12	2	-	1	-	6.52

5.2 Proportion of *S.* Typhi and Paratyphi A serotypes in bloods culture

Out of the 41 isolates, 24(58.54%) were *S.* Typhi and 17(41.46%) were *S.* Paratyphi A (fig 5.2). The distribution of these serotypes also varied among different age groups (table, 5.1).

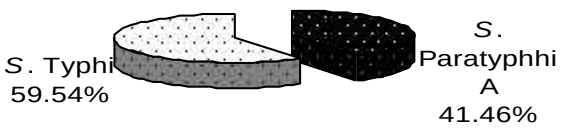


Fig 5.2: Percentage of *S.* Typhi and *S.* Paratyphi A in growth positive samples

5.3 Incubation period and growth pattern

Among all culure positive blood samples, 32(78.05%) isolates were obtained within 24 hrs incubation of blood culture bottle, and remaining 9(21.95%) isolates were obtained on the following days (fig 5.3). No isolate were obtained 120 hours onwards.

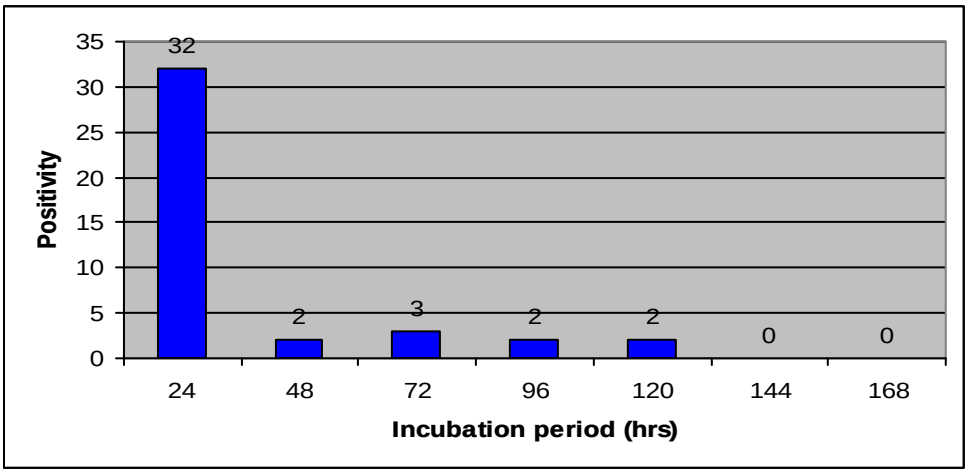


Fig 5.3: Growth pattern in different days of incubation (Positivity is the number of positive culture detected in the particular period)

5.4 Month wise distribution of *Salmonella* isolates during April to October 2008

The most of the suspected enteric fever patients visited to the National Public Health Laboratory in the month of July, August and September. Sample from these three months consisted 295(66.59%) of the total samples collected during the study period and 33(80.49%) of the total growths. Distribution of the samples and growth monthwise is shown in figure 5.4.

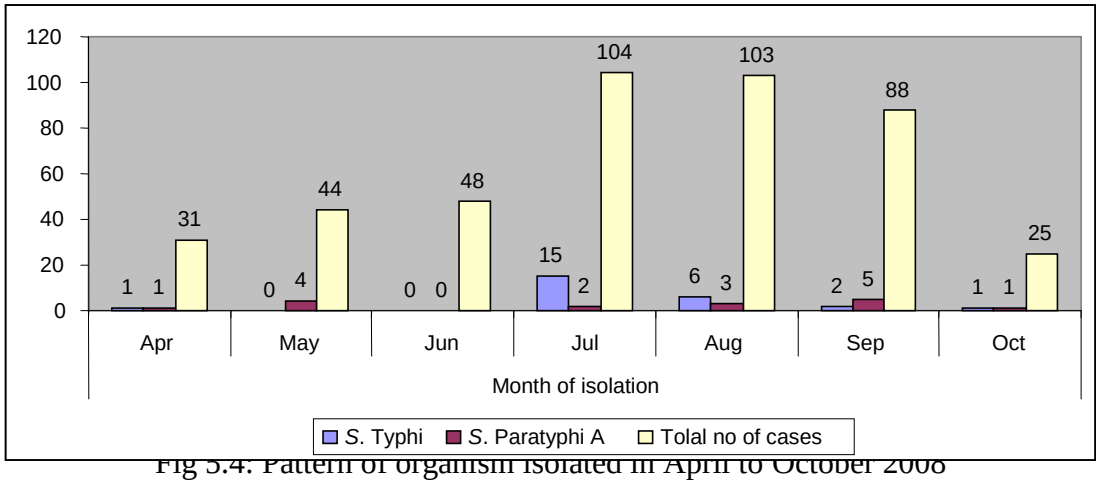


Fig 5.4: Pattern of organism isolated in April to October 2008

5.5 Antimicrobial susceptibility pattern of the *Salmonella* isolates

All the 41 isolates obtained were tested for antimicrobial susceptibility pattern using 8 antibiotics. The complete antimicrobial pattern of the isolates is shown in table 5.2.

Table 5.2: Antimicrobial susceptibility pattern of the *Salmonella* isolates

Sample ID	Organim isolated	Isolate ID	Antibiotic susceptibility pattern							
			Amp	Chl	Cot	Tet	NA	Cip	Of	CRO
008	ST	001	S	S	S	S	S	S	S	S
011	SPA	002	S	S	S	S	**R**	S	S	S
042	SPA	003	S	S	S	S	**R**	S	S	S
047	SPA	004	S	S	S	S	S	S	S	S
052	SPA	005	S	S	S	S	**R**	S	S	S
054	SPA	006	S	S	S	S	**R**	S	S	S
121	ST	007	**R**	**R**	**R**	S	**R**	S	S	S
133	ST	008	S	S	**R**	**R**	**R**	**R**	**R**	S
142	ST	009	S	S	S	S	S	S	S	S
158	ST	010	S	S	S	S	**R**	S	S	S
167	ST	011	S	S	S	S	**R**	S	S	S
168	ST	012	S	S	S	S	**R**	S	S	S
169	ST	013	S	S	S	S	**R**	S	S	S
170	ST	014	S	S	S	S	**R**	S	S	S
171	ST	015	S	S	S	S	**R**	S	S	S
172	ST	016	S	S	S	S	**R**	S	S	S
173	ST	017	S	S	S	S	**R**	S	S	S
174	ST	018	S	S	S	S	**R**	S	S	S

175	ST	019	S	S	S	S	**R**	S	S	S
176	ST	020	S	S	S	S	**R**	S	S	S
215	SPA	021	S	S	S	S	**R**	S	S	S
217	SPA	022	S	S	S	S	**R**	S	S	S
221	ST	023	S	S	S	S	**R**	S	S	S
239	ST	024	S	S	S	S	S	S	S	S
242	SPA	025	S	S	S	S	**R**	S	S	S
257	ST	026	S	S	S	S	**R**	S	S	S
262	ST	027	S	S	S	S	S	S	S	S
271	SPA	028	S	S	S	S	**R**	S	S	S
272	ST	029	S	S	S	S	**R**	S	S	S
301	ST	030	S	S	S	S	**R**	S	S	S
309	ST	031	S	S	S	S	**R**	S	S	S
324	SPA	032	S	S	S	S	**R**	S	S	S
330	ST	033	S	S	S	S	S	S	S	S
335	SPA	034	S	S	S	S	**R**	S	S	S
339	SPA	035	S	S	S	S	**R**	S	S	S
350	SPA	036	S	S	S	S	S	S	S	S
352	SPA	037	S	S	S	S	**R**	S	S	S
402	SPA	038	S	S	S	S	**R**	S	S	S
408	SPA	039	S	S	S	S	**R**	S	S	S
429	SPA	040	S	S	S	S	**R**	S	S	S
436	ST	041	S	S	S	S	S	S	S	S

Note: S, Sensitine; R, Resistant; Amp, Ampicillin; Chl, Chloramphenicol; Cot, Cotrimoxazole; Tet, Tetracycline; NA, Nalidixic acid; Cip, Ciprofloxacin; Of, Ofloxacin; CRO, Ceftriaxone

All the isolates were sensitive to ceftriaxone. Most of the isolates were also sensitive to ampicillin, chloramphenicol, cotrimoxazole and tetracycline. The percentage of isolates resistant to ampicillin, chloramphenicol, cotrimoxazole, and tetracycline were 2.43%, 2.43%, 4.87%, and 2.43%, respectively (fig 5.5).

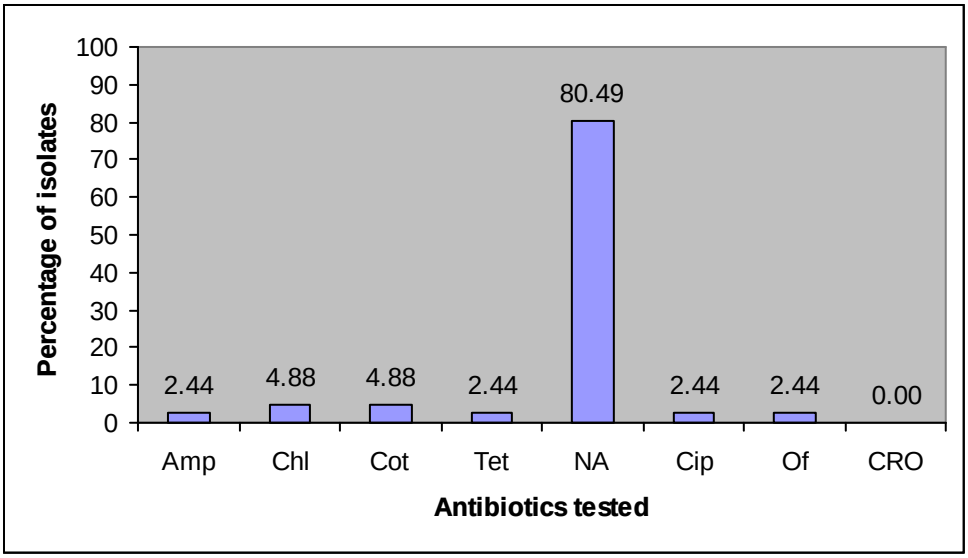


Fig 5.5: Antimicrobial susceptibility pattern of *S.* Typhi and *S.* Paratyphi A

Only 2(4.88%) isolates were resistant to two or more antibiotics (MDR), both of which were *S.* Typhi. One of the isolates showed resistance to ampicillin, chloramphenicol, cotrimoxazole (ACCo) and nalidixic acid while other showed resistance to cotrimoxazole, tetracycline, nalidixic acid, ciprofloxacin, and ofloxacin. Thirty-nine (95.12%) isolates were susceptible to all the first line drugs (ACCoT). Susceptibility to ACCoT was 100% in *S.* Paratyphi A and 91.66% in *S.* Typhi.

Antimicrobial susceptiptibility to quinolones showed that 8(19.51%) isolates were susceptible to nalidixic acid, 32(78.05%) isolates were resistant only to nalidixic acid (no zones in 30µg disc), and one *S.* Typhi (2.44%) isolates was fully resistant to fluoroquinolone.

Resistance to nalidixic acid in *S.* Typhi and *S.* Paratyphi A (fig 5.6) was 88.23% and 75%, respectively. The nalidixic acid resistance in *S.* Typhi and *S.* Paratyphi A however is statistically insignificant ($P > 0.05$).

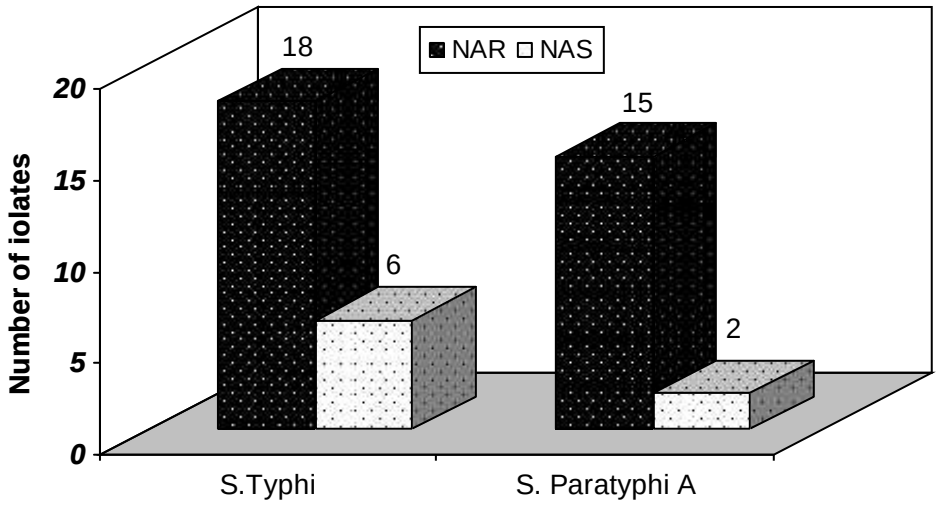


Fig 5.6: Nalidixic acid resistance pattern in *S.* Typhi and *S.* Paratyphi A

All nalidixic acid susceptible (NAS) isolates were susceptible to other first line drug, while of the 33 nalidixic acid resistant (NAR) isolates, 31(93.94%) were susceptible to all the first line drug. One of the nalidixic acid resistant isolates showed resistance to ampicillin, chloramphenicol, and cotrimoxazole (MDR-NAR).

