CHAPTER-I

INTRODUCTION AND OBJECTIVES

1.1. INTRODUCTION

Enteric fever is a systemic disease that includes typhoid fever caused by *Salmonella* serotype Typhi and less severe form of disease paratyphoid fever caused by *Salmonella* serotypes Paratyphi A, B, and C. Enteric fever is the major public health problem and important cause of morbidity and mortality in the world particularly in developing countries where water supplies and sanitation are poor (Bhatta *et al.*, 2005; Arora *et al.*, 2010). The disease is rare in resource-rich nations as a result of adequate sanitary attention, but remains a significant disease associated with travel to endemic areas (Matheson *et al.*, 2010).

Globally there are estimated 26 million cases of enteric fever, resulting over 200000 deaths each year (Arjyal *et al.*, 2011). The incidence of the disease is highest in South Central and South East Asia (Nagpal and Jairam, 2009). About 90% of typhoid-related deaths are estimated to occur in Asia (Naheed *et al.*, 2010). 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).

The disease is endemic in Nepal with peak incidence in rainy seasons as a result of flooding and fecal contamination of drinking water (Pokhrel *et al.*, 2009). The incidence of enteric fever in Nepal is estimated more than 100 / 100 000 cases per year (Crump *et al.*, 2004). In Nepal, for the fiscal year 2010/2011, enteric fever was responsible for fourth cause of hospitalization with annual morbidity of 506,183 (Mountain-38,642, Hill-257,729and Terai-209,812) (Department of Health Services, 2012).

The outbreak of typhoid fever in Bharatpur Nepal in 2002 was the largest single point source, multidrug resistance typhoid fever outbreak reported in literature. About 5963 cases of typhoid fever were confirmed or

clinically suspected during a 7-week period with a crude attack rate of 64.9 cases per 1000 population (Lewis *et al.*, 2005).

Salmonella species, the members of the family enterobacteriaceae, are gram-negative, facultative anaerobic straight rods associated with animal and human infections. *Salmonella* Typhi and *Salmonella* Paratyphi A, B and C, the causative agents of enteric fever, are the serovars of *S. enterica* subsp *enterica* (Sanchez-Vargas *et al.*, 2011).

Historically, typhoid fever is considered a more critical and predominant illness than paratyphoid fever. However, in recent decades the number of cases paratyphoid fever has been increasing. *S.* Paratyphi A is gaining prevalence in Southeast Asia and is responsible for 30-50% of enteric fever cases contributing to the emergence of multiple-drug resistance or fluoroquinolones-resistant strains of serovars *S.* Paratyphi A (Yin Ngan *et al.*, 2010).

Humans are the natural host and reservoir of salmonellae and infection occurs by ingestion of organisms in contaminated food or water or from contaminated hands. The infectious dose varies between 10^3 – 10^6 organisms given orally (Cheesbrough, 2000; Kothari *et al.*, 2008).

Infection caused by *S.* Paratyphi A is assumed to be milder than infection caused by *S.* Typhi, but in Nepal this assumption is unsupported. It is reported that the disease syndromes caused by *S.* Paratyphi A and *S.* Typhi are clinically indistinguishable (Karkey *et al.*, 2008). The illness was characterized by a history of fever, headache, diarrhea, malaise, abdominal pain, vomiting and cough and other less frequent symptoms weight loss and constipation. Fever >38 ^{0}C, abdominal tenderness, splenomegaly, rose spots (in about7% patients) and tachycardia (pulse rate >100 beats per min) during the highest temperature are other physical findings(Clark *et al.*, 2010).

Diagnosis of typhoid and paratyphoid fever requires culture of blood, bone marrow, stools or urine to confirm growth of *S.* Typhi or *S.* Paratyphi A, B or C. However, in developing countries, culture facilities are expensive and mostly

confined to hospitals. As most typhoid patients are diagnosed and treated in outpatient settings, diagnosis is usually based on the insensitive widal test or clinical presentation (Vollaard *et al.*, 2005). Microbiological culture method is the gold standard for the diagnosis of enteric fever. Due to low sensitivity and low specificity conventional widal serological test is of little value. Serological approaches to the diagnosis of *S.* Paratyphi A, B, and C have been developed but have not been evaluated for field use (Crump and Mintz, 2010). Bone marrow culture is the gold standard method for confirming case of typhoid fever. However, this requires equipment, supplies and trained laboratory personnel seldom found in primary health-care facilities in the developing countries. Blood culture is a more practical but less sensitive alternative to bone marrow culture (Keddy *et al.*, 2011).

Antibiotic treatment is not required for non-complicated *Salmonella* gastroenteritis but antibiotic therapy is essential in case of enteric fever or invasive salmonellosis (Bertrand *et al.*, 2006). Conventional first line antimicrobial agents, such as ampicillin, chloramphenicol, and trimethoprim–sulfamethoxazole (TMP-SMX), were the drugs of choice for the treatment of enteric fever in many countries up to a decade ago (Rotimi *et al.*, 2008). MDR *S.* Typhi isolates emerged from the Indian subcontinent in the early 1990s that prompted ciprofloxacin or ceftriaxone as the drug of choice for empirical treatment of typhoid fever (Saha *et al.*, 2001). Multidrug- resistance (MDR) typhoid fever is defined as typhoid fever caused by *S.* Typhi strains which are resistant to all the three antibiotics *i.e.,* chloramphenicol, ampicillin, and cotrimoxazole, the recommended first line drugs of typhoid fever (Zaki and Karande, 2011). Fluoroquinolones accumulate in high concentrations in the bile, bowel, urinary tract and macrophages which has made ciprofloxacin and ofloxacin the drugs of choice of oral administration for the treatment enteric fever with more than 90% cure rates (Joshi and Amarnath, 2007).

The first report of multidrug resistant *Salmonella enterica* serovar Typhi in Nepal was published in 1991. In subsequent years fluoroquinolones and third generation cephalosporin resistant *S.* Typhi has been reported from Nepal, Bangladesh, India and other developing countries (Acharya *et al.*, 2009). In Nepal several studies

have been conducted that focused on the prevalence of MDR and the antibiotic susceptibility patterns of *S*. Typhi that reveal spatial and temporary fluctuation affecting the treatment guideline (Acharya *et al.*, 2012).

Re-emergence of susceptibility to drugs used in the past, such as chloramphenicol, ampicillin and trimethoprim has been documented in several reports. The changing trends of *S*. Typhi resistance patterns reveal the reconsideration of conventional treatment strategy and suggest the possibility of use of these drugs for the treatment of typhoid fever (Kumar *et al.*, 2011).

Accurate estimate of the burden of enteric fever in Nepal is difficult to obtain, as many cases are treated outside hospitals in clinics with limited diagnostic facilities or by self-treatment using drugs obtained from private pharmacies and lack of resources in hospitals at rural settings (Prajapati, 2009). The antibiotic sensitivity patterns of *Salmonella* serovars are changing during recent years, and these needs to be continually monitored (Aryal, 2008).

A cross sectional study was conducted in Alka Hospital Lalitpur during July 2011 to February 2012 to determine the prevalence of enteric fever and antibiotic susceptibility pattern of the *Salmonella* serovars isolated from the blood so that appropriate strategies can be adopted in management of enteric fever. This present study would also aid physicians to recommend proper antibiotic prior to obtain blood culture report and would be the guideline for the treatment of enteric fever in rural settings, where the laboratory facilities of blood culture and antibiotic susceptibility testing is in substandard.

1.2 OBJECTIVES

1.2.1 General Objective

To determine the antibiogram pattern of the *Salmonella* serovars isolated from the blood of clinically suspected enteric fever patients visiting Alka Hospital, Lalitpur.

1.2.2 Specific Objectives

1. To determine the prevalence of enteric fever in febrile patients
2. To identify *Salmonella enterica* serovars causing enteric fever.
3. To determine the proportional contribution of *Salmonella* Typhi and *Salmonella* Paratyphi as the causative agent of enteric fever.
4. To assess the antibiotic susceptibility pattern of *Salmonella* serovars isolated from the blood of suspected enteric fever patients.

CHAPTER-II

LITERATURE RIVIEW

2.1 ENTERIC FEVER

Enteric fever is a systemic infection that includes the classic typhoid fever caused by *Salmonella* Typhi, and less severe syndrome, paratyphoid fever caused by *Salmonella* Paratyphi A, B, and C (Kudalkar *et al.*, 2004). The disease is seldom fatal, but some patients can develop life threatening complications, including hypotensive shock and perforation of the intestine (Karkey *et al.*, 2010).

2.2. HISTORY OF ENTERIC FEVER

Thomas Willis probably first described the association of the ileal ulcer with epidemic typhoid in 1659. P.C. A. Louis first used the term “typhoid fever” in 1829. The term enteric fever was proposed by Wilson in 1869 as an alternative to typhoid fever, given the anatomic site of infection, which now also includes paratyphoid fever. In 1873, Budd reported that typhoid fever is transmitted through food and water via fecal-oral route (Ellermeier and Slauch, 2006).

Karl Eberth (1880) first observed typhoid bacillus in the spleen and mesenteric lymph node of typhoid patients and Georg Gaffky (1984) successfully isolated the organisms in Germany. It was then called Eberth-Gaffky bacillus or Eberthella typhi which is nowadays recognized as serovar *S*. Typhi. Daniel E. Salmon and Theobald Smith isolated the American hog-cholera bacillus (now referred as *Salmonella* Chlolerae-suis) from the intestine of pig in1885, and in honor of discoverer, Lingnieres in 1900 suggested the name of the organism *Salmonella* (Bhatta *et al.*, 2005; Ellermeier and Slauch, 2006).

Salmonella Paratyphi A was isolated by Gwyn in 1898. Acharde and Bensaud, in 1896, first isolated *Salmonella* Paratyphi B and used the term paratyphoid fever. *Salmonella* Paratyphi C was isolated by Oblenhuth and Hubener in 1908 from the cases resembling typhoid fever (Old, 1990).

2.3 EPIDEMIOLOGY OF ENTERIC FEVER

Enteric fever is endemic in low- and middle-income countries lacking clean drinking water and having poor and inadequate sanitation and hygiene standards. However, the incidence of enteric fever in industrialized countries is generally low and invariably associated with travel to endemic regions (Parry *et al.*, 2011). The disease is endemic in the Indian subcontinent, South-East and Far East Asia, Africa, Central and South America, and the Mediterranean region (Effa and Bukirwa, 2008). Globally typhoid fever caused an estimated 21.7 million illnesses and 217,000 deaths, and paratyphoid fever caused an estimated 5.4 million illnesses in 2000 (Crump and Mintz, 2010). The incidence of typhoid fever is estimated high (>100 cases per 100,000 population per year) in South-Central Asia, South East Asia. According to WHO estimate in 2000 the annual global incidence of typhoid fever is 0.3%, and in some developing countries of Asia and Africa the annual incidence may reach 1% with case fatality rates as high as 10%. About 70% of all fatalities from typhoid fever occur in Asia (WHO, 2000).

The 1997 Global Survey of *Salmonella* serotyping estimated an incidence of 1 case of paratyphoid fever for every 4 cases of typhoid fever (Kothari *et al.*, 2008). However, *S.* Paratyphi A is now a growing emerging cause of enteric fever in Nepal, India, Pakistan, China, Vietnam and Indonesia where nearly half of the cases of enteric fever is due to *S.* Paratyphi A rather than *S*. Typhi (Wilde, 2007).

The incidence of S. Typhi infections is low (400 per 100,000 per year) in the US and mostly related the travelers. Enteric fever in UK and Canada is infrequent and most of the cases are imported from India or Pakistan. Among Asian countries the annual incidence of S. Typhi is higher in Pakistan (451.7 cases per 100,000) and India (214.2 cases per 100,000) compared with Viet Nam (21.3 cases per 100,000) and China (15.3 cases per 100,000) (Sanchez-Vargas *et al.*, 2011). Outbreaks of typhoid have been reported from Maharashtra, Bangalore, West Bengal, Pondicherry and Rajasthan in India (Anand and Ramakrishnan, 2010).

Traditionally, enteric fever is believed to be a disease of school-age children and young adults. However, a few recent studies in Asia have shown the maximum burden of the disease among children less than 5 years of age with higher rates of complications and hospitalization (Nagpal and Jairam, 2009).

Typhoid fever cases are more commonly seen in males than in females (Singh, 2001). However, a retrospective study in two hospitals in East London during 2005-2010 showed 54% male cases compared to 46% female cases (Reddy *et al.*, 2011). Typhoid fever is usually observed throughout the year. Some studies show a peak incidence of the disease in rainy season (from July to September) when the chance of water contamination is high, especially in crowded areas (Kanungo *et al.*, 2008).

2.4 ENTERIC FEVER IN NEPAL

Enteric fever is endemic in Nepal with major public health problems and the incidence of typhoid fever is estimated to be more than 100/ 100 000 cases per year. It is one of the common causes for adults to visit health facilities in Nepal (Karki and Kumar, 2011). A recent study conducted in Patan Hospital Nepal by (Kelly *et al.*, 2011) reported that *Salmonella* Typhi and *Salmonella* Paratyphi accounted for 42% of all bacteremias cases and were the most frequently cultured pathogens in children greater than 1 year of age.

Lewis *et al.* (2005) reported the largest single point source, multidrug resistance typhoid fever outbreak with 5963 confirmed or clinically suspected cases of typhoid fever during a 7-weekperiod (24 May–13 July 2002) in Bharatpur, Nepal. The outbreak was attributed to fecal contamination of municipal water supply. A retrospective study in an urban hospital in Kathmandu from1993 to 2003, recovered 9124 *Salmonella* from 12,252 positive blood cultures from 82467 suspected enteric fever patients. Among them 6447 (70.7%) were by *S.* Typhi and 2677 (29.3%) were by *S.* Paratyphi A. The percentage *Salmonella* isolated varies from 15% in 1993-1995, to 5% in 1995-2000, and again rising to 15% in 2000-2003. The proportion of *S*. Paratyphi A isolates rises from23% to 34% from the first half of the study (1993-1998) to the second half

(1999-2003), which paralleled its increased resistance to ciprofloxacin (Maskey *et al.*, 2008).

Khanal *et al.* (2007) isolated 132 *S* Typhi from 2,568 blood culture samples collected from suspected enteric fever patients and 35 of them were multidrug resistant strains. They reported 69.23% strain with reduced susceptibility to ciprofloxacin and 76% NARST strain. Khanal *et al.* (2007) reported the male-to-female ratio 2.1:1 from a study in Eastern Nepal. In an urban setting, Bhatta *et al.* (2007) isolated and identified multiple drug resistant (MDR) *S*. Typhi and *S*. Paratyphi in the drinking water supply of 14% of samples.

2.5 ETIOLOGICAL AGENT OF ENTERIC FEVER

2.5.1 GENUS *SALMONELLA*

Salmonella species are organisms that conform to the definition of the enterobacteriaceae (Old and Threlfall, 1996). Salmonellas are gram negative, facultative anaerobe, non-spore-forming, non-acid fast and non-capsulated bacilli that measure 2 to 5µm by 0.7–1.5 µm (Ellermeier and Slauch, 2006). They express common enterobacterial polysaccharide surface antigen and have a DNA of 39% -59% average G+C content (Hu and Kopecko, 2002). Salmonellas are motile due to peritrichous flagella and form type-1 (mannose-sensitive, haemagglutinating) fimbriae except serotypes *S.* Gallinarum *S.* Pullorum which are non-motile variants and form type-2 (non-haemagglutinating) fimbriae. Most *S.* Paratyphi A strains are non-fimbriate (Janda and Abbott, 2005).

2.5.2 TAXONOMY AND NOMENCLATURE

Initially one serotype-one species concept on *Salmonella* nomenclature was proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. Following the development of new technology DNA-DNA hybridization, Corsa *et al*. in 1973 demonstrated that all serotypes and subgenera I, II and IV of *Salmonella* and all serotypes of “Arizona” were related at the species level, thus belonging to a single species. And *Salmonella bongori*, formerly known as subspecies V, was identified as distinct species (Brenner *et al.*, 2000).

Initially *S. choleraesuis* was considered as the type species of *Salmonella*, and it had priority as the species name. However, the name "choleraesuis," created confusion as it referred to both a species and a serotype. Thus in 1986 CDC and other laboratories adopted *S. enterica* as the type species. In 2005 the Judicial Commission issued Opinion 80 that decided *Salmonella enterica* the type species of the genus replacing *Salmonella choleraesuis* (Tindall *et al.*, 2005).

The current nomenclature used by CDC is based on the recommendations from the WHO Collaborating Centre and according to CDC system; the genus *Salmonella* comprises two species; *Salmonella enterica* and *Salmonella bongori*, each of which contains multiple serotypes. *Salmonella enterica* is further subdivided into six subspecies that are designated by names or Roman numerals: *Salmonella enterica* subspecies *enterica* (I), *Salmonella enterica* subspecies *salamae* (II), *Salmonella enterica* subspecies *arizonae* (IIIa), *Salmonella enterica* subspecies *diarizonae* (IIIb), *Salmonella enterica* subspecies *houtenae* (IV) and *Salmonella enterica* subspecies *indica* (VI). *Salmonella* bongori, originally designated *S. enterica* subspecies V, has been determined to be a separate species of *Salmonella* (Brenner *et al.*, 2000).

The serotypes are named in subspecies I are written in Roman (not italicized) and the first letter is capitalized (for example, serotypes Typhi, and Paratyphi A) and antigenic formulae are used for serotypes subspecies II, IV, and VI and in *S. bongori*. At the first citation of a serotype the genus name is given followed by the word "serotype" or the abbreviation "ser." and then the serotype name (for example, *Salmonella* serotype or ser. Typhi). Thereafter the name may be written with the genus followed directly by the serotype name for example, *Salmonella* Typhi or *S.* Typhi (Grimont and Weill, 2007; CDC, 2008).

