CHAPTER I

INTRODUCTION AND OBJECTIVE

1.1 Background

Salmonella enterica serovars Typhi and Paratyphi A are human-adapted bacterial pathogens that cause related systemic diseases, collectively called enteric fever or typhoid (Kathryn *et al*., 2007).

Enteric fever, which embraces both typhoid and paratyphoid fever, continues to be a major public health problem in developing countries (Pokhrel *et al*., 2009). It is endemic in Nepal and afflicts local inhabitants as well as travelers (Pokhrel *et al*., 2006). The situation has been more worsen with the emergence of multidrug resistance among *Salmonella*. In the last 30 years, high rates of multiple-drug resistance (MDR) to all first-line antimicrobial agents encoded by large conjugative plasmids in *Salmonella enterica* serotype Typhi have been reported around the world, but the Indian subcontinent and Southeast Asian countries are particularly affected (Le *et al*., 2007).

It is estimated that there are approximately 22 million typhoid cases and approximately 200,000 deaths per year worldwide. *S.* Typhi causes a systemic infection, survives, and multiplies within macrophages and persists in the human body mainly by colonizing the gallbladder (Malo *et al*., 2011).

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.*, 2001).

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).

Paratyphoid fever caused by *S.* Paratyphi is considered an emerging disease, as its incidence has increased alarmingly in recent years (Thong *et al*., 1998), causing more asymptomatic infections than *S.* Typhi. In Kathmandu, Nepal, enteric fever caused by *S.* Paratyphi A is more prevalent than that caused by *S.* Typhi (Shirakawa *et al*., 2006).

Since 1997, cases of infection with nalidixic acid-resistant *Salmonella* serotype Typhi (NARST) with decreased susceptibility to ciprofloxacin have been reported in many parts of the world. Some patients with typhoid fever caused by NARST that is susceptible to fluoroquinolones *in vitro*, according to current Clinical and Laboratory Standards Institute (CLSI) interpretive criteria, can show a delayed response to ciprofloxacin or treatment failure or can even be refractory to treatment both clinically and bacteriologically (Rupali *et al*., 2004). The failure of treatment with fluoroquinolones in the cases of *S.* Typhi and *S.* Paratyphi A in the Indian subcontinent and Southeast and Central Asia due to decreased susceptibility to ciprofloxacin is now a serious concern (Threlfall and Ward, 2001).

The prospective study was carried out in Bacteriology laboratory of Alka Hospital, Lalitpur. During this period, the blood samples were investigated by culture technique, biochemical tests and antibiotic susceptibility test. Data was entered into SPSS and descriptive analysis was done. Monitoring of nalidixic acid resistant as well as multidrug resistant isolates and antibiotic susceptibility test provided information about pathogenic organisms isolated from patients and assisted in choosing the most appropriate empirical antimicrobial therapy.

The presence of *S. enterica* serovar Typhi isolates and *S. enterica* serovar Paratyphi A isolates with nalidixic acid resistant and multidrug resistant characteristics continues to be major public health problem in many countries of the world. It is necessary to alert clinicians to recognize the multidrug resistant strains. The surveillance for antimicrobial resistance of *S. enteric* serovar Typhi and *S. enterica* serovar Paratyphi A isolates should be continued, particularly to monitor the emergence of strains fully resistant to fluoroquinolones. As the incidence of antimicrobial resistance increases, the cost associated with consequences also increases and hence can be considered an economic burden to society, more so in context of developing country like Nepal. Antibiotic susceptibility profile and reporting of drug resistant strain would enlighten the appropriate antibiotic therapy and help in awareness towards misuse and overuse of antibiotics.

1.2 Objectives

General

To study incidence of Nalidixic acid resistant *Salmonella* isolates from suspected enteric fever patients.

Specific

i. To isolate and identify *Salmonella enterica* serovars causing enteric fever from the blood specimens.
ii. To study the antibiotic susceptibility patterns of the *Salmonella* isolates.
iii. To determine incidence of Nalidixic acid resistant *Salmonella* isolates.

CHAPTER II

LITERATURE REVIEW

2.1 Enteric fever

Enteric fever is a generalized infection of the reticuloendothelial system and intestinal lymphoid tissue accompanied by sustained fever and bacteremia. It embrace both typhoid and paratyphoid fever (Levine *et al*., 1982).

Typhoid and paratyphoid fever are clinically and pathologically similar, the distinction between them being a bacteriological one (Lesser and Miller, 2003). Typhoid fever is caused by *S.* Typhi and the closely related organisms *S.* Paratyphi A, *S.* Paratyphi B and *S.* Paratyphi C, cause Paratyphoid fever (Forsyth, 1998). Paratyphoid fever is clinically milder and of short duration than typhoid (Lesser and Miller, 2003).

2.2 Epidemiology

Typhoid fever affects only humans (who are the reservoir) and is spread through consumption of contaminated food and drink handled by people who shed the organism from stool or, less commonly, urine or water contaminated with sewage. Shellfish from water polluted by raw sewage and canned meat production with poor technique have caused outbreaks. Food is generally heavily contaminated - approximately 10^5 to 10^9 cells may be required to cause illness.

Typhoid fever has virtually disappeared in the developed world, but is still endemic in developing countries (Reddy *et al*., 2011). Precise incidences are difficult to obtain due to the lack of accurate diagnostic and epidemiological data in endemic countries. In 2004 it was estimated that there were 21.6 million cases and 200,000 deaths per year worldwide due to enteric fever (Crump *et al*., 2004). Most occur in Asia and Africa. In Latin America, despite there being evidence that the incidence has decreased in parallel with economic transition and with water and sanitation measures, it remains a public health problem there (Crump and Mintz, 2010). In global hot spots (eg. Indonesia and Papua New Guinea) 91% of typhoid fever occurs in children aged 3-19 years, in whom it is a common cause of death. The highest risk of

complications and death occurs in children in the first year of life, and in older adults. In the UK, an average of almost 500 cases were reported per year between 2006 and 2010. Between 2007 and 2010, the demographics of typhoid and paratyphoid disease were scrutinized by the Health Protection Agency by enhanced surveillance; 1,673 surveillance questionnaires were received; it showed that 73% of cases were of Indian, Pakistani or Bangladeshi ethnicity; that 96% of cases were acquired abroad and that the bulk of travel was to India, Pakistan or Bangladesh. 84% of those who had travelled were visiting friends and relatives. Interestingly, 7% of those who completed questionnaires had no travel history, and for most of these, there was no identifiable source of infection. 40% of non- travel cases were from London.

2.3 Classification and nomenclature of *Salmonella* species

The nomenclature of the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann on the basis of the serologic identification of 'O' (somatic) and 'H' (flagellar) antigens. Each serotype was considered a separate species (eg. *Salmonella paratyphi A, Salmonella Newport* and *Salmonella enteritidis);* this concept, if used today, would result in 2463 species of *Salmonella*. Other taxonomic proposals have been based on the clinical role of a strain or on the biochemical characteristics. The defining development in *Salmonella* taxonomy occurred in 1973 when Corsa *et al.,* demonstrated by DNA-DNA hybridization that virtually all *Salmonella* belonged in a single species name *Salmonella enterica* which is separated into 7 distinct subspecies. Most of the serotypes that cause human diseases are in sub group I. The single exception is *Salmonella bongori* which was made that there be only 2 species of *Salmonella, Salmonella enterica* (which would include the 2462 previous species) and *Salmonella bongori.*

2.4 Morphology, Culture and Biochemical characteristics

Salmonella are Gram negative, motile, non-sporing, non-capsulated bacilli measuring 2-4*0.6 μm.

Salmonella 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.

Most *Salmonella* 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 *Salmonella* 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 *Salmonella* 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 does not attack lysine (Old and Threlfall, 1998).

2.5 Antigenic characteristics

Salmonella possess three main types of antigens on the basis of which they are serologically classified.

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 suspension 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 *Salmonella* 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 by masking the agglutination by O antibodies or by causing nonspecific cross-reaction due to their presence in unrelated bacteria (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 Threfall, 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 strains of *Salmonella* may occasionally be found with flagella that are antigenically and morphologically like those of motile parent strains. Flagellate *Salmonella* 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. Beside 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 diphasic variation.

2.6 Genomics of *Salmonella*

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.