5.6 Fluoroquinolones MIC and disc diffusion test

Of the total isolates, 33(80.49%) have nalidixic acid MIC of ≥ 32 µg/mL and were classified as resistant while 8(19.51%) have MIC of ≤ 8µg/mL and were classified as susceptible according to CLSI (CLSI, 2006). For ciprofloxacin, 40(97.56 %) isolates have MIC of ≤ 1 µg/ml and were classified as susceptible while 1(2.4 %) has MIC of 16 µg/mL and was classified as resistant. Similarly for ofloxacin, 40(97.56%) isolates have MIC of ≤ 2µg/mL and classified as susceptible while 1(2.44%) has MIC of 8 µg/mL and classified as resistant. The MIC value and respective zone diameter of all isotates to three fluoroquinolones is shown in table 5.3.

Table 5.3: MIC value and respective zone diameter of nalidixic acid, ciprofloxacin and ofloxacin in all *Salmonella* isolates

Isolate ID	Sample ID	Organim isolated	Nalidixic Acid		Ciprofloxacin		Ofloxacin	
			Zone diameter (mm)	MIC (mg/L)	Zone diameter (mm)	MIC (mg/L)	Zone diameter (mm)	MIC (mg/L)
001	008	ST	24	1	34	0.0075	28	0.03
002	011	SPA	NZ	512	23	0.5	20	2
003	042	SPA	NZ	256	26	0.5	24	2
004	047	SPA	25	1	34	0.0075	32	0.06
005	052	SPA	NZ	512	23	0.5	19	2
006	054	SPA	NZ	512	24	1	21	2
007	121	ST	NZ	64	28	0.25	25	0.25
008	133	ST	NZ	>512	12	16	11	8
009	142	ST	24	0.5	34	0.0075	32	0.015
010	158	ST	NZ	128	27	0.25	25	0.5
011	167	ST	NZ	128	26	0.25	24	0.25
012	168	ST	NZ	256	28	0.25	25	0.5
013	169	ST	NZ	256	29	0.25	24	0.5
014	170	ST	NZ	128	29	0.25	24	0.25
015	171	ST	NZ	256	28	0.25	25	0.5
016	172	ST	NZ	64	28	0.25	25	0.25
017	173	ST	NZ	128	25	0.25	22	0.5
018	174	ST	NZ	256	26	0.25	23	0.5
019	175	ST	NZ	128	25	0.25	23	0.5
020	176	ST	NZ	256	26	0.25	24	0.5
021	215	SPA	NZ	512	28	1	24	2
022	217	SPA	NZ	256	30	0.25	26	1
023	221	ST	NZ	128	25	0.25	22	0.25
024	239	ST	25	1	32	0.0075	30	0.015
025	242	SPA	NZ	512	23	0.5	19	2
026	257	ST	NZ	128	26	0.25	24	0.5
027	262	ST	25	1	34	0.0075	27	0.03
028	271	SPA	NZ	512	24	0.5	19	1
029	272	ST	NZ	128	27	0.25	23	0.5
030	301	ST	NZ	256	24	0.25	21	0.5
031	309	ST	NZ	256	27	0.25	25	0.5
032	324	SPA	NZ	512	23	1	19	1
033	330	ST	24	1	32	0.015	27	0.03
034	335	SPA	NZ	512	23	0.5	22	2
035	339	SPA	NZ	512	24	1	19	2
036	350	SPA	24	1	34	0.0075	30	0.015
037	352	SPA	NZ	512	27	0.5	23	2
038	402	SPA	NZ	512	26	0.5	22	2
039	408	SPA	NZ	512	26	0.5	22	2
040	429	SPA	NZ	512	24	1	20	2
041	436	ST	20	4	27	0.06	25	0.12

Note: NZ, no zone of inhibition

The *S.* Typhi and *S.* Paratyphi A strains resistant to NA (≥ 32 µg/mL) required a higher MIC of fluoroquinolones (MIC of ciprofloxacin, 0.25 to 1 µg/mL; ofloxacin, 0.25 to 2µg/mL) compared with the strains susceptible to NA (for ciprofloxacin, 7 of 8 strains required an MIC of ≤ 0.015 and the other strain required an MIC of 0.06; for ofloxacin, 7 of 8 strains required an MIC of ≤ 0.03 and the other strain required an MIC of 0.12). The NAR *S.* Paratyphi A required increased MICs of the fluoroquinolones compared with the NAR *S.* Typhi. The difference in mean fluoroquinolone MIC in NAR *S.* Typhi and NAR *S.* Paratyphi A is stastistically significant (P< 0.001).

According to the CLSI recommendations, all isolates evaluated by means of disc diffusion tests were classified as ciprofloxacin susceptible (≥ 21mm), except one isolates which was ciprofloxacin resistant (15mm). Similarly, most isolates were classified as ofloxacin susceptible (≥ 16mm), except one which was ofloxacin resistant (12mm).

All isolates with resistance to nalidixic acid in disc diffusion have nalidixic acid MIC of ≥ 32µg/mL, whereas susceptible to nalidixic acid have MIC ≤ 8µg/mL. Of the 8 nalidixic acid susceptible isolates, 5(75%) with ciprofloxacin MIC of ≤ 0.0075µg/mL had zone of inhibition of 34 mm, one with ciprofloxacin MIC of 0.0075µg/mL had zone of inhibition of 32mm, one with ciprofloxacin MIC of 0.015µg/mL had zone of inhibition of 32mm and one with ciprofloxacin MIC of 0.06µg/mL had inhibition zone diameter of 27mm. Of the remaining, 32(83%) isolates with ciprofloxacin MIC ranging 0.25-1µg/mL had inhibition zone diameter 22-28mm and one (1.6%) isolate with ciprofloxacin MIC of 16µg/mL had inhibition zone diameter of 12mm. Similarly, of the 8 nalidixic acid susceptible isolates, 7(87.7 %) with ofloxacin MIC of ≤ 0.03µg/mL had inhibition zone diameter of ≥ 27mm and one with MIC of 0.12µg/mL had zone of inhibition of 25mm. Of the remaining, 32(83%) isolates with ofloxacin MIC ranging between 0.25 and 2µg/mL had zone diameter 19-25mm and one (1.6%) isolate with MIC 8µg/mL had a zone diameter of 11mm.

5.7 Nalidixic acid resistance and ciprofloxacin MIC

Of the total isolates, 40(97.6%) were susceptible (MIC ≤ 1µg/ml), and 1 (2.4%) was resistant (MIC 16µg/ml) to ciprofloxacin. Of the 40 ciprofloxacin susceptible isolates, 32 (MIC 0.25µg/ml to 1µg/ml) were NAR and 8 (MIC ranged between 0.0075µg/ml and 0.06µg/ml) were NAS. Based on NA susceptibility, the MIC of ciprofloxacin for susceptible isolates showed a bimodal distribution (fig 5.7).

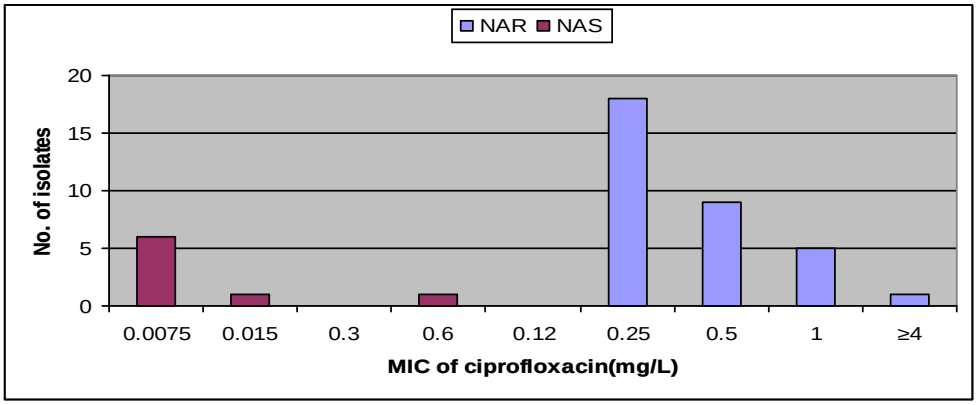


Fig 5.7: Distribution of MIC of ciprofloxacin between the NAR and NAS isolates

The scattergram correlating the MICs of ciprofloxacin and nalidixic acid illustrates the simultaneous presence of nalidixic acid resistance and reduced ciprofloxacin susceptibility (Fig.5.8). Of the 33 isolates for which the ciprofloxacin MIC was ≥ 0.25µg/mL, all were resistant to nalidixic acid (MIC, ≥ 64µg/mL).

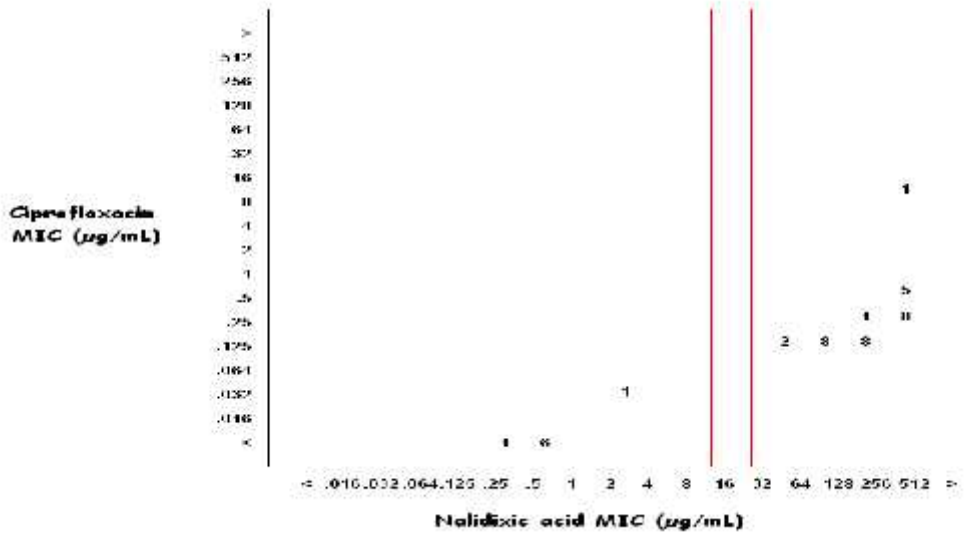


Figure 5.8: MIC scatterplots for nalidixic acid versus ciprofloxacin for *S.* Typhi and *S.* Paratyphi A (output from WHONET 5.4 after analysis)

5.8 Nalidixic acid resistance and ofloxacin MIC

Of the total isolates, 40(97.6 %) were susceptible (MIC ≤ 1 µg/ml), and 1(2.4 %) was resistant (MIC 16 µg/ml) to ofloxacin. Of the ofloxacin susceptible isolates, 32 (MIC 0.25µg/mL to 2µg/mL) were NAR and remaining 8 (MIC 0.015µg/mL to 0.12µg/mL) were NAS (100%). The MIC of ofloxacin susceptible isolates showed a bimodal distribution (fig 5.9).

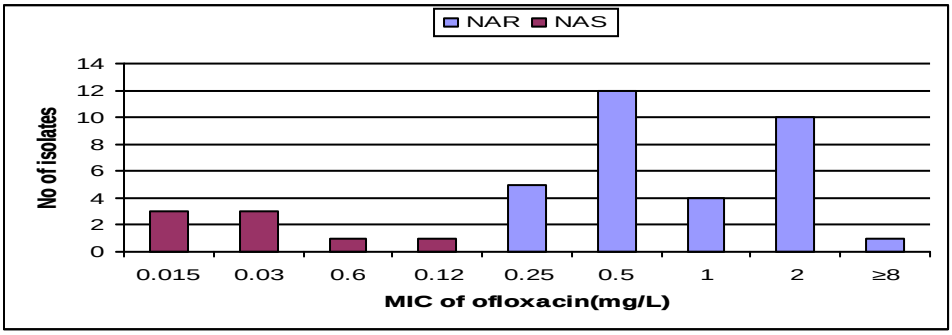


Fig: 5.9: Distribution of MIC of ofloxacin between the NAR and NAS isolates

The scattergram correlating the MICs of ofloxacin and nalidixic acid illustrates the simultaneous presence of nalidixic acid resistance and reduced ofloxacin susceptibility (Fig. 5.10). Of the 33 isolates for which the ofloxacin MIC was ≥ 0.25µg/mL, all were highly resistant to nalidixic acid (MIC, ≥ 64µg/mL).

