CHAPTER I

1. INTRODUCTION

1.1 BACKGROUND

Enteric fever (more commonly termed typhoid fever) is caused by *Salmonella enterica* subspecies *enterica* serovar Typhi and also, to a lesser extent, strains of *Salmonella enterica* subspecies *enterica* serovar Paratyphi (*S*. Paratyphi) A, B and C. *Salmonella* species have a wide spread distribution in the environment and certain host factors make human particularly susceptible to infection (Owens *et al.,* 2008).

Enteric fever most commonly begins with ingestion of bacteria in contaminated food or water but direct contact with human carrier and animal has also been implicated. About 1-5 % of patients, depending on age, become chronic carriers harbouring *S*. Typhi in gall bladder (WHO, 2003).

Typhoid fever is alone an important public health problem in many developing countries. It has been estimated that 33 millions of cases and more than half a million deaths occur throughout the developing world annually (Yap and Puthucheary, 2009). More than 90% morbidity of the disease is in Asia (Crump *et al.,* 2004). Enteric fever remains major health related problems in those underdeveloped countries where sanitary conditions are poor. It affects local inhabitants as well as travellers to the endemic areas such as Indian subcontinent, Southeast and Far East Asia, the Middle East, Africa, Central America and South America (Corales, 2004).

In Nepal, typhoid fever is prevalent in mountains, valleys and southern belts as an endemic disease with its peak incidence in May to August (Amatya *et al.,* 2007). Typhoid fever is one of the leading diagnosed fevers in most of the hospitals in Nepal (Hale, 1999; Rauniar *et al.,* 2000). Typhoid outbreak was responsible for the deaths of many Nepalese from the time it was known as one of the causes of the fever. However, typhoid fever was considered responsible for deaths of many patients admitted in hospitals in Kathmandu, the capital of Nepal, in late 1960s when the National Public Health Laboratory (NPHL) came into being (Sharma *et al.,* 2002).

Typhoid fever is characterized by the classic prolonged fever, sustained bacteraemia without endocardial or endothelial involvement, and bacterial invasion of and multiplication within mononuclear cells of liver, spleen, lymph nodes, and Payer's patches of ileum (Chart *et al*., 2007). There are four major syndromes, each with its own diagnostic and therapeutic problems. These are enteric fever, gastro-enteritidis, bacteraemia with or without metastatic infection and asymptomatic carrier state (Chart *et al*., 2006).

Investigators from the US Centers for Disease Control and Prevention estimate that there are 21.6 million typhoid cases annually, with the annual incidence varying from 100 to 1000 cases per 100 000 population. The global mortality estimates from typhoid have also been revised downward from 600 000 to 200 000, largely on the basis of regional extrapolations (Bhutta, 2006).

Salmonella spp. can usually be isolated from blood in 70-90% of the patients during the first ten days of infection and about 30% of the patients during the third week. The organisms can be isolated from 40-50% of patients during the second week of infection and from about 80% of the patients during the third week in faeces. In 25% of the patients, *Salmonella* spp. can be isolated in the urine in second week but usually urine culture is done on fourth week of infection for the isolation of *Salmonella* spp. (Cheesbrough, 1984).

Bone marrow aspirate culture is the gold standard for the diagnosis of typhoid fever and is particularly valuable for patients who have been previously treated, having long history of illness and for patients having negative blood culture with the recommended volume of blood (WHO, 2003).

For patients with enteric fever, administration of an effective antibiotic should begin as soon as clinical diagnosis is made, without recourse to results of antimicrobial sensitivity tests. After the introduction of Chloramphenicol in 1984, the fatal disease was readily treatable. However, resistance to Chloramphenicol started to develop within two years of drug introduction. Outbreaks of Chloramphenicol resistant *Salmonella* spp. occurred in Mexico, India, Vietnam, Thailand, Korea and Peru. These strains were also found to be resistant to Sulfonamide, Tetracycline and Streptomycin-amoxycillin and Trimethoprim-sulphamethoxazole were effective alternatives till the end of 1990's when strains resistant to all the first line anti- *Salmonella* drugs used at that time were reported (Thong *et al*., 2000, Connerton *et al*., 2000 and Mirza *et al*., 2000).

Threllfall and Ward (2001) reported that *Salmonella* Typhi with decreased sensitivity to Ciprofloxacin is endemic in several Asian countries, and suggested third generation Cephalosporins such as Ceftriaxone or Cefotaxime as possible alternatives since in the study all stains were sensitive to these drugs.

The present study was conducted to isolate and characterize *Salmonella* serovars from patients suspected of enteric fever and to determine the antibiotic susceptibility pattern of the isolates.

1.2 OBJECTIVES

1.2.1 GENERAL OBJECTIVE

To isolate and characterize *Salmonella* serovars from patients suspected of enteric fever and to determine the antibiotic susceptibility pattern of the isolates.

1.2.2 SPECIFIC OBJECTIVES

1) To isolate and characterize *Salmonella* serovars from patients suspected of enteric fever.
2) To determine the prevalence of enteric fever in different age groups in relation to gender.
3) To determine the antibiotic susceptibility pattern of the isolates.

CHAPTER II

2. LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF ENTERIC FEVER

The bacterial genera *Salmonella* can cause a wide spectrum of clinical illness, of which there are four major syndromes, each with its own diagnostic and therapeutic problems which are best considered separately. They include enteric fever, gastroenteritis, bacteraemia with or without meta-static infection and the asymptomatic carrier state (Greenwood *et al*., 2000).

Enteric fever is a generalized acute infectious disease characterized by cyclic course, definitive temperature curve, general intoxication, bacteraemia and affection of the lymphatic apparatus of the small intestine through which the infection implants itself in the organism upon entrance of the causative agent into gastrointestinal tract (Bunin, 1980). Enteric fever is potentially life threatening systemic illness characterized by high fever and abdominal complaints. The term enteric fever encompasses both typhoid and paratyphoid fever (Kumari and Ichhpujani, 2000). Typhoid and paratyphoid fever are clinically and pathologically similar, the distinction between them being a bacteriological one. However, paratyphoid fever is clinically milder and of short duration than typhoid (Lesser and Miller, 2003).

Enteric fever is a septicaemic disease caused by member of certain *Salmonella* serotypes such as Typhi, Paratyphi A, Paratyphi B, Sendai (Old, 1990). Certain other *Salmonella* serotypes that cause septicaemia also frequently give rise to pyaemic lesion in the internal organ. The chief of these are member of serotypes Choleraesuis, Paratyphi C, Dublin and certain rare serotypes related to Enteritidis. The distinction between *Salmonella* septicaemia or enteric fever and *Salmonella* pyaemia is useful but by no means absolute and some of the common effects of typhoid fever arise from localization of bacteria in individual organs (Parker and Collier, 1990).

An estimated 12.5 million cases of typhoid fever occur globally each year and it is a significant cause of morbidity and mortality worldwide. Case fatality rates of 10-50% have been reported from endemic countries when diagnosis is delayed or in cases of severe typhoid fever not treated with high dose of corticosteroid therapy and antibiotics (Corales, 2004).

In India the annual incidence rate was as high as 760/100000; 980/100000 in Delhi alone (Sinha *et al*., 1995). In Indonesia there were a mean of 900000 cases per year and more than 20000 deaths; 3-19 years old accounted 91% of cases with an attack rate of blood culture positive enteric fever of 1026 per 100000 per year (Ivanoff *et al*., 1994).