S. Typhi CT18 harbors two plasmids. The larger conjugative plasmid, pHCM1. The pHCM1 plasmid encodes resistance to chloramphenicol (*catI*), ampicillin (TEM-1, *Bla)*, trimethoprim (*dhfr1b*), sulfonamides (*sulII*), and streptomycin (*strAB*). The smaller plasmid is pHCM2.

2.7 Typing and molecular characterization

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.

a. 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).

b. Phage typing: Based on the underlying principle of host specificity of bacteriophages several phage typing schemes have been developed for some of the *Salmonella* 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 worldwide. 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.

c. 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 *Salmonella* serotype: Agona, Livingstone, Crichton, Montevideo and Paratyphi B.

d. Molecular typing: A range of molecular typing methods based on characterization of the genotype of the organism by analysis of plasmid and chromosomal DNA has now been developed for typing *Salmonella*. Molecular typing methods based on the characterization of plasmid DNA include plasmid profile typing, plasmid fingerprinting and identification of plasmid mediated virulence genes. Chromosome 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).

The methods used more extensively in support of epidemiological investigation of Salmonella are plasmid typing, ribotyping, RCCS tying, fingerprinting and more recently, PFGE.

2.8 Pathothegenesis

Salmonella infection is initiated by ingestion of a sufficient dose of organism varied from 10^6 to 10^9 organism to overcome the body's defences in particular gastric acidity and to the immune system of the patients. The organisms must be swallowed for infection (Lewis, 1982). Transmission of *Salmonella* to a susceptible host usually occurs by consumption of contaminated foods and water. Moreover, human to human transmission can occur (Corales, 2004).

Pathogenesis of enteric infection can be described under three headings:

a. Incubation stage

Infection is transmitted by the fecal oral route through contaminated food and water. The ingested organisms are mostly destroyed in the stomach. Sufficient number of bacilli pass through the gastric acid barrier and reach the duodenum, where they multiply in alkaline medium (Ananthanarayan and Paniker, 2000).

The M cell, epithelial cell that overlie the Peyer's Patches are the potential sites where the bacilli is internalized and transported to underlying lymphoid tissue. Once phagocytosed, the bacteria are protected from polymorphonuclear leucocytes, the complement system and the acquired immune response. *Salmonella* have evolved mechanisms to avoid or delay killing by macrophages. Upon phagocytosis, the bacteria form a "spacious phagosomes" and alter the regulation of ~200 bacterial proteins. The best characterized regulatory system is phoP/phoQ, a two component regulon that sense changes in bacteria location and alters bacterial protein expression. The alterations

mediated by phoP/phoQ include modification in LPS and in the synthesis of outer membrane protein, these changes presumably remodel the bacteria's outer surface such that the organisms can resist microbicidal activities and possibly alter host cell signaling. phoP/phoQ also mediates the synthesis of divalent cationic transporters that scavenges magnesium (Lesser *et al*., 2003). From the submucosa, the organism travels the mesenteric lymph node, multiply and then enter the blood stream through the thoracic duct (transient primary bacteraemia) to seed other tissues (Chakraborty, 2000).

Blood stream is rapidly cleared of by the cells of the mononuclear phagocytic system (MPS) in the liver, bone marrow, spleen, lung and lymph node. Thus, the internal organs are infected during primary bacteremia in first 7-10 days (Chakraborty, 2000).

b. Septicemic stage

During primary bacteremia the bacilli are able to live and multiply in cells of mononuclear phagocytic system and by the 10^{th} day the parasitized cells undergo necrosis and the bacilli pass into blood leading to a secondary and heavier bacteremia, which corresponds with the onset of clinical illness at about 14^{th} day after ingestion (infection). During this period some organisms undergo lysis liberating endotoxin in the circulation. The bacteremia and toxaemia causes pyrexia and other signs of clinical illness (Chakraborty, 2000).

c. Stage of localization

Some organisms localize in organs from the blood stream example: gall bladder, liver, spleen, bone etc. Some bacilli are discharged from the gall bladder into the intestine which cause inflammation of payer's patches into the intestine and lymphoid follicles procuding necrosis and slouging of the affected follicles with resultant typhoid ulcers which may lead to hemorrhage and perforation (Chakraborty, 2000).

2.9 Presentation

Typhoid is one of the most common febrile illnesses seen by practitioners in the developing world.

a. Symptoms

i. Initially there may be intermittent diarrhoea.

ii. Fever which develops in steps over 2 or 3 days with temperatures up to 39°C or 40°C during the bacteremic phase (early antibiotics may modify the presentation) (Siddiqui*et al*., 2006).

iii. Headaches, non-productive cough and constipation may also occur.

b. Signs

i. Rose spots - caused by bacterial emboli - are crops of macules 2-4 mm in diameter that blanch on pressure.

ii. Relative bradycardia- the pulse is slower than would be expected from the degree of temperature.

iii. Eye complications may occur (usually only with associated systemic illness) which include corneal ulcers, uveitis, abscesses (eyelid or orbit), vitreous or retinal haemorrhage, retinal detachment, optic neuritis, extraocular muscle palsies, and orbital thromboses.

c. Subsequent course

i. During the second week the patient has a toxic appearance with apathy and sustained pyrexia. The abdomen is distended slightly and splenomegalyis common.

ii. By the third week there is considerable weight loss. Pyrexia persists and a delirious state may occur. Marked abdominal distension develops and liquid, foul, green-yellow 'pea soup diarrhoea' is common.

iii. The patient is weak with a weak pulse and raised respiratory rate, and crackles may develop over the lung bases. Death can occur at this stage from overwhelming toxaemia, myocarditis, intestinal haemorrhage, or perforation of the gut, usually at Peyer's patches.

iv. In the untreated patient the fourth week sees the fever, mental state and abdominal distension slowly improve over a few days, but intestinal complications may still occur. Convalescence is prolonged, and most relapses occur at this stage.

2.10 Complications

i. The two most common complications are haemorrhage (including disseminated intravascular coagulation) and perforation of the bowel. Before antibiotics, perforation had a mortality of around 75%.
ii. Jaundice may be due to hepatitis, cholangitis, cholecystitis or haemolysis.
iii. Pancreatitis with acute renal failure and hepatitis with hepatomegaly are rare.
iv. Toxic myocarditis occurs in 1-5% of patients (ECG changes may be present). It is a significant cause of death in endemic areas.
v. Toxic confusional states and other neurological and psychiatric disturbances have been reported.

2.11 Carriers of Enteric infection

There are two types of carriers:-

a. Chronic carriers

Chronic carriers are those patients who excrete the disease producing organisms for indefinite period of several years and sometimes for the rest of one's life (Chakraborty, 2000).

b. Temporary or Transient carrier

Those carriers who excrete typhoid bacilli for not more than a year are known as temporary or transient carrier. Temporary excreters can be defined under two headings (Ananathanarayan and Paniker, 2000).

i. Covalescent carier

Those who exerete the organism for a limited period of time after apparent clinical cure are known as convalescent excreter.

ii. Asymtomatic carrier

They are those who do not have a clinically recognizable attack of disease.

2.12 Use of antibiotics and drug resistance

Due to the emergence of resistance on the classic first lines of drug, qinolones became the alternative drug of choice to treat *Salmonella* Typhi infection. But over the past decade, there have been increasing reports of treatment failure using fluoroquinolones for patient whose isolates are susceptible to fluoroquinolones and resistant to Nalidixic acid, the prototypic quinolone in

vitro (Crump *et al*., 2003; Threfall *et al*., 1999). These strains are called Nalidixic acid resistant *Salmonella* Typhi (NARST).

Salmonella Typhi strains with reduced susceptibility to fluoroquinolones have become a major problem in Asia and other parts of the world (Kapil, 2002). Although they were reported to be susceptible to fluoroquinolones, by disc diffusion test with use of recommended break points, these organisms were resistant to Nalidixic acid and the MIC of fluoroquinolones for these strains was 10 times higher than that for fully susceptible strains. This reduction in susceptibility results in a poor clinical response to treatment (Manchanda *et al*., 2006).