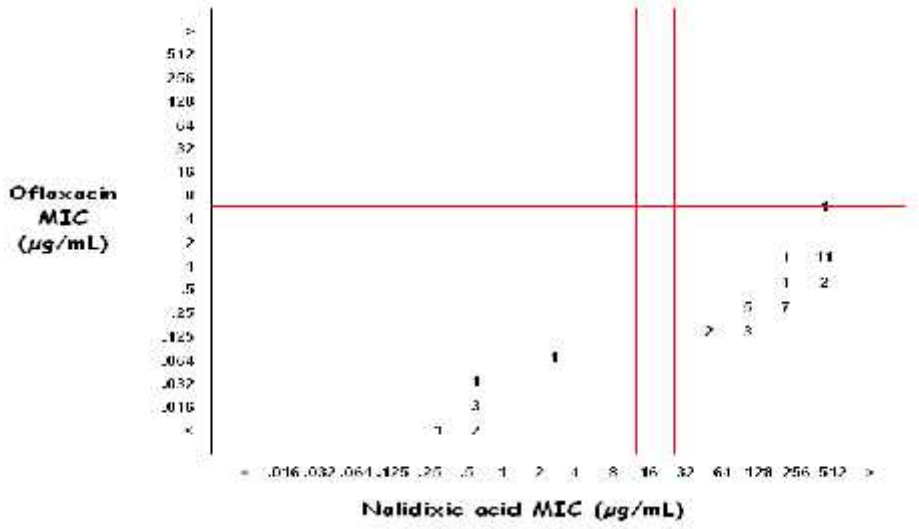


Figure 5.10: MIC scatterplots for nalidixic acid versus ofloxacin for *S.* Typhi and *S.* Paratyphi A (output from WHONET 5.4 after analysis)

5.9 Nalidixic acid screening test to determine reduced susceptibility to fluoroquinolones

Of the 33 NAR isolates, 32 were susceptible to ciprofloxacin and one was resistant. Of the 8 NAS isolates, all were ciprofloxacin susceptible. Nalidixic acid susceptibility showed a predictive value of 100% for ciprofloxacin susceptibility, whereas nalidixic acid resistance showed a predictive value of 3.03% for ciprofloxacin resistance. This shows that a substantial proportion (97%) of NAR isolates showed ciprofloxacin MIC in the susceptible zone. Predictive value of nalidixic acid susceptibility for ofloxacin susceptibility and nalidixic acid resistance for ofloxacin resistance was similar to that of ciprofloxacin.

All of the 32 isolates with reduced susceptibility have ciprofloxacin MIC of 0.25- 1µg/mL and ofloxacin MIC of 0.25- 2µg/mL. The difference in MIC values of ciprofloxacin and

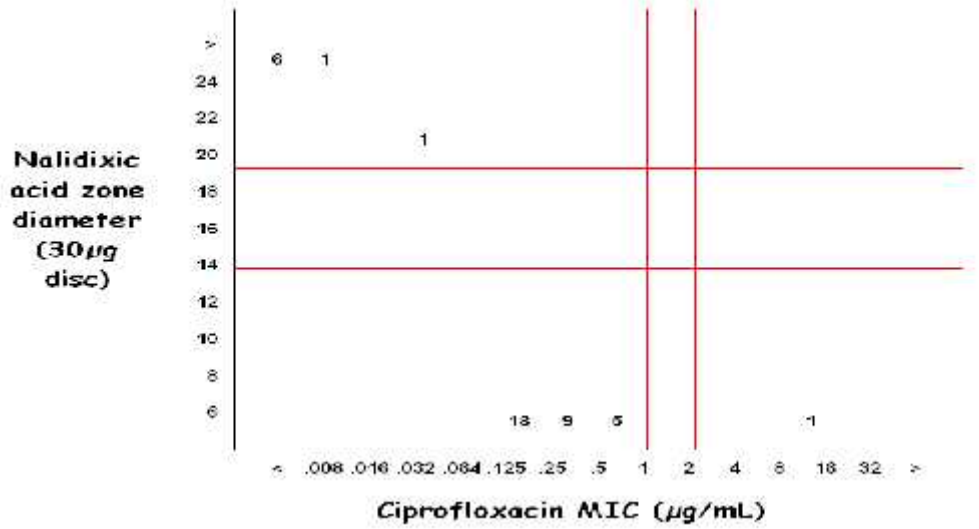
ofloxacin in nalidixic acid susceptible and nalidixic acid resistant population is statistically significant ($P < 0.001$).

The relevance of using the resistance to nalidixic acid as a marker for reduced fluoroquinolones susceptibility was evaluated by comparing the MICs of ciprofloxacin and ofloxacin with that of nalidixic acid for the 40 *Salmonella* isolates. When an ciprofloxacin MIC of ≥ 0.125µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC, ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ciprofloxacin susceptibility (MIC, ≥ 0.125µg/mL) and none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100%.

Similarly, when an ofloxacin MIC of ≥ 0.25µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC, ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ofloxacin susceptibility (MIC, ≥ 0.25µg/mL) and none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100%.

Based on the MICs of ciprofloxacin and zone diameters around 30µg nalidixic acid disc for the 40 isolates tested (Fig. 5.11), screening for nalidixic acid resistance (inhibition zone diameter, ≤ 13mm) led to the detection of all isolates for which the MICs were ≥ 0.125µg/mL. When this MIC was used as a breakpoint of reduced ciprofloxacin

susceptibility, the sensitivity and specificity of the nalidixic acid disc screening was



100%.

Figure 5.11: Scatterplots for nalidixic acid zone diameter versus ciprofloxacin MIC for *S.* Typhi and *S.* Paratyphi A (output from WHONET 5.4 after analysis)

Similarly, based on the MICs of ofloxacin and zone diameters around 30µg nalidixic acid disc for the 40 *Salmonella* isolates tested (Fig. 5.12), screening for nalidixic acid resistance (inhibition zone diameter, ≤ 13mm) led to the detection of all isolates for which the oflxacin MICs of ≥ 0.25- 2µg/mL. When this MIC was used as a breakpoint of reduced ofloxacin susceptibility, the sensitivity and specificity of the nalidixic acid disc screening was 100%.

The difference in ciprofloxacin and ofloxacin zone diameter in NAR and NAS isolates was statistically significant (P> 0.001). Based on this difference in zone diameter, the applicability of the 5µg ciprofloxacin and 5µg ofloxacin disc diffusion test in detecting reduced ciprofloxacin and ofloxacin susceptibility was assessed by scatterplot analysis (Fig 5.13 and 5.14**).**

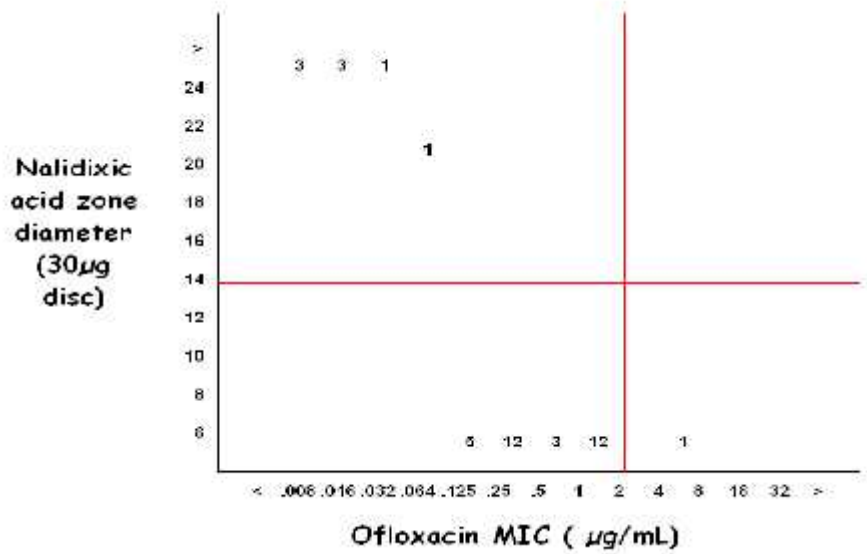


Figure 5.12: Scatterplots for nalidixic acid zone diameter versus ofloxacin MIC for *S.* Typhi and *S.* Paratyphi A (output from WHONET 5.4 after analysis)

The MICs of ciprofloxacin for 32 nalidixic acid resistant isolates with the ciprofloxacin inhibition zone diameter of ≤ 28mm were ≥ 0.125µg/mL, whereas for all the nalidixic acid sensitive isolates except one (zone diameter 27mm, MIC 0.06µg/mL) with ciprofloxacin zone diameter of ≥ 32mm, the MICs were ≤ 0.015µg/mL. Thus, when an MIC of ≤ 0.125µg/mL was adopted as a breakpoint, the ciprofloxacin inhibition zone diameter of ≥ 32mm yielded 100% sensitivity and 87.5% specificity in screening for full ciprofloxacin susceptibility.

Similarly, when an MIC of ≤ 0.25µg/mL was adopted as a breakpoint, the ofloxacin inhibition zone diameter of ≥ 27mm yielded 100% sensitivity and 87.5% specificity in screening for full ofloxacin susceptibility.

5.10 Regression analysis of log MIC versus zone diameter

Based on the current CLSI recommended guidelines for MIC of *Enterobacteriaceae*, the regression analysis of log MIC vs zone diameter was performed and regression lines were plotted (fig 5.12 and 5.13).

According to regression analysis, to accommodate a susceptible MIC of ≤ 1µg/mL, the zone diameter of 5µg ciprofloxacin disc for susceptible organism increased to about 25 mm from 21mm with corresponding increase in zone diameter for resistant from ≤ 15mm to about 23mm for resistant MIC of ≥ 4µg/mL.

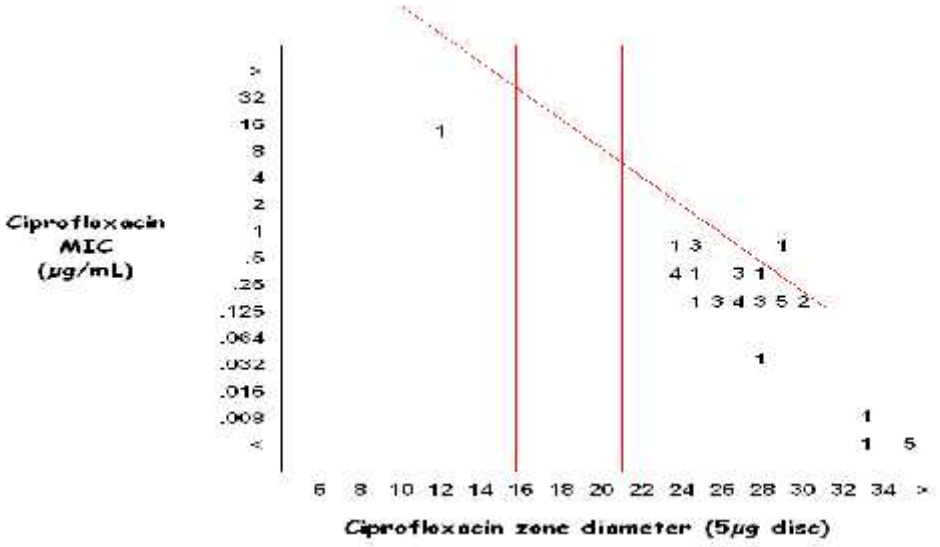


Figure 5.13: Scatterplots of ciprofloxacin MIC versus 5µg disc zone diameter with regression line (output from WHONET 5.4 after analysis)

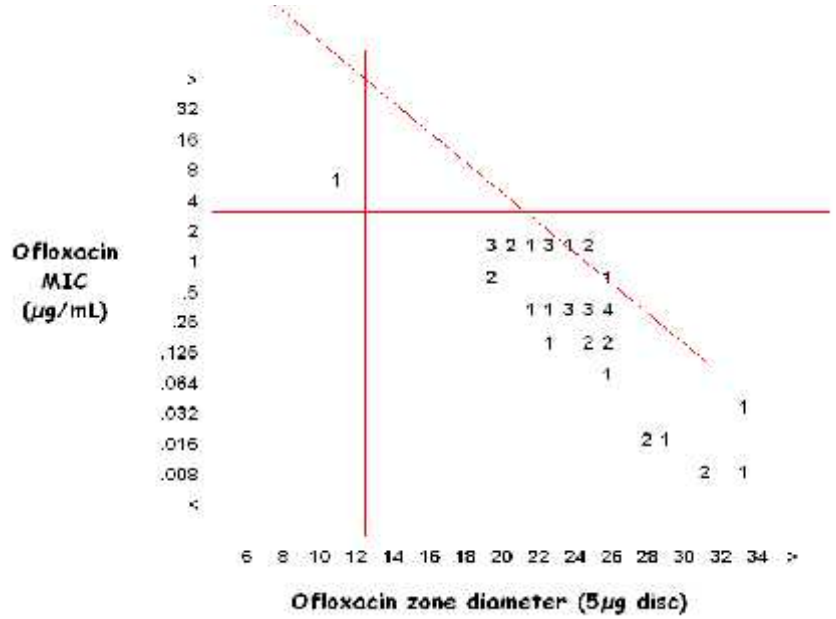


Figure 5.14: Scatterplots of ofloxacin MIC versus 5µg disc zone diameter with regression line (output from WHONET 5.4 after analysis)

Similarly, to accommodate a susceptible MIC of ≤ 2µg/mL, the zone diameter of 5µg ofloxacin disc for susceptible increased to about 22mm from 16mm with corresponding increase in zone diameter for resistant from ≤ 12mm to about 19mm for resistant MIC of ≥ 4µg/mL.