It is difficult to estimate the world wide impact about enteric fever because the clinical picture resemblances many other febrile infections and because of the limited capacity for bacteriological diagnosis in most areas of the developing countries owing to lack of man power, fund and other facilities (Ivanoff *et al*., 1994).

In developed countries, the incidence of cases and death has been decreased by a combination of improved sanitation, hygiene, vaccination and effective antimicrobial therapy. Improved sanitation and hygiene are difficult to implement in many developing countries. The effectiveness of antimicrobial therapy is also being a challenge by the emergence of antibiotic resistance (Chandra, 1992; Mirza, 1995).

Global incidence is about 0.5% but incidence rate as high as 2% have been reported in hot spots such as Indonesia and Paupa New Guinea, where typhoid fever ranks among five most common cause of death (Curtis, 2005).

2.2 HISTORY

Although cases of typhoid fever were known in early in antiquity, the first correct clinical description on the disease was made by the French physician Britannia only in 1813 while the course of the disease was set forth in detailed by PCA Louis in his monograph in 1829. He first named the *Salmonella* bacillus as "typhoid" meaning "typhus-like", derived from the Greek word "typhus" meaning "smoke" which refers to the apathy and confusion associated with the fully developed clinical syndrome of typhoid fever (Bunin, 1980; Old, 1990).

In 1880 KJ Ebert and NP Solomon for the first time described the causative agent of typhoid fever they had discovered in the histological sections of mesenteric lymph nodes and spleen but the pure culture of typhoid bacillus was first isolated by GTA Gaffkey only in 1884 (Bunin, 1980; Thong and Hoffman, 1998). Hence this bacillus is also called as Eberth-Gaffkey bacillus or Eberthela typhi. In 1886 William Budd concluded from his astute clinical observation that typhoid fever was transmitted from patient to patient and it was water borne disease (Bunin, 1980; Old, 1990).

In 1896 Archard and Bensaude isolated *Salmonella* Paratyphi B and first used the term paratyphoid fever. In the year 1896 itself, Wright from England and Pfeiffer from Germany described Widal test along with the development of first typhoid vaccine (Agarwal *et al*., 2004). Salmon and Smith in 1885 together isolated the American hog cholera bacillus (*Salmonella* Cholerasuis). Hence the term *Salmonella* is derived from first author Salmon (Old, 1990).

2.3 HABIT AND HABITAT

Salmonellae are primarily intestinal parasites of man and animals, both domestic and wild. They may also be isolated from their blood and internal organs. They are frequently found in sewage, river and other waters and soil in which they do not multiply significantly. Provided the environmental conditions are suitable, they may survive for weeks in waters and for years in soil. Some serotypes are adapted to specific hosts; e.g. Abortusovis, Gallinarum, Typhi and Typhisuis are confined respectively to sheep, fowl, man and swine (or closely related species). The majority, however, do ubiquitous serotypes inhabit a wide range of hosts (Old, 1990).

Salmonellae are Gram-negative bacilli, non-acid fast, non-capsulated, non-sporing bacilli, most serotypes are motile with peritrichous flagella, but *Salmonella* Gallinarum and *S.* Pullorum are non-motile variants (OH-O variation) are occasionally found in other serotypes, most strains of most serotypes form type-1 (mannose sensitive, haemagglutinating) fimbriae; *S.* Gallinarum, *S.* Pullorum and a few strains of other serotypes form type-2 (non-haemagglutinating) fimbriae and most *S.* Paratyphi A strains are non-fimbriate (Janda and Abbott, 2005).

Salmonella enterica serotype Typhi, *Salmonella enterica* serotype Paratyphi 'A' and 'B' and *Salmonella enterica* serotype Cholerasuis are primarily infective for humans and infections with these organisms implies acquisition from a human source (Brooks *et al*., 2002).

S. Typhi can be identified in the laboratory by several biochemical and serological tests. One of the most specific is that of polysaccharide capsule Vi, which is present in about 90% of all freshly isolated *S.* Typhi and has a protective effect against the bactericidal action of the serum of infected patients. This capsule provides the basis for one of the commercially available vaccines. Vi antigen is present in some other bacteria (*Citrobacter freundii*, *Salmonella* Paratyphi C and *Salmonella* Dublin) but not in exactly the same genetic context.

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 (Bhutta, 2006)

Typhi: It is the cause of typhoid fever in man and is very rarely isolated from other animals which it does not appear to infect under natural conditions (Old, 1990). It is serologically positive for LPS antigen O9 and O12, protein flagellar antigen H and polysaccharide capsular antigen V_i (Agarwal *et al.*, 2004).

Paratyphi A: It was first isolated by Gwyn in 1898. *Salmonella* Paratyphi A is an important cause of enteric fever in Asia, the Middle East, Africa and South America. It occurs naturally in H1 phase. Among subspecies-I salmonellae, it is unusual in being H_2S negative, xylose non-fermenting, lysine-decarboxylase negative and often anaerogenic (Ewing, 1969).

Paratyphi B: *Salmonella* Paratyphi B has been isolated from cases of enteric fever in man. Some strains lack O antigen 5. Two broad categories of strain exist: (1) strains which cause an enteric-type fever, form a mucoid (slime) wall, do not ferment D-tartarate, are diphasic and are rarely isolated from animals, i.e. the group called '*Salmonella* Paratyphi B' by Kauffmann; (2) strains which cause an acute but mild enteritis, do not form a mucoid wall, are D-tartarate fermenting and are not uncommonly isolated from animals and food (Old, 1990).

Choleraseuis: When first isolated by Salmon and Smith (1885), it was thought to be the agent of hog cholera, which is now known to be a virus disease. The natural reservoir of this host-adapted serotype is pigs in which it commonly causes enteritis, though generalized infections also occurs. It is occasionally isolated from other animals, including man, for whom it is an important pathogen, not because it gives rise to many infections, but because it is highly invasive and its effects are severe. About 50% of recorded human infections are associated with prolonged pyrexia, usually of the septic type. Over one-third result in local pus formation and about 20% are fatal. The common pyaemic manifestations include pneumonia, septic arthritis, osteomyelits, meningitis and endocarditis (Old, 1990).

2.4 ANTIGENIC STRUCTURE OF *SALMONELLA* SPP.

O (Somatic) Antigens: These somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide layer on the surface of the bacterial cell wall. They are hydrophilic and enable the bacteria to form stable, homogenous

suspensions in saline (0.85% NaCl) solution. Over 60 different O antigens have been recognized and they are designated by Arabic numerals. The O antigens are heat stable, being unaffected by heating for 2.5 hours at 100°C and alcohol-stable withstanding treatment with 96% ethanol at 37°C for 4 hours. The O antigens are unaffected by suspension of the bacteria in 0.2% formaldehyde but if flagella are present; their fixation by the formaldehyde renders the bacteria inagglutinable by O antibodies. The O antigens are liable to be changed in character by form variation and lysogenic conversion and to be lost from the bacteria in S → R mutation (Old, 1996).

H (Falgellar) Antigens: These antigens represent determinant groups on the flagellar protein. They are heat-labile and alcohol-labile but are well-preserved in 0.04-0.2% formaldehyde. Heating at temperatures above 60°C detaches the flagella from the bacteria and detachments of all flagella are achieved by heating for 30 minutes at 100°C. The deflagellated bacteria are inagglutinable by H antibodies but the detached flagella remain immunogenic and suspensions of bacteria to be used for production of O antisera should be freed from detached flagella by centrifugation and washing or by inactivation by heating for 2.5 hours at 100°C (Old, 1996).