2.5.3 HABITAT

The Salmonellas live primarily in the intestinal tracts of man and animals, both domestic and wild. They are frequently found in sewage, river and other waters and soil. They may survive for weeks in water (89 days in tap water and 115 days in pond water) and for years in soil (Janda and Abbott, 2005). *Salmonella enterica* subsp. *enterica* inhabit warm-blooded animals, whereas all other *S.*

enterica subspecies and *S. bongori* are commensals of cold-blood animals and only rarely infect humans. The *S. enterica* subsp. e*nterica* serovars can be further divided into those that are “host adapted” (primarily infecting one host but capable of causing disease in others), “host restricted” (infecting only a single host), and “generalists” (capable of infecting many hosts). *Salmonella* infection of many of these hosts often leads to a self-limiting gastroenteritis except typhoid and paratyphoid fever (Ellermeier and Slauch, 2006).

2.5.4 ANTIGENIC STRUCTURE OF *SALMONELLA* SPS.

Salmonella express polysaccharide O antigen, flagellar H antigen, and capsular Vi antigen which determine strain pathogenicity (CDC, 2008) and the agglutination reaction of these antigens with specific antisera determine serotype identification (Hu and Kopecko, 2002). About 2579 serotypes of *Salmonella* have identified by WHO Collaborating Center for Reference and Research on *Salmonella* to the date 2007, among which 2557 serotypes belongs to *S. enterica* (Grimont and Weill, 2007).

O (SOMATIC) ANTIGEN

The O antigen is the lipopolysaccharides (LPS) of the outer membrane, similar to the O antigen of other enterobacteriaceae (Hu and Kopecko, 2002).The structure of the O antigen is extremely variable among strains of *Salmonella* and differs in monosaccharide components, linkage between monosaccharides, and other minor modifications, such as acetylation. These subtle chemical alterations in O-antigen structure have profound effects on antibody recognition that attribute different serogroups (Ellermeier and Slauch, 2006; CDC, 2008).

H (FLAGELLAR) ANTIGEN

H antigen is heat labile proteins composed of subunits flagellin that make up the filamentous portion of peritrichous flagella (Ellermeier and Slauch, 2006). Some Salmonellas exhibit unique characteristic of diphasic variation in H antigen; Phase 1 and Phase 2. Each strain can spontaneously and reversibly vary between these two phases with different sets of H-antigens, but only one antigen is expressed at time in a single bacterial cell. Monophasic isolates express only a single flagellin type and occur naturally for some serotypes (e.g., serotypes

Enteritidis, Typhi, and most subspecies IIIa and IV serotypes are monophasic), or can occur through the inactivation of a flagellin gene (CDC, 2008).

VI (CAPSULAR) ANTIGEN

The Vi antigen is a capsular polysaccharide produced by serovar Typhi and Paratyphi C, and occasionally by strains of serovar Dublin. The presence of the capsule can block antibody recognition of the O-antigen. Therefore, if a strain is positive for Vi antigen, the strain should be heated in boiling water for 15 minute, cooled, and tested again. Upon heating, a serovar Typhi strain should be negative for Vi antigen and positive for O group D (Ellermeier and Slauch, 2006).

M ANTIGEN

M antigen is an extracellular polysaccharide antigen consisting of colanic acid and is produced by those strains of *S.* Paratyphi B and other serotypes that form mucoid colonies when cultures are held for several days at room temperature after incubation for 1 day at 37ºC. It resembles the Vi antigen in preventing agglutination by O antiserum (Old, 1996).

FIMBRIAL ANTIGEN

The type-1 fimbriae formed by most strains of *Salmonella* species, bear antigens that determine agglutination by sera containing anti-fimbrial antibodies. Fimbriae are not found in young (6-24 hours old) broth cultures, but can be found in 24-48 hours old broth cultures. Five type-1 fimbrial antigens have been described by Duguid and Campbell in 1969. The type-1 fimbrial determinants of some representative serotypes are: Paratyphi B and Typhimurium (1,2,3), Choleraesuis \(1,2,4) and Typhi (1,5) (Old, 1990).

R ANTIGEN

In some mutant strain of *Salmonella* mutational loss of one enzyme that links the polysaccharide core or side chains of the cell-wall lipopolysaccharides leads to an absence of the hydrophilic side chains that determine O antigen specificity and new 'R' antigens are exposed at the bacterial surface. The exposed incomplete (R_I) complete (R_{II}) core polysaccharide constitutes the R antigen (Old, 1996).

2.5.5 KAUFFMANN- WHITE SCHEME CLASSIFICATION

Kauffmann-White scheme, first proposed by White and expanded by Kauffman in 1934, is the typing method of *Salmonella* species on the basis of the antigenic structure of O antigen, H antigen, and Vi antigen if present (Ellermeier and Slauch, 2006) and each *Salmonella* serotype is designated by antigenic formulae (CDC, 2008).O antigens are designated by numbers and are divided into O serogroups, also called O groups. O groups are designated by the primary O factor(s) that are associated with the group. Additional O factors are associated with some O groups and are often variably present or variably expressed (CDC, 2008).Historically, O groups were first designated by capital letters A to Z and continued with numbers 51 to 67 to designate those discovered later. Now each O group is designated using the characteristic O factor and letters are provisionally kept into brackets. Therefore, new designation for O groups become: O:2 (A), O:4 (B), O:6,7 (C_1-C_4), O:8 (C_2-C_3), O:9 (D1), O9,46 (D2), O:9,46,27 (D_3), O:3,10 (E_1-E_2-E_3) etc. (Grimont and Weill, 2007). Within each O group the different serotypes are distinguished by their particular H antigen or combination of H antigens i.e. identification of phase 1 and phase 2 flagellar antigens (Janda and Abbott, 2005).

2.5.6 CULTURAL AND BIOCHEMICAL CHARACTERISTICS

Salmonellae grow over a wide temperature range from 7-48 ^{0}C and pH 4-8, but optimum temperature for the growth is 37^{0}C. They grow readily on ordinary culture media in aerobic and anaerobic **c**onditions and produce colonies similar to those of enterobacteriaceae. Some serotypes like Paratyphi B produce mucoid colonies at low temperatures, humidity and high osmolarity (Old and Threlfall, 1996).

On nutrient agar salmonellae produce grey white moist, convex and entire colony (Janda and Abbott, 2005). On blood agar, *S*. Typhi and *S*. Paratyphi usually produce non-haemolytic smooth white colonies. On MacConkey agar, Salmonellae produce non lactose fermenting smooth colonies. On Salmonella-Shigella agar and Deoxycholate agar they produce non lactose fermenting colonies with black center but *S*. Paratyphi A produces colonies without black centers. On Xylose Lysine Deoxycholate agar and Hektoen Enteric agar,

salmonellae produce transparent red colonies with black centers and transparent green colonies with black centers respectively (except *S*. Paratyphi A, which produces colonies without black centers). On Bismuth sulfite agar medium, salmonellae produce black colonies. Selenite F broth is used as enrichment media to isolate salmonellae from stool sample (WHO, 2003).

Salmonellae are catalase positive, oxidase negative and reduce nitrates to nitrites, phenylalanine deaminase negative, and ferment glucose, maltose, mannitol and sorbitol with production of acid and gas except *S*. Typhi which do not produce gas. Generally they are indole negative, methyl red positive, vogue-proskaeur negative, citrate negative, urease negative. On tipple sugar iron agar slant medium, *S*. Typhi gives alkaline/acidic reaction with production of weak H_2S but no gas. However, *S*. Paratyphi A is gas positive and but H_2S negative (Cheesbrough, 2000).

2.6 TRANSMISSION OF ENTERIC FEVER

Human are the only natural host and reservoir for enteric fever, being excreted in the faeces and urine of infected individuals and carriers (Cheesbrough, 2000). The infection is acquired through faecal-oral route via contaminated water, food and hand (Kothari *et al.*, 2008; Malisa and Nyaki, 2010). Transmission efficiency has been coupled to increased levels of fecal bacteria with high-shedding hosts. About 1.0-5.0% of the infected individuals becomes carriers (Pokharel *et al.*, 2009) and persistently shed Salmonella in their feces for long periods of time, thereby serve as a reservoir for the pathogen, transmitting *Salmonella* to new hosts by contamination of food or water sources (Gopinath *et al.*, 2012). Food worker who are asymptomatic Typhoid and Paratyphoid carriers shed large number of Salmonellae, are the common sources of contaminated of food (Hu and Kopecko, 2002). Shellfish taken from sewage contaminated beds, unwashed raw fruits or vegetables fertilized by night soil, or milk contaminated by carriers also serve as the vehicles of transmission of disease (Avery *et al.*, 2008).

2.7 RISK FACTORS OF ENTERIC FEVER

Exposure of the individual to contaminated food or water correlates closely with the risk for enteric fever. Poor housing with inadequate facilities for personal

hygiene, drinking contaminated water having a close contact or relative with recent typhoid fever, eating food prepared outside the home, such as ice cream or flavored iced drinks from street vendors, low socio economic conditions, and recent use of antimicrobial drugs are identified risk factors of enteric fever in endemic areas (Parry *et al.*, 2002; Kanungo *et al.*, 2008).

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). Karkey *et al.* (2010) reported the uneven distribution of enteric fever patients in Kathmandu is associated with local variation in risk factors such as contaminated drinking water.

Factor like heavy rainfall and higher temperatures contribute to increase in outbreaks of enteric pathogens, usually due to the contamination of water supplies and transmission probabilities of surface water bodies (Wang *et al.*, 2012). HIV/AIDS is clearly associated with an increased risk of non typhoidal as well as typhoidal *Salmonella* species (Monack *et al.*, 2004; Gordon *et al.*, 2008). Other risk factors for clinical *S.* Typhi infection include various genetic polymorphisms and often predispose to other intracellular pathogens (Ali *et al.*, 2006).

2.8 PATHOGENESIS OF FEVER

The infectious dose of S. Typhi in volunteers varies between 1000 and 1 million organisms. Vi-negative strains of *S.* Typhi are less infectious and less virulent than Vi-positive strains (Parry *et al.*, 2002). The bacteria must survive the low gastric acid barrier, the important defense mechanism to reach the small intestine. In the small intestine, bacteria move across the intestinal epithelial cell and reach and invade the M cells, the specialized epithelial cells overlying Peyer's patches, and are rapidly internalized (Kaur and Jain, 2012).

Virulence genes involved in invasion and required for intracellular survival are clustered in large chromosomal DNA regions designated Salmonella pathogenicity islands (SPIs). SPI-1 and SPI-2 encode type III secretion systems, consisting of multiprotein complexes that build a contiguous channel across both

the bacterial and epithelial cell membranes, resulting in efficient translocation of bacterial effectors directly into the epithelial cell cytoplasm. The secreted effectors interact with eukaryotic proteins to activate signal transduction pathways and rearrange the actins cytoskeleton and lead to membrane ruffling and bacterial engulfment (Sanchez-Vargas *et al.*, 2011).

After penetration, the invading microorganisms translocate to the intestinal lymphoid follicles, mesenteric lymph nodes, and the reticuloendothelial cells of the liver and spleen. However, *Salmonella* are able to survive and multiply within the mononuclear phagocytic cells of the lymphoid follicles, liver, and spleen during the asymptomatic incubation phase of typhoid fever (Parry *et al.*, 2002). After reaching the threshold level, the bacteria are released into blood initiating continuous bacteraemia with secretion of cytokines by macrophages during symptomatic phase of typhoid fever. This secondary and persistent bacteraemia is characterized by dissemination of the organisms (Huang and DuPont, 2005). The most common sites of secondary infection are the liver, spleen, bone marrow, gallbladder and Peyer's patches in the terminal ileum. Gall bladder invasion occurs either directly from the blood or by retrograde spread from the bile. Organisms excreted in the bile either reinvade the intestinal wall or are excreted in the feces (Parry *et al.*, 2002).

2.9 CLINICAL MANIFESTATION OF ENTERIC FEVER

The clinical symptoms in patients with typhoid fever are correlated to cellular microbiological phenomena. The bacterial invasion of several host cells and the inflammatory response with high cytokine production are responsible for the clinical manifestations (Kaur and Jain, 2012). The asymptomatic incubation period of enteric fever is 7-14 days, with range 3-60 days. The onset of bacteraemia is marked by fever and malaise (Connor and Schwartz, 2005). Initially there is low fever, that rises progressively, and by the second week it is often high and sustained (39–40 ^{0}C). The fever occurs in more than 80% of patients (Kaur and Jain, 2012).The patients typically present other nonspecific influenza-like symptoms with chills, dull frontal headache, malaise, anorexia, and nausea (Connor and Schwartz, 2005). In some cases, patients may complain the symptoms of headache, diarrhea or constipation and abdominal pain before the

onset of fever. Headache is a frequent symptom while diarrhea is most common in adults with HIV and children (Sanchez-Vargas *et al.*, 2011). The other symptoms include sore throat, coated tongue, hepatomegaly, splenomegaly and relative bradycardia, psychosis and mental confusion in 5-10 % cases. In the second week, a few rose spots, blanching erythematous maculapapular lesions, approximately 2–4 mm in diameter, appear in 5–30 percent of cases. Without prompt treatment or correct diagnosis, the typhoid fever may prolong to the third week and the inflammatory lesions become intense in Peyer's patches and intestinal lamina propria that may lead to ulceration and necrosis with subsequent gastrointestinal bleeding or intestinal perforation. Further complications in 10–15 percent of patients may result in death, after the third week of disease. The fever declines in the 4th week of disease in 90% of the survivors, without antibiotic therapy. However, weakness and weight loss may persist for many months (Kaur and Jain, 2012).

Intestinal perforation and intestinal hemorrhages are the most common complications of typhoid fever. Other complications reported with typhoid fever include typhoid meningitis, encephalomyelitis, Guillain-Barré syndrome, cranial or peripheral neuritis, psychotic symptoms, haemorrhages, hepatitis, myocarditis, pneumonia, disseminated intravascular coagulation, thrombocytopenia and haemolytic uraemic syndrome. About 1-5% of patients, depending on age, become chronic carriers harbouring *S.* Typhi in the gallbladder (WHO, 2003). These complications are life threatening and required advanced medical care which are often lacking in typhoid endemic region (Gonzalez-Escobedo *et al.*, 2011). Ahmed *et al.* (2012) reported abdominal lymphadenopathy associated with enteric fever. Case fatality rates in endemic countries have been reported as high as 30%. *S.* Paratyphi A causes clinically indistinguishable disease from that of *S.* Typhi, but less severe than typhoid fever (Connor and Schwartz, 2005).

2.10 LABORATORY DIAGNOSIS OF ENTERIC FEVER

Typhoid and paratyphoid fever most often present similar clinical illness, and the accurate diagnosis is made on laboratory confirmation (Crump and Mintz, 2010). The definitive diagnosis of enteric fever depends on the isolation of *S.* Typhi or *S.* Paratyphi (A, B, and C) from blood, bone marrow, stool or a specific anatomical

lesion (WHO, 2003; Parry *et al.*, 2011). Blood or stool cultures followed by conventional microbiological identification and serology are the mainstay salmonella infection diagnostic testing (Sanchez-Vargas *et al.*, 2011).

2.10.1 CULTURE

A. BLOOD CULTURE

Blood culture is positive in 75-80% of the patients during the first ten days of infection and 30% of patients during the third week (Cheesbrough, 2000) depending on the amount of blood sampled, the bacteremic level of *S*. Typhi, the type of culture medium used, and the length of incubation period (Song *et al.*, 1993). The number of bacteria circulating in blood is usually quite low in these diseases (Escamilla *et al.*, 1986). So the volume of blood cultured is one of the most important factors in isolation *Salmonella* spp. from enteric fever patient. About 10-15 ml should be taken from schoolchildren and adults and 2-4 ml is required from toddlers and preschool children (WHO, 2003). Traditional blood culture media include of BHI broth or tryptic soy broth. The optimum ratio of the volume of blood to culture broth should be 1:10. About 45 ml of tryptic soy broth or brain heart infusion broth is inoculated with 5 ml of fresh blood. In the laboratory, blood culture bottles should be incubated at 37 ^{0}C aerobically and checked for turbidity, gas formation and other evidence of growth after 1, 2, 3 and 7 days. For days 1, 2 and 3, only bottles showing signs of positive growth are sub cultured on BA and MA plates. On day 7 all bottles should be sub cultured before being discarded as negative. The subculture plates should be incubated at 37 ^{0}C for 18-24 hours in an aerobic incubator. The growth if any is identified by biochemical reactions and confirmed (WHO, 2003).

B. BONE MARROW CULTURE

Enteric fever is the only bacterial infection of humans for which bone marrow examination is routinely recommended (Wain *et al.*, 2001). Bone marrow culture is more accurate and successful with positive response rate up to 90% (Cheesbrough, 2000). It is particularly valuable for patients who have been previously treated, who have a long history of illness and for whom there has been a negative blood culture with the recommended volume of blood (WHO, 2003). Bone marrow culture highly is sensitive technique and the current gold

standard method for confirming a case of typhoid fever. However, this requires equipment, supplies and trained laboratory personnel seldom found in primary health-care facilities in the developing world, thus rarely performed in routine laboratory (Keddy *et al.*, 2011; Parry *et al.*, 2011).

C. STOOL CULTURE

Organisms can usually be isolated from 40-50% of patients during the second week of infection and from about 80% of patients during the third week from faeces (Cheesbrough, 2000). Children have a higher incidence of positive stool cultures compared with adults. Stools can be collected from acute patients and they are especially useful for the diagnosis of typhoid carriers. Specimens should preferably be processed within two hours after collection, and delay is anticipated the specimens should be stored in a refrigerator at 4 ^{0}C. One gm of stool is inoculated into 10 ml of Selenite F broth and incubated at 37 ^{0}C for 18-48 hours. Then sub cultured on a selective agar from the surface of the broth without disturbing the sediment. The choice of agar media includes MA, DCA, XLD agar, and HE agar or SS agar. The plate is incubated at 37 ^{0}C for 24 hours and growth is identified on by biochemical reaction and confirmed (WHO, 2003).