CHAPTER-VI

6. DISCUSSION

Among the 443 blood specimens subjected to culture, only 41(9.11%) showed growth. Lack of growth in blood culture resulting low isolation of the pathogen is common mainly because of use of antibiotics prior to blood collection for culture (Parry *et al.*, 1999; Vallenas *et al.,* 1985) as availability and misuse of antibiotics even for mild cases of fever is common in Nepal (Khanal *et al.*, 2007; Malla *et al.*, 2006; Wachter *et al.,* 1999). Besides that, low concentration of circulating *Salmonella* in blood and difficulties of obtaining enough volume of blood for culture (Wain *et al.,* 2001) also play important role in the sensitivity of the blood culture. The result of culture is obatained by 30-70% even in well-equipped laboratories (Gassem *et al.,* 1995). Furthermore, because of our reliance upon a

single blood culture for diagnosis, the observed rate of enteric fever may undoubtedly be an underestimate. Although blood cultures remains a gold standard for diagnosis, the poor sensitivity of these methods have been described (Hoffman *et al.,* 1986). In a large study conducted with 21847 samples with febrile episode in five Asian countries, *S.* Typhi was isolated only in 2% by blood culture (Ochiai *et al.*, 2008).

This study showed the higher prevalence of enteric fever among males. Out of the 41 enteric fever cases, 32 (78.05%) were male and 9(21.95%) were female. Among 305 male and 138 female, 32(10.49%) and 9(6.52%) showed growth on blood culture. This genderwise difference in prevalence of enteric fever and growth positive rate may be due to the higher number of male patients involved in the study than female patients or due to relatively small sample size. Besides that, other behavioural and socio-economic factors may also play important role.

In contrast to the highest number of 265(57.78%) samples from the age group 19- 45 years, the blood culture positive rate (enteric fever) was highest (20%) in the age group 5-18 years consisting of only 130 samples. In the age group < 5 years with only 7 samples, 1(9.09%) showed growth on blood culture. The age group of 5-18 years consisted of the 63.61% of all the enteric fever cases. This finding is similar to the findings of the previous study in Nepal (Shrestha B, 1996; Shrestha S, 2004). In a recent study carried out in five Asian countries, 75% of the growths were from the age group of 5-15 years (Ochai *et al,* 2008). Where typhoid is endemic, most cases in health facilities are persons aged 3-19 years (WHO, 2003). Studies from India, Indonesia, and Vietnam suggest that in some settings typhoid fever is common in 1–5 year-old children (Simanjuntak *et al.*, 1991; Sinha *et al.*, 1999; Lin *et al.*, 2000). Data from hospital-based studies in Bangladesh (Saha *et al.,* 2001) and Thailand (Thisyakorn *et al.*, 1987) support these findings. Surveillance data from the USA indicate that the proportion of cases of typhoid is constant over the first 25 years of life (Crump *et al.*, 2004).

Although most of the causative agents in present study were isolated within 24 hours of incubation of blood culture bottle, there were also significant number of isolates in the following days. The volume of blood and the ratio of blood to broth determines blood culture yield. In children who have a greater concentration of bacteria in their blood, 2–4 mL of blood is sufficient for isolation of causative agent, but for optimum isolation 10 to 15ml of blood is required for adults (Vallenas *et al.*, 1985). As causative agent could be isolated 24hrs onwards, blood culture bottle should be incubated for upto 10 days before reporting negative, which is not followed in resource poor laboratory in developing countries including Nepal.

In the present study, most of the febrile cases and diagnosed enteric fever cases were from July, August and September. This may be possibly due to the sewage-mediated contamination of water sample during the rainy seasons. The poor microbiological quality of the urban water supply is among the major risk factor for transmission of enteric fever in Nepal (Bhatta *et al*., 2007).

The percentage of *S.* Paratyphi A and *S.* Typhi in culture positive cases in the present study was 41.46% and 58.54%, respectively. Based on the report from different part of the world, an estimated one case of paratyphoid fever occurs for every four cases of typhoid fever (Crump *et al.*, 2004). When compared with this estimates, this study indicated more cases of paratyphoid fever caused by *S.* Paratyphi A. The percentage of *S*. Paratyphi A over total *Salmonella* isolates in blood culture increased from 23% in 1993-1998, to 34% during 1999-2003 (Maskey *et al.*, 2008). Other studies from India and Nepal suggest that in some settings and times, paratyphoid fever caused by *S.* Paratyphi *A* can contribute up to half of all cases of enteric fever (Shlim *et al.*, 1995; Sood *et al.*, 1999).

A possible reason proposed for the higher incidence for paratyphoid, which needs higher inocula, is more likely involvement of food from street vendors as waterborne transmission of *S.* Typhi usually involves small inocula, whereas foodborne transmission is associated

with large inocula (WHO, 2003). In 2004, Vollaard *et al.* found a higher risk for transmission within the household for typhoid fever while paratyphoid fever seemed to be transmitted more frequently outside the household. Sur *et al.* in 2007 found differences in socio economic status, with a more depressed status among paratyphoid patients. Being focused mainly in the laboratory aspects, the possible involvement of different risk factor in the transmission of typhoid and paratyphoid fever is beyond the limit of this study.

S. Paratyphi A is now a emerging cause of enteric fever in Nepal, India, Pakistan, China, Vietnam and Indonesia where up to half the cases of enteric fever were due to this organism rather than *S.* Typhi (Wood *et al.*, 2006; Kapil *et al.*, 1997*).* The fact that current typhoid vaccines have no efficacy against *S.* Paratyphi A may interfere to expand typhoid vaccination campaigns for regions with a high incidence of confirmed *S.* Typhi disease as this is not likely to solve the problem alone or to make a significant contribution if outbreaks are due to *S.* Paratyphi A (Wilde, 2007). Starting such programs without better surveillance could undermine confidence in vaccination programs, should one encounter outbreaks with *S.* Paratyphi in a vaccinated population (Wilde, 2007). However, typhoid vaccination is not in well practice and has little value on emerging *S*. Paratyphi A cases in Nepalese context.

In the present study, rate of nalidixic acid resistance, which is a phenotypic marker for reduced susceptibility to fluoroquinolones (Crump *et al.*, 2003; Hakanen *et al.*, 1999) was high (80.48%). *S.* Paratyphi A strains showed the higher rate (88.23%) of nalidixic acid resistance than *S.* Typhi (75%). In a hospital based study from Nepal in 2003, 49% isolates of *S*. Typhi and 86% of *S*. Paratyphi A were resistant to nalidixic acid (Maskey et al., 2008). Chau *et al.* found that in 2002- 2004, 51% of the *S.* Typhi strains isolated from Nepal were resistant to nalidixic acid (Chau *et al.*, 2007). Khanal *et al.* in 2007 reported that 76% of *S.* Typhi strains isolated during 2000 to 2004 in eastern Nepal were resistant to nalidixic acid. In another study carried out in Nepal in 2005, it was found that 73.3% and 94.9% of *S.* Typhi and *S.* Paratyphi A strains showed the resistance to nalidixic acid (Shirakawa *et al.*, 2006). The emergence of these strains is worrying given that ciprofloxacin and ofloxacin are

the most widely used antibiotic for the treatment of enteric fever in Nepal, where it is available from pharmacies without prescription by physicians (Wachter *et al.*, 1999).

The percentage of isolates of *S.* Typhi in the UK with reduced susceptibility to ciprofloxacin doubled from 2·7% in 1995 to about 5% in 1997, but in 1998 such strains were responsible for 21% of *S.* Typhi infections (Threlfall *et al.*, 1999). From 2001 to 2004, the proportion of patients infected with such strains of *S.* Typhi increased from 35% to 49 % (Threlfall *et al.*, 2006). For *S.* Paratyphi A, the increase in isolates with reduced susceptibility to ciprofloxacin was even more striking, with 84% of isolates in 2004 showing such resistance (Threlfall *et al.*, 2006). In a recent study carried out in eight Asian countries (Chau *et al.*, 2007), there was a dramatic increase in nalidixic acid resistance between 1993 (4%) and 2005 (97%). Recent report from India showed that 91.1% *S.* Typhi and 97.5% *S.* Paratyphi A showed resistance to nalidixic acid (Raveendran *et al.*, 2008).

Over the past decade, increasing antibiotic resistance in *S. enterica* has lead to a shift in the antibiotics used against this organism from chloramphenicol, ampicillin, cotrimoxazole, to fluoroquinolones (ofloxacin, ciprofloxacin), and ceftriaxone. In 1993, during the initial outbreak of MDR *S.* Typhi in Vietnam, the fluoroquinolone antibiotics were introduced for the treatment of typhoid fever (Nguyen *et al.*, 1993). In Nepal, ciprofloxacin replaced chloramphenicol in 1994, but from 2000, increasing failures of treatment with this drug were reported (Ansari *et al.,* 2002). Since 1994, the proportion of MDR *S.* Typhi has decreased and there has been a dramatic increase in nalidixic acid resistance (Maskey et al., 2008). This reflects the local practice of widespread use, and probably misuse and overuse, of ciprofloxacin and ofloxacin. In addition, over-the-counter availability of these antibiotics, self prescription by patients, and incomplete courses of treatment are probable additional factors contributing to the development of resistance (Pokhrel *et al.*, 2006).

In contrast to the nalidixic acid resistance, this study found re-emergence of sensitivity to first line drug. Among the 41 isolates, 95.12% of the isolates were susceptible to all the first

line antibiotics (ACCoT) tested. An intresting observation in the present study is that MDR in *S.* Typhi has come down to 4.87% in comparision to previous report (Maskey et al., 2008; Pokhrel *et al.*, 2006). In a study from Nepal, compared to period 1993-1998 to the 1999-2003: susceptibility to *S.* Typhi increased from 65 to 85% for tetracycline, from 51 to 86% for amoxicillin, from 77 to 88% for cotrimoxazole, and from 79 to 85% for chloramphenicol and in *S.* Paratyphi susceptibility increased from 77 to 92% for tetracycline, from 72 to 94% for amoxicillin, from 95 to 98% for cotrimoxazole, and from 95 to 98% for chloramphenicol (Maskey et al., 2008). By contrast with *S.* Typhi, multidrug resistance in *S.* Paratyphi A isolates with reduced ciprofloxacin susceptibility was rare with none of strains showing such resistance (Raveendran *et al.*, 2008; Manchanda *et al.*, 2006).

While in most of the Asian countries the MDR phenotype of *S.* Typhi has remained at high levels over the last 10 years, there have been reports of a return sensitivity to first line drug in most of the regions (Dutta *et al.*, 2005; Mohanty *et al.*, 2006). Wafsy *et al.* in their study from 1987 through 2000 observed resurgence of chloramphenicol susceptibility and suggested reuse of this drug for the treatment of typhoid fever in Egypt. Analysis of *S.* Typhi strains isolated from Vietnam in 2002 revealed that 94.5% of the isolates were fully susceptible to ACCoT, with a clear decline in the occurrence of MDR *S.* Typhi isolates (4.4%) compared with 30.8-100% in the period 1995-1999 (Weil *et al.*, 2007). A hospital in India, reported that the incidence of MDR *S.* Typhi isolates decreased from 40% in 2000 to 17% in 2002 (Rodrigues *et al*., 2003). In a study on children in India, all isolates were MDR during 1990 to 1992 (Saha *et al*., 2002) but in 2000, 50% were susceptible to chloramphenicol and 40% to cotrimoxazole and ampicillin. Report from most of the countries (Maskey et al., 2008; Weil *et al.*, 2007; Rodrigues *et al*., 2003; Saha *et al*., 2002) showed the incidence of MDR isolates appears to have decreased with significant increase in the isolates with reduced susceptibility to fluoroquinolones.

This study found that, only 2 (4.88%) of the isolates were MDR. One of the MDR isolates showed resistance to Ampicillin, chloramphenicol, cotrimoxazole, and nalidixic acid (MDR-

NAR). Plasmid-mediated chloramphenicol resistance in *S.* Typhi was first reported in India (Paniker and Vimla 1972) and has been isolated sporadically from different parts of the world (Goldstein *et al.*, 1986). The first major outbreaks of typhoid fever caused by MDR strains occurred in 1972 in Mexico City (Olarte & Galindo 1973) and in South India (Paniker & Vimla 1972). Since then the occurrence of MDR strains of *S.* Typhi has been reported from Britain (Ward *et al.*, 1982), India (Threlfall *et al.*, 1992), South Africa (Coovadia *et al.*, 1992), Egypt (Farid 1992), Bangladesh (Ahasan *et al.*, 1993), Pakistan (Bhutta *et al.*, 1994), and many other places. The first report of MDR *S.* Typhi in Nepal was published in 1991 (Watson and Pettibone, 1991). The combination of MDR and NAR (MDR-NAR) is a particular problem, because it severely restricts the therapeutic options for patients with typhoid fever. During the last decades, the increased prevalence of MDR-NAR strains of *S.* Typhi and *S.* Paratyphi has posed a therapeutic challenge. However, the proportion of such strains (2.43%) in the present study) has been continually reducing from Nepal (Maskey et al., 2008; Pokherel *et al.*, 2006) and other endemic countries.