In many salmonellae i.e. other than serotypes of subspecies IIIa, IV and V, the production of flagellar antigens is diphasic, each strain varying spontaneously and reversibly between two phases with different sets of H antigens. In phase 1, the bacteria from flagella with one or more antigens form a set of over 70 antigen designated by the small letters of the alphabet 'a to z', a series that is complete except for 'j' then z_1, z_2, z_3, etc. In phase 2, the bacteria form flagella with one or more antigens from a mainly different set of antigens. The first discovered of these were designated by Arabic numerals (not implying any relationship with the similarly numbered O antigens) but later certain phase-1 antigens, especially e, n, x, z, l and w were found in the phase 2 of some serotypes. Phase 2 used to be termed the 'group' or 'non-specific' phase because numerous serotypes of salmonellae share the same antigens when in this phase. The presumptive identification of serotypes therefore mainly depends on the identification of the H antigens in phase 1 which are relatively specific (Old, 1996).

2.4.1 OTHER SURFACE ANTIGENS

Although the serotype of an enterobacterium is defined mainly by its O and H antigens, there may be other antigens at the bacterial surface that determine agglutination with homologous antibodies. These include the capsular or K antigens including the Vi antigen; the slime (mucus) or M antigen and the fimbrial or F antigens. Such antigens may cause difficulty in

the serological identification of bacteria either by masking the O antigens so that the bacteria are inagglutinable by O antibodies or by causing non-specific cross-reaction due to their presence in unrelated bacteria (Old, 1996).

Vi Antigen: The Vi antigen is a surface polysaccharide of beta (1-4) linked N-acetyl-D-galactosaminuronic acid, the C_3 groups of which are variably acetylated. Almost all recently isolated strains of Typhi form Vi antigen as a covering layer outside their cell wall. This heat-labile antigen is an acidic polysaccharide. When fully developed, it renders the bacteria agglutinable by Vi antibody and inagglutinable by O antibody. Freshly isolated strains of Typhi rich in Vi antigen (V forms) produce more opaque colonies than strains lacking Vi antigens (W forms). Vi rich strains maintained by subculture on conventional media re-rapidly replaced by spontaneously originating Vi deficient mutants (V $\rightarrow$ W variation) (Old, 1996).

M Antigen: This antigen is a loose extra-cellular polysaccharide slime consisting of colanic acid. It occurs in a serologically similar form in various unrelated enterobacteria including serotypes of *Salmonella* and many strains of *Escherichia coli* and resembles the Vi antigen in preventing agglutination by O antibodies. Heating for 2.5 hours at 100°C removes the M antigen and renders the bacteria agglutinable by O antiserum (Old, 1996).

Fimbrial Antigen: The antigenic determinants of the type-1 fimbriae of salmonellae are unrelated to those of most other Enterobacteriaceae. Five type-1 fimbrial antigens were described by Duguid and Campbell (1969). The type-1 fimbriae of *Salmonella* serotypes from different subspecies shared common fimbrial antigen (1). Strains of the same serotype possess the same type-1 fimbrial antigens but that from different serotypes possesses one or more different additional fimbrial components (2-5). Thus, type-1 fimbrial determinants of some representative serotypes are: Montevideo (1 only), Senftenberg (1, 2), Paratyphi B and Typhimurium (1, 2, 3), Cholerasuis (1, 2, 4), Enteritidis (1, 2, 5), Typhi (1, 5) and Newport (1, 2, 4, 5). There was some sharing of type-1 fimbrial antigens between salmonellae and *Citrobacter freundii* but not with *Citrobacter amalonaticus* or other type-1 fimbriae Enterobacteriaceae. Type-1 fimbrial antigen is synonymous with X-antigen of earlier workers (Old, 1990).

R Antigen: In S $\rightarrow$ R mutation the O antigens are lost and new 'R' antigens are revealed at the bacterial surface. Mutational loss of an enzyme required for the formation of one of the links in the polysaccharide core or side chains of the cell-wall lipopolysaccharide leads to an absence of the hydrophilic side chains that determine O antigen specificity. The exposed incomplete (R_I) or complete (R_{II}) core polysaccharide constitutes the R antigen. The R

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

2.4.2 ANTIGENIC VARIATION

Variation in the O-antigen

a) Rough mutant strains with defects in the biosynthesis of the O polysaccharide lack the specific side chains responsible for the O specificity and some of them have additional abnormalities of the core structure. They can be classified in a series from Ra, which lacks the side chains only, through Rb to Re which show a progressive loss of sugar constituents from the core. The term 'semi rough' has been applied to organisms that form less than the normal number of repeating units. Rough mutant strains are not agglutinated by homologous O antiserum (Old, 1990).

b) Form variation: Form variation is a spontaneous reversible variation in the amount of one of the O antigens e.g. factor 1, 6_1, 12_2, 22, 23, 24 or 25. Different amounts of the antigen are found in different colonies in plating from the culture (Old, 1996).

c) Lysogenic Conversion: The specificities of O antigens may be modified in the process of lysogenic conversion by phages which genetically determine subtle changes in the chemistry of the repeating unit of the polysaccharide. The new specificities appear in salmonellae within minutes of their infection with phage (Lindberg and Le Minor, 1984).

Variation in the H-antigens

a) Non-motile (Mot^-) strains of salmonellae may occasionally be found with flagella that are antigenically and morphologically like those of motile parent strains. The failure of Mot^- strains to rotate the flagella is thought to be associated with defects in the mechanism of energy transduction. Flagellate salmonellae sometimes give rise to non-flagellate H^- strains. This kind of change observed in both laboratory and field strains and originally described as the OH→H variation results from defects in *fla*, *flb* genes (MacNab, 1987).

b) Diphasic variation: Most serotypes of salmonella of subspecies other than IIIa, IV and V express alternately 2 kinds of H flagellar specificity; this phenomenon of diphasic variation was first demonstrated by Andrews in 1922. The genera possess two set of gene coding for flagellar antigen viz. H_1 coding for phase 1 flagellar antigen and H_2 encoding for phase 2 flagellar antigens (Old, 1996).

2.5 THE KAUFFMANN-WHITE SCHEME CLASSIFICTION

This system of classification scheme, first developed in 1934, classifies the Salmonellae into different O groups, O serogroups, each of which contains a number of serotypes possessing a common O antigen not found in other O groups. The O groups first defined were designated by capital letters A to Z and those discovered later by the number (51-67) of the characteristic O antigen (Old, 1996).

It is now considered more correct to designate each O group by its characteristic O factor i.e. to abandon the letters A-Z used to designate early O groups. Hence, O groups become: O2 (A), O4 (B), O7 (C_1), O8 (C_2-C_3), O9, 12 (D_1), O9, 46 (D_2), O3, 10 (E_1) etc. Groups O2 to O3, 10 (A-E_1) contain nearly all the salmonellae that are important pathogens in man and animals (Popoff and Le Minor, 1992). Within each O group the different serotypes are distinguished by their particular H antigen or combination of H antigens (Old, 1996).

2.6 PATHOGENESIS OF ENTERIC FEVER

Studies performed in human volunteers suggest that infection may be inoculum dependent. In healthy previously unvaccinated men, ingestion of 10^5 organisms led to the clinical disease in 25% of volunteers, ingestion of 10^7 organisms caused disease in 50% and 10^9 organisms caused diseases in 95%. As the number of organism increase, the incubation period decrease. Although the infectious dose varies among strains, a large inoculum is thought to be necessary to overcome stomach acidity and to compete with normal intestinal flora. However, lower infectious doses may be adequate to cause infection if these organisms are co-ingested with foods that rapidly transit the stomach (e.g. liquids) or that raise gastric pH (e.g. cheese, milk) if antacids are used concomitantly or if these organisms are ingested by individuals with impaired immune systems (Corales, 2004).