2.12.1 Mechanisms of Nalidixic Acid Resistance

Bacteria most commonly developed resistance to quinolones by nontransmissible, spontaneously occurring point mutations in chromosomal genes (gyrA, gyrB, parC and parE). These point mutations alter the enzymes (DNA gyrase and topoisomerase) that are targets for quinolone drugs. Altered permeability of bacterial cell membranes and efflux pumps also play a role in quinolone resistance for some isolates. More recently, a multi-drug resistance plasmid was discovered that encodes transferable resistance to quinolones via the qnv gene. The qnv gene product has been demonstrate to directly protect DNA gyrase from quinolone inhibition (Crump *et al*., 2003).

Chromosomally point mutations resulting in alterations of the A subunit of DNA gyrase that lead to quinolone resistance have been defined in a substantial number of clinical and laboratory isolates of *Enteriobacteriaceae*, including *Escherichia coli.* These alterations of the target enzyme are clustered between amino acids 67 and 106 in amino terminus of the A protein known as the quinolone resistance-determining region (Crump *et al*., 2003). Similar chromosomal mutation and changes in the A subunit have been documented for isolates of *Salmonella enterica* (Nakaya *et al*., 2003). Single chromosomal point mutations have been demonstrated to be sufficient to cause an amino acid change and to result in Nalidixic acid resistant. Two or more chormsomal point mutations are usually necessary to result in Ciprofloxacin resistance, on the basis of current NCCLS interpretive criteria (Crump *et al*.,

2003). For the high levels all resistance to fluoroqinolones, the presence of additional mutations in gyrA and/or in another target such as parC is required. Thus, it has been proposed that the MIC of Nalidixic acid could be used as a generic marker of resistance for the quinolone family in Gram-negative bacteria (Ruiz, 2003).

2.13 Laboratory diagnosis

Microbiological procedure

a. Specimens used for detection

For the detection of enteric infection following types of specimens are used:

i. Blood: Microorganisms can usually be detected in 75-90% of patients during the ten days of infection and in about 30% of patients during the third week. In chronic salmonellosis, it has been reported that *S.* Typhi can be more rapidly and successfully isolated from bone marrow than from blood, especially if the patient has been treated with antibiotics.

ii. Faces: Microorganisms can usually be isolated from urine in about 25% of patients after the second week of infection and from about 80% of patients during the third week from faeces.

iii. Urine: Microorganisms can usually be isolated from urine in about 25% of patients after the second week. The bacteria are not excreted continuously and therefore several specimens may need to be cultured before the organisms are isolated (Cheesbrough, 1984).

b. Blood collection

i. Timing of blood collection

Whenever possible, blood should be taken before antibiotics are administered. The best time is when the patient is expected to have chills of a temperature spike.

ii. Quantity of blood

10 ml per veinpucture for adults, 2-5 ml for children, who usually have higher levels of bacteremia, for infants and neonates, 1-2 ml is often the most that can be obtained (Vandepitte *et al*., 2004).

iii. Types of Broth media

The most frequently used liquid media are either nutrient, glucose of Brain Heart infusion Broth, but some laboratories use a biphasic medium (Phillips and Eykyn, 1990).

iv. Blood Dilution

The blood sample should be diluted between 1 in 5 and 1 in 10 in the culture medium in order to reduce the concentration of natural antibacterial constituents such as serum complement, immunoglobulin, lysozyme, monocytes and PMNs to a sub-effective level. The dilution also reduces the concentration of any therapeutically administered antibiotic (Collee *et al*., 1996). Clotting of blood is also prevented by dilution (Strokes *et al*., 1993).

v. Anticoagulant

The use of Sodium Polyanethol Sulfonate (SPS) as an anticoagulant is recommended because it also inhibits the antibacterial effect of serum and phagocytes. If the blood is immediately added to a sufficient volume (50ml) of broth and throughly mixed to prevent clotting, no anticoagulant is needed. It is recommended that blood culture bottles be available at hospitals and major health centers (Vandepitte *et al*., 2004).

If blood culture bottles are not available, blood may be transported to the laboratory in a tube containing a sterile anticoagulant solution (Citrate, heparin or SPS). Upon receipt in the laboratory, such blood samples must be transferred immediately to blood-cultures bottles using a strict aseptic technique. Where blood is taken without anticoagulant, the clot can be aseptically transferred to broth in the laboratory and the serum used for certain serological tests (e.g. Widal test) (Vandepitte *et al*., 2004).

vi. Subculture from Blood Cultures

The most important recent advances in blood culture technology have been in methods for the detection of growth in the liquid medium after in has been incubated at 37^{o}C. Such growth may be visible as turbidity or as colonies and, with haemolytic organisms, the blood may be haemolysed. However, the most

sensitive means to detect is subculture of a small volume on appropriate solid medium (Phillips and Eykyn, 1990).

vii. Identification

The isolated organisms are identified by Gram staining procedure, biochemical tests and agglutination tests (Chakraborty, 2000).

2.14 Antimicrobial Susceptibility Test

Bacterial susceptibility test to antimicrobial agents is done in vitro by Kirby-bauer disc diffusion technique using 24 hour broth culture of isolates in Mueller Hinton agar Medium and commercially available antibiotic discs. On the basis of zone – size compared with that of control strains, the result is interpreted (Greenwood *et al*., 2000).

In this method the broth culture of test organism (Comparable to McFarand 0.5 standard, inoculum density $1.5*10^8$organisms per ml) is uniformly carpeted on the surface of Mueller Hinton agar. Then antibiotic discs are evenly distributed over the lawn culture of organism. After incubation at 37^oC for 24hrs, zone of inhibition of each antibiotics interpreted using the interpreting chart and the organism is reported as resistant, intermediates or susceptible (Vandepitte *et al*., 2004).

2.15 Recommended antibiotic treatment for typhoid fever (WHO, 2003; Sachez-Vargas *et al*., 2011)

a. Uncomplicated typhoid fever:

i. Empirical treatment if infection is likely to have originated in Asia - azithromycin.

ii. Fully sensitive - fluoroquinolone (eg ofloxacin or ciprofloxacin) for 5-7 days.

iii. Multiple-resistant - fluoroquinolone (for 5-7 days), or cefixime (7-14 days).

iv. Quinolone-resistant - azithromycin (7 days) or ceftriaxone (10-14 days).

b. Severe typhoid fever requiring parenteral treatment:

i. Empirical treatment if the origin of infection is likely to be Asia - ceftriaxone.

ii. Fully sensitive - fluoroquinolone (such as ofloxacin) for 10-14 days.

iii. Multiple-resistant - fluoroquinolone (such as ofloxacin) for 10-14 days.

iv. Quinolone-resistant - ceftriaxone *or* cefotaxime (10-14 days).

2.16 Prevention and control

The first step in prevention is always limiting the infectivity of sources by proper treatment and management of acute cases, undiagnosed of convalescent excreter or chronic carriers. The next step in prevention is by preventing transmission by possible routes like contaminated food or drinking (WHO, 2003). Vaccination is an additional tool for prevention of typhoid fever. Following vaccines are available against typhoid fever:

i. Acetone inactivated parental vaccine: This vaccine is currently available in US armed force. The efficacy rate of this vaccine ranges from 75- 94%. Booster doses should be administered every three years.

ii. Live oral vaccine (Ty21a): This is an oral vaccine containing live attenuated "*Salmonella* Typhi Ty21a" strains in an enteric-coated capsule. Although the mechanism is not known, it elicits both serum and cell-mediated immune response. It is recommended that the vaccine should not be given children younger than 6 years old and the optimal booster's dose has been determined.

iii. Purified Vi capsular is composed of purified Vi antigen. This vaccine is given intramuscularly above 2 years of age. This offers 64-72% protection within 2 weeks of administration and the effects lasts for a minimum of 2 years (Corales, 2004).

CHAPTER III

MATERIALS AND METHODS

3.1 Materials and equipments

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

3.2 Methods

- Study site and study period

The study was carried out prospectively at Alka hospital from May through October 2012.

- Study population

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

- Case definitions

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.

- Sample size and inclusion/exclusion criteria

A total of 949 blood samples from patients following case definition of suspected enteric fever were included in the study. Samples with improper labeling, insufficient blood volume, inappropriate collection and transport were excluded.

- Collection of blood sample and culture

Bacteria are normally present on the skin surface. It is imperative for quality test results that bacteria are not introduced into the specimen being collected.