Other MDR isolates reported in the present study are the strains of *S.* Typhi with complete resistance to ciprofloxacin (MIC, 16µg/mL) and ofloxacin (MIC, 8µg/mL) which also showed resistance to tetracycline and cotrimoxazole. Although, high level ciprofloxacin resistant *S.* Typhi strain were rare before 2005, the prevalence of such strains in India increased to 6.78% in 2006 from 1.52% in 2005 (Raveendran *et al.*, 2008). In a study carried out in Nepal, all the *S.* Typhi and *S.* Paratyphi A isolates were reported as susceptible until 1998 but during 1999 to 2003 ciprofloxacin resistance increased 5% in the *S.* Typhi and 13% in *S.* Paratyphi A (Maskey *et al.*, 2007). This increase in the high-level ciprofloxacin probably reflects the overuse or irrational use of ciprofloxacin in the treatment of enteric fever as well as other unrelated infections. Incomplete treatment may be another factor contributing to development of resistance. The emergence of complete resistance to ciprofloxacin in *S.* Typhi or *S.* Paratyphi A would severely limit the choice of antimicrobial therapy for treating enteric fever.

The ciprofloxacin-resistant strain of *S.* Typhi with additional resistance to tetracycline and cotrimoxazole has not been previously characterized from Nepal. Similar report of ciprofloxacin-resistant strains of *S.* Typhi conferring three mutations: two in gyrA (Ser-83 to Phe and Asp-87 to Asn or Asp-87 to Gly) and one in *parC* and harbouring a 50 plasmid borne class 1 integron (dfrA15/aadA1) that confers resistance to co-trimoxazole and tetracycline had been characterized by Gaind *et al.* in 2006 for the first time in India. The presence of integrons in these ciprofloxacin-resistant *S.* Typhi isolates is a threat as integrons represent the main vehicle of antibiotic resistance and possibly play a role in the development and dissemination of new MDR strains (Fluit, 2003). The strains of *S.* Typhi described in the present study, with multiple resistance pattern, probably having class 1 integron on a plasmid, and associated chromosomally mediated resistance to fluoroquinolones, thus have the possibility of becoming resistant to the third generation cephalosporins which are currently the last line drugs for treating enteric fever. This is possible by the acquisition of gene cassettes such as *veb-1* or *blaVIM*, by the plasmid-borne integron thus leading to untreatable enteric fever in the near future (Gaind *et al*., 2006). The prevalence of clone of *Salmonella enterica* resistant to third generation cephalosporins has already reported (Pokherel *et al*., 2006) from Nepal.

All strains with reduced susceptibility to ciprofloxacin were fully susceptible to cephalosporin such as ceftriaxone. One of the isolate which was fully resistant to ciprofloxacin with additional resistance to cotrimoxazole and tetracycline was also susceptible to ceftriaxone. There have been sporadic reports of high-level resistance to ceftriaxone *S.* Typhi and *S.* Paratyphi A, although these strains are very rare. Pokhrel *et al.* in 2006 has reported ceftriaxone resistance and ESBL production by *S.* Paratyphi A in Nepal. Ceftriaxone remains the last line drug against infections with ciprofloxacin resistant *Salmonella* which is also resistant to other first line drug. The usage of this drug in the empiric therapy, misuse, and overuse should be discouraged.

Analysis of the MIC values of three fluoroquinolones in the present study found that 80.48% isolates were resistant to nalidixic acid (MIC ≥ 32), 2.4 % resistant to ciprofloxacin (MIC 16 µg/ml), and 2.4% resistant to ofloxacin (MIC 8 µg/ml). The NAR *S.* Paratyphi A strains required increased MICs of the fluoroquinolones (MIC [mean ± SD] for nalidixic acid 477.8667±87.023µg/mL, ofloxacin, 1.8±0.6324µg/mL; ciprofloxacin, 0.25µg/mL) compared with the NAR *S.* Typhi (MIC [mean ± SD] for nalidixic acid 173.1765±72.03 µg/mL, ofloxacin, 0.4264±0.1139 µg/mL; ciprofloxacin, 0.6166±0.3 µg/mL). The higher MIC for various fluoroquinolones against *S.* Paratyphi A than against *S.* Typhi in our study is similar to the findings observed in an earlier study in Nepal (Shirakawa *et al.*, 2006).

The ciprofloxacin susceptible isolates showed two populations based on nalidixic acid susceptibility, with the ciprofloxacin MICs ranging from 0.0075 to 0.06µg/mL for the NAS population and from 0.25 to 1 µg/mL for the NAR population. Similar bimodal distributions were also observed for ofloxacin susceptible population. All NAR isolates (MIC, ≥ 32 µg/mL) with reduced susceptibility to fluoroquinolone (increased MIC value) were classified as susceptible to ciprofloxacin and ofloxacin (MIC, ≤ 1 µg/mL and ≤ 4 µg/mL; inhibition zone diameter, ≥ 21mm and ≥ 16mm, respectively) according to the CLSI recommendations. The accepted CLSI breakpoints for *Enterobacteriaceae* of ciprofloxacin are ≤ 1 and ≥ 4µg/mL and of ofloxacin is ≤ 4 and ≥ 8 µg/mL respectively for susceptible and resistant (CLSI, 2006). Testing for resistance at these levels resulted in reduced susceptibility to ciprofloxacin not being detected.

In the present study, the reduced susceptibility to fluoroquinolones in *S.* Typhi and *S.* Paratyphi A was strongly correlated with resistance to nalidixic acid. The differences in the MIC values of fluoroquinolones and inhibition zone diameters between the two study groups (NAR and NAS isolates) were statistically significant ($P < 0.001$) regarding every fluoroquinolone tested, supporting the association between nalidixic acid resistance and reduced fluoroquinolone susceptibility. The scattergram correlating the MICs of ciprofloxacin and ofloxacin with nalidixic acid (fig 5.11 and 5.12) illustrates the

simultaneous presence of nalidixic acid resistance and reduced ciprofloxacin and ofloxacin susceptibility in study population. Based on the MICs of ciprofloxacin and zone diameters around 30µg nalidixic acid discs, screening for nalidixic acid resistance led to the detection of all isolates (specificity and specificity of 100%) with reduced ciprofloxacin and ofloxacin susceptibility (MICs ≥ 0.125µg/mL) and none of the susceptible isolates.

Hakanan *et al.*, in 1999 reported the 100% sensitivity and 87.5% specificity of nalidixic acid screening test for isolates for which the MICs of ciprofloxacin were ≥ 0.125µg/mL. In contrast to the high sensitivity of nalidisic acid screening test, the emergence of new quinolone resistance pattern in *Salmonella enterica* which are susceptible to nalidixic acid but exhibit reduced susceptibility to ciprofloxacin (Hakanen *et al.*, 2005) was also reported from South Asia.

Besides this, the applicability of the 5µg ciprofloxacin and 5µg ofloxacin disc diffusion test in respectively detecting ciprofloxacin and ofloxacin susceptibility has also evaluated. When an MIC of ≤ 0.125µg/mL was adopted as a breakpoint, the ciprofloxacin inhibition zone diameter of ≥ 32mm and ofloxacin zone diameter of ≥ 27mm yielded sensitivity and specificity of 100% and 87.5%, respectively, in detecting fully susceptible isolates.

British Society for Antimicrobial Chemotherapy (BASC) has stabilized new susceptibility breakpoint for ciprofloxacin and ofloxacin (Andrews, 2008). According to current BASC recommendation, susceptible, intermediate and resistant breakpoints for ciprofloxacin and ofloxacin are ≤ 0.5, 1 and > 1 (respective inhibition zone diameter to 1µg ciprofloxacin are ≥ 20mm, 17–19mm and 16mm; and 5µg ofloxacin disc are 29mm, 26-28mm and 25mm). Because the clinical response to fluoroquinolones in patients infected with nalidixic acid–resistant strains is greatly inferior to the response in those infected with nalidixic acid–susceptible strains, Threlfall *et al.* (2001) and Crump *et al.* (2003) suggested that the break points for the classification of *S.* Typhi strains according to their susceptibility to fluoroquinolones should be changed. *S.* typhi and *S.* Paratyphi A strains that are resistant to

nalidixic acid but susceptible to fluoroquinolones according to current disc susceptibility testing criteria shoud be classified as resistant to quinolones or nonsusceptible to fluoroquinolones. Regression analysis of Log MIC versus zone diameter in the present study revealed that, to accommodate a susceptible MIC of ≤ 1µg/mL, the zone diameter for susceptible has to be increased to about 25mm from 21mm with a corresponding increase in the zone diameter for resistant isolates from 15mm to about 22mm for resistant MIC of ≥ 4µg/mL. Rodriguez *et al.* in 2008 have also recommended to revise ciprofloxacin breakpoint and susceptibility criteria. Thus, taking into consideration of such reports, the CLSI guidelines for MIC breakpoint and zone diameter susceptibility criteria of fluoroquinolones for *Salmonella* needs re-evaluation.

S. Typhi and *S.* Paratyphi A with reduced susceptibility to fluoroquinolones and resistance to nalidixic acid require higher MICs of ciprofloxacin and other fluoroquinolones (Crump *et al.*, 2003), although they are still considered susceptible according to current CLSI interpretive criteria. Before the widespread emergence of nalidixic acid resistant *S.* Typhi, demonstrates that fluoroquinolones treatment courses as short as 2 days were > 90% effective in treating enteric fever patients (Hien *et al.,* 1995; Vinh *et al.,* 1996) led to the wide adoption of short course treatment strategies to minimize the likelyhood of adverse events associated with fluoroquinolones use in children, to reduce cost, and to improve patient's compliance. Clinical treatment failures in typhoid fever with reduced ciprofloxacin-susceptible *S.* Typhi with short course and standard long course treatment are common (Nkemngu *et al.,* 2005; Bhan *et al.*, 2005; Rupali *et al.*, 2004). Thus, clinicians should be aware of the possibility of treatment failures when fluoroquinolones are used against infections with nalidixic acid resistant *S.* Typhi and *S.* Paratyphi A considering the fact that ciprofloxacin and ofloxacin are the most commonly prescribed antibiotics for the treatment of enteric fever in Nepal.

Besides that, the emergence of the full fluproquinolones resistance *S.* Typhi and Paratyphi A isolates showing resistance to chloramphenicol, ampicilin, cotrimoxazole and tetracycline have limited the use antibiotics therapy. In many tropical countries, including Nepal and

other Indian subcontinent, there is widespread availability and uncontrolled use of fluoroquinolones. As isolates with reduced susceptibility to fluoroquinolones could become highly resistant upon sequential accumulation of mutations in topoisomerase genes, the use of fluoroquinolones as first-line drugs for empirical therapy and management of enteric fever in areas where these strains are endemic is questionable and requires an urgent review. Low exposure to fluoroquinolones reducing the selective pressure on a large bacterial population would definitely lessen the likelihood of selecting mutants.

6.2 CONCLUSION

Enteric fever still remains the major diagnosis among the febrile patients presenting to hospitals of Nepal. The high rate nalidixic acid-resistant *S.* Typhi and *S.* Paratyphi A, emergence of strains with full resistance to fluoroquinolones and persistence of MDR strains constitute a major problem in Nepal.

Ciprofloxacin and ofloxacin can no longer be considered the drug of choice in treating *Salmonella* infections due to its high level reduced susceptibility to fluoroquinolones and increasing report of full fluoroquinolone resistant strains. Based on our observation of re-emergence of susceptibility, conventional first line antimicrobials may play important role in the treatment of enteric fever. Ceftriaxone remains the last line drug against ciprofloxacin resistant *S.* Typhi and *S.* Paratyphi A which are also resistant to ACCoT.

Testing for resistance at currently accepted CLSI breakpoints for ciprofloxacin and ofloxacin could result in reduced sensitivity to these drug not being detected. These isolates had all been reported as susceptible with the disc diffusion technique. The current study suggests that in such cases nalidixic acid screening and determination of MICs of fluoroquinolone should be performed and that if strains show reduced fluoroquinolone sensitivity possible alternative antibiotics such as conventional first line drug (if susceptible) or third generation cephalosporins (eg ceftriaxone or cefotexime) should be used.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 Summary

- 41(9.26 %) of the specimens were positive for the *Salmonella enterica*. The percentage of growth was 20% in the age group 5-18 years.
- Out of the 41 blood culture positive samples, 24(58.54%) were *S.* Typhi and 17(41.46%) were *S.* Paratyphi A.
- On daywise subculture from the blood culture bottle, 32(78.05%) of the total growth were obtained within 24 hrs incubation.