D. URINE CULTURE

The urine culture sensitivity is also low and gives 25% positive rate after the second week of infection. The bacteria are not excreted continuously urine and therefore repeated culture is needed before the organisms are isolated (Cheesbrough, 2000). Positive cultures in urine or stools may indicate an acute infection or may occur in chronic carriage (Sanchez-Vargas *et al.*, 2011).

Besides these *S.* Typhi has been successfully isolated from culture of bile, rose spots, cerebrospinal fluid, peritoneal fluid, mesenteric lymph nodes, resected intestine, pharynx, tonsils, abscess, and bone (Cheesbrough, 2000).

2.10.2 SEROLOGICAL DIAGNOSIS

A. WIDAL TEST

Widal agglutination test, developed by Georges Fernand Isadora Widal in 1896, is an alternative laboratory test widely used for serological diagnosis of enteric

fever in developing countries. Widal test utilizes a suspension of killed *Salmonella enterica* as antigen to detect the presence of agglutinin(antibody) in the serum of an infected patient, against the H (flagellar) and O (somatic) antigens of *Salmonella enterica* serotype Typhi, Paratyphi A and Paratyphi B, during the acute and convalescent period of infection (Pokhrel *et al.*, 2009). Usually O antibodies (IgM) appear on day 6-8 & H antibodies (IgG) on days 10-12 after the onset of the disease. The test has only moderate sensitivity and specificity with negative response in up to 30% of culture-proven cases of typhoid fever (WHO, 2003).

Two types of agglutination techniques are available: the slide test and the tube test. The slide test is rapid and is used as a screening procedure. A drop of the suspended commercially available antigens of *S.* Typhi is added to an equal amount of previously prepared serum. After an initial positive screening the strength of the antibody determined by adding together equal amounts of antigen suspension and serially diluted serum from the suspected patient. Agglutinations are visualised as clumps. The tube agglutination test is a macroscopic test, and requires much more technical work than the rapid slide test. It also serves as a means of confirming the results of the slide test. A mixture of suspended antigen and antibody is incubated for up to 20 h at 37°C in a water bath. Agglutinations are visualised in the form of pellets, clumped together at the bottom of the test tube (Olopoenia and King, 2000). *S.* Typhi shares O and H antigens with other *Salmonella* serotypes and has cross-reacting epitopes with other enterobacteriaceae which give false-positive results. Such results may also occur in other clinical conditions, e.g. malaria, typhus, bacteraemia caused by other organisms, and cirrhosis. Therefore it is imperative to establish cut off value for a positive result if a single acute sera is taken. If paired sera are available a fourfold rise in the antibody titer between convalescent and acute sera is diagnostic (WHO, 2003).

B. NEW DIAGNOSTIC TESTS

New typhoid rapid antibody tests as an alternative to the Widal test have been evaluated in Asian countries having high endemicity of typhoid fever but have shown variable performance (Keddy *et al.*, 2011). Recent advances include the

IDL Tubex® test, Typhidot-M®, and the dipstick test. Tubex® test can detect IgM O9 antibodies from patients within a few minutes. Another rapid serological test, Typhidot®, takes three hours to perform detect specific IgM and IgG antibodies against a 50 kD antigen of *S.* Typhi. A newer version of the test was recently developed to detect specific IgM antibodies only. Evaluations of Typhidot® and Typhidot-M® in clinical settings showed that they performed better than the Widal test and the culture method. Typhidot-M® can replace the Widal test when used in conjunction with the culture method for the rapid and accurate diagnosis of typhoid fever. The predictive value of the test suggests that Typhidot-M® would be useful in areas of high endemicity high negative (WHO, 2003).

The typhoid IgM dipstick assay detects of S. Typhi-specific IgM antibodies in serum or whole blood samples. Evaluations of the dipstick test in laboratory-based studies in Indonesia Kenya, Viet Nam and Egypt have shown consistent results indicating sensitivities of 65% to 77% for samples collected at the time of first consultation from culture-confirmed patients and specificities of 95% to 100%. The dipstick test provides a rapid and simple alternative for the diagnosis of typhoid fever, particularly in situations where culture facilities are not available (WHO, 2003).

2.11 ANTIMICROBIAL SUSCEPTIBILITY TEST

Antimicrobial susceptibility testing is crucial for the guidance of clinical management (WHO, 2003). The establishment of appropriate antimicrobial therapy requires the knowledge of the prevalence of *S*. Typhi and their antimicrobial susceptibility patterns (Khanal *et al.*, 2007). WHO recommended that susceptibility tests be performed against fluoroquinolones (e.g. ciprofloxacin, ofloxacin), third-generation cephalosporins (e.g. ceftriaxone, cefotaxime), a monobactum beta-lactam (aztreonam) and a macrolide (azithromycin), nalidixic acid (for determining reduced susceptibility to fluoroquinolones), and the previous first-line antimicrobials (ampicillin, chloramphenicol, sulfonamide, trimethoprim, streptomycin and tetracycline) to which the strains could be resistant (WHO, 2003). In vitro bacterial susceptibility test to antimicrobial agents is performed by Kirby-Bauer disc diffusion technique using fresh broth culture of isolates in Mueller Hinton agar medium and

commercially available antibiotics discs. The result is interpreted on the basis of zone-size compared with that of control strains (Greenwood *et al.*, 2000).

2.12 TREATMENT, PREVENTION AND CONTROL

Drug-susceptible uncomplicated typhoid and paratyphoid can effectively be managed in outpatient settings with oral therapy of chloramphenicol, amoxycillin, trimethoprim-sulfamethoxazole, ciprofloxacin, ofloxacin (Levine *et al.*, 2011). The third generation cephalosporins like ceftriaxone, cefotaxime, cefixime and ceftazidime are good alternatives against MDR and fluoroquinolones resistant *S.* Typhi and *S.* Paratyphi (Pokharel *et al.*, 2006). Oral azithromycin and cefixime are used as first-line therapy for MDR and NARST strain. Severe or complicated typhoid should be managed in hospital with parenteral administration of ciprofloxacin or ofloxacin for fluoroquinolones susceptible strain and cefotaxime or ceftriaxone for fluoroquinolones resistant or decreased susceptibility (NARST) strain (Levine *et al.*, 2011). Azithromycin can be used for the treatment of uncomplicated cases of typhoid fever (Crump and Mintz, 2010).

The disease can be prevented by use of safe water and food. Only boiled or treated water should be consumed the foods that may be fecally contaminated (e.g., uncooked salad vegetables) should be avoided. Ty21a live oral vaccine and Vi Polysaccharide Parenteral Vaccine are the two vaccines used in the prevention of enteric fever (Levine *et al.*, 2011).

2.13 ANTIMICROBIAL SUSCEPTIBILITY PATTERN

Chloramphenicol was the drug of choice for the treatment of typhoid for more than 40 years until the emergence of multidrug-resistant *S.* Typhi in the late 1980s in the Indian subcontinent (Kariuki *et al.*, 2004). The incidence of multidrug-resistant *S.* Typhi is reported to be as high as 60%, although there are some reports noting its decline. MDR *Salmonella* Typhi have caused typhoid fever outbreaks throughout the world, especially in South America, Indian subcontinent, Africa, and South-East Asia having significant mortality and

morbidity over years (Khanal *et al.*, 2007). Resistance to chloramphenicol, ampicillin, trimethoprim, sulfonamides, and tetracycline is often encoded by large (180 kb) and conjugative plasmids belonging to the incompatibility complex group *Inc*HI, and are originated from Southeast Asia (Shanahan *et al.*, 1998).

Over 90% of the sporadic or epidemic serotype Typhi isolates in the north, central, and south regions of Vietnam were MDR from 1995 to 2002 (Le *et al.*, 2007). Watson and Pettibone (1991) first documented multidrug-resistant *S.* Typhi in Nepal (Maskey *et al.*, 2008). Pokharel *et al.* (2006) reported a 5% prevalence of multidrug resistance among S. *enterica* at a tertiary care hospital in Kathmandu, Nepal, with a higher rate of resistance among the serotype Paratyphi A. It also showed a low but definite presence of ESBL-producing strains (0.5%) of Paratyphi A. Hundred percent prevalence of MDR *Salmonella* reported in Egypt in 1993 and it later declined to 5% in 2000 (Pokharel *et al.*, 2006). A study based on the investigation of an outbreak of typhoid fever occurred in Nek Muhammad village, situated Karachi-Pakistan show the presence of multidrug resistant strain of *Salmonella enterica* serovar Typhi in 100% well water, 65% household water samples and 2% food items (Farooqui *et al.*, 2009).

Emergence of MDRST led the widespread use of fluoroquinolones, such as ciprofloxacin and ofloxacin (Kariuki *et al.*, 2010). However, during early 1990s nalidixic acid-resistant *S.* Typhi (NARST) with decreased susceptibility to ciprofloxacin [defined as a minimum inhibitory concentration (MIC) of 0.125–1 g/ml] emerged in developing countries particularly in South Asia, Southeast Asia and Africa with delayed therapeutic response or treatment failure (Kim *et al.*, 2010). Nalidixic acid susceptibility has been validated as a screening test for reduced susceptibility to ciprofloxacin, and nalidixic acid resistance is associated with a high MIC of ciprofloxacin which in turn is associated with treatment failure (Madhulika *et al.*, 2004). Le *et al.* (2007) reported that nalidixic acid resistance in most of the isolates (94%) was due to a mutation in the quinolones resistance-determining chromosomal region (QRDR) of *gyrA* that led to the amino acid substitution Ser83Phe. The reduced ciprofloxacin MIC in *S.* Typhi

and *S*. Paratyphi A is also solely due to an amino acid substitution in the QRDR 'cluster' of the gyrA gene (Hassing *et al.*, 2011).

There are several reports of nalidixic acid resistant and with decreased susceptibility to ciprofloxacin from Bangladesh, India, Nepal, Thailand, Vietnam, and Tajikistan (Shirakawa *et al.*, 2006; Acharya *et al.*, 2012). Various studies in India report that the MDRST isolates are decreasing and NARST isolates are increasing which notify the reversal of antibiotic resistant pattern in favour of chloramphenicol, ampicillin and trimethoprim-sulfamethoxazole (Rai *et al.*, 2012).

CHAPTER-III

MATERIALS AND METHODS

The cross sectional study was carried out during July 2011 to February 2012 in clinically suspected enteric fever patients visiting Alka Hospital Jawalakhel, Lalitpur. A total of 2012 blood specimens were collected and processed in the Microbiology Laboratory.

3.1 MATERIALS

All the media, equipment, apparatus, chemicals and reagents and antibiotic discs used in this study are mentioned in appendix B.

3.2 INCLUSION AND EXCLUSION CRITERIA

A case defined by physicians as a 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 was included in this study. The cases of the patients who have already started antibiotic therapy prior to sample taken were excluded from the study.

3.3 METHODS

3.3.1 BLOOD SPECIMEN COLLECTION

Blood samples were collected from clinically suspected enteric fever patients either by nurses or laboratory technician using standard aseptic techniques described as in appendix D (Cheesbrough, 2000).

For adult person 5ml of blood sample was collected and for children 2 ml of blood was collected per vein puncture.

3.3.2 PROCESSING OF BLOOD SPECIMEN

About 5 ml of blood for adult and about 2 ml of blood for children were transferred in the culture bottles containing about 50 ml and 20 ml of BHI broth respectively. Immediately after the blood culture bottles were received, they were labeled with laboratory identification numbers and processed further.

The culture bottles were incubated at 37 ^{0}C for 7 days. The day of sample collection was defined as day one in this study. The culture bottles were checked daily for turbidity, gas formation and any other visual evidence of growth to make the presumptive diagnosis of positive culture. The samples showing signs of growth on BHI broth after day 1, 2 or 3 were sub cultured in Blood Agar and MacConkey Agar and on day 7 all bottles were sub cultured before being discarded as negative. The MacConkey agar and Blood agar plates were incubated at 37°c for 24 hours.

The subculture plates were examined after overnight incubation. Blood Agar plates were observed for the growth non-haemolytic smooth white colonies and MacConkey agar plates were observed for growth of non-lactose fermenter.

3.3.3 IDENTIFICATION OF *SALMONELLA* SEROTYPES

Presumptive identification of *Salmonella* species from positive culture plates were made with the use of standard microbiological techniques like colony morphology, Gram stain, biochemical reaction and serotyping. A single colony of suspected pathogen was picked up by sterile loop and inoculated in Nutrient Broth and incubated at 37°C for 4 hours, and then gram staining and various biochemical tests were performed. The following biochemical tests were performed to identify the organism.

i. Catalase test
ii. Oxidase test
iii. Methyl Red (MR) test
iv. Voges-Proskauer (VP) test
v. Citrate utilization test
vi. Triple sugar iron (TSI) test
vii. Sulphide Indole Motility (SIM) test
viii. Urea hydrolysis test (Urease test)

3.3.4 PURITY PLATE

The purity plate was maintained to see whether the biochemical tests were processed in an aseptic condition or not. It was performed by inoculating half portion of nutrient agar plate before preceding the test and rests half after completing the test procedure, in order to know the contamination for culture from outer sources during the experiment.

3.3.5 SEROTYPING

Salmonella species were confirmed by performing serological agglutination test with specific antisera. Isolated *Salmonella* species were subjected for serotyping using kit (Murex, UK) as per kit's instruction (Appendix E).

3.3.6 ANTIBIOTIC SUSCEPTIBILITY TEST

In vitro antimicrobial susceptibility test of isolated *Salmonella* serovar was performed by modified Kirby-Bauer disc diffusion method using fresh broth culture of isolates in Mueller Hinton agar medium. The antibiotic discs used were amoxycillin (30µg), chloramphenicol (30µg), cotrimoxazole (25µg), nalidixic acid (30µg), ciprofloxacin (5µg), ofloxacin (5µg), cefixime (5µg), ceftriaxone (30µg), azithromycin (15µg), gentamicin (10µg) (Hi-Media Laboratory Ltd., Mumbai, India). The procedures were followed and results were interpreted as sensitive, intermediate or resistant based on definition of CLSI (2007) mentioned in Appendix F.

3.3.7 QUALITY CONTROL

Quality control was applied in various areas during study period that ensured accuracy, reliability and reproducibility of the information generated by study.

Laboratory equipment like incubator, hot air oven, autoclave, refrigerator etc. were regularly monitored for their performance. Reagents and biochemical media were checked for manufacture, expiry date and proper storage. The media and reagent were prepared strictly following the manufacturer’s instruction and stored in proper conditions. Sterility testing and performance testing were carried out for each batch of prepared media using standard control strains.

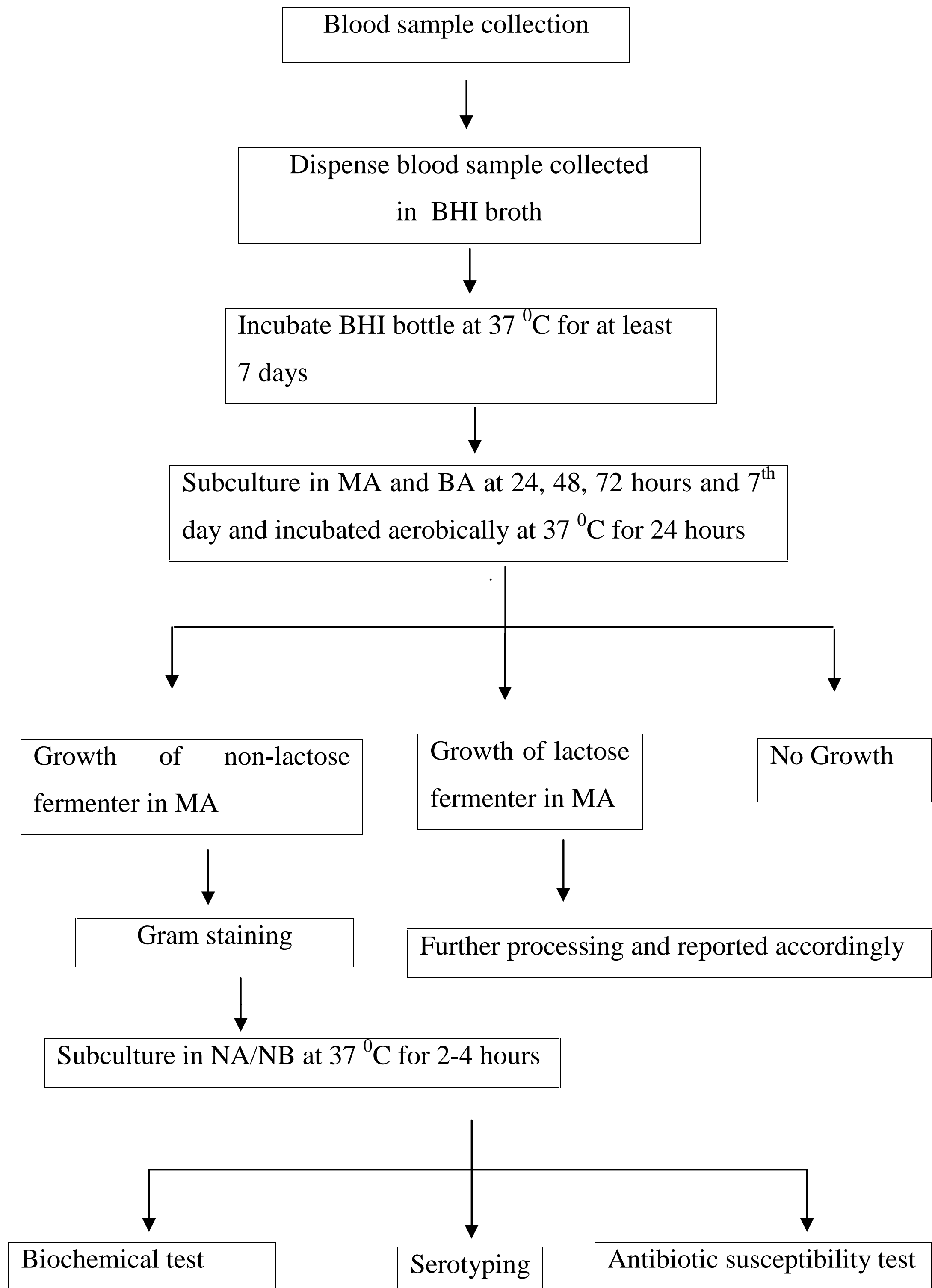
Aseptic method was followed during sample collection using sterile syringe and needle, disinfecting the skin over vein of the patient and collecting the blood in sterile bottles in order to avoid contamination. The sample was also processed in aseptic condition. Purity plate was maintained to know the contamination during biochemical testing and serotyping was performed aseptically following manufacturer's instruction.