The veinpunture site was cleansed with a 2 percent tincture of iodine solution. Before any cleansing of the site begun, the patients were asked about any allergy to iodine. If the patient had an iodine allergy, the only recourse was to cleanse thoroughly with 70 percent alcohol. The cleansing was done with a circular motion, starting at the site of the puncture and moving in concentric circles outward.

The seals on the bottles were broken off. This seal usually consists of a metal flip off cap. Under the seal was a rubber septum through which the blood was

injected. Once the flip off cap was removed, the septum was cleaned with an alcohol pad. The proper amount of blood was drawn with a syringe. The alcohol pad was removed from the bottle cap. Without changing needles, the blood was then injected about 3ml of blood from children and 5ml of blood from adult was collected and aseptically inoculated into BHI bottles containing a solution that enhances the growth of significant microorganisms if they were present, and also anticoagulant was present in the bottle to avoid coagulation. If blood cultures were to be collected after antimicrobial treatment has started, the blood culture must be drawn in a special bottle containing a resin solution to inactivate the antimicrobial agent. After proper labeling the blood culture specimens, then the culture bottles were incubated at 37^0C for 48 hours. The culture bottles with the cloudiness of bacterial growth were sub-cultured on MacConkey agar whereas, the culture bottles with no turbidity were further incubated at 37^0C for 7 day and discarded. Then antibiotic susceptibility test was performed for the isolate obtained.

- Subculture and identification of *Salmonella* isolates

a. Subculture

Causative agent of enteric fever was isolated by culture of blood in BHI broth, incubated and BHI broth was routinely examined 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).

b. Biochemical tests

Isolates from BHI on MacConkey agar were identified by standard conventional biochemical methods using different biochemical tests: TSI, SIM, Urease test and Citrate test (appendix II).

3.3 Antimicrobial susceptibility testing

Disc diffusion method

The antibiotic sensitivity tests of the pathogens isolated from the clinical specimen against different antibiotics were determined by Kirby-Bauer method of disk diffusion technique as recommended by NCCLS using Mueller Hinton agar (MHA). At least three to five well isolated colonies of the same

morphological types were selected from the MacConkey plate. The base of each colony was touched with a inoculating wire and the growth was transferred into a tube containing 5ml of nutrient broth and was incubated at 37°C (usually 2 to 6 hours) until it achieved the McFarland tube number 0.5. In case overgrowth, the broth was diluted with sterile physiological saline to match with McFarland tube number 0.5. The suspension was then adjusted to match with McFarland tube number 0.5. A sterile cotton swab was dipped into the broth and the swab was rotated several times and pressed firmly on the inner side wall of the tube above the fluid level to remove excess inoculums from the swab. Then the dried surface of a MHA plate was inoculated by streaking the swab over the entire agar surface three times, turning the plate 60^{o} between streaking. Finally the inoculum was left to dry for a few minutes at room temperature with the lid closed. Then the antimicrobial disks were placed on the surface of the prior inoculated agar plate such that there was 25mm distance from disk to disk. The antibiotic discs used were nalidixic acid (30µg), ofloxacin (5µg), ciprofloxacin (5µg), chloramphenicol (30µg), cotrimoxazole (30µg) and ceftriaxone (30µg) to ensure reproducibility, each tests and measurement were carried out twice. The disks were pressed down to ensure complete contact with the agar surface. For about 15minutes of applying the disks, the plates were left at room temperature to allow antimicrobials to diffuse from the disk. Then they were incubated aerobically at 37°C overnight. After overnight incubation, the diameter of zone of inhibition (ZOI) of each disk was measured (including the diameter of the disk) and recorded in millimeter. It is then compared with Standard Chart developed by Kirby-Bauer to determine bacterial susceptibility towards different antimicrobial agents in terms of ‘sensitive’, ‘resistant’ and ‘moderately sensitive (intermediate)’. The results obtained were recorded in the worksheet (appendix III).

3.4 Analysis of data

All the results were recorded in the format as included in the appendix I to V and entered into SPSS 16.0. Statistical analysis was done by using SPSS 16.0.

3.5 Quality control

a. 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.

b. 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.

c. Quality control during antimicrobial susceptibility testing: Muller Hinton agar and antibiotic disc were checked for each lot number, manufacture date, 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 Mcfarland solutions) was used.

CHAPTER IV

RESULTS

A total of 949 patients suspected with enteric fever were included in the study conducted during May to October 2012 at Alka Hospital, Kathmandu.

Table No.1: Age and gender wise distribution of patients

Age	Gender						Total	%
	Male			Female				
	Out patient	In patient	Total	Out patient	In patient	Total		
0-10	84	27	111	48	20	68	179	18.9
10-20	93	26	119	53	22	75	194	20.4
20-30	83	32	115	84	29	113	228	24.0
30-40	53	12	65	51	13	64	129	13.6
40-50	35	5	40	34	12	46	86	9.1
50-60	26	9	35	17	9	26	61	6.4
60-70	12	7	19	16	5	21	40	4.2
70-80	7	5	12	11	2	13	25	2.6
80-90	1	2	3	2	0	2	5	0.5
90-100	0	0	0	0	2	2	2	0.2
Total	394	125	519	316	114	430	949	100
%	41.5	13.2	54.7	33.3	12.0	45.3	100	

Among the 949 blood samples collected for culture, 519 (54.7%) were from male and 430 (45.3%) were from female. The adults having age 20-30 years were most frequently suspected to enteric fever and requested for blood culture (24.0%) than any other age groups. The age

group 10-20 was second with 20.4% requests. Only two i.e. 0.2% blood culture was requested for age group 90-100 years.

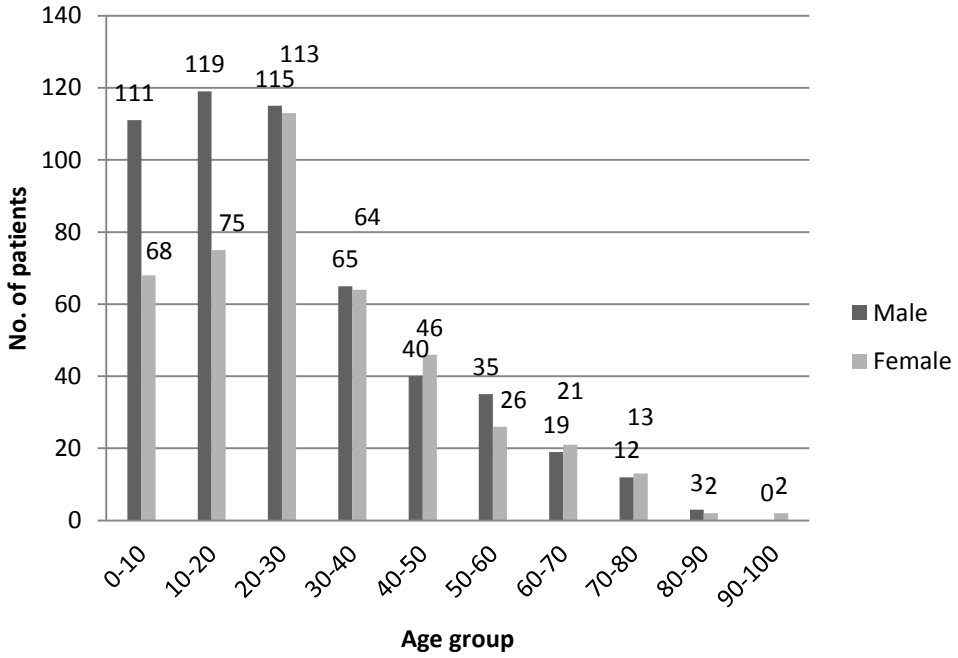

Figure No. 1: Age and gender wise distribution of the patients requesting for blood culture