- Among the eight antibiotics tested ceftriaxone was susceptible to all the strain tested. Percentage of isolates resistant to ampicillin, chloramphenicol, cotrimoxazole and tetracycline were 2.43%, 2.43%, 4.87% and 2.43%, respectively.
- 33(80.48%) isolates were resistant to nalidixic acid with higher rate in *S.* Paratyphi A (88.23%) compared to *S.* Typhi (75%).
- Only 2(4.88%) isolates were resistant to two or more antibiotics (MDR).
- 39(95.12%) of the isolates were susceptible to first line drugs (ACCoT).
- Of the total isolates, 33 isolates have nalidixic acid MIC of ≥ 32 µg/mL and were classified as resistant while 8 isolates have MIC of ≤ 8µg/mL and were classified as susceptible according to CLSI (CLSI, 2006). For ciprofloxacin, 40 (97.6 %) have MIC of ≤ 1 µg/ml and were classified as susceptible while 1 (2.4 %) have MIC of 16 µg/mL and was classified as resistant. Similarly for ofloxacin, 40(97.6%) have MIC of ≤ 2µg/mL and classified as susceptible while 1(2.4%) have MIC of 8µg/mL and classified as resistant according to CLSI.
- The difference in zone diameter and MIC values of ciprofloxacin and ofloxacin in nalidixic acid susceptible and nalidixic acid resistant population was statistically significant ($P < 0.001$).
- The NAR *S.* Paratyphi A strains required increased MICs of the fluoroquinolones (MIC [mean ± SD] for nalidixic acid 477.8667±87.023µg/mL, ofloxacin, 1.8±0.6324µg/mL; ciprofloxacin, 0.25µg/mL) compared with the NAR *S.* Typhi (MIC [mean ± SD] for nalidixic acid 173.1765±72.03µg/mL, ofloxacin, 0.4264±0.1139µg/mL; ciprofloxacin, 0.6166±0.3µg/mL).
- When an ciprofloxacin MIC of ≥ 0.125µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC, ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ciprofloxacin susceptibility (MIC, ≥ 0.125µg/mL) and, none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100%.

- When an ofloxacin MIC of ≥ 0.125µg/mL was adopted as a breakpoint, screening for nalidixic acid resistance (MIC, ≥ 32µg/mL) led to the detection of all 32 isolates with reduced ciprofloxacin susceptibility (MIC, ≥ 0.125µg/mL) and, none of the susceptible isolates. Thus, the sensitivity and specificity of the approach was 100%.
- When an MIC of ≤ 0.25µg/mL was adopted as a breakpoint, the ofloxacin inhibition zone diameter of ≥ 27mm yielded 100% sensitivity and 87.5% specificity in screening for reduced ofloxacin susceptibility.
- When an MIC of ≤ 0.125µg/mL was adopted as a breakpoint, the ciprofloxacin inhibition zone diameter of ≥ 32mm yielded 100% sensitivity and 87.5% specificity in screening for reduced ciprofloxacin susceptibility.
- According to regression analysis, to accommodate a susceptible and resistant MIC breakpoint of ciprofloxacin and ofloxacin, corresponding inhibition zone diameter intreprative criteria is increased.

7.2 Recommendations

- Molecular analysis of the nalidixic acid resistant isolates, conventional MDR isolates, and fully fluoroquinolone resistant isolates play important role in further evaluation and characterization of the drug resistant isolates.
- Nalidixic acid susceptibility test to screen reduced susceptibility to fluoroquinolones in *Salmonella* should be routinely performed in clinical microbiology laboratory. Isolates showing nalidixic acid resistance should be further evaluated by MIC determination.

- Clinicians should be aware of the possibility of treatment failures when fluoroquinolones are used against infections with *S.* Typhi and *S.* Paratyphi A with reduced susceptibility to these drugs.
- Education of health professionals to ensure appropriate antibiotic prescriptions and education of patients to avoid self-medication should be emphasized.
- The current breakpoints and susceptibility criteria of ciprofloxacin and ofloxacin need to be re-evaluated.
- Use of conventional first line drug, if susceptible, is recommended to use in the treatment of enteric fever. Ceftriaxone is adviced to be a reserve drug for treating MDR and ciprofloxacin–resistant cases.
- An investigation of the extent of full fluoroquinolone resistance problem in *Salmonella* is urgently needed in Nepal.

APPENDICES

Appendix I: Patient's request form

Name: ..... Lab No.: .....
Age/Gender: ..... Date: .....
Culture request for: .....
Brief Clinical History: .....

Current antibiotic treatment: a) Yes b) No
If Yes, Antibiotics (s) taken: ……..
Duration of treatment: .....

Appendix II: Worksheet for blood culture, isolation and identification

A. Visual observation for change in turbidity, appearance in blood culture bottle

Sample ID	Age/ sex	Change in turbidity, appearance in blood culture bottle				
		2^{nd} day (24 hrs)	3^{rd} day (48 hrs)	4^{th} day (72 hrs)	5^{th}day (96 hrs)	6^{th} day (120 hrs)

B. Observation of growth after subculture on MA

Sample ID	Age/ Sex	Growth				
		3^{rd} day	4^{th} day	5^{th} day	6^{th} day	7^{th} day

C. Colony characteristics and Gram staining reaction (organism grown on MA after subculture from blood culture bottle)

Sample ID	Isolate ID	Colony characteristics							Grams' reaction
		Media	size	shape	margin	elevation	opacity	consistency	

D. Biochemical test

Sample ID	Isolate ID	Catalase	Oxidase	Indole	Motility	MR	VP	TSI	Citrate	Lysine	Urease	Isolated organism

Note: MR, methyl red; VP, Voges proskauer; TSI, triple sugar iron

E. Serological identification of microorganisms

Sample ID	Isolate ID	Anti-sera used for slide agglutination						Organism isolated
		Salmonella poly A- S	*S.* Typhi			*S.* Paratyphi A		
			O9	H_1: d	Vi	O2	H_1: a	

Appendix III: Worksheet for antimicrobial susceptibility testing

A. Worksheet for Determination of antimicrobial susceptibility testing by disc diffusion method

Test Date: ……
Antimicrobial agent: …… Disc content: ……
Disc Manufacturer: …… Mfd date: …… Exp. Date: ….
Agar preparation date: ……

Sample ID	Isotated Organism	Reference zone of diameter (mm)				Observed zone of diameter (mm)		Remarks
		Sen.	Int.	Res.	E. coli ATCC 25922	Isolates	E. coli ATCC 25922	

Note: S = susceptible, I = Intermediate. R = Resistance

B. Worksheet for antimicrobial susceptibility pattern

Antimicrobial disc manufacturer: …… Mfd date: …… Exp. Date: ….
Mueller Hinton agar manufacturer: …… Mfd date: …… Exp. Date: ….

Sample ID	Isolate ID	Age/sex	Organim isolated	Antimicrobial susceptibility pattern								Remarks
				A	C	Co	T	Cf	Of	Na	Ci	

Note: S = Susceptible, I = Intermediate. R = Resistance

Appendix IV: Working procedures

A. Gram staining

Procedure

- A thin film of the material to be examined is prepared on a clean, grease free glass slide and dried. The smear is heat fixed and allowed to cool before staining.
- The slide is flooded with crystal violet stain and allowed to remain without drying for 10-30 seconds. The slide is then rinsed with distilled water.
- The slide is flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface. The slide is rinsed with tap water, shaking off excess.
- The slide is flooded with decolorizer (acetone alcohol) for 10 seconds and rinsed immediately with distilled water until no further colour flows from the slide with the decolorizer.
- The slide is flooded with counter stain (safranine) for 30 seconds and washed off with distilled water.
- The slide is blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 1000X.

Quality control

Test laboratory staining procedure and reagents prior to use of new lots of each staining and decolorizing agents and at least weekly thereafter, using a gram positive and gram negative microorganism (*E. coli* ATCC 25922 and *S. aureus* ATCC 25923).

B. Slide agglutination test

Procedures

- A dense suspension of the organism is made on normal saline. Always take the organism from nonselective media.
- A drop of respective antiserum is added to the organism suspension and mixed well with an applicator stick.
- The slide is observed for the clumping of the suspension. Positive test is indicated by rapid complete agglutination of the organism.
- For Vi antigen producing *Salmonella*, the suspension of the organism was prepared in normal saline and boiled before testing with O antisera.

Quality control

Each new batch of the antisera is tested with standard organism. The suspension without adding antisera should be kept as a control during slide agglutination to detect auto agglutination.

C. Disc Diffusion method for the antimicrobial susceptibility testing

PRINCIPLE

A standardized inoculum of bacteria is swabbed onto the surface of a Mueller Hinton agar (MHA) plate. Filter paper disc impregnated with antimicrobial agents are placed on the agar. After overnight incubation, the diameter of the zone of inhibition is measured around each disc. By referring to the tables in the CLSI disc diffusion standard, a qualitative report of susceptible, intermediate or resistant is obtained.

MATERIALS

Requirements for the disc diffusion susceptibility testing were described in appendix

QUALITY CONTROL

A. QC strains

1. *Escherichia coli* ATCC 25922 2. *Staphylococcus aureus* ATCC 25923
3. *Enterococcus faecalis* ATCC 29212

B. Monitoring accuracy

1. Test QC strains by following routine procedure, and record results. Record lot number and expiration date of discs and agar.
2. Compare to expected results (CLSI QC tables). Note any out of control result and document; proceed with corrective action, if necessary**.**
3. Perform daily and weekly QC testing.

PROCEDURE

A. Brings agar plates and canisters of discs to room temperature before use. Agar plates may be removed from refrigerator and placed in a 35 C ambient air incubator with lids slightly ajar to evaporate excess moisture. Do not leave in incubator for longer than 30 min.

B. Inoculum preparation

Using a loop or swab, transfer colonies as follows

1. Direct colony suspension method: - pick several colonies from a fresh (18 – 24 hr) nonselective agar plate to broth or 0.9% NaCl.
2. log phase method
    a. Pick four or five isolated colonies to 3.0 to 5.0 ml of broth.
    b. Incubate at 35 C for 2 to 8 hr until growth reaches the turbidity at or above that of a 0.5 McFarland standards.
3. For either the log phase or direct colony suspension method, vortex well and adjust turbidity visually with sterile broth or 0.9% NaCl to match a 0.5% McFarland standard.

C. Inoculation of agar plates

1. Within 15 minutes of adjusting turbidity, dip a sterile cotton swab into the inoculum and rotate against the wall of the tube above the liquid to remove excess inoculum.

2. Swab entire surface of agar plate three times, rotating plates approximately 60˚ between streaking to ensure even distribution. Avoid hitting the slides of the plate to avoid aerosols. Finally, run swab around the edge of the agar to remove any excess moisture.
3. Allow inoculated plate to stand for 3 to 15 min before applying discs.

D. Application of discs
1. Apply disc to agar surface with dispenser or manually with a sterile forceps.
2. Apply gentle pressure with sterile forceps or needle to ensure complete contact of disc with agar.
3. Do not place discs closer than 24mm from center to center (no more than 12 discs on 150 mm plates and 5 discs on 100 mm plates.
4. Do not relocate a disc once it has made contact with agar surface. Instead, place a new disc in another location on the agar.

E. Incubation
1. Invert plates and incubate within 15 min of disc application.
2. Incubate for 16 to 18 at 35˚C in an ambient air incubator.

F. Reading plates
1. Read plates only if lawn of growth is confluent or nearly confluent.
2. Hold inverted plate a few inches above a black nonreflecting surface.
3. Illuminate plate with reflected light.
4. Use a sliding caliper or ruler held on the back of the plate to measure the diameter of inhibition zone to nearest whole millimeter.
5. When measuring zones for sulfonamides, trimethoprim, or trimethoprim-sulfamethoxazole, disregard light growth (20% less of lawn of growth) and measure edge of the more obvious margin of the zone.
6. Discrete colonies growing within the inhibition zone may represent a mixed culture or resistant variants; subculture single colonies from the primary culture plate, re-identify, and retest for susceptibility. If the discrete colonies are still apparent, measure the colony – free inner zone.

G. Interpretation and Reporting

Use criteria specified by the CLSI to interpret the zone of inhibition for each antimicrobial agents and report categorical result as either susceptible(S), intermediate (I), or resistant (R).

PRECAUTIONS

The following common sources of error should be investigated to verify that:

- Zone diameters were measured and transcribed correctly;
- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use;
- All materials used were within their expiration dates and stored at the proper temperature;
- The incubator is at proper temperature and atmosphere;
- Other equipment used (e.g., pipettors) are functioning properly;

- Discs are stored desiccated and at proper temperature;
- The control strain has not changed and is not contaminated;
- Inoculum suspensions were prepared and adjusted correctly; and inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

REFERENCES

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2. NCCLS (2003) Performance Standards for Antimicrobial Disc Susceptibility Tests; Approved Standard- Eighth Edition. NCCLS document M2-A8 [ISBN 1-56238-485-6]. NCCLS, 940 West Valley Road, Suite 1400, Wayne, Pennsylvania 19087-1898 USA)

D. Determination of Minimum Inhibitory Concentrations of antibiotics (Fluoquinolones)

PRINCIPLE

Minimum inhibitory concentration are considered the gold standard for determining the susceptibility of organisms to antimicrobials and therefore used to judge the performance of all other methods of susceptibility testing. MICs are widely used to give a definitive answer when a borderline result is obtained by other methods of testing or when disc diffusion methods are not appropriate and are important in the evaluation of antibiotics breakpoints.