Muller Hinton agar and antibiotic disc were checked for each lot number, manufacture date and expiry date and stored properly. Quality control of the disc susceptibility testing was maintained by monitoring performance quality of Muller Hinton Agar and each antibiotic disc with reference strain *E. coli* ATCC 25922 for each test strain. The standard inoculum that matched with 0.5 Mc-Farland solutions was used.

3.3.8 DATA ANALYSIS

Results obtained were recorded in standard sheet "Clinical and Microbiological Profile" (Appendix A). All the recorded data were entered in the worksheet of SPSS software (Version 17) and analyzed. Chi-square test was used to determine significant association of dependent variables to different independent variables.

3.4 FLOWCHART FOR METHODOLOGY



CHAPTER- IV

RESULTS

A total 1202 blood specimen from clinically suspected enteric fever patients were studied during July 2011 to February 2012 at Alka Hospital Jawalakhel, Lalitpur. Among which 660 (54.9 %) blood specimens were from male patients and 542 (45.1%) blood specimens were from female patients. The age of the patients under study ranged from 1 year to 90 year old with mean age ± standard deviation of 31.16 ± 18.32.

4.1 CULTURE POSITIVITY

Out of the 1202 blood specimens subjected for culture, 86 (7.2%) blood specimens were blood culture positive. Out of 660 male patients, 46 (7.0%) cases showed positive blood culture result and out of 542 female patients, only 40 (7.4%) cases showed positive culture result. The male to female ratio in culture positive cases was 1.15: 1. There was no significance association of presence of enteric fever with gender of the patient (p=0.7844).

Table 1 Gender wise distribution of blood culture positive cases

Gender	Blood culture positive		Blood culture negative		Total	
	Number	Percentage	Number	Percentage	Number	Percentage
Male	46	7.0	614	93.0	660	54.9
Female	40	7.4	502	92.6	542	45.1
Total	86	7.2	1116	92.8	1202	100

4.2 AGE WISE DISTRIBUTION OF BLOOD CULTURE POSITIVE CASES

The highest blood culture positive rate i.e. 12.7% (28/220) was found in age group 11-20 years followed by the age group 21-30 years with 9.5% (32/336) blood culture positive rate. The age group below 10 years has 8.5% blood culture positive rate whereas only 2.3% blood culture positive rate was found in above 70 years age.

Table 2: Age wise distribution of blood culture positive cases

Age Group (years)	Suspected enteric fever cases	Blood culture positive cases	Percentage
1-10	130	11	8.5
11-20	220	28	12.7
21-30	336	32	9.5
31-40	194	6	3.1
41-50	126	3	2.4
51-60	94	2	2.1
61-70	58	3	5.2
Above 70	44	1	2.3
Total	1202	86	7.2

4.3 DISTRIBUTION PATTERN OF BACTERIAL ISOLATES

A total of 86 *Salmonella* species were isolated from different blood specimens. Among which 56 (65.1%) isolates were *Salmonella* Typhi and 30 (34.9%) isolates were *Salmonella* Paratyphi A. None of *Salmonella* Paratyphi B and *Salmonella* Paratyphi C was isolated. The case of mixed infection was also not identified.

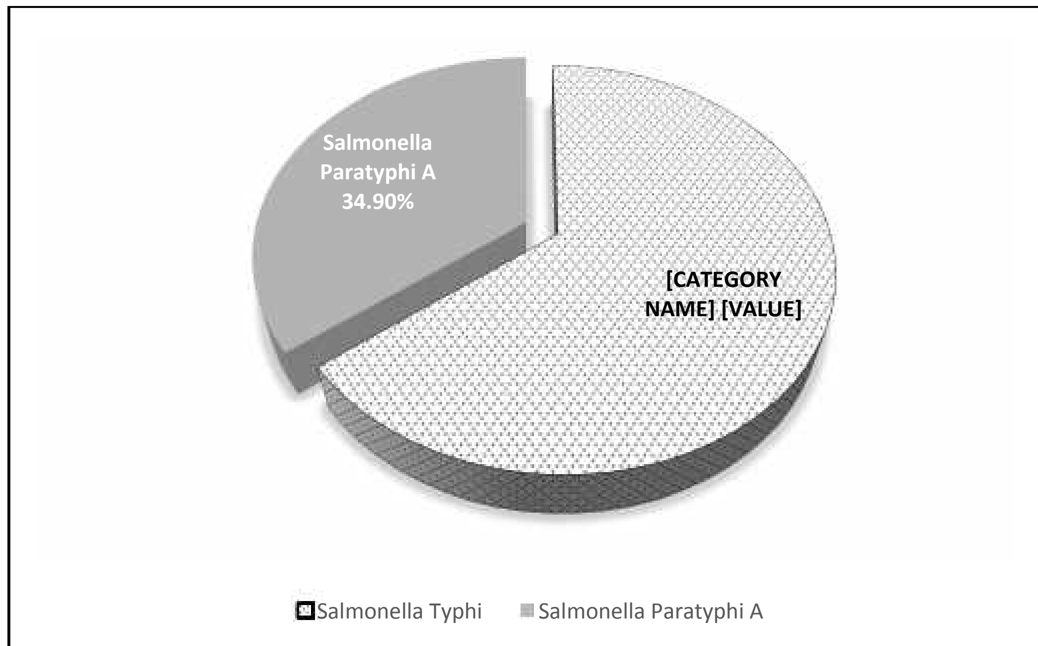


Figure 2: Proportion of *Salmonella* serovars in positive growth samples

4.4. DISTRIBUTION OF *SALMONELLA* ISOLATES

Among 56 *Salmonella* Typhi isolates, 29 (51.79%) were found in male patients and 27 (48.21%) were found in female patients. The highest number i.e. 22(39.29%) of *S.* Typhi isolates was found in age group 11-20 years followed by 17 (30.36%) *S.* Typhi isolates in age group 21-30 years. The age group below 10 years includes only 8 (14.29%) *S.* Typhi isolates whereas no *S.* Typhi was isolated from patients above 70 years age.

Table 3: Age and gender wise distribution of *Salmonella* Typhi

Age Group (years)	Male	Female	Total	Percentage
1-10	5	3	8	14.29
11-20	9	13	22	39.29
21-30	10	7	17	30.36
31-40	1	2	3	5.36
41-50	2	1	3	5.36
51-60	1	0	1	1.79
61-70	1	1	2	3.57
Above 70	0	0	0	0
Total (%)	29 (51.79)	27 (48.21)	56 (65.12)	100

Among 30 *Salmonella* Paratyphi A isolates, 17 (56.67%) were found in male patients and 13 (43.33%) were found in female patients. The highest number i.e. 15 (50%) *S.* Paratyphi A was found from age group 21-30 years followed by 6 (20%) *S*. Paratyphi A in the age group 11-20 years. Only 3 (10%) isolates were found from age below 10 years and no isolates were found in age group 41-50 years. The group above 70 years include 1 (3.33%) isolates.

Table 4: Age and gender wise distribution of *Salmonella* Paratyphi A

Age Group (years)	Male	Female	Total	Percentage
1-10	2	1	3	10
11-20	4	2	6	20
21-30	7	8	15	50
31-40	2	1	3	10
41-50	0	0	0	0
51-60	0	1	1	3.33
61-70	1	0	1	3.33
Above 70	1	0	1	3.33
Total (%)	17 (56.67%)	13 (43.33%)	30 (34.88%)	100

4.5 SEASONAL DISTRIBUTION OF *SALMONELLA* ISOLATES

Most of *Salmonella* species were isolated in the month of July (n=24) and August (n=21) that correspond to rainy season. Least number of *Salmonella* species was recovered in the month of January (n=3).

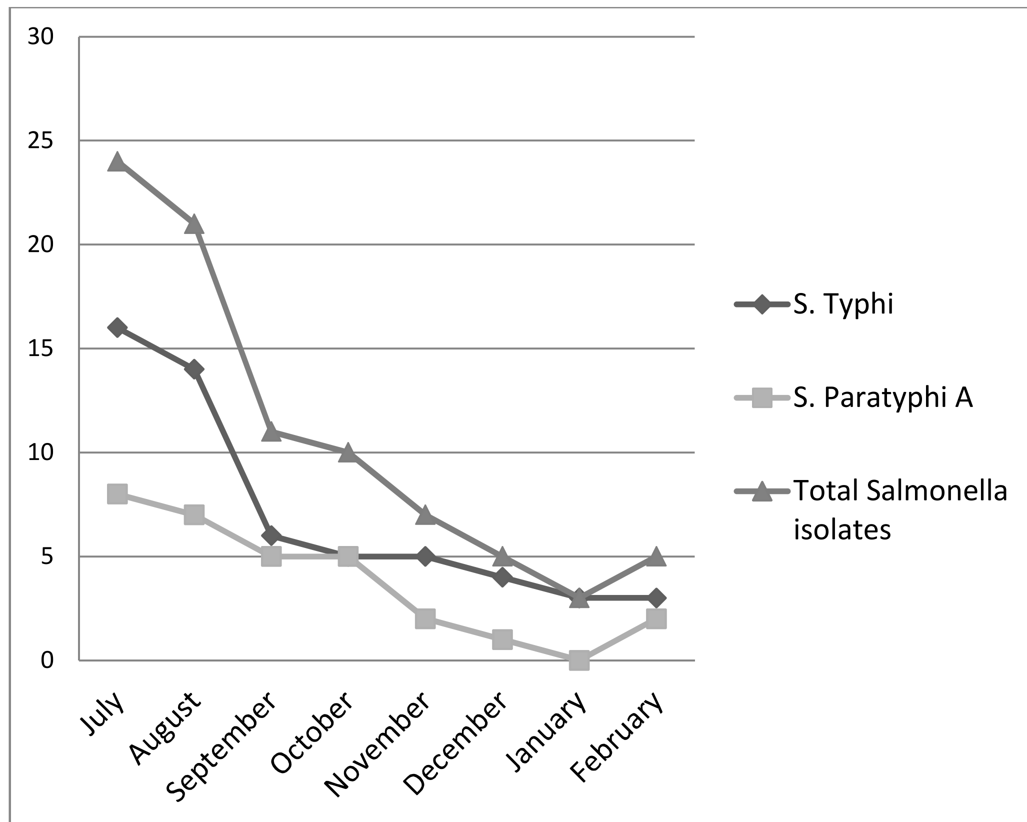


Figure 3: Seasonal distribution of *Salmonella* isolates during July 2011 to February 2012

4.6 ANTIBIOGRAM OF *SALMONELLA* TYPHI

Almost 100% *Salmonella* Typhi isolates were sensitive to antibiotics chloramphenicol, cotrimoxazole, cefixime, ceftriaxone, ciprofloxacin and gentamicin. No resistant strains were found against these antibiotics. About 98.2% isolates were sensitive and 1.2% isolates were resistant to amoxycillin and ofloxacin. Against the antibiotic azithromycin 89.3% isolates were sensitive and only 10.7% were resistant. Most of the isolates of *Salmonella* Typhi were nalidixic acid resistant which show 91.1% resistant isolates and only 8.9% sensitive isolates. None of the isolates were found MDR. Among 56 isolates 6 (8.93%) isolates were resistant to at least one other antibiotic along with nalidixic acid.

Table 5: Antibiotic susceptibility pattern of *Salmonella* Typhi (N=56)

Antibiotic used	Antibiotic susceptibility			
	Susceptible		Resistance	
	Number	Percentage	Number	Percentage
Amoxycillin	55	98.2	1	1.8
Chloramphenicol	56	100	-	-
Cotrimoxazole	56	100	-	-
Cefixime	56	100	-	-
Ceftriaxone	56	100	-	-
Ciprofloxacin	56	100	-	-
Ofloxacin	55	98.2	1	1.8
Nalidixic Acid	5	8.9	51	91.1
Azithromycin	50	89.3	6	10.7
Gentamicin	56	100	-	-

4.7 ANTIBIOGRAM OF *SALMONELLA* PARATYPHI A

Among 30 *Salmonella* Paratyphi A isolates subjected for antibiotic susceptibility test, 100% isolates were sensitive to amoxycillin, cotrimoxazole, ceftriaxone and gentamicin. About 96.7% isolates were sensitive to each of the drug chloramphenicol, cefixime, ciprofloxacin and ofloxacin. About 86.7% *S.*

Paratyphi A isolates were found sensitive to azithromycin. Most of the isolates were resistant to nalidixic acid i.e. 90% isolates were resistant. None of the isolates were found multidrug resistant; however, 7 (23.33%) isolates were resistant to at least one of the tested drugs along with nalidixic acid.

Table 6: Antibiotic susceptibility pattern of *Salmonella* Paratyphi A (N=30)

Antibiotic used	Antibiotic susceptibility			
	Susceptible		Resistance	
	Number	Percentage	Number	Percentage
Amoxycillin	30	100	-	-
Chloramphenicol	29	96.7	1	3.3
Cotrimoxazole	30	100	-	-
Cefixime	29	96.7	1	3.3
Ceftriaxone	30	100	-	-
Ciprofloxacin	29	96.7	1	3.3
Ofloxacin	29	96.7	1	3.3
Nalidixic Acid	3	10	27	90
Azithromycin	26	86.7	4	13.3
Gentamicin	30	100	-	-

4.8 CIPROFLOXACIN SUSCEPTIBILITY PATTERN OF NALIDIXIC ACID RESISTANT *SALMONELLA* ISOLATES

Out of 86 *Salmonella* isolates 78 (90.7%) were nalidixic acid resistant. A total of 51 (91.1%) *S.* Typhi isolates and 27 (90.0%) of total *S.* Paratyphi A isolates were nalidixic acid resistant. NAR *S.* Typhi isolates were 100% susceptible to ciprofloxacin. Similarly 26 (96.3%) of nalidixic acid resistant *S.* Paratyphi A isolates were sensitive to ciprofloxacin and 1 (3.7%) were resistant to ciprofloxacin.

Table 7: Ciprofloxacin susceptibility pattern of nalidixic acid resistant *Salmonella* isolates.

Nalidixic acid resistant *Salmonella* isolates	Ciprofloxacin susceptibility				Total	
	Sensitive		Resistant			
	Number	%	Number	%	Number	%
S. Typhi	51	100	-	-	51	91.1
S. Paratyphi A	26	96.3	1	3.7	27	90.0
Total	77	98.7	1	1.3	78	90.7

CHAPTER-V

DISCUSSION

Enteric fever is the major public health problem in developing country including Nepal. The disease remains endemic in capital city Kathmandu and is attributed to lack of clean drinking water, poor sanitary condition, and cross contamination of water supply with sewerage (Pokharel *et al.*, 2009).

In the present study all together 1202 blood samples were collected from enteric fever suspected patient visiting Alka Hospital Jawalakhel, Lalitpur during July 2011 to February 2012. The proportion of male patients was slightly higher (54.9%) than female patients (45.1%) and most of the patients belonged to age group 21- 30 years.

Out of the 1202 blood samples of patients subjected for culture, 7.2% (86) samples were culture positive. Similar results have been reported from other studies conducted in Nepal. Sharma *et al.* (2006) reported the blood culture positive rate of 6.9% in a similar study conducted at Dhulikhel Hospital and Bhatta *et al.* (2005) reported the 8.97% blood culture positivity. Pokharel *et al.* (2009) in a study conducted at a Teaching Hospital in Kathmandu Valley reported the overall prevalence rate of 5.4%. Khanal *et al.* (2007) from a teaching hospital in Eastern Nepal reported 5.1% culture positive rate in suspected enteric fever cases. In another similar study conducted at National Public Health Laboratory, Nepal Acharya *et al.* (2011) reported 8.99% culture positivity in suspected enteric fever cases. In a recent study conducted at Bir Hospital, Dahal (2012) observed 8.17% culture positive rate in enteric fever suspected patients.

Some of the studies have revealed high blood culture positive rate than the present study. Shrestha (1996) observed 14.83% culture positivity, Shrestha (2004) reported 16.26% culture positivity and Khatiwada (2006) reported 14.61% culture positivity in suspected enteric fever cases. In a study done at Kathmandu Model Hospital Kunwor (2007) reported 19.28% culture positivity and Amatya *et al.* (2007) reported slightly higher rate of culture positivity i.e. 23.12%. Prajapati

(2009) showed 12.29% culture positivity in suspected enteric fever cases from a study at Patan Hospital.

By comparing different studies conducted in Nepal at different time intervals it has been observed that the result of blood culture positivity in case of enteric fever is in decreasing trend in recent years that indicates the low incidence of enteric fever. Most of the reports published in previous years showed high incidence of enteric fever with blood culture positivity rate greater than 10%, however, several reports published in recent years including this study showed decrease in the incidence with culture positivity below 10%. This significant decrease in the incidence of enteric fever cases may be associated with the provision of safe drinking water, increasing awareness in general public, and advancement in the health services and facilities.