The M cell, epithelial cell that overlie the Peyer's patches are the potential sites where the bacilli is internalized and transported to underlying lymphoid tissue. Once phagocytosed, the bacteria are protected from polymorphoneuclear leukocytes, the complement system and the

acquired immune response. Salmonellae have evolved mechanisms to avoid or delay killing by macrophages. Upon phagocytosis, the bacteria form a "spacious phagosomes" and alter the regulation of ~200 bacterial proteins. The best characterized regulatory system is PhoP/PhoQ, a two component regulon that sense changes in bacterial location and alters bacterial protein expression. The alterations mediated by PhoP/PhoQ include modification in LPS and in the synthesis of outer membrane protein; these changes presumably remodel the bacteria's outer surface such that the organisms can resist microbicidal activities and possibly alter host cell signalling. PhoP/PhoQ also mediates the synthesis of divalent cationic transporters that scavenges magnesium (Lesser and Miller, 2003).

Virulence factors of salmonellae are complex and encoded both on the organism's chromosome and large plasmids that are 34-120 kd. Some areas of active investigation include the means by which salmonellae attach to and invade the intestine, survive within phagosomes, effect massive efflux of electrolytes and water into the intestinal lumen and develop drug resistance (Zapor, 2002).

During this bacteraemic phase, the microbes may invade any organ but most commonly found in reticuloendothelial tissue of liver, spleen, bone marrow and gall bladder and Peyer's patch of terminal ileum. The gall bladder is infected through liver and resultant cholesystitis is subclinal. Invasion of Peyer's patch occur during either primary bacteraemia or secondary bacteraemia and further seeding occurs through infected bile. The Peyer's patch become hyperplastic with infiltration of chronically inflamed cell which may lead to necrosis of superficial layer and ulcer formation with potential haemorrhage from blood vessel erosion or peritonitis from transmural perforation. The pathogenesis of prolonged fever and toxaemia of enteric fever is not well understood. Pyrogen and mediators produced at the sites of inflammation have been postulated as factor responsible for the prolonged fever (Corales, 2004).

2.7 TRANSMISSION OF ENTERIC FEVER

Transmission of salmonellae to susceptible host usually occurs from food and beverages handled by chronic carrier who sheds bacteria in stool or less commonly urine. Transmission also occurs from sewage-contaminated water or shellfish especially in the developing world (Ram *et al*., 2007), hand to mouth transmission after using contaminated toilets and neglecting hand hygiene (WHO, 2003).

Infective doses of as small as 10^5 organisms can cause infection in more than 50% of healthy volunteers. Investigations of outbreak suggest that in natural infection the infective dose might be below 10^3 viable organisms (Chart *et al*., 2006).

2.8 CLINICAL DIAGNOSIS OF ENTERIC FEVER

Typhoid fever begins 7-14 days after ingestion of *S*. Typhi. The fever pattern is stepwise, characterized by a rising temperature over the course of each day that drops by subsequent morning. The peaks and troughs rise progressively over time. Over the course of the first week of illness, the notorious gastrointestinal manifestations of the disease develop. Monocytic infiltration inflames Peyer's patches and narrows the bowel lumen, causing constipation that lasts the duration of the illness. The individual then develops a dry cough, frontal headache, delirium, and malaise (Christie, 1987; Brusch *et al*., 2008).

At the end of first week of illness, the fever plateaus at 103-104°F (39-40°C). The patient develops rose spots, which are salmon-colored, blanching, truncal and usually 1-4 cm wide; these generally resolve within 2-5 days (Christie, 1987; Brusch *et al*., 2008).

In the third week, the febrile individual grows more toxic and anorexic with significant weight loss. Some patients experience foul, green-yellow, liquid diarrhea. Necrotic Peyer's patches may cause bowel perforation and peritonitis (Rahaman *et al*., 1977; Cunha *et al*., 2005).

If the individual survives to the fourth week, fever, mental state, and abdominal distension slowly improve. Intestinal and neurologic complications may still occur in surviving untreated individuals. Some survivors become asymptomatic carriers and have potential to transmit the bacteria indefinitely (Christie, 1987; Parry *et al*., 2002; Dutta *et al*., 2001; Cunha *et al*., 2005).

2.8.1 ACUTE NON-COMPLICATED DISEASE

Acute typhoid fever is characterized by prolonged fever, disturbances of bowel function (constipation in adults, diarrhoea in children), headache, malaise and anorexia. Bronchitic cough is common in the early stage of the illness. During the period of fever, up to 25% of patients show exanthem (rose spots) on the chest, abdomen and back (WHO, 2003).

2.8.2 COMPLICATED DISEASE

Acute typhoid fever may be severe. Depending on the clinical setting and the quality of available medical care, up to 10% of typhoid patients may develop serious complications. Since the gut-associated lymphoid tissue exhibits prominent pathology, the presence of occult blood is a common finding in the stool of 10-20% of patients, and up to 3% may have melena. Intestinal perforation has also been reported in up to 3% of hospitalized cases. Abdominal discomfort develops and increases. It is often restricted to the right lower quadrant but may be diffuse. The symptoms and signs of intestinal perforation and peritonitis sometimes follow, accompanied by a sudden rise in pulse rate, hypotension, marked abdominal tenderness, rebound tenderness and guarding, and subsequent abdominal rigidity. A rising white blood cell count with a left shift and free air on abdominal radiographs are usually seen. Altered mental status in typhoid patients has been associated with a high case-fatality rate. Such patients generally have delirium or obtundation, rarely with coma (WHO, 2003).

Typhoid meningitis, encephalomyelitis, Guillain-Barré syndrome, cranial or peripheral neuritis, and psychotic symptoms, although rare, have been reported. Other serious complications documented with typhoid fever include haemorrhages (causing rapid death in some patients), hepatitis, myocarditis, pneumonia, disseminated intravascular coagulation, thrombocytopenia and haemolytic uraemic syndrome. In the pre-antibiotic era, which had a different clinical picture, if patients did not die with peritonitis or intestinal haemorrhage, 15% of typhoid fever cases died with prolonged persistent fever and diseases for no clear reason. Patients may also experience genitourinary tract manifestations or relapse, and/or a chronic carrier state may develop (WHO, 2003).

2.8.3 CARRIER STATE

1-5% of patients, depending on age, become chronic carriers harbouring *S.* Typhi in the gallbladder (WHO, 2003)

2.8.4 CASE DEFINITION

Confirmed case of typhoid fever: A patient with fever (38°C and above) that has lasted for at least three days, with a laboratory-confirmed positive culture (blood, bone marrow, bowel fluid) of *S.* Typhi (WHO, 2003).

Probable case of typhoid fever: A patient with fever (38°C and above) that has lasted for at least three days, with a positive serodiagnosis or antigen detection test but without *S.* Typhi isolation (WHO, 2003).

Chronic carrier: Excretion of *S.* Typhi in stool or urine (or repeated positive bile or duodenal string cultures) for longer than one year after the onset of acute typhoid fever. Short-term carriers also exist but their epidemiological role is not as important as that of chronic carriers. Some patients excreting *S.* Typhi have no history of typhoid fever (WHO, 2003).