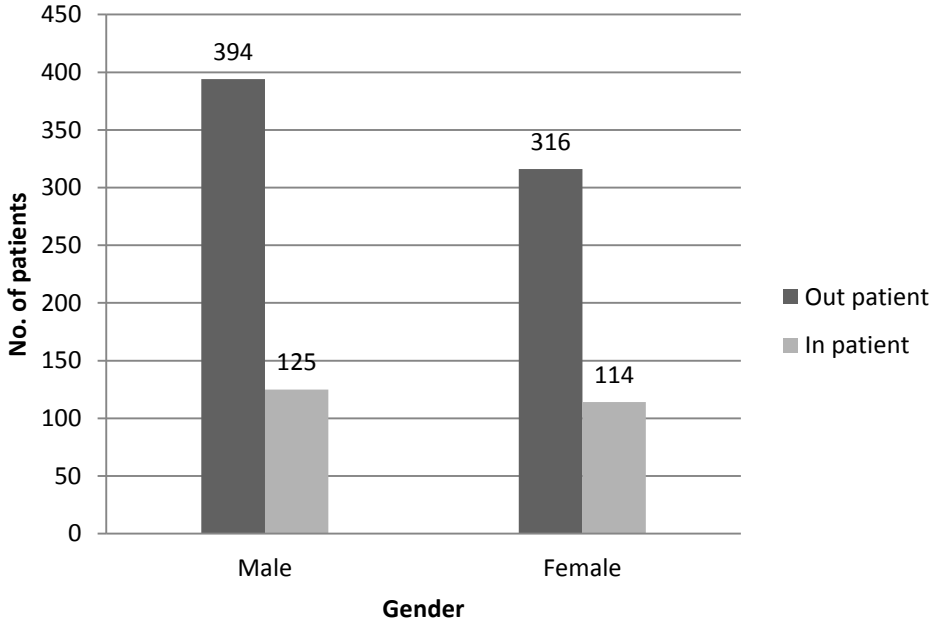

Figure No. 2: In/out patients and gender wise distribution of patients requesting for blood culture

Table No.2: Growth pattern in blood culture with relation to age and sex

Age	Male	Female	Culture positive male	Culture positive female	Total positive culture
0-10	111	68	9 (8.1%)	6 (8.8%)	15 (8.4%)
10-20	119	75	17 (14.3%)	7 (9.3%)	24 (12.4%)
20-30	115	113	13 (11.3%)	5 (4.4%)	18 (7.9%)
30-40	65	64	2 (3.1%)	-	2 (1.6%)
40-50	40	46	3 (7.5%)	1 (2.2%)	4 (4.7%)
50-60	35	26	2 (5.7%)	1 (3.8%)	3 (4.9%)
60-70	19	21	-	-	-
70-80	12	13	-	-	-
80-90	3	2	-	-	-
90-100	-	2	-	-	-
Total	519	430	46 (8.9%)	20 (4.7%)	66 (6.95%)

As given in the table 2, out of 66 culture positive samples, 24 were found in age group 10-20 years. The next was age group 20-30 years in which 18 culture positive samples were found. The highest percentage of positive culture was found in age group 10-20 years i.e. 12.4 percent. The total culture positive males were 46 (8.9% of total male patients i.e 519) and females were 20 (4.7% of total female patients i.e 430). The age group 60-70, 70-80, 80-90 and 90-100 years were not found with positive growth results.

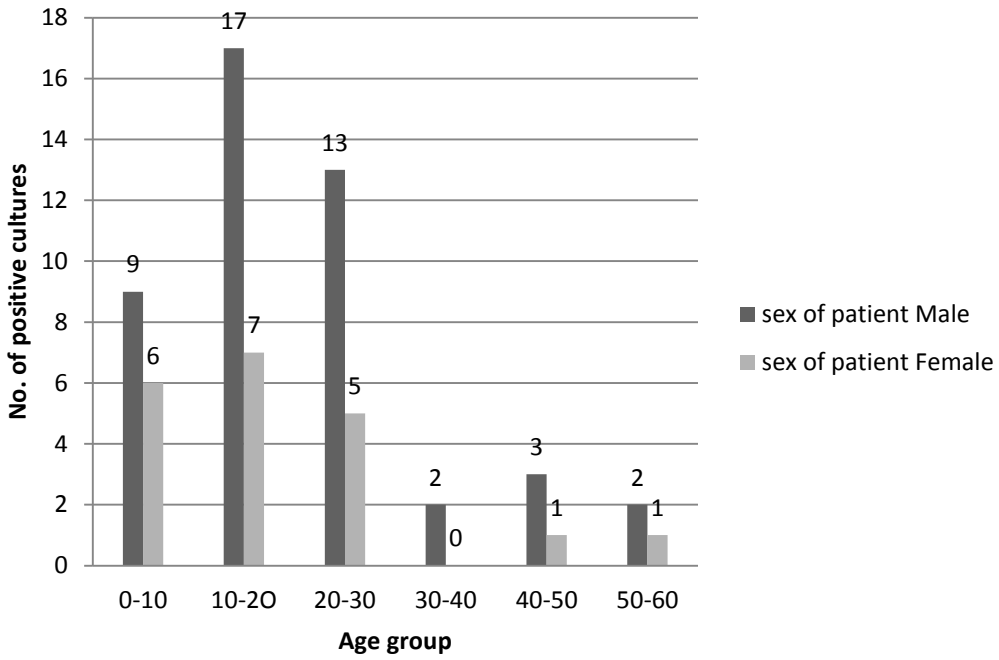

Figure No. 3: Age and gender wise distribution of culture positive patients

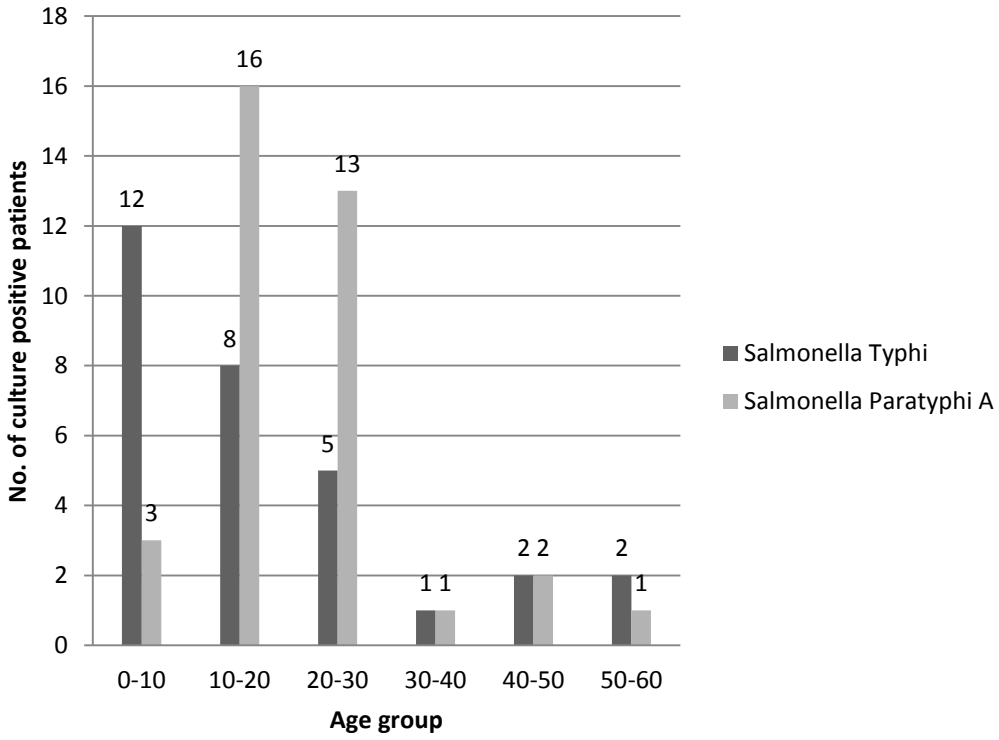

Figure No. 4: Distribution of bacterial isolates in different age group

Table No.3: Distribution of the bacterial isolates in outpatient and in patient

Origin	No. of patients	Bacterial isolates		Total
		Salmonella Typhi	*Salmonella* Paratyphi A	
Outpatient	710	24 (80%)	29 (80.6%)	53(80.3%)
Inpatient	239	6 (20.0%)	7 (19.4%)	13(19.7%)
Total	949	30 (45.5%)	36 (54.5%)	66

Table 3 showed that among 66 growth 53 were from outpatient and 13 were from inpatient. In outpatient out of the total growth 66, 24 were *S.*Typhi and 29 were *S.* Paratyphi A. In inpatient the number of *S.* Paratyphi A was 7 and *S.* Typhi was 6. Among 66 isolates from blood culture positive cases 30 (45.5%) isolates were identified as *S.* Typhi and 36 (54.5%) isolates were identified as *S.* ParatyphiA.