Blood culture and microbiological characterization is the mainstay of enteric fever diagnosis, yet it is only positive in approximately 40–60% of presumptive cases (Parry *et al.*, 2011). The isolation rate of *Salmonella* Typhi or *Salmonella* Paratyphi from blood cultures depends on many factors, including the volume of blood cultured, the ratio of volume of blood to volume of culture broth (ideally, the ratio should be 1: 8), inclusion of anti-complementary substances in the broth (e.g., sodium polyanethol sulfonate or bile), and whether the patient has already received antibiotics (Levine *et al.*, 2011).

Out of the 86 enteric fever cases, 46 (53.5%) were male patients and 40 (46.5%) were female patients. Although number of enteric fever cases were more in male than in female, the culture positive rate was found slightly higher in female (7.4%) compared to male (7.0%). In contrast to previous report (Bhatta *et al.*, 2005; Sharma *et al.*, 2006; Aryal, 2008; Prajapati *et al.*, 2008; Acharya *et al.*, 2011), the present study reveals that the incidence of disease is slightly higher in female than in male. The reason for such gender wise discrepancy requires further investigation. Pokharel *et al.* (2009) also reported higher prevalence in female compared to male.

The enteric fever was more prevalent in age group 11-20 years. Gurung (2008) also reported higher prevalence of typhoid fever in age group of 11- 20 years with 12.85% (9/41) blood culture positivity. Similar report consistent to our finding was
documented by Aryal (2008) that showed 14.8% culture positivity in the age group 11-20 years. The higher prevalence in age group of 11-20 years which include school going children might be contributed by lack of health awareness and eating contaminated food and water in school or open places.

Pokharel *et al.* (2009) reported blood culture positive rate higher in children (15 and less than 15 years old) and decreasing culture positivity rate with increasing age. Amatya (2005) documented maximum culture positivity in patients between age group 20-30 years which covered 51.22% of enteric fever cases. Bhatta *et al.* (2005) in a hospital based study in Kathmandu found age group (21-40) with the maximum number 185 (47.43%) of enteric fever cases. In a study conducted at Dhulikhel Hospital, Sharma *et al.* (2003) found that 71% typhoid fever cases were less than 30 years of age group. Bhatia *et al.* (2007) in a study in northern India showed maximum culture positive enteric fever patients in the 20-25 years age group. Similarly Walia *et al.* (2006) found that the majority of cases occurred in children aged 5-12 years and 24.8% of cases were in children up to 5 years of age in India. (Naheed *et al.*, 2010) in a study in Bangladesh reported highest incidence in children below 5 years of age. In Indonesia, people aged 3 to19 years accounted for 91% of cases of typhoid fever where as in South America the peak incidence occurred in school students aged 5 to 19 years and in adults aged (WHO, 2003).

In this study, among 86 *Salmonella* species isolated 56 (65.1%) were *Salmonella* Typhi and 30 (34.9%) were *Salmonella* Paratyphi A. This indicates that *Salmonella* Typhi is the most prevalent cause of enteric fever but the proportional contribution of *Salmonella* Paratyphi A as a causative agent of enteric fever is in increasing trend. Among total 56 *Salmonella* Typhi isolates 29 (51.79%) were found in male patients and 27 (48.21%) were found in female patients. Similarly among 30 *Salmonella* Paratyphi A isolates 17 (56.67%) isolates were isolated from male patients and 13 (43.33%) isolates were isolated from female patients.

Salmonella Typhi was found most prevalent in age group 11-20 years and *Salmonella* Paratyphi A was found most prevalent in age group 21-30 years.

The 1997 Global Survey of *Salmonella* serotyping estimated an incidence of 1 case of paratyphoid fever for every 4 cases of typhoid fever (Kothari *et al.*, 2008). According to WHO (2003) the ratio of disease caused by *S.* Typhi to that caused by *S.* Paratyphi is about 10 to 1 in most of the countries where this matter has been studied. However the above mentioned situation was not found in our study.

Various reports have been published in Nepal supporting the increasing causative role of *Salmonella* Paratyphi A. The increasing trend of this organism was observed by Maskey *et al.* (2008), and in their observation, *S.* Paratyphi A as a proportion of all *Salmonella* isolates rose significantly from 23.0% during 1993-1998 to 34.0% in 1999-2003. Karkey *et al.* (2010) by analyzing retrospective data extracted from blood culture-confirmed enteric fever cases from Patan Hospital in Lalitpur, between June 2005 and May 2009 showed a considerable burden of enteric fever caused by *S*. Typhi (68.5%) and *S*. Paratyphi A (31.5%) which is comparable to the finding of our study. Similarly Khatiwada (2006) reported 76.3% *S*. Typhi and 23.7% *S*. Paratyphi A isolates in blood cultured confirm enteric fever cases. Amatya *et al.* (2007) reported that in bacteraemia cases the most frequent isolated bacteria were *S.* Typhi 78 (63.4%) followed by *S.* Paratyphi A (35.8%) and *E. coli* (0.8%). In a similar study, Prajapati (2009) showed that among *Salmonella* isolates 69.21% were *Salmonella* Typhi 30.67% were *Salmonella* Paratyphi A and 0.06% each of *Salmonella* Paratyphi B and C. In other studies conducted in Nepal, the proportion of *Salmonella* Typhi and *Salmonella* Paratyphi A was reported to be 69.89% and 29.03% respectively by Thapa (1991); 71% and 29% respectively by Ghimire (1995); 63.63% and 35.06% respectively by (Shrestha, 1996).

In contrast to this study, Pokharel *et al.* (2009) documented that *S.* Paratyphi A was more commonly isolated than *S.* Typhi which includes 65.4% and 34.6% isolates respectively. Woods *et al.* (2006) reported *S*. Paratyphi A as a major cause of enteric fever in Kathmandu where he observed almost half enteric fever cases caused by this organism. Acharya *et al.* (2011), in a study at National

Public Health Laboratory (NPHL) Nepal, recovered 49.15% *S*. Typhi and 50.85% *S*. Paratyphi A isolates from enteric fever suspected patient discovering the fact that *Salmonella enterica* serovar Paratyphi A as an emerging cause of febrile illness in Nepal. Some reports stated *S*. Paratyphi A as emerging cause of enteric fever in most part of India and some provinces of China (Gupta *et al.*, 2009; Bekur *et al.*, 2010; Dong *et al.*, 2010).

There is no currently available licensed vaccines against *S.* Paratyphi A and may interfere with the mass immunization programs held in a bid to control enteric fever. This fact might be the reason for the high incidence of the disease along with outbreaks associated with the involvement of *S*. Paratyphi A.

In this study most of *Salmonella* serovars were isolated in the month of July and August. Enteric fever is most prevalent in July-August due to possible sewage-mediated contamination of water sample during the rainy seasons. Karkey *et al.* (2008) reported the heaviest burden of enteric fever during monsoon months from June to August and is correlated with rainfall which is consistent to our findings. The isolation of *Salmonella* spp. were significantly high in March to August than September to February in study conducted by Prajapati (2009). Typhoid fever is endemic in Nepal and prevalent in mountains, valleys and southern belts with its peak incidence in May to August (Sharma *et al.*, 2003). According to hospital based surveillance report the number of typhoid fever patients was higher in the summer months than that in the winter months in Uzbekistan (Srikantiah *et al.*, 2007). Typhoid fever in India is usually observed throughout the year with peak of the disease from July to September, as it coincides with the rainy season when the chance of water contamination is high, especially in crowded areas (Kanungo *et al.*, 2008).

In this study, all the *Salmonella* isolates were tested against the 10 antibiotic discs. Based on the in-vitro sensitivity test cotrimoxazole, ceftriaxone and gentamicin were found the most effective drugs for the treatment of enteric fever.

Salmonella Typhi isolates showed 100% susceptibility to chloramphenicol, cotrimoxazole, cefixime, ceftriaxone, ciprofloxacin and gentamicin confirming

them as the drugs of choice in the treatment of typhoid fever. Amoxycillin and ofloxacin were second effective drugs with efficacy rate of 98.2%. Azithromycin was found to be less effective drug with 89.3% effectiveness.

Similar reports were found in other studies conducted in Nepal in similar settings. In study conducted by Prajapati (2009), *Salmonella* Typhi was found to be most sensitive to cefotaxime, gentamicin, chloramphenicol and amoxycillin. Shrestha (2004) found 100% efficacy rate of the antibiotics ofloxacin, ciprofloxacin, chloramphenicol, ceftriaxone and cotrimoxazole. Gurung (2008) reported that *S.* Typhi were 100% susceptible to chloramphenicol, amoxycillin, cotrimoxazole and ceftriaxone. Aryal (2008) reported that *S.* Typhi were 98% susceptible to ceftriaxone, followed by ofloxacin (96%), ciprofloxacin (94.2%), cotrimoxazole (88.5%) and ampicillin (5.7%). She reported extremely low sensitivity of ampicillin in contrast to our present studied. In a recent study conducted at Bir Hospital, Dahal (2012) reported *Salmonella* Typhi showed 100% susceptibility towards ofloxacin and ceftriaxone, followed by ciprofloxacin (95.83%), chloramphenicol (95.83%), amoxycillin (93.75%) and cotrimoxazole (91.67%). Bhatta *et al.* (2005) reported ciprofloxacin as highly sensitive (98.718%) followed by ofloxacin (98.208%), cephalexin (96.411%), chloramphenicol (95.898%), cotrimoxazole (94.103%) and ampicillin (87.18%). In a study conducted by Ashok R *et al.* (2010) in Rwanda all the *S.* Typhi isolates were 100% sensitive to ceftriaxone, levofloxacin and ciprofloxacin. In a study conducted in Chennai, India Muthu *et al.* (2011) reported ceftriaxone the most effective drug with efficacy 98.5% followed gentamicin (97.7%), chloramphenicol (97.5%), cotrimoxazole (97.5%), cefotaxime (79%) and ampicillin (67.5%).

Various reports claimed the increasing burden of multidrug resistant *Salmonella* Typhi in various parts of the world like India, Nigeria, Uzbekistan including Nepal (Gautam *et al.*, 2002; Akinyemia *et al.*, 2005; Pokharel *et al.*, 2006; Srikantiah *et al.*, 2007; Kumar *et al.*, 2008; Acharya *et al.*, 2009). In contrast to such claims no multidrug resistant *Salmonella* Typhi (MDRST) was isolated in this study. The present study revealed that 8.93% S. Typhi isolates were resistant to at least one other antibiotic along with nalidixic acid. Prajapati (2009) reported

that 0.44% of the *Salmonella* Typhi isolates were multi drug resistant (MDR). Aryal (2008) reported 2 (3.84%) MDR S. Typhi. Naheed *et al.* (2010), in a study in a densely populated urban community, Dhaka, Bangladesh, reported 40% of *Salmonella* Typhi isolates were MDR. Arora *et al.* (2010) reported the cases of MDRST in North India increases from 2003 to 2005 and decreases from 2006 onwards. An outbreak of enteric fever occurred in Mumbai garrison where all strains isolated were resistant to four primary drugs i.e. ampicillin, chloramphenicol, cotrimoxazole and tetracycline (Misra *et al.*, 2005).
We concluded from our study that amoxycillin, cotrimoxazole, ceftriaxone and gentamicin were the drug of choice for *Salmonella* Paratyphi A infection with efficacy rate of 100%. Chloramphenicol, cefixime, ciprofloxacin, and ofloxacin were drug of second choice with efficacy rate of 97.7%. Like in case of *S*. Typhi azithromycin was found least effective drug which was only 87.7% effective.

In a recent study, Dahal (2012) reported *Salmonella enterica* serovar Paratyphi A isolates were found to be 100% sensitive to ceftriaxone, ofloxacin, chloramphenicol followed by ciprofloxacin (94.87%), cotrimoxazole (94.87%) and amoxycillin (92.31%). Similarly the study performed by Khatiwada (2006) reported that *S*. Paratyphi A isolates were susceptible to tetracycline, ciprofloxacin, ceftriaxone and cotrimoxazole. Prajapati (2009) reported that *Salmonella* Paratyphi A were highly sensitive to gentamicin (99.60%), cephotaxime (99.20%), amoxycillin (98.81%), and cotrimoxazole (98.81%). Aryal (2008) reported that *S*. Paratyphi A were 100% susceptibility to ceftriaxone, ofloxacin, chloramphenicol, ciprofloxacin followed by cotrimoxazole (95.5%). Study conducted by Amatya *et al.* (2007) reported that *S*. Paratyphi A was 100% susceptible to three antibiotics, chloramphenicol, amoxycillin and cotrimoxazole.

None of the *S*. Paratyphi A isolated were found to be multi drug resistant (MDR), however, 7 (23.33%) isolates were resistant to at least one of the tested drugs along with nalidixic acid. Our reports was consistent with that of Kunwor (2007) and Aryal (2008) who reported none of the isolates to be MDR. As consistent to our finding Amatya *et al.* (2007) did not isolate any *S*. Paratyphi A resistant to more than 2 antibiotics. However, in contrast to our reports several cases of MDR

S. Paratyphi A were isolated from Nepal by other researchers. Gurung (2008) reported 8.7% *S*. Paratyphi A resistant to 3 or more antibiotics. Likewise KhattriChhetri (2008) isolated 8.8% *S*. Paratyphi A resistance to 3 or more drugs. Only one isolate of *S*. Paratyphi A was found to be MDR in a study conducted by Prajapati (2009). In a study conducted at Tribhuvan University Teaching Hospital, Pokharel *et al.* (2006) reported 7% of the *Salmonella* Paratyphi isolates to be MDR.

The first report of MDR *S*. Typhi in Nepal was published in 1991 (Watson and Pettibone, 1991). The emergence of MDR S. Typhi (resistant to ampicillin, chloramphenicol and cotrimoxazole- ACCo) led to the use of quinolones (ciprofloxacin, ofloxacin) as the first line drug for treatment.

In this study 90.7% of total *Salmonella* isolates were nalidixic acid resistant (NAR) among which 51(91.1%) of total *Salmonella* Typhi isolates and 27 (90.0%) of total *Salmonella* Paratyphi A isolates were nalidixic acid resistant. NAR *S*. Typhi isolates were 100% susceptible to ciprofloxacin. But the efficacy of ciprofloxacin to NAR *S*. Paratyphi was found to be 96.3% and 3.7% isolates were resistant to ciprofloxacin. Nalidixic acid resistant strain indicates reduced susceptibility to ciprofloxacin and is associated with treatment failure to ciprofloxacin. Very high frequency of NAR *Salmonella* isolates were found in this study which suggests the alarming threat in the use of fluoroquinolones in the treatment of enteric fever.

Similar to our findings the higher frequency of NAR *Salmonella* isolates were reported in previous studies. Prajapati (2009) reported 68.19% *Salmonella* Typhi and 94.83% *Salmonella* Paratyphi A isolates to be resistant to nalidixic acid whereas Aryal (2008) reported 79% *Salmonella* Typhi and 82.2% *Salmonella* Paratyphi A isolates to be resistant to nalidixic acid which is close to our finding.

For the United States, the Centers for Disease Control and Prevention reported that in the year 2001, 30% of serovar Typhi isolates were nalidixic acid resistant as opposed to 19% in 1999 (Shakespeare *et al.*, 2005). In a study conducted at

Chennai, India Muthu *et al.* (2011) documented that 95.5% of *S*. Typhi and 92.5% *S*. Paratyphi A isolates were resistant nalidixic acid by disc diffusion technique which was slightly higher than our findings.

Several reports of treatment failure of fluoroquinolones (ciprofloxacin) or reduced susceptibility to ciprofloxacin in enteric fever patients infected with nalidixic acid resistant *Salmonella* isolates have been documented (Butt *et al.*, 2003; Kadhiravan *et al.*, 2005; Nkemngu *et al.*, 2005; Dimitrov *et al.*, 2007). In a report of Capoor *et al.* (2006) conducted in India, nalidixic acid resistance was observed in 51% *Salmonella* Typhi isolates, of which 98.9% had decreased susceptibility (MIC> or = 0.125-4 μg/ml) to ciprofloxacin.

Isolates with decreased susceptibility to ciprofloxacin appear susceptible with routine disc diffusion tests. Routine application of these tests for each strain is not convenient, and the literature suggests that resistance to nalidixic acid may be an indicator of decreased susceptibility to ciprofloxacin (Kownhar *et al.*, 2007). Although fluoroquinolones may still be useful as antibiotics for the treatment of typhoid fever, clinicians should be aware of the possibility of treatment failures of infections with *S*. Typhi and *S*. Paratyphi A strains with decreased susceptibility to fluoroquinolones (Shirakawa *et al.*, 2006).

Chloramphenicol had been the gold standard of therapy since its introduction in 1948 (Bhatia *et al.*, 2007). Ciprofloxacin is the drug of choice for treating typhoid fever in areas where multidrug-resistant (MDR) *Salmonella* Typhi strains are prevalent since 1991 when the resistance of *Salmonella* Typhi strains to chloramphenicol and other antimicrobial agents reached its peak. Recently, however, there have been reports of ciprofloxacin resistance being detected among *Salmonella* Typhi strains, leading many clinicians to question the efficacy of this drug (Mehta *et al.*, 2001). Somily (2010) recommended that all *Salmonella* systemic infections resistant to nalidixic acid with in vitro but decreased susceptibility to fluoroquinolones be treated with other antibiotics like third generation cephalosporins or azithromycin.

Re-emergence of susceptibility to drugs used in the past, such as chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole has been documented in several reports. The changing trends of *S*. Typhi resistance patterns reveal the reconsideration of conventional treatment strategy and suggest the possibility of use of these drugs for the treatment of typhoid fever and give fluoroquinolones a respite. (Joshi and Amarnath, 2007; Arora *et al.*, 2010; Kumar *et al.*, 2011). Similar conclusion could be drawn from our study which revealed high proportion of nalidixic acid *Salmonella* isolates and none MDR *Salmonella* isolates.