2.9 LABORATORY DIAGNOSIS OF ENTERIC FEVER

2.9.1 SPECIMENS USED FOR DETECTION

Definitive diagnosis of enteric fever requires the isolation of *Salmonella* Typhi and *Salmonella* Paratyphi (A, B or C). Cultures of blood, stool, urine, aspirate from rose spots, bone marrow and gastric or intestinal secretions may each be useful in establishing the diagnosis (Pillay *et al.*, 1975). For the detection of enteric infection following types of specimens are used:

Blood: Organisms can usually be detected in 75-90% of patients during the ten days of infection and in about 30% of patients during the third week (Cheesbrough, 1984).

Faeces: 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, 1984).

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

2.9.2 MICROBIOLOGICAL PROCEDURE

Blood culture system comprise (1) aseptic collection, (2) culture of this in a liquid medium to allow organisms present in small numbers to multiply, (3) a system or combination of systems to detect them and (4) a final phase of subcultures so that the bacteria can be identified and their antibiotic susceptibility determined (Phillips and Eykyn, 1990).

SPECIMEN COLLECTION

Blood must be collected with sterile disposable syringe. The vein from which blood is to be collected must be chosen before skin is cleaned with 70% ethanol followed by tincture of iodine solution. It is less desirable to draw blood through a vascular shunt of catheter because these prosthetic devices are difficult to decontaminate completely (Forbes *et al.,* 2002).

i). Timing of blood collection: The number of bacteria found in the blood varies from time to time in the same patient and it is clearly desirable to take sample when many are present (Stokes *et al.,* 1993). If a regular periodicity of fever can be established, then the most appropriate time to draw blood culture will be just before raise in the temperature. Blood cultures should always be collected before antibiotic treatment has begun (Reimer *et al.,* 1997).

ii). Volume of blood: Because the number of bacteria per ml of blood is usually low, it is important to take reasonable quantity of blood. Ten ml per veinpuncture for adults, 2-5 ml may suffice for children, who usually have higher levels of bacteraemia for infants and neonates, 1-2 ml is often the most that can be obtained (Vandepitte *et al.,* 2004).

TYPES OF BROTH MEDIA

The most frequently used liquid media are either nutrient, glucose, Tryptic Soya or Brain Heart Infusion Broth but some laboratories use bile salt broth for enteric fever (Phillips and Eykyn, 1990).

BLOOD DILUTION

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

ANTICOAGULANT IN BROTH MEDIA

The anticoagulant in blood culture media must not harm the bacteria and must prevent clotting of the blood which would entrap the bacteria and prevent their detection. The most commonly used preparation in blood culture media is 0.025-0.03% solution of Sodium Polyanethol Sulphonate (SPS). In addition to its anticoagulant properties, SPS is also anticomplementary, antiphagocytic and interferes with the activity of certain antimicrobial agents, notably the aminoglycosides (Forbes *et al.*, 2002).

If blood is immediately added to a sufficient volume (50 ml) of broth and thoroughly mixed to prevent clotting, no anticoagulant is needed. It is recommended that blood culture bottles be available at hospitals and major health centres (Vandepitte *et al.*, 2004).

SUBCULTURE FROM BLOOD CULTURES

The most important recent advances in blood culture technology have been in methods for the detection of growth in the liquid medium after it has been incubated at 37°C. Such growth may be visible as turbidity or as colonies and with haemolytic organisms, the blood may be haemolysed. However, the most sensitive means of detection is subculture of a small volume on appropriate solid medium (Phillips and Eykyn, 1990).

Initial subculture may include chocolate agar, 5% sheep blood agar, MacConkey agar, anaerobic supplemented blood agar and a selective medium for *Bacteroides* spp., such as bacteroides-bile aesculin agar. Bottles are then re-incubated for 5-7 days unless the patient's conditions require special consideration (Forbes *et al.*, 2002).

IDENTIFICATION

The isolated organisms are identified by Gram staining procedure, biochemical tests and agglutination tests (Chakraborty, 1998).

2.9.3 INSTRUMENT BASED SYSTEMS

Conventional blood culture techniques are labour intensive and time consuming. During these times of cost constraints in health care and a corresponding requirement for clinically relevant care, the development of instrumentation for blood cultures has been accomplished. Instruments can rapidly and accurately detect organism in blood specimens. By using such

instrumentation, laboratories that process many blood cultures can also provide results cost effectively (Forbes *et al.*, 2002). Some of them are:

BACTEC SYSTEMS: Many laboratories use the BACTEC system (Becton Dickinson Microbiology Systems, Md.), which measures the production of carbon dioxide (CO_2) by metabolizing organisms. Blood or sterile body fluid for routine culture is inoculated into bottles that contain the substrates (Forbes *et al.*, 2002).

Bac T/ALERT MICROBIAL DETECTION SYSTEM: Other laboratories use the Bac T/Alert System (Organon Teknika, Durham, NC) which measures CO_2 derived pH changes by a colorimetric sensor in the bottom of each bottle (Forbes *et al.*, 2002).

ESP SYSTEM: The ESP Culture System II (Trek Diagnostic Systems, Inc, Westlake, Ohio) differ the other previously discussed systems in that microbial growth is detected by the consumption and/or production of gases as organisms metabolize nutrients in the culture medium (Forbes *et al.*, 2002).

VITAL: Another continuous monitoring blood culture system is the Vital (bioMerieux, Inc., Hazelwood, Mo.,USA). A fluorescent molecule that decreases its fluorescent output in the presence of CO_2, changes in pH or modification of oxidation-reduction is incorporated in the broth solution and serves as an indicator, detecting any organism present in the culture (Forbes *et al.*, 2002).

ANTIMICROBIAL SUSCEPTIBILITY TEST

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

Once a pathogen has been isolated and its antibiotic susceptibility is known, definitive therapy should be given (Phillips and Eykyn, 1990). Laboratory monitoring of therapy is necessary and for which the minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) of the antimicrobial agent for the isolated organisms must be determined. The routine disc diffusion technique with a fixed concentration of drug is not adequate for guiding treatment of infective endocarditis. The measurement of MIC and MBC

of antibiotic helps to determine adequate dose of the antibiotic to be used for ensuring the serum levels that can penetrate the valves and kill the organisms (Chakraborty, 1998).

TREATMENT

For patients with enteric fever, administration of an effective antibiotic should begin as soon as clinical diagnosis is made without recourse to results of antimicrobial susceptibility tests. From 1948 to the mid-1970s, Chloramphenicol was the first-line drug of choice and in developed countries its use resulted in a reduction in mortality rates from 10-15% to less than 1%. After extensive outbreaks of typhoid fever occurred in Mexico and India in the early and mid-1970s in which epidemic strains were resistant to Chloramphenicol, the efficacy of this antimicrobial agent was in doubt (Threlfall and Ward, 2001).

Alternative drugs for enteric fever are Ampicillin and Trimethoprim. However, following outbreaks in the Indian subcontinent, the Arabian Gulf, the Philippines and South Africa in the late 1980s and early 1990s in which causative strains were resistant to Ampicillin and Trimethoprim in addition to Chloramphenicol, the efficacy of these antimicrobial agents has also been impaired (Rowe *et al*., 1997).

Ciprofloxacin, a Fluoroquinolone, was then introduced for the treatment of enteric fever. Physicians have also suggested another Fluoroquinolone "Ofloxacin" for the treatment of multi-drug resistant (MDR) isolates. Both drugs were found to be effective against MDR strains. However, several treatment failures with these Quinolones have been reported due to development of Fluoroquinolone resistance in *Salmonella* serotypes (Mandal *et al*., 2003).