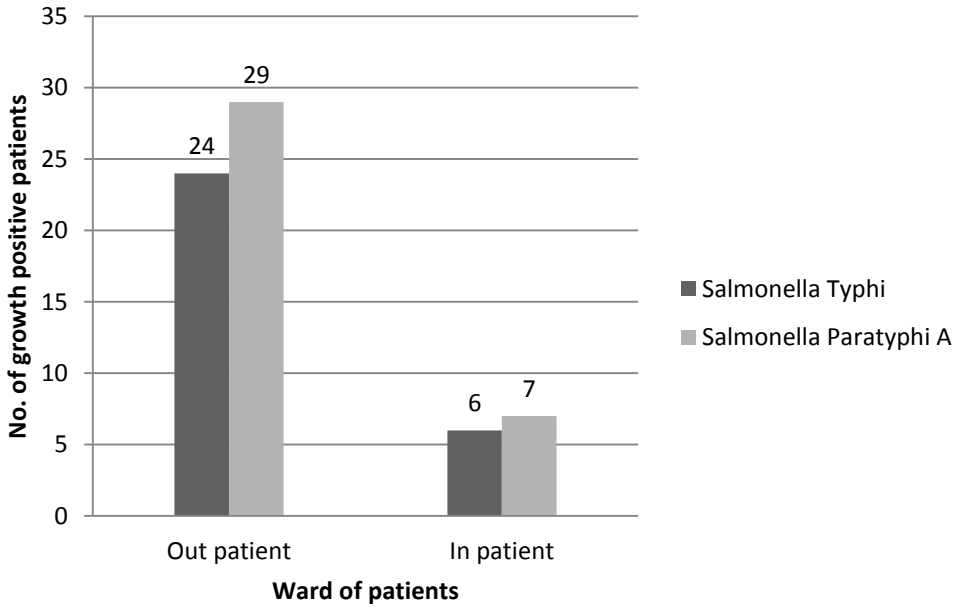

Figure No. 5: Bacterial growth pattern in blood culture with relation to in patients and out patients

Table No.4: Antibiotic susceptibility pattern of *Salmonella* Typhi

Antibiotics	Sensitive		Intermediate		Resistant		Total
	Number	%	Number	%	Number	%	
Nalidixic acid	4	13.3	-		26	86.7	30
Ciprofloxacin	30	100	-	-	-	-	30
Ofloxacin	29	96.7	-	-	1	3.3	30
Cotrimoxazole	28	93.3	-	-	2	6.7	30
Ceftriaxone	0	100	-	-	-	-	30
Chloramphenicol	29	96.7	-	-	1	3.3	30

As given in table 4, among the isolated *S.*Typhi (30), 100% (30) sensitivity was found to the antibiotics ciprofloxacin and ceftriaxone. 100 percent isolates were susceptible towards Ciprofloxacin. Out of 30 *S.* Typhi 3.3% (1) of *S.* Typhi was resistant to ofloxacin, 86.7% (26) to nalidixic acid, 6.7% (2) to cotrimoxazole and 3.3% (1) to chloramphenicol.

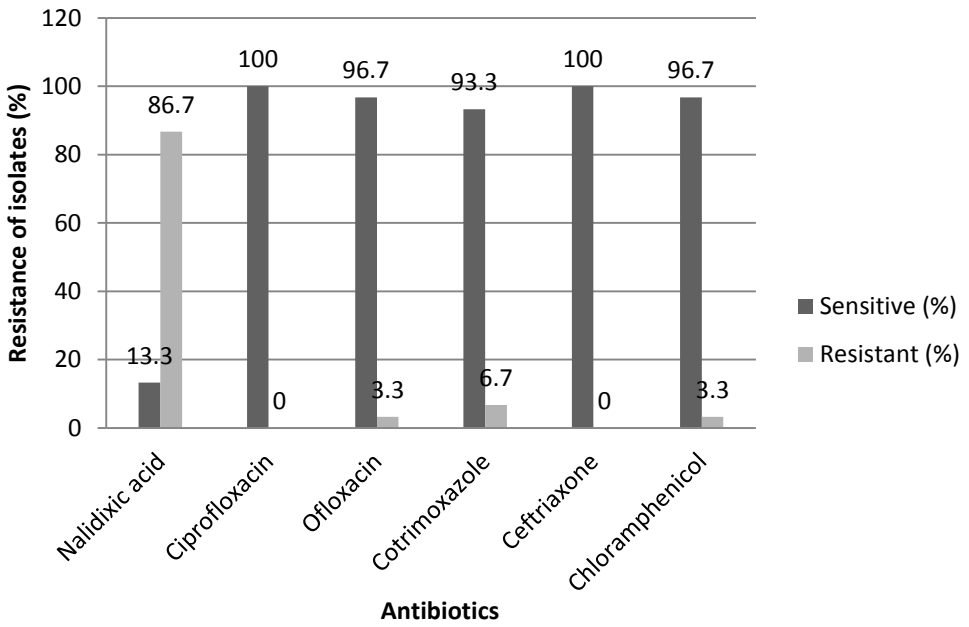

Figure No. 6: Antibiotic susceptibility pattern of *Salmonella* Typhi

Table No.5: Antibiotic susceptibility pattern of *Salmonella* Paratyphi A

Antibiotics	Sensitive		Intermediate		Resistant		Total
	Number	%	Number	%	Number	%	
Nalidixic acid	-	-	-	-	36	100	36
Ciprofloxacin	36	100	-		-	-	36
Ofloxacin	34	94.4	1	2.8	1	2.8	36
Cotrimoxazole	36	100	-	-	-	-	36
Ceftriaxone	36	100	-	-	-	-	36
Chloramphenicol	36	100	-	-	-	-	36

As given in the table 5, among the *S.* Paratyphi A isolates (36), none of the isolates were found to be resistant to the antibiotics Ceftriaxone, Ciprofloxacin, Chloramphenicol and Cotrimoxazole. Out of 36 *S.* Paratyphi A 100% (36) Nalidixic acid and 2.8% (1) Ofloxacin resistant *S.* Paratyphi A isolates were found.