CHAPTER-VI

CONCLUSION AND RECOMMENDATIONS

6.1. CONCLUSION

Enteric fever remained the major public health problem in Nepal with high incidence rate and *Salmonella* Typhi was found to be more prevalent causative agent of enteric fever than *Salmonella* Paratyphi A i.e. typhoid fever was more prevalent than paratyphoid fever in patients suspected of enteric fever. However the cases with *Salmonella* Paratyphi A were found in increasing trend in subsequent years. The incidence of the enteric was higher in females than in males, and this was statistically insignificant. The enteric fever was more prevalent in age group 11-20 years. The disease was most frequently occurred in monsoon season (July-August) that correspond rainy season.

Chloramphenicol, cotrimoxazole, cefixime, ceftriaxone, ciprofloxacin and gentamicin proved to be most effective drugs against *Salmonella* Typhi isolates and amoxycillin, cotrimoxazole, ceftriaxone and gentamicin proved to be most effective drugs against *Salmonella* Paratyphi A isolates with 100% sensitivity in vitro disc diffusion test. Azithromycin was least effect drug for both isolates. No MDR isolates were reported in this study and increasing sensitivity of *Salmonella* isolates towards traditional first line drug amoxycillin, chloramphenicol and cotrimoxazole was observed. The isolation of highest frequency (>90%) of nalidixic acid resistant *Salmonella* isolates which act as a marker for decreased fluoroquinolones susceptibility indicate that resistant to quinolones (ciprofloxacin) will occur in near future and it will jeopardize the use of ciprofloxacin as the first line of drug in empirical therapy of enteric fever.

This study revealed the increasing frequency of nalidixic acid resistant *Salmonella* isolates and re-emergence of susceptibility to conventional first line drugs.

6.2. RECOMMENDATIONS

1. Since the study was confined to a one hospital, the finding might not necessarily represent the true figure of the country, so nationwide surveillance system considering wide geographic region, ethnic diversity, and seasonal variation should be carried out that could reflect the burden of enteric fever and antibiotic profile of *Salmonella* isolates in true sense.

2. 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 of ciprofloxacin.

3. The current break points and susceptibility criteria of ciprofloxacin and ofloxacin need to be re-evaluated for nalidixic acid resistance *Salmonella* isolates.

4. Conventional first line drug amoxycillin, chloramphenicol and cotrimoxazole, if susceptible, should be recommended for the treatment of enteric fever.

REFERENCES

Acharya D, Bhatta DR, Malla S, Dumre SP, Adhikari N and Kandel BP (2011). *Salmonella enterica* serovar Paratyphi A: an emerging cause of febrile illness in Nepal. *Nepal Med Coll J,* **13:** 69-73.

Acharya D, Malla S, Bhatta DR and Dumre SP (2009). Multidrug resistant *Salmonella enterica* serovar Typhi. *J Nepal Med Assoc,* **48:** 196-197.

Acharya D, Trakulsomboon S, Madhup SK and Korbsrisate S (2012). Antibiotic susceptibility pattern and the indicator of decreased ciprofloxacin susceptibility of *Salmonella enterica* serovar Typhi isolated from Dhulikhel Hospital, Nepal. *Jpn J Infect Dis,* **65:** 264-267.

Ahmed N, Saeed ZI and Tariq M (2012). Abdominal lymphadenopathy: an atypical presentation of enteric fever. *Asian Pac J Trop Biomed***:** 409-410.

Akinyemia KO, Smith SI, Oyefolua AOB and Cokerc AO (2005). Multidrug resistance in *Salmonella enterica* serovar Typhi isolated from patients with typhoid fever complications in Lagos, Nigeria. *Public Health,* **119:** 321-327.

Ali S, Vollaard A, Widjaja S, Surjadi C, Vosse E and Dissel J (2006). PARK2/PACRG polymorphisms and susceptibility to typhoid and paratyphoid fever. *Clin Exp Immunol,* **144:** 425-431.

Amatya N, Shrestha B and Lekhak B (2007). Etiological agents of bacteraemia and antibiotic susceptibility pattern in Kathmandu Model Hospital. *J Nepal Med Assco,* **46:** 112-118.

Amatya NM (2005). A study on etiological agents of bacteraemia and antibiotic susceptibility pattern of isolates, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Anand PK and Ramakrishnan R (2010). Investigation of the outbreak of typhoid in a village of Thar Desert Rajasthan, India. *Indian J Med Res,* **131:** 799-803.

Arjyal A, Basnyat B, Koirala S, Karkey A, Dongol S, Agrawaal KK, Shakya N, Shrestha K, Sharma M, Lama S, Shrestha K, Khatri NS, Shrestha U, Campbell JI, Baker S, Farrar J, Wolbers M and Dolecek C (2011). Gatifloxacin versus chloramphenicol for uncomplicated enteric fever: an open-label, randomised, controlled trial. *Lancet Infect Dis,* **11:** 445-454.

Arora D, Singh R, Kaur M and Ahi RS (2010). A changing pattern in antimicrobial susceptibility of *Salmonella enterica* serotype isolated In North India. *Afr J Microbiol Res,* **4:** 197-203.

Aryal M (2008). Isolation, identification and antibiotic sensitivity testing of *Salmonella* serovars from enteric fever suspected patients visiting Bir Hospital, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Ashok R, Peter K, Joselyne N and Emma N (2010). Antimicrobial susceptibility patterns of *Salmonella* Typhi from Ki-gali, Rwanda. *Shiraz E Med J,* **11**.

Avery C, Baumbach J, Ettestad P, Ewers C, Jungk J, Reynolds P, Selvage D and Smelser C (2008). Manual for Investigation and Control of Selected Communicable Diseases, New Mexico Department of Health, Epidemiology and Response Division, Infectious Disease Epidemiology Bureau,

Bekur R, Vandana KE, Shivashankara KN, Valsala R and Sathyanarayanan V (2010). Paratyphoid fever- emerging problem in South India. *Asian Pacific J Trop Med***:** 815-817.

Bertrand S, Weill F-X, Cloeckaert A, Vrints M, Mairiaux E, Praud K, Dierick K, Wildemauve C, Godard C, Butaye P, Imberechts H, Grimont PAD and Collard J-M (2006). Clonal emergence of extended-spectrum -Lactamase (CTX-M-2)-producing *Salmonella enterica* serovar Virchow isolates with reduced susceptibilities to ciprofloxacin among poultry and humans in Belgium and France (2000 to 2003). *J Clin Microbiol,* **44:** 2897-2903.

Bhatia JK, Mathur AD and Arora MM (2007). Reemergence of chloramphenicol sensitivity in enteric fever. *Med J Armed Forces India,* **63:** 212-214.

Bhatta CP, Bhuyan KC and Maharjan A (2005). Antibiotic sensitivity pattern of *Salmonella* species isolated from blood culture. *J Nepal Health Res Counc,* **3:** 35-38.

Bhatta DR, Bangtrakulnonth A, Tishyadhigama P, Saroj SD, Bandekar JR, Hendriksen RS and Kapadnis BP (2007). Serotyping, PCR, phage-typing and antibiotic sensitivity testing of *Salmonella* serovars isolated from urban drinking water supply systems of Nepal. *Lett Appl Microbiol,* **44:** 588-594.

Brenner FW, Villar RG, Angulo FJ, Tauxe R and Swaminathan B (2000). Salmonella nomenclature. *J Clin Microbiol,* **38:** 2465-2467.

Butt T, Ahmad RN, Mahmood A and Zaidi S (2003). Ciprofloxacin treatment failure in typhoid fever case, Pakistan. *Emerg Infect Dis,* **9**.

Capoor MR, Nair D, Hasan AS, Aggarwal P and Gupta B (2006). Typhoid fever: narrowing therapeutic options in India. *Southeast Asian J Trop Med Public Health,* **37:** 1170-1174.

CDC (2008). Salmonella surveillance: annual summary, 2006, US Department of Health and Human Services, Center for Disease Control and Prevention Atlanta, Georgia

Chau TT, Campbell JI, Galindo CM, Van Minh Hoang N, Diep TS, Nga TTT, Van Vinh Chau N, Tuan PQ, Page AL, Ochiai RL, Schultsz C, Wain J, Bhutta ZA, Parry CM, Bhattacharya SK, Dutta S, Agtini M, Dong B, Honghui Y, Anh DD, Canh DG, Naheed A, Albert MJ, Phetsouvanh R, Newton PN, Basnyat B, Arjyal A, La TTP, Rang NN, Phuong LT, Van Be Bay P, von Seidlein L, Dougan G, Clemens JD, Vinh H, Hien TT, Chinh NT, Acosta CJ, Farrar J and Dolecek C (2007). Antimicrobial drug resistance of *Salmonella enterica* serovar Typhi in Asia and molecular mechanism of reduced susceptibility to the fluoroquinolones. *Antimicrob Agents Chemother,* **51:** 4315-4323.

Cheesbrough M (2000). District Laboratory Practice in Tropical Countries, Cambridge Low Price edn, Cambridge University Press, United Kingdom

Clark TW, Daneshvar C, Pareek M, Perera N and Stephenson I (2010). Enteric fever in a UK regional infectious diseases unit: a 10 year retrospective review. *J Infect,* **60:** 91-98.

Connor BA and Schwartz E (2005). Typhoid and paratyphoid fever in travellers. *Lancet Infect Dis,* **5:** 623-628.

Crump JA, Luby SP and Mintz ED (2004). The global burden of typhoid fever. *Bull World Health Organ,* **82:** 346-353.

Crump JA and Mintz ED (2010). Global trends in typhoid and paratyphoid fever. *Clin Infect Dis,* **50:** 241-246.

Dahal S (2012). Isolation and characterization of *Salmonella* serovars from enteric fever suspected patients attending Bir Hospital, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Department of Health Services (2012). Annual Report 2011/2012, Department of Health Services Kathmandu, Nepal

Dimitrov T, Udo EE, Albaksami O, Kilani AA and Shehab el DM (2007). Ciprofloxacin treatment failure in a case of typhoid fever caused by *Salmonella enterica* serotype Paratyphi A with reduced susceptibility to ciprofloxacin. *J Med Microbiol,* **56:** 277-279.

Dong B-Q, Yang J, Wang X-Y, Gong J, Seidlein Lv, Wang M-L, Lin M, Liao H-Z, Ochiai RL, Xu Z-Y, Jodarc L and Clemensc JD (2010). Trends and disease burden of enteric fever in Guangxi province, China, 1994–2004. *Bull World Health Organ,* **88**.

Effa EE and Bukirwa H (2008). Azithromycin for treating uncomplicated typhoid and paratyphoid fever (enteric fever). *Cochrane Database Syst Rev*: CD006083.

Ellermeier CD and Slauch JM (2006). The Genus *Salmonella*. *In:* Dworkin M, Falkow S, Rosenberg E, Schleifer K-H and Stackebrandt E (eds.) *The Prokaryotes -A Handbook on the Biology of Bacteria.* 3rd edn, Springer, Singapore

Escamilla J, Florez-Ugarte H and Kilpatrick ME (1986). Evaluation of blood clot cultures for isolation of *Salmonella* Typhi, *Salmonella* Paratyphi A, and *Brucella melitensis*. *J Clin Microbiol,* **24:** 388-390.

Farooqui A, Khan A and Kazmi S (2009). Investigation of a community outbreak of typhoid fever associated with drinking water. *BMC Public Health,* **9:** 476.

Gautam V, Gupta NK, Chaudhary U and Arora DR (2002). Sensitivity pattern of *Salmonella* serotypes in Northern India. *Braz J Infect Dis,* **6:** 281-287.

Ghimire D (1995). Bacteriological profile of bacteraemia and septicaemia among the patients visiting Patan Hospital, M.Sc. Dissertation Submitted to the Central Department of Microbiolgy, Tribhuvan University, Kathmandu.

Gonzalez-Escobedo G, Marshall JM and Gunn JS (2011). Chronic and acute infection of the gall bladder by *Salmonella* Typhi: understanding the carrier state. *Nat Rev Micro,* **9:** 9-14.

Gopinath S, Carden S and Monack D (2012). Shedding light on *Salmonella* carriers. *Trends Microbiol,* **20:** 320-327.

Gordon MA, Graham SM, Walsh AL, Wilson L, Phiri A and E M (2008). Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *S. enteric* Serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clin Infect Dis,* **46:** 963-969.

Greenwood D, Slack R and Puetherer J (eds.) 2000. Medical Microbiology, A Guide to Microbial Infections: Pathogenesis, Immunity, Laboratory Diagnosis and Control, 15th edn, Churchill Livingstone, USA

Grimont PAD and Weill FX (2007). Antigenic formulae of the *Salmonella* serovars. 9th edn. Paris, France: WHOCC-Salm, Institute Pasteur.

Gupta V, Kaur J and Chander J (2009). An increase in enteric fever cases due to *Salmonella* Paratyphi A in & around Chandigarh. *Indian J Med Res,* **129:** 95-98.

Gurung B (2008). Antibiotic susceptibility patterns of *Salmonella* isolates from blood samples of patients visiting Shree Birendra Hospital Chhauni, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Hassing RJ, Menezes GA, Van Pelt W, Petit PL, Van Genderen PJ and Goessens WHF (2011). Analysis of mechanisms involved in reduced susceptibility to ciprofloxacin in *Salmonella enterica* serotypes Typhi and Paratyphi A isolates from travellers to Southeast Asia. *Int J Antimicrob Agents,* **37:** 240-243.

Hu L and Kopecko DJ (2002). Salmonella Typhi and Paratyphi. *In:* Max S (ed.) *Molecular Medical Microbiology.* Academic Press, London

Huang DB and DuPont HL (2005). Problem pathogens: extra-intestinal complications of *Salmonella enterica* serotype Typhi infection. *Lancet Infect Dis,* **5:** 341-348.

Janda MJ and Abbott SL (2005). The Enterobacteria, 2nd edn, ASM press,

Joshi S and Amarnath SK (2007). Fluoroquinolone resistance in *Salmonella* Typhi and *S*. Paratyphi A in Bangalore, India. *Trans R Soc Trop Med Hyg,* **101:** 308-310.

Kadhiravan T, Wig N, Kapil A, Kabra SK, Renuka K and Misra A (2005). Clinical outcomes in typhoid fever: adverse impact of infection with nalidixic acid-resistant *Salmonella* Typhi. *BMC Infect Dis,* **5:** 37.

Kanungo S, Dutta S and Sur D (2008). Epidemiology of typhoid and paratyphoid fever in India. *J Infect Dev Ctries,* **2:** 454-460.

Kariuki S, Revathi G, Kiiru J, Mengo DM, Mwituria J, Muyodi J, Munyalo A, Teo YY, Holt KE, Kingsley RA and Dougan G (2010). Typhoid in Kenya is associated with a dominant multidrug-resistant *Salmonella enterica* serovar Typhi haplotype that is also widespread in Southeast Asia. *J Clin Microbiol,* **48:** 2171-2176.

Kariuki S, Revathi G, Muyodi J, Mwituria J, Munyalo A, Mirza S and Hart CA (2004). Characterization of multidrug-resistant typhoid outbreaks in Kenya. *J Clin Microbiol,* **42:** 1477-1482.

Karkey A, Arjyal A, Anders KL, Boni MF, Dongol S, Koirala S, My PV, Nga TV, Clements AC, Holt KE, Duy PT, Day JN, Campbell JI, Dougan G, Dolecek C, Farrar J, Basnyat B and Baker S (2010). The burden and characteristics of enteric fever at a healthcare facility in a densely populated area of Kathmandu. *PLoS ONE,* **5**.

Karkey A, Aryjal A, Basnyat B and Baker S (2008). Kathmandu, Nepal: still an enteric fever capital of the world. *J Infect Dev Ctries,* **2:** 461-465.

Karki S and Kumar K (2011). Study on the possible use of Vi polysaccharide typhoid fever vaccine to control endemic typhoid fever in Nepal. *Asian Pac J Trop Dis*: 76-79.

Kaur J and Jain SK (2012). Role of antigens and virulence factors of *Salmonella enterica* serovar Typhi in its pathogenesis. *Microbiol Research,* **167:** 199-210.

Keddy KH, Sooka A, Letsoalo ME, Hoyland G, Chaignat CL, Morrissey AB and Crump JA (2011). Sensitivity and specificity of typhoid fever rapid antibody tests for laboratory diagnosis at two sub-Saharan African sites. *Bull World Health Organ,* **89:** 640-647.

Kelly DF, Thorson S, Maskey M, Mahat S, Shrestha U, Hamaluba M, Williams E, Dongol S, Werno AM, Portess H, Yadav BK, Adhikari N, Guiver M, Thomas K, Murdoch DR and Pollard AJ (2011). The burden of vaccine-preventable invasive bacterial infections and pneumonia in children admitted to hospital in urban Nepal. *Int J Infect Dis,* **15:** 30.

Khanal B, Sharma SK, Bhattacharya SK, Bhattarai NR, Deb M and Kanungo R (2007). Antimicrobial susceptibility patterns of *Salmonella enterica* serotype Typhi in eastern Nepal. *J Health Popul Nutr,* **25:** 82-87.

Khatiwada S (2006). Study of prevalence of enteric fever and assessment of widal test in diagnosis of typhoid fever, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

KhattriChhetri M (2008). Study of prevalence of enteric fever and assessment of single Widal test in the diagnosis of Typhoid fever, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Kim DM, Neupane GP, Jang SJ, Kim SH and Lee BK (2010). In vitro efficacy of the combination of ciprofloxacin and cefotaxime against nalidixic acid-resistant *Salmonella enterica* serotype Typhi. *Int J Antimicrob Agents,* **36:** 155-158.

Kothari A, Pruthi A and Chugh TD (2008). The Burden of Enteric Fever. *J Infect Dev Ctries,* **2:** 253-259.

Kownhar H, Shankar EM, Rajan R and Rao UA (2007). Emergence of nalidixic acid-resistant *Salmonella enterica* serovar Typhi resistant to ciprofloxacin in India. *J Med Microbiol,* **56:** 136-137.