Third generation Cephalosporins (Ceftriaxone, Cefixime, Cefotaxime and Cefoperazone) and Macrolides such as Azithromycin are also effective drugs for typhoid. With use of Ceftriaxone and Cefixime, the fever clearance time averaged one week and the rates of treatment failure were 5-10%. The relapse rates were 3-6% (Agarwal *et al*., 2004).

CONTROL

This is possible only by: Observing proper sanitation, ensuring a safe water supply, public health legislation designed to ensure uncontaminated food materials (Agarwal *et al*., 2004).

VACCINATION

There are three types of vaccines currently available against typhoid fever:

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

administered every three years if continued of renewed exposure is expected (Corales, 2004).

ii) Live oral vaccine (*Ty* 21a): This is an oral vaccine containing live attenuated "*S.* Typhi Ty21a" strains in an enteric-coated capsule. Although the mechanism is not known, it elicits both serum and cell-mediated immune response. It is recommended that the vaccine should not be given children younger than 6 years old and the optimal booster's dose has not been determined (Corales, 2004).

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

2.9.4 EMERGING DRUG RESISTANCE PROBLEM

The overuse and misuse of the antimicrobials have led to the death of the sensitive strains leaving resistant strains to survive, multiply and infect new hosts. Bacteria most likely to cause hospital infection are usually highly resistant to antimicrobials The fact that bacteria which are multiple drug resistant are uncommon outside the hospital means that they are not biologically dominant, without the aid of antibiotics to depress resident bacteria, they are unable to establish themselves (Stokes *et al.*, 1993).

The most frequent antimicrobial resistance mechanism in bacteria is plasmid mediated enzymatic inactivation. Plasmids are extra-chromosomal DNA that represents a reasonable stable but dispensable gene pool in bacteria. They impart considerable additional genetic information to their bacterial host. The kind of antibiotic inactivation is unique to R-plasmids (Hugo and Russel, 1993). The result of a study carried out by Khor and Jegathesan (1983) in Malaysia demonstrated that transferable resistant plasmids play a major role in antibiotic resistance in Enterobacteriaceae.

Chloramphenicol was used universally to treat typhoid fever from 1948 until 1970s, when widespread resistance occurred, Ampicillin and Trimethoprim-sulphamethoxazole (TMP-

SMZ) then became treatments of choice. However, in the late 1980s, some *S.* Typhi and *S.* Paratyphi A strains (multidrug resistant [MDR] *S.* Typhi and *S.* Paratyphi A) developed simultaneous plasmid-mediated resistance to all three agents (WHO, 2003).

Fluoroquinolones and third-generation Cephalosporins are currently the first-line treatments and yield a better cure rate than Cephlaosporins. However, resistance to Fluoroquinolones is widespread in some areas, and sporadiac resistance to Cephalosporins has been reported (Brusch *et al*., 2008).

Fluoroquinolone use has driven the clonal expansion of a Nalidixic acid-resistant serovar Typhi halotype, H58, in Southeast Asia, The emergence of resistance of serovar Typhi to Ciprofloxacin (6/149 isolates; 4%) in Nepal, together with reports of high-level Ciprofloxacin resistance in India and Bangladesh, might prelude to a worsening drug resistance problem in Asia. The rate of Fluoroquinolone resistance in Southeast Asia is generally high. Susceptibility to Cloramphenicol, TMP-SMZ, and Ampicillin in these areas is rebounding. In Southeast Aisa, MDR strains remain predominant (Chau *et al*., 2007).

2.10 MAGNITUDE OF ENTERIC FEVER

Enteric fever is a global health problem. Its real impact is difficult to estimate because the clinical picture is confused with those of many other febrile infections. Additionally, the disease is underestimated because there are no bacteriology laboratories in most areas of developing countries. These factors are believed to result in many cases going undiagnosed. It has been estimated that approximately 17 million cases of typhoid fever and 600 000 associated deaths occur annually (Ivanoff, 1994). Preliminary results from recent studies conducted in Bangladesh by ICDDRB show an incidence of approximately 2000 per 100 000 per year (Punjabi, 1998).

In Indonesia there is a mean of 900 000 cases per year with over 20 000 deaths. In Indonesia, people aged 3 to19 years accounted for 91% of cases of typhoid fever and the attack rate of blood-culture-positive typhoid fever was 1026 per 100 000 per year. A similar situation was reported from Papua New Guinea. When typhoid fever was highly endemic in certain countries in South America the incidence of clinical typhoid fever in children aged less than 3 years was low.

In Chile, however, single blood cultures for all children aged less than 24 months who presented at health centres with fever, regardless of other clinical symptoms, showed that 3.5% had unrecognized bacteraemic infections caused by *S.* Typhi or *S.* Paratyphi (Ferrecio *et al.*, 1984).

Between 1% and 5% of patients with acute typhoid infection have been reported to become chronic carriers of the infection in the gall bladder, depending on age, sex and treatment regimen. The propensity to become a carrier follows the epidemiology of gall bladder disease, increasing with age and being greater in females than in males. The role of chronic carriers as a reservoir of infection was studied in Santiago, Chile, where a crude rate of 694 carriers per 100 000 inhabitants was found (Levine *et al.*, 1982).

In a prospective study by Maskey *et al*. (2006) in Patan Hospital found confirmed cases of enteric fever in which 409 (67.16%) were typhoid fever cases and 200 (32.84%) were paratyphoid fever.

Faecal contamination of urban water supplies in Nepal has been reported; an outbreak of *S.* Typhi infecting 5,936 people in Bharatpur in 2002 was traced to the municipal water supply (Lewis *et al*., 2005). In an urban setting, Bhatta *et al.* (2007) isolated and identified multidrug resistant (MDR) *S.* Typhi and *S.* Paratyphi A in the drinking water supply of 14% of the samples.

CHAPTER III

3. MATERIALS AND METHODS

This study was conducted at Microbiology Laboratory of Bir Hospital from June 2011 to September 2011. During this period 1542 blood samples from patients suspected of enteric fever were processed in the microbiology laboratory. The samples were processed according to the standard protocol.

3.1 MATERIALS

Different materials required for present work are mentioned in Appendix B.

3.2 METHODS

3.2.1 COLLECTION OF SAMPLE

Blood samples were collected by standard aseptic techniques. Each patient was provided with a culture bottle containing Bile broth. The blood collection procedure was as follows (Cheesbrough, 1984):

- Using a pressure cuff, a suitable vein was located in the arm.
- The skin over the vein was cleansed in a circle approximately 5 cm in diameter with 70% alcohol, rubbing vigorously.

- Starting in the centre 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.
- Using a sterile disposable syringe and size 21 gauge needles, appropriate volume (5 ml) of blood was withdrawn by inserting the needle into the vein.
- After the needle was removed, the site was cleansed with 70 % alcohol again, as many patients were sensitive to iodine.
- One culture bottle containing about 45 ml of bile broth was provided and 5 ml of patient's blood was collected and dispensed in the culture bottle.
- 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.

3.2.2 PROCESSING OF SAMPLE

Immediately after the blood culture bottles were received in the laboratory, they were provided with laboratory identification numbers and further processed.

3.2.3 INCUBATION OF SAMPLE

The culture bottles were incubated at 37°C. Incubation was continued upto 7 days and subcultured at 24 hrs, 48 hrs, 72 hrs or unless the visible growth was obtained. The day of collection of sample was defined as the first day in this study.

3.2.4 MACROSCOPIC EXAMINATION OF BROTH CULTURE

The culture bottles were examined daily for visual evidence of microbial growth such as turbidity, haemolysis, formation of gas bubbles to make presumptive diagnosis of positive culture.