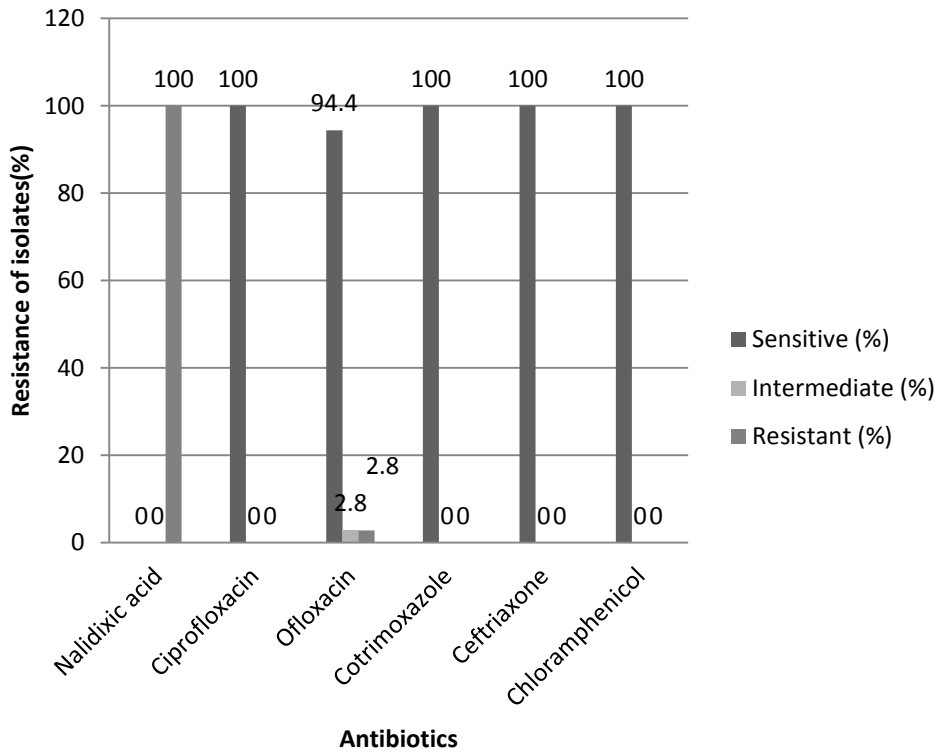

Figure No. 7: Antibiotic susceptibility pattern of *Salmonella* Paratyphi A

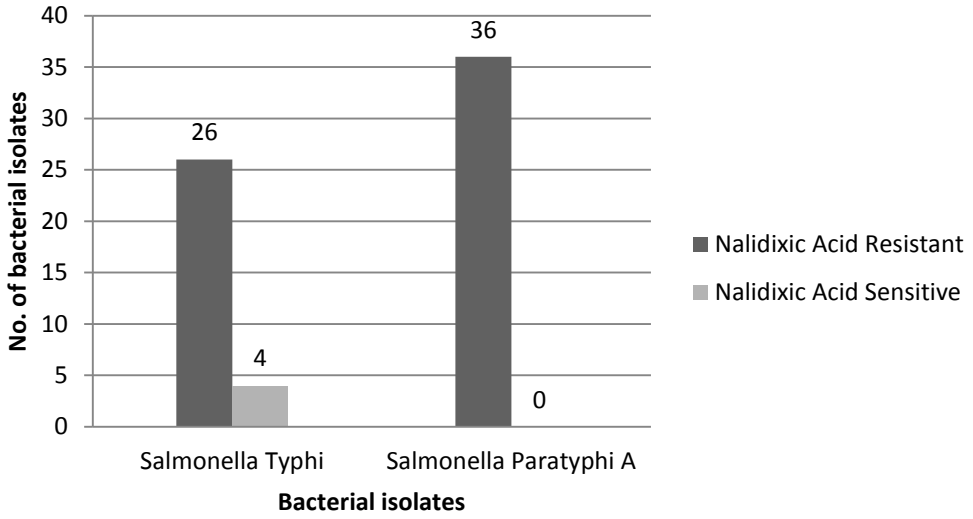

Figure No. 8: Nalidixic acid susceptibility pattern of *Salmonella* isolates

Table No.6: Nalidixic acid susceptibility pattern of *Salmonella* isolates with relation to in patient and out patient

Bacterial isolates	AST pattern of Nalidixic acid									Total
	Resistant			Intermediate			Sensitive			
	Out patient	In patient	Total	Out patient	In patient	Total	Out patient	In patient	Total	
S. Typhi	22	4	26	-	-	-	2	2	4	30
S. Paratyphi A	29	7	36	-	-	-	-	-	-	36
Total	51	11	62	-	-	-	2	2	4	66

Table 6 showed that among 53 culture positive outpatient 51 (96.22%) were Nalidixic acid resistant and among 13 inpatient 11 (84.61%) were Nalidixic acid resistant. The incidence of Nalidixic acid resistant *S.* Typhi and *S.* Paratyphi A among total outpatient (710) and inpatient (239) was 7.2% (51) and 4.6% (11) respectively. The incidence of Nalidixic acid resistant *S.* Typhi and *S.* Paratyphi A among total population (949) was 2.7% (26) and 3.8% (36) respectively.

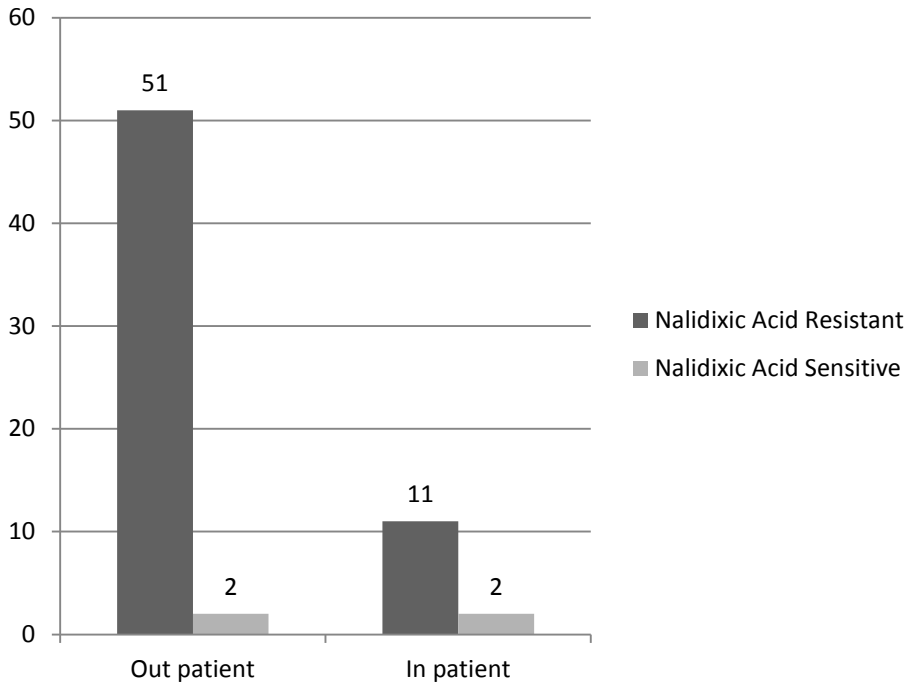

Figure No. 9: Nalidixic acid susceptibility pattern of *Salmonella* isolates with relation to in patient and out patient

Table No.7: Nalidixic acid susceptibility pattern of *Salmonella* isolates with relation to Gender of patient

Bacterial isolates	AST pattern of Nalidixic acid									Total
	Resistant			Intermediate			Sensitive			
	Male	Female	Total	Male	Female	Total	Male	Female	Total	
ST	18	8	26	-	-	-	4	-	4	30
SPA	24	12	36	-	-	-	-	-	-	36
Total	42	20	62	-	-	-	4	-	4	66

Note: ST, *Salmonella* Typhi; SPA, *Salmonella* Paratyphi A

Table 7 showed that among 46 culture positive male patients 42(91.3%) were Nalidixic acid resistant and among 20 culture positive female all 20 (100%) were Nalidixic acid resistant. The incidence of Nalidixic acid resistant *Salmonella* isolates among male (519) and female (430) was 8.1% (42) and 4.7% (20) respectively.

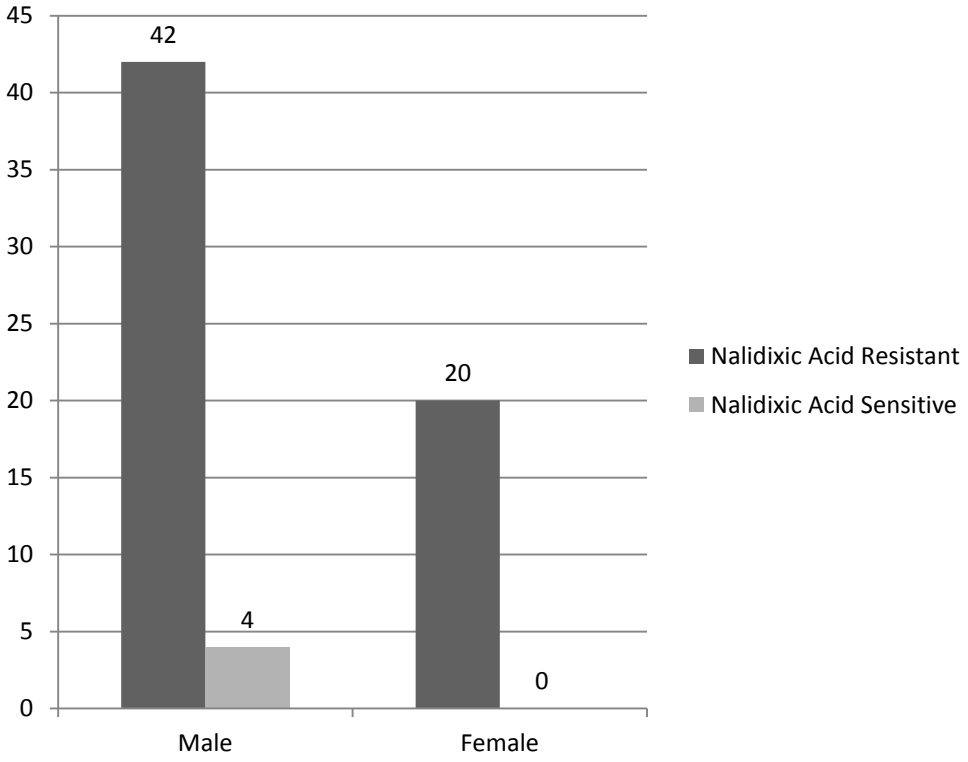

Figure No. 10: Nalidixic acid susceptibility pattern of *Salmonella* isolates with relation to Gender of patient