Kudalkar D, Thermidor M and Cunha BA (2004). *Salmonella* Paratyphi A enteric fever mimicking viral meningitis. *Heart & Lung: J Acute Crit Care,* **33:** 414-416.

Kumar S, Rizvi M and Berry N (2008). Rising prevalence of enteric fever due to multi drug resistant *Salmonella*: an epidemiological study. *J Med Microbiol,* **57:** 1247-1250.

Kumar Y, Sharma A and Mani KR (2011). Re-emergence of susceptibility to conventionally used drugs among strains of *Salmonella* Typhi in central west India. *J Infect Dev Ctries,* **5:** 227-230.

Kunwor R (2007). Nalidixic acid resistant *Salmonella* with decreased ciprofloxacin susceptibility, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Le TAH, Fabre L, Roumagnac P, Grimont PAD, Scavizzi MR and Weill F-X (2007). Clonal expansion and microevolution of quinolone-resistant *Salmonella enterica* serotype Typhi in Vietnam from 1996 to 2004. *J Clin Microbiol,* **45:** 3485-3492.

Levine MM, Tapia MD and Zaidi AKM (2011). Typhoid and Paratyphoid (Enteric) Fever. *In:* Guerrant RL, Walker DH and Weller PF (eds.) *Tropical Infectious Diseases: Principles, Pathogens and Practice.* 3rd edn, Elsevier,

Lewis MD, Serichantalergs O, Pitarangsi C, Chuanak N, Mason CJ, Regmi LR, Pandey P, Laskar R, Shrestha CD and Malla S (2005). Typhoid fever: a massive, single-point source, multidrug-resistant outbreak in Nepal. *Clin Infect Dis,* **40:** 554-561.

Madhulika U, Harish BN and Parija SC (2004). Current pattern in antimicrobial susceptibility of *Salmonella* Typhi isolates in Pondicherry. *Indian J Med Res,* **120:** 111-114.

Malisa A and Nyaki H (2010). Prevalence and constraints of typhoid fever and its control in an endemic area of Singida region in Tanzania: Lessons for effective control of the disease. *J Public Health Epidiomiol,* **2:** 93-99.

Maskey AP, Basnyat B, Thwaites GE, Campbell JI, Farrar JJ and Zimmerman MD (2008). Emerging trends in enteric fever in Nepal: 9124 cases confirmed by blood culture 1993-2003. *Trans R Soc Trop Med Hyg,* **102:** 91-95.

Matheson N, Kingsley RA, Sturgess K, Aliyu SH, Wain J, Dougan G and Cooke FJ (2010). Ten years experience of Salmonella infections in Cambridge, UK. *J Infect* **60:** 21-25.

Mehta GM, Randhawa VR and Mohapatra NM (2001). Intermediate susceptibility to ciprofloxacin in *Salmonella* Typhi strains in India. *Eur J Clin Microbiol Infect Dis,* **20:** 760-761.

Misra RN, Bawa KS, Magu SK, Bhandari S, Nagendra A and Menon PK (2005). Outbreak of multi-drug resistant *Salmonella* Typhi enteric fever in Mumbai Garrison. *MJAFI,* **6f**.

Monack DM, Mueller A and Falkow S (2004). Persistent bacterial infections: the interface of the pathogen and the host immune system. *Nat Rev Microbiol,* **2:** 747-765.

Muthu G, Suresh A, Sumathy G and Srivani R (2011). Studies on antimicrobila susceptibility pattern of *Salmonella* isolates from Chennai, India. *Int J Pharma Bio Sci,* **2**.

Nagpal AK and Jairam A (2009). Enteric fever: emerging trends. *Med J Armed Forces India,* **65:** 298-299.

Naheed A, Ramb PK, Brooks WA, Hossain MA, Parsons MB, Talukder KA, Mintz E, Luby S and Breiman RF (2010). Burden of typhoid and paratyphoid fever in a densely populated urban community, Dhaka, Bangladesh. *Int J Infect Dis,* **14S:** e93–e99.

Nkemngu NJ, Asonganyi ED and Njunda AL (2005). Treatment failure in a typhoid patient infected with nalidixic acid resistant *S. enterica* serovar Typhi with reduced susceptibility to ciprofloxacin: a case report from Cameroon. *BMC Infect Dis,* **5:** 49.

Old DC (1990). *Salmonella*. *In:* Parker M.T. and Buerden (eds.) *Topley and Wilson's Principles of Bacteriology, Virology and Immunology.* 8th edn, B.C. Decker Inc., Philadephia, USA

Old DC (1996). *Salmonela*. *In:* Colle J, Marmion B, Fraser A and Simmon A (eds.) *Mackie and McCartney Practical Medical Microbiology.* 14th edn, Churchill Livingstone, New York

Old DC and Threlfall EJ (1996). *Salmonella*. *In:* Collier L, Balaws A and Sussman M (eds.) *Topley and Wilson's Microbiology and Microbial infections.* 9th edn, Arnold, Oxford University Press, Inc., New York

Olopoenia LA and King AL (2000). Widal agglutination test - 100 years later: still plagued by controversy. *Postgrad Med J,* **76:** 80-84.

Parry CM, Hien TT, Dougan G, White NJ and Farrar JJ (2002). Typhoid fever. *N Engl J Med,* **347:** 1770-1782.

Parry CM, Wijedoru L, Arjyal A and Baker S (2011). The utility of diagnostic tests for enteric fever in endemic locations. *Expert Rev Anti Infect Ther,* **9:** 711–725.

Pokharel BM, Koirala J, Dahal RK, Mishra SK, Khadga PK and Tuladhar NR (2006). Multidrug-resistant and extended-spectrum beta-lactamase (ESBL)-producing *Salmonella enterica* (serotypes Typhi and Paratyphi A) from blood isolates in Nepal: surveillance of resistance and a search for newer alternatives. *Int J Infect Dis,* **10:** 434-438.

Pokharel P, Rai S, Karki G, Katuwal A, Vitrakoti R and Shrestha S (2009). Study of enteric fever and antibiogram of *Salmonella* isolates at a Teaching Hospital in Kathmandu Valley. *Nepal Med Coll J,* **11:** 176-178.

Pokhrel BM, Karmacharya R, Mishra SK and Koirala J (2009). Distribution of antibody titer against *Salmonella enterica* among healthy individuals in nepal. *Ann Clin Microbiol Antimicrob,* **8:** 1.

Prajapati B, Rai GK, Rai SK, Upreti HC, Thapa M, Singh G and Shrestha RM (2008). Prevalence of *Salmonella* Typhi and Paratyphi infection in children: a hospital based study. *Nepal Med Coll J,* **10:** 238-241.

Prajapati KG (2009). Antibiotic susceptibility pattern of *Salmonella* from blood of suspected enteric fever patients attending Patan Hospital, M.Sc. Dessertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Rai S, Jain S, Prasad KN, Ghoshal U and Dhole TN (2012). Rationale of azithromycin prescribing practices for enteric fever in India. *Indian J Med Microbiol,* **30:** 30-33.

Reddy S, Rangaiah J, Addiman S, Wareham D, Wilson P and Sefton A (2011). Epidemiology, antibiotic resistance trends and the cost of enteric fever in East London, 2005–2010. *Travel Med Infect Dis,* **9:** 206-212.

Rotimi VO, Jamal W, Pal T, Sonnevend A, Dimitrov TS and Albert MJ (2008). Emergence of multidrug-resistant *Salmonella* spp. and isolates with reduced susceptibility to ciprofloxacin in Kuwait and the United Arab Emirates. *Diagn Microbiol Infect Dis,* **60:** 71-77.

Saha SK, Darmstadt GL, Baqui AH, M.Hanif, Ruhulamin M, Santosham M, Nagatake T and Black RE (2001). Rapid identification and antibiotic susceptibility testing of *Salmonella enterica* serovar Typhi isolated from blood: implications for therapy. *J Clin Microbiol,* **39:** 3583.

Sanchez-Vargas FM, Abu-El-Haija MA and Gomez-Duarte OG (2011). Salmonella infections: an update on epidemiology, management, and prevention. *Travel Med Infect Dis,* **9:** 263-277.

Shakespeare WA, Davie D, Tonnerre C, Rubin MA, Strong M and Petti CA (2005). Nalidixic acid-resistant *Salmonella enterica* serotype Typhi presenting as a primary psoas abscess: case report and review of the literature. *J Clin Microbiol,* **43:** 996-998.

Shanahan PM, Jesudason MV, Thomson CJ and Amyes SG (1998). Molecular analysis of and identification of antibiotic resistance genes in clinical isolates of *Salmonella* Typhi from India. *J Clin Microbiol,* **36:** 1595-1600.

Sharma N, Koju R, Karmacharya B, Tamang MD, Makaju R, Nepali N, Shrestha P and Adhikari D (2003). Typhoid fever in Dhulikhel hospital, Nepal. *Kathmandu Univ Med J,* **2:** 188-192.

Sharma NP, Peacock SJ, Phumratanaprapin W, Day N, White N and Pukrittayakamee S (2006). A hospital-based study of bloodstream infections in febrile patients in Dhulikhel Hospital Kathmandu University Teaching Hospital, Nepal. *Southeast Asian J Trop Med Public Health,* **37:** 351-356.

Shirakawa T, Acharya B, Kinoshita S, Kumagai S, Gotoh A and Kawabata M (2006). Decreased susceptibility to fluoroquinolones and gyrA gene mutation in the *Salmonella enterica* serovar Typhi and Paratyphi A isolated in Katmandu, Nepal, in 2003. *Diagn Microbiol Infect Dis,* **54:** 299-303.

Shrestha B (1996). Isolation of *Salmonella* spp. from blood and study of its antibiotic sensitivity pattern, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Shrestha S (2004). Study on the prevalence of *Salmonella* spp. from blood sample of the patients visiting National Public Health Laboratories, Teku, Nepal, M.Sc. Dissertation Submitted to the Central Department of Microbiology, Tribhuvan University, Kathmandu.

Singh B (2001). Symposium: typhoid fever epidemiology. *Indian Acad Clin Med,* **2:** 12.

Somily AM (2010). An imported enteric fever caused by a quinolone-resistant *Salmonella* Typhi. *Ann Saudi Med,* **30:** 313–316.

Song JH, Cho H, Park MY, Na DS, Moon HB and Pai CH (1993). Detection of *Salmonella* Typhi in the blood of patients with typhoid fever by polymerase chain reaction. *J Clin Microbiol,* **31:** 1439-1443.

Srikantiah P, Vafokulov S, Luby SP, Ishmail T, Earhart K, Khodjaev N, Jennings G, Crump JA and Mahoney FJ (2007). Epidemiology and risk factors for endemic typhoid fever in Uzbekistan. *Trop Med Int Health,* **12:** 838-847.

Thapa BJ (1991). Drug sensitivity of enteric fever organisms. *J Inst Med,* **13:** 327-330.

Tindall BJ, Grimont PAD, Garrity GM and Euzeby JP (2005). Nomenclature and taxonomy of the genus *Salmonella*. *Int J Syst Evol Microbiol,* **55:** 521-524.

Vollaard AM, Ali S, Widjaja S, Asten HAGHv, Visser LG, Surjadi C and van Dissel JT (2005). Identification of typhoid fever and paratyphoid fever cases at presentation in outpatient clinics in Jakarta, Indonesia. *Trans R Soc Trop Med Hyg,* **99:** 440-450.

Wain J, Bay PVB, Vinh H, Duong NM, Diep TS, Walsh AL, Parry CM, Hasserjian RP, Ho VA, Hien TT, Farrar J, White NJ and Day NPJ (2001). Quantitation of bacteria in bone marrow from patients with typhoid tever: Relationship between counts and clinical features. *J Clin Microbiol,* **39:** 1571-1576.

Walia M, Gaind R, Paul P, Mehta R, Aggarwal P and Kalaivani M (2006). Age-related clinical and microbiological characteristics of enteric fever in India. *Trans R Soc Trop Med Hyg,* **100:** 942-948.

Wang LX, Li XJ, Fang LQ, Wang DC, Cao WC and Kan B (2012). Association between the incidence of typhoid and paratyphoid fever and meteorological variables in Guizhou, China. *Chin Med J,* **125:** 455-460.

WHO (2000). Weekly epidemiological report. Geneva.

WHO (2003). Background document : the diagnosis, treatment and prevention of typhoid fever, Department of Biologicals and Vaccines, World Health Organization Geneva

Wilde H (2007). Enteric fever due to *Salmonella* Typhi and Paratyphi A: a neglected and emerging problem. *Vaccine,* **25:** 5246-5247.

Woods CW, Murdoch DR, Zimmerman MD, Glover WA, Basnyat B, Wolf L, Belbase RH and Reller LB (2006). Emergence of *Salmonella enterica* serotype Paratyphi A as a major cause of enteric fever in Kathmandu, Nepal. *Trans R Soc Trop Med Hyg,* **100:** 1063-1067.

Yin Ngan GJ, Ng LM, Lin RTP and Teo JWP (2010). Development of a novel multiplex PCR for the detection and differentiation of *Salmonella enterica* serovars Typhi and Paratyphi A. *Res Microbiol,* **161:** 243-248.

Zaki SA and Karande S (2011). Multidrug-resistant typhoid fever: a review. *J Infect Dev Ctries,* **5:** 324-337.

APPENDICES

APPENDIX A

CLINICAL AND MICROBIOLOGICAL PROFILE OF THE PATIENTS

A. Personal Information
Name: Date:
Patient No: Age/Sex:
Address: Patient: OPD /Emergency
Ward: Bed No.
Culture request for:

B. Clinical Manifestation
Fever:
Duration of fever:
Intense headache:
Abdominal Pain:
Vomiting:
Diarrhea:
Constipations:
Others
Current antibiotic treatment: Yes/No
If yes, Antibiotic(s) taken: 1)………………….. 2)……………………
Duration of treatment: ………………………

C. Microbiological profile
I. Evidence of microbial growth on BHI within: Day 1 / Day 2/ Day 3
II. Microbial growth on MA and BA (after subculture):
III. Colony characteristics

Media	Size	Shape	Color	Margin	Elevation	Opacity	Consistency
MA							
BA							

IV. Gram's reaction:
V. Biochemical characterization
Catalase: Oxidase: SIM: MR:
VP: TSI: Citrate: Urease:
VI. Serological Test
Antisera used: 1……………….. 2………………….. 3………………..
VII. Microorganism identified as: ……………………………………..
VIII. Antibiotic susceptibility test

Antibiotics used	Zone of inhibition (mm)	Interpretation

Amoxycillin		
Chloramphenicol		
Cotrimoxazole		
Nalidixic acid		
Ciprofloxacin		
Ofloxacin		
Cefixime		
Ceftriaxone		
Azithromycin		
Gentamicin		

Performed By: Checked By:

APPENDIX B

LIST OF EQUIPMENT, MATERIALS AND SUPPLIES

A. EQUIPMENTS

Autoclave
Incubator (Memmert, Germany)
Hot air oven (Universal, India)
Microscope (Olympus, Japan)
Refrigerator (Sanyo, Japan)
Gas burners
Weighing machine (Scaltec instruments, Germany)
Glasswares
Inoculating wire and loops

B. MICROBIOLOGICAL MEDIA

Blood agar
Brain Heart Infusion broth
Nutrient agar
Nutrient broth
Triple Sugar Iron agar
MacConkey agar
Simmon’s Citrate agar
Sulphur Indole Motility agar

Mueller Hinton agar
Urea broth base

C. CHEMICALS AND REAGENTS

Barritt's reagent
Catalase reagent (3% H_2O_2)
Oxidase reagent (1% Tetramethyl p-phenylene diamine dihydrochloride)
Kovac's reagent
Crystal violet
Gram's iodine
Absolute (95%) alcohol
Safranine
Normal saline
Sulphuric acid
Barium chloride

D. ANTIBIOTICS DISCS

All the antibiotics discs used for the susceptibility tests were from HiMedia Laboratories Pvt. Limited, Bombay, India. The antibiotics used were as follows

Amoxycillin (30 mcg)
Chloramphenicol (30 mcg)
Cotrimoxazole (1.25/23.75mcg)
Ciprofloxacin (5 mcg)
Ofloxacin (5mcg)
Nalidixic acid (30 mcg)
Cefixime (5mcg)
Ceftriaxone (30mcg)
Azithromycin (15 mcg)
Gentamicin (10mcg)

E. MISCELLANEOUS

Conical flasks
Cotton
Distilled water
Droppers
Forceps
Glass slides and cover slips
Immersion oil
Inoculating loop, Inoculating wire
Lysol,
Measuring cylinder
Petri dishes
Pipettes
Plastic containers
Spatula
Test tubes
Wooden applicator stick

APPENDIX C

A. COMPOSITION AND PREPARATION OF DIFFERENT TYPES OF CULTURE MEDIA

The culture media used were from Hi-Media Laboratories Pvt. Limited, Bombay, India.

(Note: All compositions are given in grams per liter and at 25°C temperature.)

1. Blood Agar (BA)

Blood agar base (infusion agar) + 5-10% sheep blood

Ingredients	gram/liter
Beef heart infusion	500.0
Tryptose	10.0
Sodium chloride	5.0
Agar	15.0
Final pH at 25° C	7.3±0.2

Preparation: 42.5 gm of the blood agar base medium was dissolved in 1000 ml distilled water. The medium was then sterilized by autoclaving at 15 lbs pressure (121 ºC) for 15 min. Then the prepared medium was cooled to about 40-50 ºC, to which 50 ml of sterile defibrinated blood was added aseptically. Then the medium was poured into petriplates.