3.2.5 SUBCULTURE

The broth cultures were subcultured on MacConkey agar (MA) and Blood agar (BA) plates. Repeated subcultures were made at different times during their incubation from 24 to 96 hrs.

The composition and preparation of media are mentioned in the Appendix C.

3.2.6 INCUBATION OF SUBCULTURE PLATES

The MA and BA plates were incubated at 37°C aerobically and in carbondioxide jar for 24 hours.

3.2.7 EXAMINATION OF SUBCULTURE PLATES

The subculture plates were examined after overnight incubation. MA plates were examined for the growth of non-lactose fermenters.

3.2.8 IDENTIFICATION OF ISOLATES

Identification of bacteria from positive culture plates was done by using standard microbiological techniques as described in the Bergey's manual which involves morphological appearance of the colonies, staining reactions, biochemical properties and serotyping (Forbes *et al.*, 2002; Cheesbrough, 1984; Collee *et al.*, 1996).

PURITY PLATE

A purity plate was included in the test system inoculating half portion of nutrient agar plate before proceeding the test and rest half after completing the test procedure, in order to know the contamination for culture from outer sources during the experiment.

BIOCHEMICAL TEST

Appropriate biochemical tests were performed for the identification of the bacterial isolates. A single colony of suspected pathogen was inoculated in Nutrient Broth and incubated at 37°C for 4 hrs. After incubation Gram’s staining was performed. The pure colonies on the media plates were inoculated onto different biochemical media.

The biochemical tests used for the identification of bacterial isolates include Catalase test, Oxidase test, Indole test, Methyl red test, Voges Proskauer test, Citrate utilization test, Oxidation Fermentation test, Triple Sugar Iron (TSI) test, Urease test, Motility test, Sulphide production test and Gas production test.

The composition and preparation of biochemical media and reagents used in the biochemical tests are described in the Appendix C. The procedure for performing biochemical tests are mentioned in Appendix E.

SEROTYPING OF *SALMONELLA* ISOLATES

Salmonella isolates were subjected for serotyping, as per instructions mentioned in Appendix D.

3.2.9 ANTIBIOTIC SUSCEPTIBILITY TEST

The antimicrobial susceptibility testing of the isolates towards various antimicrobial discs was done by modified Kirby-Bauer disk diffusion method as recommended by Clinical Laboratory Standards Institute (CLSI-M100-S16) using Mueller Hinton agar (MHA).

Mueller Hinton Agar was prepared and sterilized as instructed by the manufacturer. The pH of the medium 7.2-7.4 and the depth of the medium at 4 mm (about 25 ml per plate) were maintained in petridish. Using a sterile wire loop, a single isolated colony of which the sensitivity pattern is to be determined was touched and inoculated into Mueller Hinton broth tube and was incubated at 37ºC for 2-4 hrs. After incubation, the turbidity of the suspension was matched with the turbidity standard of Mc Farland tube number 0.5. A sterile cotton-wool swab was dipped into the suspension and the excess liquid was removed by turning the swab against the side of the tube. The inoculum was spread evenly over the entire surface of the MHA plate by swabbing in three directions. The plate was allowed to dry before applying discs and the discs should be applied to the surface of the agar within 15 min of inoculation. Using sterile forceps, appropriate antibiotic discs (6mm diameter) were placed into the organism carpeted MHA plate at a distance of 15 mm away from the edge and 24 mm apart from each other, not more than 6 discs were placed on a 90 mm diameter petridish. After overnight incubation, the plates were examined and the diameter of zone of inhibition in mm was measured and compared with standardized zone interpretative chart provided by the company.

The preparation and composition of Mueller Hinton Agar medium is mentioned in the Appendix C. The detailed about antibiotic discs used and its interpretative chart are mentioned in Appendix F.

3.2.10 QUALITY CONTROL

To obtain reliable microbiological result, it is necessary to maintain quality control. During this study, quality control was applied in various areas.

- During sample collection, aseptic technique was followed using sterile syringe and needle, disinfecting the skin over the vein of the patient and collecting blood in sterile bottles in order to avoid contamination.
- During sample processing, all the tests were carried out appropriately in aseptic conditions.
- While using readymade dehydrated media, the manufacturer's instructions for preparation, sterilization and storage were followed to prevent the alteration of the nutritional, selective, inhibitory and biochemical properties of the media.
- The performance of newly prepared media was tested using control species of bacteria (i.e. known organisms giving positive and negative reactions).
- For stains and reagents, whenever a new batch of them were prepared, a control smear was stained to ensure correct staining reaction.
- Control strains of *E. coli* (ATCC 25922) and *Staphylococcus aureus* (ATCC 25923) were used for the standardization of the Kirby-Bauer test and also for correct interpretation of zone of diameter.

3.2.11 DATA ANALYSIS

Chi- square test was applied to find significance of the values. Significant test of present work are shown in appendix G.

CHAPTER IV

4. RESULTS

4.1 AGE AND GENDER WISE DISTRIBUTION OF THE PATIENTS

During study period, all together 1542 blood samples were collected for culture in which 990 (64.20%) were from male patients. The highest number of blood samples was collected from age group 21-30 with 546 (35.41%) requests. The least number of samples were taken from age group 91-100 with only 3 (0.19%) requests and they were from male patients.

Table 1 Age and gender wise distribution of patients

Age Group (yrs)	Male		Female		Total	
	Number	%	Number	%	Number	%
11-20	253	25.56	123	22.28	376	24.38
21-30	365	36.87	181	32.79	546	35.41
31-40	125	12.63	84	15.22	209	13.55
41-50	90	9.09	77	13.95	167	10.83
51-60	71	7.17	43	7.79	114	7.39
61-70	38	3.84	30	5.43	68	4.41
71-80	35	3.54	9	1.63	44	2.85
81-90	10	1.01	5	0.91	15	0.97
91-100	3	0.30	0	0	3	0.19
Total	**990**	**64.20**	**552**	**35.80**	**1542**	**100**

4.2 PATTERN OF BLOOD CULTURE RESULT

Out of 1542 cases requested for blood culture, 126 (8.17%) of cases showed positive culture result. In 990 male patients, 86 (8.69%) cases showed culture positive result. Similarly, out of total 552 female patients, 40 (7.25%) cases showed positive blood culture result. The results are shown in Table 2.

Table 2 Gender wise distribution of culture positive patients

Gender	Culture positive		Culture negative		Total	
	Cases	%	Cases	%	Cases	%
Male	86	8.69	904	91.31	990	64.20
Female	40	7.25	512	92.75	552	35.80
Total	**126**	**8.17**	**1416**	**91.8**	**1542**	**100**

4.3 GROWTH PATTERN IN RELATION TO ORIGIN OF THE SAMPLE

Out of 1212 samples collected from outdoor patients, 103 (8.5%) cases showed culture positive. Similarly, 330 blood samples were collected from indoor patients and 23 (6.97%) showed positive culture result. The results are shown in Table 3.

Table 3 Growth pattern in relation to origin of the sample

Origin	Culture positive		Culture negative		Total
	Cases	%	Cases	%	
Outdoor	103	8.50	1109	91.50	1212
Indoor	23	6.97	307	93.03	330
Total	**126**	**8.17**	**1416**	**91.83**	**1542**

4.4 PATTERN OF BACTERIAL ISOLATES

Out of 126 positive growths obtained, 87 (69.05%) were *Salmonella* isolates and remaining were other bacterial species. The other bacterial species isolated were *Acinetobacter* spp.