CHAPTER V

DISCUSSION

Blood samples were collected from 949 enteric fever suspected patients visiting Alka Hospital, Lalitpur. Outpatients were higher (74.8%) than hospitalized patients (25.2%). Male patients were higher in number (54.7%) than the females (45.3%). The majority of patients belonged to age group 20-30 years (with 24.02% of the studied population). Among the total 949 enteric fever suspected patients, bacterial growth on culture was observed only in 6.95% (66) cases. Lack of growth in blood culture resulting low isolation of the pathogen is common mainly because of use of antibiotics prior to blood culture collection for culture (Parry *et al*., 1999; Vallenas *et al*., 1985) as availability and misuse of the antibiotics even for mild cases of fever is common in Nepal (Khanal *et al*., 2007; Wachter *et al*., 1999). Besides that, low concentration of circulating *Salmonella* in blood and difficulties of obtaining enough volume of blood for culture also play important role in the sensitivity of the blood culture.

In this study, the incidence rate of *Salmonella* isolates in outpatient and inpatient was 7.5% and 5.4% respectively. The incidence rate of *Salmonella* isolates in male patients (8.8%) was higher than that of female patient (4.7%). Similarly the blood culture positive rate was found to be highest in the age group 10-20 years in both male and female patients.

Out of 66 blood culture positive cases 36 (54.54%) were *Salmonella* Paratyphi A and remaining 30 (45.46%) were *Salmonella* Typhi, indicating higher incidence of paratyphoid cases than typhoid cases. *S.* Paratyphi B and C were not isolated during the study period. Paratyphoid fever caused by *S*. Paratyphi is considered an emerging disease, as its incidence has increased alarmingly in recent years (Thong *et al*., 1998), causing more asymptomatic infections than *S*. Typhi. In Kathmandu, Nepal, enteric fever caused by *S*. Paratyphi A is more prevalent than that caused by *S*. Typhi (Shirakawa *et al*., 2006).

The incidence of Nalidixic acid resistant *Salmonella* isolates among outpatient and inpatient was 7.2% and 4.6% respectively. The incidence of Nalidixic acid

resistant *S.* Typhi and *S.* Paratyphi A among total population was 2.7% and 3.8% respectively. The incidence of Nalidixic acid resistant *Salmonella* isolates among male and female was 8.1% and 4.7% respectively. Among the isolated *Salmonella* Typhi, 100 percent sensitivity was found to the antibiotics ciprofloxacin and ceftriaxone. 100 percent isolates were susceptible towards Ciprofloxacin. Altogether 3.3 percent of *Salmonella* Typhi were resistant to ofloxacin, 86.7 percent to Nalidixic acid, 6.7 percent to cotrimoxazole and 3.3 percent to chloramphenicol.

Among the *Salmonella* Paratyphi A isolates, none of the isolates were found to be resistant to the antibiotics Ceftriaxone, Ciprofloxacin, Chloramphenicol and Cotrimoxazole. Altogether 100 percent Nalidixic acid and 2.8 percent Ofloxacin resistant *Salmonella* ParatyphiA isolates were found.

Isolates of *Salmonella* with decreased susceptibility to ciprofloxacin have been found to be nalidixic acid resistant. According to CLSI guidelines, fluroquinolones-susceptible strains of *Salmonella* that test resistant to nalidixic acid may be associated with clinical failures or delayed response in fluroquinolone treated patients with extra intestinal salmonelosis. So the extra intestinal isolates of *Salmonella* should also be tested for resistant to nalidixic acid or else, false susceptibility reports may be issued. The uncertain clinical response of fluoroquinolone susceptible, Nalidixic acid resistance strains has prompted debate over changing the established CLSI break points for the fluoroquinolones. Some of the researchers feel that it is important to identify Nalidixic acid resistance in *Salmonella* as a predictor for decreased fluoroquinolone susceptibility. The current CLSI breakpoints may have to be reevaluated for *Salmonella*. This implies that quinolones may no longer be the drug of choice in treating enteric fever (Joshi *et al*., 2004).

The result of this study is silent on the above comment as none of the isolates were ciprofloxacin resistant. To agree or disagree on the above statement, it requires more investigation.

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 up to 10 days before reporting negative, which is not followed in resource poor laboratory in developing countries including Nepal.

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 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 resistant to nalidixic acid (Raveendra *et al*., 2008).

In contrast to the nalidixic acid resistant, this study found re-emergence of sensitivity to first line drug. In a study from Nepal, compared to period 1993-1998to 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 79-85% 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).

Over past decade, increasing antibiotic resistant in *S. enterica* has lead to a shift in the antibiotics used against this organism from chloramphenicol, ampicillin, cotrimoxazole, to fluroquinolones (ofloxacin, ciprofloxacin), and ceftriaxone.

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 resistant (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 resistant (Pokhrel *et al*., 2006).

The combination of MDR *S*. Typhi and NAR (MDR-NAR) is a particular problem, because it severely restricts the therapeutic options for patients with typhoid fever.

CHAPTER VI

CONCLUSION AND RECOMMENDATION

6.1 Conclusion

Enteric fever caused by *Salmonella* Typhi and *Salmonella* Paratyphi is one of the most common clinical disease presented to hospitals. The fluoroquinolone drugs are the first choice for the treatment of enteric fever but in present study, a large number of NARS was observed resulting question in the efficacy of fluoroqinolones used for the treatment of the enteric fever.

Therefore the third generation cephalosporin drugs like ceftriaxone remains the better choice of drug against fluoroquinolone resistant *Salmonella* which is also resistant to first line antibiotics (Ampicillin, cotrimoxazole and chloramphenicol) as resistant to third generation cephalosporins has not been detected from enteric fever patients in present study.

6.2 Recommendation

Following recommendation can be put forward for further research.

- More extensive studies with larger sample numbers on Nalidixic acid resistant *Salmonella* strains should be carried out on other parts of the country as the study conducted in Alka Hospital may represent small focus of the diverse geography of the country.

- In this study only *Salmonella* isolates from blood culture were considered. Surveillance with *Salmonella* isolates from urine, stool, food, water and other animals may be more representative.

- Molecular analysis may reveal more lights in the mechanism and genes for Nalidixic acid resistance.

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APPENDICES

Appendix I: Patient's request form

Name: ..... Lab No.: .....

Age/Gender: ..... Date: .....

Culture request for: .....

Brief Clinical History: .....

Recurrence of infection:……

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

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

C. Biochemical test

Sample ID	Isolate ID	Catalase	Oxidase	Indole	Motility	TSI	Citrate	Urease	others	Isolated organism

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	Organism isolated	Antimicrobial susceptibility pattern								
				C	COT	OF	NA	CTR	CIP			

Appendix IV: Working procedures

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 100X.

Quality control

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

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 V.

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 expiry 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. Bring 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. 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).

Appendix V: Media, reagents and equipments

Culture media and biochemical media: Nutrient agar, MacConkey agar, Nutrient broth, Muller Hinton agar, BHI broth, Muller Hinton broth, SIM media, Simmon citrate media, Triple iron agar, Urea broth

Reagents: Catalase reagent, Oxidase Reagent, Kovac's Reagent, Gram's reagent

Equipments, materials, and supplies

General Microbiology laboratory Equipments: Autoclave, Incubator, Hot air oven, Microscope, Refrigerator, Weighing machine, Gas burner , Glasswares, Inoculating wire and loops

Materials for the disc diffusion method

Media and reagents(a) Agar plates (90 mm; depth approximately 4 mm) (b) MHA (c) Mueller Hinton broth or 0.9% NaCl (d) Antimicrobial discs from HiMedia Laboratories Pvt. Limited (chloramphenicol 30µg, cotrimoxazole 30µg, 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.