MATERIALS

Media equipment and reagent for determination of MIC were as described in appendix

QUALITY CONTROL

A. QC strains

1. *Escherichia coli* ATCC 25922 2. *Staphylococcus aureus* ATCC 25923

B. Monitoring accuracy

1. Test QC strains by following routine procedure, and record results. Record lot number and expiration date of antibiotic powder.
2. Compare to expected results (CLSI QC tables). Note any out of control result and document; proceed with corrective action, if necessary.
3. Perform daily and weekly QC testing

PROCEDURE

1. Antibiotic powder, solvents and diluents

1.1. Obtain standard powder from the pharmaceutical company or a reputable supplier such as Sigma (Poole, Dorset, UK).

1.2. Obtain information from the supplier regarding expiry date, potency, solubility, stability as a powder and in solution, storage conditions and any relevant information.
1.3. Always prepare stock solutions following the manufacturer's recommendations.
1.4. Freeze and thaw stock solutions only once and then discard them.
1.5. Appendix X shows solvent, diluents and storage conditions for antibiotics.

2. Preparation of antibiotic stock solutions and dilution range

2.1. Choose a suitable range of antibiotic concentrations for the organisms to be tested (see suggested ranges Appendix X).
2.2. Prepare stock solutions using the formula 1000/P x V x C = W. Where P = potency given by the manufacturer (µg/mg), V = volume required (mL), C = final concentration of solution (multiples of 1000) (mg/L), and W = weight of antibiotic in mg to be dissolved in volume V (mL).
2.3. For preparation of further stock solutions and dilution range, from the solution, prepare as described in table 3.

Dilution range for each antibiotic is prepared similarly. Solvents, diluents, dilution range and storage condition for antibiotic solution is described in appendix.

4. Preparation of agar dilution plates

4.1 Prepare Mueller-Hinton agar following the manufacturer's instructions.
4.2 Add 1 ml of working antibiotic solution to each container containing 19 ml of cooled molten agar (ensure that the medium is cooled to 45°C before adding to the antibiotic), including the antibiotic-free control. Mix well before pouring into 90 mm Petri dishes.
4.3 Allow agar to set and then dry surface of the plates for 10 min in a fan assisted drying cabinet (without ultraviolet light) or in an incubator (time needed depend on the efficiency of the incubator).
4.4 Store plates at 4-8°C protected from light until inoculated. Ideally, plates should be used on the day of preparation. If plates are to be stored at 4 -80˚C before use, the stability of the drug must be determined by individual laboratories as part of the routine quality control programme.

5. Preparation of Inoculum

The inoculum should be adjusted so that 10^4 cfu/spot are applied to the plates. The following procedure describes a method for preparing the desired inoculum by comparison with a 0.5 McFarland standard.

5.1. *Preparation of the McFarland standard*

Add 0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.2H_2O$) to 99.5 ml of 0.18 M H_2SO4 (1% v/v) with constant stirring. Distribute the standard into screw cap tubes of the same size and with the same volume as those used in growing the broth cultures. Seal the tubes tightly to prevent loss by evaporation. Store protected from light at room temperature. Vigorously agitate the turbidity standard on a vortex mixer before use. Standards may be stored for up to six months after which time they should be discarded.

5.2. *Preparation of inoculum*

Touch at least four morphologically similar colonies with a sterile loop. Transfer growth into Mueller-Hinton agar or equivalent that has been shown not to affect the performance of the test and incubate broth with shaking at 35-37°C until the visible turbidity is equal to or greater than the 0.5 McFarland standard. Alternatively an overnight broth culture can be used. *Direct colony suspension method can also be used.*

5.3. *Adjustment of the organism suspension to the density of the 0.5 McFarland standards.*

Adjust the density of the organism suspension prepared to equal that of the 0.5 McFarland standards by adding sterile distilled water. To aid comparison, compare the test and standard against a white background with a contrasting black line. Suspensions should contain between 10^7 and 10^8 cfu/ml depending on genera. For the agar dilution method further dilution of suspension in sterile distilled water (1:10 for *Enterobacteriaceae*) is carried out before inoculation.

6. Quality Control

Appropriate controls, depending on genera, must be included with every batch of MIC determinations.

7. Inoculation

Use a multipoint inoculator to deliver 1-2 µl of suspension on to the surface of the agar. Allow the inoculum to be absorbed into the agar before incubation.

8. Incubation conditions

Incubation 35-37°C in air for 18-20 h

9. Reading and intrepration

After incubation ensure that all of the organisms have grown on the antibiotic-free control plate. The MIC is defined as the lowest concentration of antibiotic at which there is no visible growth of the organism. The growth of one or two colonies or a fine film of growth should be disregarded. The MIC for the control strain should be within plus or minus one two-fold dilution of the expected MIC (see appendix).

PRECAUTIONS

- The turbidity standard has not expired, is stored properly, meets performance requirements, and was adequately mixed prior to use.
- All materials used were within their expiration dates and stored at the proper temperature;
- The incubator is at proper temperature and atmosphere.
- The control strain has not changed and is not contaminated.
- Inoculum suspensions were prepared and adjusted correctly.
- Inoculum for the test was prepared from a plate incubated for the correct length of time and in no case more than 24 hours old.

REFERENCES

1. Andrews JM (2001) Determination of minimum inhibitory concentrations. *Journal of Antimicrobial Chemotherapy*. Suppl. 48, SL, 5-16

2. National Committee for Clinical Laboratory Standards (2003) Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically. 6th ed: Approved Standard M7-A6. Wayne, PA: NCCLS.

Appendix V: Preparation of media and reagents

A. composition and preparation of different culture media

1. Nutrient agar ((HiMedia, M 001)

Composition	gm/lit
Peptic digest of animal issue	5.00
Beef extract	1.50
Yeast extract	1.50
NaCl	5.00
Agar	15.00
Final pH at 25°C 7.4 ± 0.2	

Preparation: As directed by manufacturer, 28mg of the media was dissolved in 1000ml of the distilled water. The media was then sterilized by autoclaving at 15 lbs pressure (121°C) for 15min. The sterilized media was then poured in sterilized Petri-plate and then allowed to cool.

3. Macconkey agar ((HiMedia, M 008)

Composition	gm/lit
Peptic digest of animal tissue	17.00
Proteose peptone	3.00
Lactose	10.00
Bile salt	1.50
NaCl	5.00
Neutral red	0.03
Agar	15.00
Final pH at 25° 7.1 ± 0.2	

Preparation: As directed by manufacturing company, 51.53gm of the media was dissolved in 1000ml of the distilled water. The media was then sterilized by autoclaving at 151bs pressure (121°C) for 15min. The sterilized media was then poured in sterilized petriplate then allowed to cool.

4. Nutrient broth (HiMedia, M 002)

Composition	gm/lit
Peptic digest of animal tissue	5.00
NaCl	5.00

Beef extract	1.50
Yeast extract	1.50
Final pH at 25° 7.4 ± 0.2	

Preparation: As directed by manufacturing company, 13gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tube in amount of 3ml in each and sterilized by autoclaving at 151bs pressure (121°C) for 15mikn. The sterilized media was then cooled to room temperature.

5. Mueller Hinton agar (HiMedia, M 173)

Composition	gm/lit
Beef infusion form	300.0
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00
Final pH at 25° 7.3 ± 0.2	

Preparation: As directed by manufacturing company, 38gm of the media was dissolved in 1000ml of the distilled water. The media was then sterilized by autoclaving at 15lbs pressure (121°C) for 15min. The sterilized media was then poured in sterilized petriplate then allowed to cool.

6. Bile broth (Nutrient broth: HiMedia, M 002; Bile salts: Oxoid, LP 0055)

Composition	gm/lit
Peptic digest of animal tissue	5.00
NaCl	5.00
Beef extract	1.50
Yeast extract	1.50
Bile salt	10.0
Final pH at 25° 7.4 ± 0.2	

Preparation: As directed by manufacturing company, 13 gm of the media was dissolved in 1000ml of the distilled water and 10.0 gm of bile salts was added. The media was the dispensed in blood culture bottle in amount of 45ml in each, sterilized by autoclaving at 151bs pressure (121°C) for 15min.

7. Mueller Hinton Broth (Oxoid, CM 0405)

Composition	gm/lit
Beef	300.00
Casein Hydroxylate	17.50
Starch	1.50
Calcium	0.003665
Magnesium	6.29
Final pH at 25° 7.3 ± 0.1	

Preparation: As directed by manufacturing company, 21g media was added to 1 litre of D/W, mixed well to dissolve and sterilized by autoclaving at 151bs pressure (121°C) for 15min.

B. Composition and preparation of different biochemical media

1. Hugh and Leifson media (HiMedia, M 826)

Composition	gm/lit
Peptic digest of animal tissue	2.00
NaCl	5.00
K_2PO_4	0.30
Glucose	10.00
BTB	0.05
Agar	2.00

Final pH at 25° 6.8 ± 0.2

Preparation: As directed by manufacturing company, 19.40gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min.

2. SIM media (HiMedia, M 181)

Composition	gm/lit
Peptic digest of animal	30.00
Beef extract	3.00
Peptonized iron	0.20
NaS_2O_3	0.025
Agar	3.00

Final pH 7.3 ± at 25°C

Preparation: As directed by manufacturing company, 36.23gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min.

3. Simmon citrate media (HiMedia, M 099)

Composition	gm/lit
$MgSO_4$	0.20
$NH_4H_2PO_4$	1.00
K_2PO_4	1.00
Na-citrate	2.00
NaCl	5.00
BTB	0.08
Agar	15.00

Final pH 6.8 ± 0.5 at 25°C

Preparation: As directed by manufacturing company, 24.2gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min. The sterilized media in the test tube was then allowed to set in slopes.

4. Triple sugar iron agar (HiMedia, M 021)

Composition	gm/lit
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00
Dextrose	1.00
NaCl	5.00
Ferrous sulphate	0.20
Sodium thio-sulphate	0.30
Phenol red	0.024
Agar	12.00

Final pH 25°C 7.4 ± 0.2

Preparation: As directed by manufacturing company, 65gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min. The sterilized media in the test tube was then allowed to set in sloped form with a butt of about 1 inch.

5. Urea broth (HiMedia, M 112)

Composition	gm/lit
Peptic digest of animal tissue	1.00
Dextrose	1.00
NaCl	5.00
Na_2PO_4	1.20
KPO_4	0.80
Phenol red	0.012

Preparation: As directed by manufacturing company, 24gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min. The sterilized media in the test tube was then allowed to cool at 45°C, added sterilized 40% urea, and then set in sloped form with a butt of about 1 inch.

6. Decarboxylase test media (M 912)

Composition	gm/lit
Peptic digest of animal tissue	5.00
Yeast extract	3.00
Dextrose	1.00
Bromo cresol purple	0.02

Preparation: As directed by manufacturing company, 9gm of the media was dissolved in 1000ml of the distilled water. The media was then dissolved by heating to boil and divided into 4 equal parts. One part was dispensed in test tubes without adding amino acid, to the remaining part three amino acids (L- Lysine, L-Arginine and L- Ornithine) were separately added to a final concentration of 0.5% and dispensed in tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min.

C. Composition and preparation of different reagent

1. Gram staining reagent

a) Hucker's Crystal violet stain

Prepare crystal violet stock solution by dissolving 40 g of crystal violet (90- 95% dye content) in 400ml of ethanol (95%), filter it and store at room temperature. For the preparation of working solution of crystal violet, add 40 ml of stock solution to 160 ml of filtered ammonium oxalate solution (1%).

b) Gram's iodine

To prepare stock solution of Lugol's iodine, mix 25 g of iodine crystals and 50 g of potassium iodide in 500ml of distilled water in a brown glass bottle. Fore the preparation of working solution, mix 60ml of Lugol's iodine stock solution with 220ml of distilled water and 60 ml 5% sodium bicarbonate solution.

c) Safranine

Prepare stock solution by dissolving 5 g of safranine in 200ml of 95% ethanol. To prepare safranine working solution, mix 20ml of stock solution with180ml of distilled water.

d) Acetone alcohol (1:1)

Mix 50ml 0f ethanol (95%) with 50ml acetone in a brown bottle. Label the date of preparation and store at room temperature.

2. Catalase reagent

To make 100ml

Hydrogen peroxide	3ml
Distilled water	97ml

Preparation: To 97ml distilled water, 3ml hydrogen peroxide was added and mixed well.

3. **Oxidase Reagent**

To make 100ml

Tetra methyl paraphenylenediamine dihydrochloride	1.00gm
Distilled water	100ml

Preparation: This reagent was prepared by dissolved by 1gm of reagent in 100ml of distilled water. To that solution stripes of Whatman No. 1 filter paper was soaked and drained for about 30sec. Then these stripes were completely dried and stored in dark bottle tightly sealed with a screw cap.

4. **Kovac's Reagent**

To prepare 40ml reagent

4-dimethyl aminobenzyldehyde	2gm
Isoamyl alcohol	30ml
Con. HCl	10ml

Preparation: In 30ml of isoamyl alcohol, 2gm of reagent was dissolved in clean brown bottle. Then to it, 10ml of conc. HCl was added and mixed well.

5. **Methyl Red**

To make 50ml solution

Methyl red	0.05gm
Ethanol (absolute)	28gm
Distilled water	22ml

Preparation: To 28ml ethanol, 0.05gm of methyl red was dissolved and transferred to clean brown bottle. Then 22ml distilled water was added to that bottle and mixed well.