2. Brain Heart Infusion Broth (BHI) (M210)

Ingredients	grams /litre
Calf brain, infusion from	200.0
Beef heart, infusion from	250.0
Proteose peptone	10.0
Dextrose	2.0
Sodium chloride	5.0
Disodium phosphate	2.5
Final pH (at 25°C)	7.4±0.2

Preparation: 37gm of the media was suspended in 1000ml of the distilled water. Also added 0.25gm of sodium polyanethol sulphonate. 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.

3. MacConkey Agar (MA)

Ingredients	gram/liter
Peptone	20.0
Lactose	10.0
Sodium taurocholate	5.0
Sodium chloride	5.0
Agar	20.0
Neutral red	0.04
Final pH at 25 ºC	7.4±0.2

Preparation: 55 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^{0}C (15 lbs pressure) for 15 minutes.

4. Mueller Hinton Agar (MHA)

Ingredients	gram/liter
Beef extract	300.0
Casein acid hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH at 25ºC	7.4±0.2

Preparation: 38 gm of the medium was dissolved in 1000 ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121º C) for 15 minutes.

5. Nutrient agar (NA)

Ingredients	gram/liter
Beef extract	10.0
Peptone	10.0
Sodium chloride	5.0
Yeast extract	1.5
Agar	12.0
Final pH at 25º C	7.4±0.2

Preparation: 37 grams of the medium was suspended in 1000 ml of distilled water and then boiled to dissolve completely. Then the medium was sterilized by autoclaving at 121^{0}C (15 lbs pressure) for 15 minutes.

6. Nutrient Broth (NB)

Ingredients	gram/liter
Peptone	5.0
Sodium chloride	5.0
Beef extract	1.5
Yeast extract	1.5
Final pH at 25ºC	7.4±0.2

Preparation: 13 gm of the medium was dissolved in 1000ml distilled water and was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes.

B. COMPOSITION AND PREPARATION OF DIFFERENT TYPES OF BIOCHEMICAL MEDIA

1. MR-VP Medium

Ingredients gram/litre

Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 250C)	6.9±0.2

Direction: 15 gm powder was dissolved in 1000 ml of distilled water & mixed well. 3 ml of medium was distributed in each test tube and autoclaved at 1210C for 15 minutes.

2. Christensen Urea Agar

Ingredients	gram/liter
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Disodium phosphate	1.2
Monopotassium phosphate	0.8
Phenol red	0.012
Agar	15
Final pH at 25°C	6.9±0.2

Preparation: 24 grams of the medium was suspended in 950 ml distilled water and sterilized by autoclaving at121^{0}C for 15 minutes. After cooling to about 45^{0}C, 50 ml of 40% urea was added and mixed well. Then 5 ml was dispensed in test tube and set at slant position.

3. Simmon's Citrate Agar

Ingredients	gram/liter
Magnesium sulphate	0.2
Mono-ammonium phosphate	1.0
Dipotassium phosphate	1.0
Sodium citrate	2.0
Sodium chloride	5.0
Agar	15.0
Bromothymol blue	0.08
Final pH at 25° C	6.8±0.2

Preparation: 24.2 gm of the medium was dissolved in 1000 ml distilled water. 3 ml medium was then dispensed in test tubes and sterilized by autoclaving at 15 lbs pressure (121° C) for 15 minutes. The sterilized medium in the tubes were tilted to form slopes.

4. **Sulphide Indole Motility Medium (SIM)**

Ingredients	gram/liter
Beef extract	3.0
Peptone	30.0
Peptonized iron	0.2
Sodium Thiosulphate	0.025
Agar	3.0
Final pH at 25ºC	7.3±0

Preparation: 36 gm of the medium was dissolved in 1000 ml distilled water and distributed into tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121° C) for 15 minutes

5. Triple Sugar Iron Agar (TSI)

Ingredients	gram/liter
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Lactose	10.0
Sucrose	10.0
Dextrose	1.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12
Final pH at 25°C	7.4±0.2

Preparation: 65 gm of the medium was dissolved in 1000 ml distilled water and then distributed into test tubes. The medium was sterilized by autoclaving at 15 lbs pressure (121°C) for 15 minutes. The medium was allowed to set in a slope form with a butt of about 1 inch.

C. COMPOSITION AND PREPARATION OF DIFFERENT REAGENTS

1. Gram staining reagents

(a) Crystal violet solution

Crystal violet	20.0 g
Ammonium oxalate	9.0 g
Ethanol or methanol, absolute	95ml
Distilled water (D/W) to make	1 litre

Preparation: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown paper. Then, 95 ml of ethanol was added and mixed until the dye was completely dissolved. To the mixture, 9 gm of ammonium oxalate dissolved in 200 ml of D/W was added. Finally the volume was made 1 litre by adding D/W.

(b) Lugol's Iodine

Potassium iodide	20.0 g
Iodine	10.0 g
Distilled water	1000 ml

Preparation: To 250 ml of D/W, 20 gm of potassium iodide was dissolved. Then 10 gm of iodine was mixed to it until it was dissolved completely. Finally the volume was made 1 litre by adding D/W.

(c) Acetone-alcohol decoloriser

Acetone	500 ml
Ethanol (absolute)	475 ml
Distilled water	25 m

Preparation: To 25 ml distilled water, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then 500 ml acetone was added and mixed well.

(d) Counter stain solution (Safranine)

Safranine	10.0 gm
Distilled water	1000 ml

Preparation: In a piece of clean paper, 10 gm of safranine was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranine dissolves completely.

2. Normal saline

Sodium Chloride	0.85 g
Distilled Water	100 ml

Direction: The sodium chloride was weighed and transferred to a leak-proof bottle pre marked to hold 100 ml. Distilled water was added to the 100 ml mark, and mixed until the salt was fully dissolved. The bottle was labeled and stored at room temperature.

3. Test reagents

(a) For Catalase test

Catalase Reagent (3% H_2O_2)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

Preparation**:** To 97 ml of D/W, 3 ml of hydrogen peroxide was added and mixed well.

(b) For Oxidase Test

Oxidase Reagent (impregnated in Whatman's No. 1 filter paper)

Tetramethyl *p*-phenylene diamine dihydrochloride (TPD)	1 gm
Distilled Water	100 ml

Preparation: This reagent solution was made by dissolving 1 gm of TPD in 100 ml D/W. To that solution strips of Whatman's No. 1 filter paper were soaked and drained for about 30 seconds. Then these strips were freeze dried and stored in a dark bottle tightly sealed with a screw cap.

(c) For Indole Test

Kovac's Indole Reagent

Isoamyl alcohol	30 ml
p-dimethyl aminobenzaldehyde	2.0 g
Hydrochloric acid	10 ml

Preparation: In 30 ml of isoamylalcohol, 2 g of *p*-dimethyl aminobenzaldehyde was dissolved and transferred to a clean brown bottle. Then to that, 10 ml of conc. HCl was added and mixed well.

(d) Mac Farland standard 0.5

0.5 ml of 0.048 M $BaCl_2$ (1.17% w/v $BaCl_2.H_2O$) was added to 99.5 ml of 0.18 M H_2SO_4 (1% w/v) with constant stirring. The McFarland standard was thoroughly mixed to ensure that it is evenly suspended. Using matched cuvettes with a 1 cm

light path and water as a blank standard, the absorbance was measured in a spectrophotometer at a wavelength of 625 nm. The acceptable range for the turbidity standard is 0.08-0.13. The standard was distributed into screw-cap tubes of the same size and volume as those used to prepare the test inoculum. The tubes were sealed tightly to prevent loss by evaporation and stored protected from light at room temperature. The turbidity standard was then vigorously agitated on a vortex mixer before use. Standards may be stored for up to 6 months, after which time they should be discarded.

APPENDIX D

PROCEDURE FOR BLOOD SPECIMEN COLLECTION

Blood sample was collected as follows (Cheesbrough, 2000)

1. Using a pressure cuff, a suitable vein was located in the arm.
2. The skin over the vein was cleansed in a circle approximately 5 cm in diameter with 70% alcohol, rubbing vigorously.
3. Starting in the center of the circle, 2% tincture iodine was applied in ever widening circles until the entire circle was saturated with iodine. The iodine was allowed to remain on the skin for at least 1 minute.
4. Using a sterile disposable syringe and size 21 gauge needles, appropriate volume (5 ml / 2 ml) of blood was withdrawn by inserting the needle into the vein.
5. After the needle was removed, the site was cleansed with 70 % alcohol again, as many patients were sensitive to iodine.
6. One culture bottle containing about 50 ml/ 20 ml of BHI broth was provided and 5 ml/ 2ml of patient's blood was collected and dispensed in the culture bottle.
7. Inoculation of blood sample into the culture broth was done immediately after collection i.e. in laboratory, in wards and emergency rooms wherever the sample was collected.

APPENDIX E

SLIDE AGGLUTINATION TEST FOR IDENTIFICATION OF *SALMONELLA* SPP.

1. A drop of normal saline was kept on a clean glass slide.
2. A dense suspension of the organism was made. The organism was taken from nutrient agar.
3. A drop of respective antiserum was added to the organism suspension and mixed well with a wooden stick.
4. The slide was observed for the clumping of the suspension. Positive test was indicated by rapid complete agglutination of the test organism. The suspension without adding antisera should be kept as a control to detect auto agglutination.

APPENDIX F

A. PROCEDURE OF ANTIBIOTIC SENSITIVITY TEST (KIRBY-BAUER'S DISC DIFFUSION METHOD)

i. Preparation of inoculum: For inoculum preparation, 4-5 pure culture colonies were transferred into Nutrient Broth and incubated at 37°C for 2-4 hours to obtain turbidity equivalent to 0.5 MaFarland.

ii. Inoculation: A dry sterile non-toxic cotton swab was dipped into the standardized inoculum and rotated the soaked swab firmly against the upper inside wall of tube to remove excess fluid (inoculum). The entire agar surface of MHA plate was streaked with the swab three times, turning the plate at 60 ^{0}C angle between each streaking. Then MHA plate was allowed to dry for 5-10 minutes with lead in place.

iii. Application of discs: With the help of flamed forceps, disc were carefully placed on the agar surface at least 15 mm away from the edge and no closer than about 25 mm from disc to disc to prevent the overlapping of the zone of inhibition .The disc were pressed lightly to make contact with

the surface of the medium and plates were allowed to stand at room temperature for few minutes (pre-diffusion time).

iv. Incubation: The plates were incubated at 37°C for 24 hrs.

v. Interpretation of result: After incubation, diameter of ZOI was measured and interpreted comparing with the zone size interpretive chart as per CLSI.

B. ZONE SIZE INTERPRETIVE CHART FOR ANTIBIOTICS AS PER CLSI GUIDELINE

Antimicrobial Agent	Symbol	Disc content (mcg)	Diameter of Zone of inhibition (in mm)		
			Resistant	Intermediate	Sensitive
Amoxycillin	AMX	30	13	14-16	17
Chloramphenicol	C	30	12	13-17	18
Cotrimoxazole	COT	1.25/23.75	10	11-15	16
Ciprofloxacin	CIP	5	15	16-20	21
Ofloxacin	OF	5	12	13-15	16
Nalidixic Acid	NA	30	13	14-18	19
Cefixime	CFM	5	15	16-18	19
Ceftriaxone	CTR	30	13	14-20	21
Azithromycin	AZM	15	13	14-17	18
Gentamicin	GEN	10	12	13-14	15

Source: Product Information Guide, HiMedia Laboratories Pvt. Limited, Mumbai, India

C. TABLE FOR ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA* TYPHI

Isolate code	Antibiotic used									
	AMX	C	COT	CFM	CTR	OF	CIP	NA	AZM	GEN
ST314	S	S	S	S	S	S	S	R	S	S
ST430	S	S	S	S	S	S	S	R	S	S
ST435	S	S	S	S	S	S	S	R	S	S
ST447	S	S	S	S	S	S	S	R	S	S
ST449	S	S	S	S	S	S	S	R	S	S
ST831	S	S	S	S	S	S	S	R	S	S
ST950	S	S	S	S	S	S	S	R	S	S
ST1210	S	S	S	S	S	S	S	R	S	S
ST1336	S	S	S	S	S	S	S	R	S	S
ST1383	S	S	S	S	S	S	S	R	S	S
ST1427	S	S	S	S	S	S	S	R	S	S
ST1436	S	S	S	S	S	S	S	R	S	S
ST1701	S	S	S	S	S	S	S	R	S	S
ST1753	S	S	S	S	S	S	S	R	S	S
ST2054	S	S	S	S	S	S	S	R	S	S
ST2121	S	S	S	S	S	S	S	R	S	S
ST2250	S	S	S	S	S	S	S	R	S	S
ST2329	S	S	S	S	S	S	S	R	S	S
ST2343	S	S	S	S	S	S	S	R	S	S
ST2415	S	S	S	S	S	S	S	R	S	S
ST2437	S	S	S	S	S	S	S	R	S	S
ST2659	S	S	S	S	S	S	S	R	S	S
ST2667	S	S	S	S	S	S	S	R	S	S
ST2733	S	S	S	S	S	S	S	R	S	S
ST3425	S	S	S	S	S	S	S	R	S	S
ST3532	S	S	S	S	S	S	S	S	S	S
ST60406	S	S	S	S	S	S	S	S	S	S
ST61068	S	S	S	S	S	S	S	R	S	S
ST61137	S	S	S	S	S	S	S	S	S	S
ST61155	S	S	S	S	S	S	S	R	S	S
ST61625	R	S	S	S	S	S	S	R	S	S
T61694	S	S	S	S	S	S	S	S	R	S

Isolate code	Antibiotic used									
	AMX	C	COT	CFM	CTR	OF	CIP	NA	AZM	GEN
ST61913	S	S	S	S	S	S	S	R	S	S

ST61937	S	S	S	S	S	S	S	R	S	S
ST62008	S	S	S	S	S	S	S	R	S	S
ST62149	S	S	S	S	S	S	S	R	S	S
ST62243	S	S	S	S	S	S	S	R	S	S
ST62256	S	S	S	S	S	S	S	R	S	S
ST62669	S	S	S	S	S	S	S	R	S	R
ST63231	S	S	S	S	S	S	S	R	S	S
ST63586	S	S	S	S	S	S	S	R	S	S
ST63761	S	S	S	S	S	S	S	S	S	S
ST63867	S	S	S	S	S	R	S	R	R	S
ST63933	S	S	S	S	S	S	S	R	S	S
ST64152	S	S	S	S	S	S	S	R	R	S
ST64160	S	S	S	S	S	S	S	R	S	S
ST64281	S	S	S	S	S	S	S	R	S	S
ST64633	S	S	S	S	S	S	S	R	S	S
ST65244	S	S	S	S	S	S	S	R	R	S
ST65370	S	S	S	S	S	S	S	R	S	S
ST65506	S	S	S	S	S	S	S	R	S	S
ST65690	S	S	S	S	S	S	S	R	R	S
ST66184	S	S	S	S	S	S	S	R	S	S
ST66310	S	S	S	S	S	S	S	R	S	S
ST66989	S	S	S	S	S	S	S	R	S	S
ST67254	S	S	S	S	S	S	S	R	S	S

AMX: Amoxycillin, C: Chloramphenicol, COT: Cotrimoxazole, CFM: Cefixime, CTR: Ceftriaxone, OF: Ofloxacin, CIP: Ciprofloxacin, NA: Nalidixic acid, AZM: Azithromycin, GEN: Gentamicin, S: Sensitive, R: Resistant

D. TABLE FOR ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA* PARATYPHI A

Isolate code	Antibiotic used									
	AMX	C	COT	CFM	CTR	OF	CIP	NA	AZM	GEN
SP24	S	S	S	S	S	S	S	R	S	S
SP186	S	S	S	S	S	S	S	R	S	S
SP625	S	S	S	R	S	S	S	R	S	S
SP690	S	S	S	S	S	R	R	R	S	S
SP1590	S	S	S	S	S	S	S	R	S	S

SP2713	S	S	S	S	S	S	S	R	S	S
ST3034	S	S	S	S	S	S	S	R	S	S
SP3230	S	S	S	S	S	S	S	R	S	S
SP3403	S	S	S	S	S	S	S	R	S	S
SP3803	S	S	S	S	S	S	S	R	S	S
SP3903	S	S	S	S	S	S	S	R	S	S
SP4656	S	S	S	S	S	S	S	R	S	S
SP5656	S	S	S	S	S	S	S	R	S	S
SP60297	S	S	S	S	S	S	S	S	S	S
SP60761	S	S	S	S	S	S	S	R	R	S
SP61202	S	S	S	S	S	S	S	R	R	S
SP61408	S	S	S	S	S	S	S	R	S	S
SP61745	S	S	S	S	S	S	S	R	S	S
SP62056	S	S	S	S	S	S	S	S	S	S
SP62176	S	S	S	S	S	S	S	R	S	S
SP62312	S	S	S	S	S	S	S	S	S	S
SP62579	S	S	S	S	S	S	S	R	S	S
SP62918	S	S	S	S	S	S	S	R	S	S
SP63089	S	R	S	S	S	S	S	R	S	S
SP63369	S	S	S	S	S	S	S	R	S	S
SP64088	S	S	S	S	S	S	S	R	R	S
SP64156	S	S	S	S	S	S	S	R	R	S
SP64935	S	S	S	S	S	S	S	R	S	S
SP66651	S	S	S	S	S	S	S	R	S	S
SP67176	S	S	S	S	S	S	S	R	S	S

AMX: Amoxycillin, C: Chloramphenicol, COT: Cotrimoxazole, CFM: Cefixime, CTR: Ceftriaxone, OF: Ofloxacin, CIP: Ciprofloxacin, NA: Nalidixic acid, AZM: Azithromycin, GEN: Gentamicin, S: Sensitive, R: Resistant

APPENDIX G

DATA ANALYSIS (CHI SQUARE TEST)

Association of disease with gender

Sex	Blood culture result		Total
	Negative	Positive	
Female	502	40	542
Male	614	46	660
Total	1116	86	1202

There was no significance association of presence of enteric fever with gender of the patient (p=0.784).