6. **Voges-proskauer Reagent (Barritt's Reagent**)

a) VP Reagent A:

To make 100ml

Alpha-naphthol	5gm
Ethanol (absolute)	100ml

Preparation: To 28ml distilled water, 5gm of alpha naphthol was dissolved and transferred to a clean brown bottle. Then final volume was made 100ml by adding distilled water.

b) VP reagent B:

To make 100ml

Potassium hydroxide	40gm
Distilled water	100ml

Preparation: Potassium hydroxide was dissolved and transferred to a clean brown bottle. Then final volume was made 100ml by adding distilled water.

Appendix VI: Equipments, materials, and supplies

A. General Microbiology laboratory Equipments

(a) Autoclave (Stermite, Japan) **(b)** Incubator (Sakura, Japan) **(c)** Hot air oven (Memmert, Germany; and Gallencamp) **(d)** Microscope (Olympus) **(e)** Refrigerator 4-8˚C (Sanyo, Japan) **(f)** Refrigerator -20˚C (Videocon) **(g)** Refrigerator -75˚C (Sanyo, Japan) **(h)** Weighing machine (Ohaus Corporation, USA) **(g)** Gas burner **(h)** Glasswares **(i)** Inoculating wire and loops

B. Salmonella Antisera (Denka Seiken Co. Ltd. Tokyo, Japan)

a) Group O antiera: Polyvalent A-S, group O2, group O9
b) Group H antiera: H-a, H-d
c) Vi antisera

C. Materials for the disc diffusion method

Media and reagents

(a) Agar plates (150 or 100 mm; depth approximately 4 mm) (b) MHA (c) Mueller Hinton broth or 0.9% NaCl (d) Antimicrobial discs from HiMedia Laboratories Pvt. Limited (ampicillin 10µg, tetracycline 30µg, chloramphenicol 30µg, cotrimoxazole 1.25/23.75µg, ciprofloxacin 5µg, ofloxacin 5µg, nalidixic acid 30µg, ceftriaxone 30µg) store with desiccant at -4 to 8˚C.

Supplies

(a) Sterile cotton tipped swabs (b) Sterile plastic pipettes (c) McFarland 0.5 turbidity standard

Equipments

(a) Forceps (b) Ruler, template or sliding caliper (c) Movable light source (d) Black nonreflecting surface(e. g., sheet of black paper or black counter surface) (e) Vortex mixer (f) 35 ambient air incubator (g) Multidisc disc dispensig apparatus and container (optional)

C. Materials for determination of MIC by agar dilution method

a. Media and reagents

(a) Agar plates (150 or 100 mm, depth approximately 4 mm) (b) MHA (c) Nutrient broth or 0.9% NaCl (d) Solvents and diluents for antibiotics (e) Antimicrobial powder (ciprofloxacin, ofloxacin, nalidixic acid)

b. Supplies

(a) Sterile plastic pipettes (b) Sterile cotton tipped swab (c) McFarland 0.5 turbidity standards

c. Equipments

(a) Sterile graduated pipettes of 10ml, 5ml, 2ml and 1ml (b) Sterile capped 7.5 x 1.3 cm tubes / small screw-capped bottles (c) Pasteur pipettes (d) Vortex mixer (e) 35 ambient air incubator

Appendix VII: Antibiotics Stock Solution and Dilution Range

Table 1: Preparation and storage of antibiotic solutions (stored solutions should contain ≥ 1000 mg/L)

Anibiotic	Solvent	Diluent	4˚C	-20˚C	-70˚C	Storage of powder
ciprofloxacin	water	water	2 weeks	3 months	3 months	+4 - 25˚C
Nalidixic acid	c	water	-	-	-	+4˚C
ofloxacin	a	water	-	-	-	+4˚C

a= Saturated NaHCO3 solution, b= Ethanol, *c*= Water and 0.1 M NaOH drop wise to dissolve

Table 2: Suggested ranges of antibiotic concentrations for MIC determinations for *Enterobacteriaceae* (mg/L)

Organism	Ciprofloxacin	Ofloxacin	Nalidixic acid
Enterobacteriaceae	0.004-128 µg/mL	1-128 µg/mL	0.06-128 µg/mL

Appendix VIII: Inhibition zone diameter size interpretive standards with equivalent MIC breakpoint for *Enterobacteriaceae* (CLSI, 2006)

Antimicrobial agent(Disc potency)	Diameter of zone of inhibition (mm) and Antimicrobial Disc equivalent MIC breakpoint (µg/ml)			NCCLS QC strain agent potency *E. coli* ATCC 25922
	Susceptible	Intermediate	Resistant	

Ampicillin (10 µg)	≥ 17 mm (≤ 8 µg/ml)	14 – 16 mm (16 µg/ml)	≤ 13 mm (≥ 32 µg/ml)	16 – 22 mm (2-8 µg/ml)
Chloramphenicol (30 µg)	≥ 18 mm (≤ 8 µg/ml)	13 -17 mm (16 µg/ml)	≤ 12 mm (≥32 µg/ml)	21- 27 mm (2-8 µg/ml)
Cotrimoxazole (1.25/23.75 µg)	≥16 mm (≤ 2/38 µg/ml)	11- 15 mm (4/76 µg/ml)	10 mm (≥8/152 µg/ml)	23- 29 mm (≤ 0.5/9.5 µg/ml)
Nalidixic acid (30 µg)	≥19 mm (≤ 8 µg/ml)	14- 18 mm (16 µg/ml)	13 mm (≥32 µg/ml)	22- 28 mm (1- 4 µg/ml)
Ciprofloxacin (5 µg)	≥21 mm (≤ 1 µg/ml)	16- 20 mm (2 µg/ml)	15 mm (≥4 µg/ml)	30- 40 mm (0.004- 0.016 µg/ml)
Ofloxacin (5 µg)	≤12mm ≤2 µg/ml	13-15 mm 4 µg/ml	≥16mm ≥8 µg/ml	29- 33 mm 0.03 µg/ml

Appendix IX: Statistical Analysis

1. Culture positive (enteric fever) in male and female

Growth	Males	Females	Total
Positive	32	9	41
Negative	273	129	402
Total	305	138	443

Null Hypothesis (H_0): There is no difference in growth rate among male and female.
Alternative Hypothesis (H_1): There is a significant difference in growth rate among male and female.

Test Statistic: $\chi 2 = \frac{(ad- bc)^2 N}{(a+b)(c+d)(a+c)(b+d)}$

$\chi 2$ cal =1.7830

But we know that
$\chi 2$ tab at (2-1)(2-1) degree of freedom =1and 95% confidence level, is equal to 3.841

***Decision*:** Since $\chi 2$ cal (1.7830) < $\chi 2$ tab (3.841), the null hypothesis is accepted, which means there is a no significant difference of enteric fever among male and female.

2. Enteric fever in the age group of 5-18 year

Growth	5-18 years	Other age	Total
Positive	26	15	41
Negative	104	298	402
Total	130	313	443

Null Hypothesis (H_0): There is no difference in growth rate in the age group 5-18 years and other age group.

Alternative Hypothesis (H_1): There is a significant difference in growth rate in the age group 5-18 and other age group.

Test Statistic: $\chi 2 = \frac{(ad- bc)^2 N}{(a+b)(c+d)(a+c)(b+d)}$

$\chi 2$ cal =25.2933

But we know that

$\chi 2$ tab at (2-1)(2-1) degree of freedom =1and 95% confidence level, is equal to 3.841

***Decision*:** Since $\chi 2$ cal (25.2933) < $\chi 2$ tab(3.841), the null hypothesis is rejected, which means there is a significant difference of enteric fever in the age group 5-18 years and other age group.

3. Nalidixic acid susceptibility and susceptibility to other drugs

Nalidixic acid	Other drugs		Total
	susceptible	resistant	
susceptible	8	0	8
resistant	31	2	33
Total	39	2	41

Null Hypothesis (H_0): There is no difference in susceptibility to nalidixic acid and susceptibility to other drug.

Alternative Hypothesis (H_1): There is a significant difference susceptibility to nalidixic acid and susceptibility to other drug.

Test Statistic: $\chi 2$ with Yate's correction = $\frac{[(|ad- bc|)^2 N/2] N}{(a+b)(c+d)(a+c)(b+d)}$

$\chi 2$ cal =0.737

But we know that

$\chi 2$ tab at (2-1)(2-1) degree of freedom =1and 95% confidence level, is equal to 3.841

***Decision*:** Since $\chi 2$ cal (0.737) < $\chi 2$ tab(3.841), the null hypothesis is accepted, which means there is no significant difference bet nalidixic acid susceptibility and susceptibility to other drug.

4. Association of nalidixic acid susceptibility pattern among *S.* Typhi and *S.* Paratyphi A

Isolates	NAR	NAS	Total
S. Typhi	18	6	24
S. Paratyphi A	15	2	17
Total	33	8	41

Ho: There is no significant association of nalidixic acid susceptibility among *S.* Typhi and *S.* Paratyphi A.

H_1: There is significant association of nalidixic acid susceptibility among *S.* Typhi and *S.* Paratyphi A.

Test Statistic: $\chi 2 = \frac{(ad- bc)^2 N}{(a+b)(c+d)(a+c)(b+d)}$

$\chi 2 = 1.5187$

$\chi^2_{0.05, 1} = 3.841$

***Decision*:** Since the tabulated value of χ^2 is greater than the corresponding calculated χ^2 value, the null hypothesis is accepted i.e. there is no significant association of nalidixic acid susceptibility among *S.* Typhi and *S.* Paratyphi A.

5. Significance of mean zone diameter of fluoroquinolones in NAR and NAS population

Table: Fluoroquinolones zone diameter in NA sensitive and resistant isolates

Agent(Disc content)	NA resistant (n=32)		NA sensitive (n=8)	
	Mean	SD* (mm)	Mean	SD (mm)
Nalidixic acid(30µg)	6	0	23.875	1.5360
Ciprofloxacin(5µg)	25.8125	1.875	32.625	2.2875
Ofloxacin(5µg)	22.5625	2.090	28.875	2.3684

***Note**: NA resistant isolates gave no zone on 30µg disc, which is considered as 6mm. In this case standard deviation couldnot be calculated

Ho: Ho: $\mu 1=\mu 2$; ***H_1***: $\mu 1 \neq \mu 2$

Calculated t- value: $t_{\text{nalidixic acid}} = 39.088$, $t_{\text{ciprofloxacin}} = 8.0165$, $t_{\text{ofloxacin}} = 6.5562$

Tabulated t-value: $t_{0.001, 38\ df} = 3.29$

***Decision*:** Since the tabulated value of t is higher than critical value of t, it falls in the rejection region. Hence the null hypothesis is rejected and it may be concluded that there is the significant difference in mean zone diameter of ciprofloxacin, ofloxacin and nalidixic acid in nalidixic acid resistant and nalidixic acid sensitive isolates

6. Significance of mean MIC of fluoroquinolones in NAR and NAS population

Table: Fluoroquinolones zone diameter in NA sensitive and resistant isolates

Agent	NA resistant (n=32)		NA sensitive(n= 8)	
	Mean (µg/mL)	SD (µg/mL)	Mean (µg/mL)	SD (µg/mL)
Nalidixic acid	316	171.53	1.312	1.0288
Ciprofloxacin	0.4375	0.2651	0.015	0.0710
Ofloxacin	1.0703	0.7427	0.0393	0.0334

Ho: Ho: µ1=µ2;***H*$_1$**: µ1≠µ2

Calculated t- value: $t_{\text{nalidixic acid}} = 5.051$, $t_{\text{ciprofloxacin}} = 4.352$, $t_{\text{ofloxacin}} = 3.825$

Tabulated t-value: $t_{0.001,\ 38\ df} = 3.29$

***Decision*:** Since the tabulated value of t is higher than critical value of t, it falls in the rejection region. Hence the null hypothesis is rejected and it may be concluded that there is the significant difference in mean MIC ciprofloxacin, ofloxacin and nalidixic acid in nalidixic acid resistant and nalidixic acid sensitive isolates

7. Significance of difference in mean MIC of fluoroquinolones in NAR *S*. Typhi and *S*. Paratyphi A

Table: Fluoroquinolones zone diameter in NA sensitive and resistant isolates

Agent	NARST (n=17)		NARSPA(n= 15)	
	Mean (µg/mL)	SD (µg/mL)	Mean (µg/mL)	SD (µg/mL)
Nalidixic acid	173.1765	72.03	477.8667	87.023
Ciprofloxacin	0.25	0	0.6166	0.3
Ofloxacin	0.4264	0.1139	1.8	0.6324

Ho: Ho: µ1=µ2;***H*$_1$**: µ1≠µ2

Calculated t- value: $t_{\text{nalidixic acid}} = 10.478$, $t_{\text{ciprofloxacin}} = 4.874$, $t_{\text{ofloxacin}} = 8.516$

Tabulated t-value: $t_{0.001, 38\ df} = 3.29$

Decision: Since the tabulated value of t is higher than critical value of t, it falls in the rejection region. Hence the null hypothesis is rejected and it may be concluded that there is the significant difference in mean MIC ciprofloxacin, ofloxacin and nalidixic acid in nalidixic acid resistant *S*. typhi and nalidixic acid resistant *S*. Paratyphi A