(15.87%), *Enterobacter* spp. (3.97%), *Staphylococcus aureus* (3.97%), *Pseudomonas aeruginosa* (3.17%), *Escherichia coli* (2.38%) and *Klebsiella* spp. (1.59%).

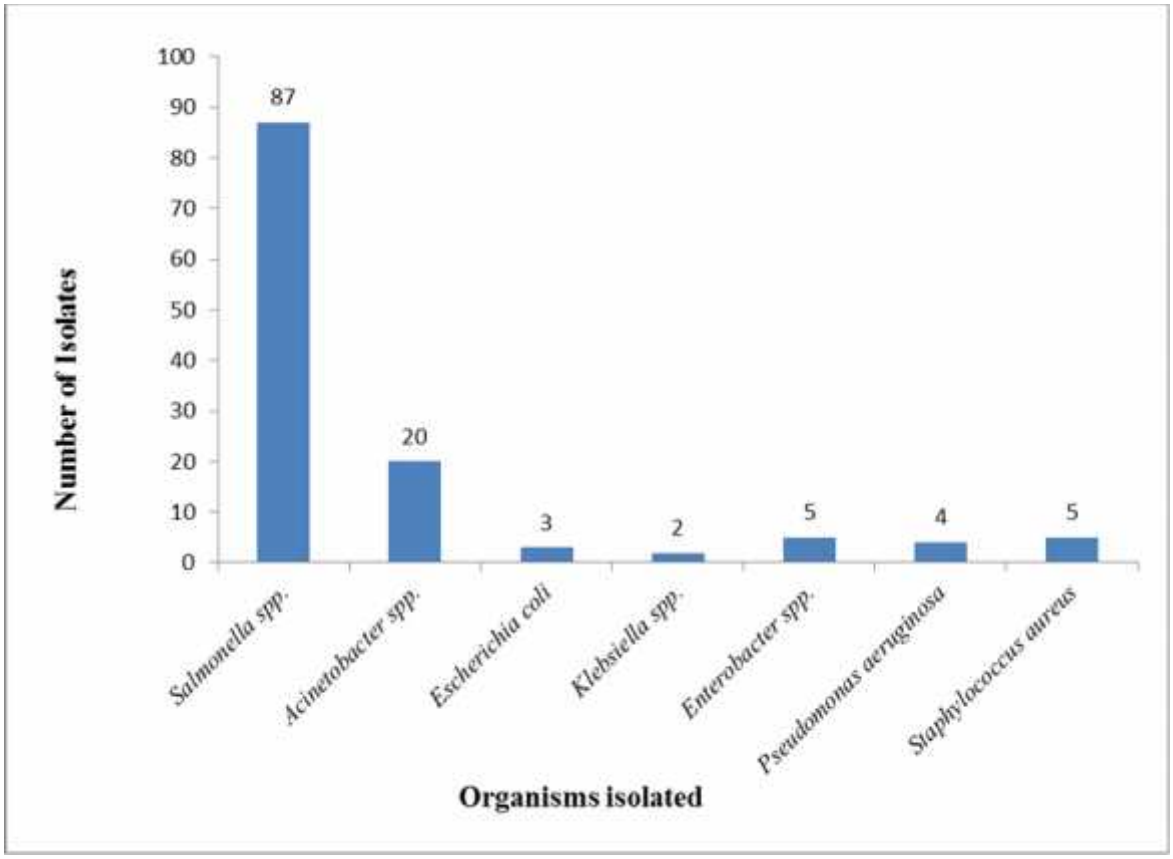

Figure 2 Pattern of bacterial isolates

4.5 GENDER WISE DISTRIBUTION OF *SALMONELLA* ISOLATES

Out of 87 *Salmonella* isolates, 58 isolates were obtained from suspected male patients whereas remaining 29 isolates were obtained from suspected female patients.

Table 4 Gender wise distribution of *Salmonella* isolates

Gender	*Salmonella* isolates	
	N	%
Male	58	66.67
Female	29	33.33
Total	87	100.00

4.6 AGE AND GENDER WISE DISTRIBUTION OF *SALMONELLA* ISOLATES

Altogether there were 48 (55.17%) *Salmonella* Typhi isolates 39 (44.83%) *Salmonella* Paratyphi A isolates. Occurrence of *Salmonella* was found to be highest among the age group 11-20 yrs (50.58%) followed by age group 21-30 yrs (40.23%). *Salmonella* was seen least in the age group 61-70 yrs (1.15%) whereas there were no isolates from patients above 70 yrs.

Isolates of *S.* Typhi and *S.* Paratyphi A were 37.93% and 28.74% respectively in male patients which were comparatively higher than in female patients. In female patients, *S.* Typhi and *S.* Paratyphi were found to be 17.24% and 16.09% respectively.

Table 5 Age and gender wise distribution of *Salmonella* isolates

Age group	*Salmonella* Typhi			*Salmonella* Paratyphi A			Total	
	Male	Female	Total	Male	Female	Total	*Salmonella* isolates	%
11-20	15	6	21	14	9	23	44	50.58
21-30	16	6	22	8	5	13	35	40.23
31-40	1	0	1	2	0	2	3	3.45
41-50	0	1	1	1	0	1	2	2.30
51-60	1	1	2	0	0	0	2	2.30
61-70	0	1	1	0	0	0	1	1.15
71-80	0	0	0	0	0	0	0	0
81-90	0	0	0	0	0	0	0	0
91-100	0	0	0	0	0	0	0	0
Total (%)	**33 (37.93)**	**15 (17.24)**	**48 (55.17)**	**25 (28.74)**	**14 (16.09)**	**39 (44.83)**	**87**	**100**

4.7 OUTDOOR AND INDOOR DISTRIBUTION OF *SALMONELLA* ISOLATES

Higher occurrence of *Salmonella* isolates was found in outdoor patients. Out of total 87 isolates of *Salmonella* isolates, 84 (96.55%) isolates were obtained from outdoor patients. Only 3 (3.45%) isolates of *Salmonella* Typhi were isolated from indoor patients. No isolates of *Salmonella* Paratyphi A was obtained from indoor patients.

Table 6 Outdoor and indoor distribution of *Salmonella* isolates

Origin	*Salmonella* Typhi	*Salmonella* Paratyphi A	Total	
			N	%
Outdoor	45	39	84	96.55
Indoor	3	0	3	3.45
Total	**48**	**39**	**87**	**100**

4.8 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA* TYPHI

The antibiotic susceptibility pattern of *Salmonella* Typhi showed that 100% of the isolates were susceptible to Ofloxacin and Ceftriaxone, followed by, Ciprofloxacin (95.83%), Chloramphenicol (95.83%), Amoxycillin (93.75%) and Cotrimoxazole (91.67%). Nalidixic acid resistance was in found in 75% of the isolates.

Table 7 Antibiotic susceptibility pattern of *Salmonella* Typhi (N= 48)

Anitibiotics used	Antibiotic Susceptibility Pattern					
	Resistant		Intermediate		Susceptible	
	N	%	N	%	N	%
Ceftriaxone	0	0	0	0	48	100.00
Ofloxacin	0	0	0	0	48	100.00
Ciprofloxacin	0	0	2	4.17	46	95.83
Amoxycillin	3	6.25	0	0	45	93.75
Chloramphenicol	2	4.17	0	0	46	95.83
Cotrimoxazole	4	8.33	0	0	44	91.67
Nalidixic acid	36	75.00	0	0	12	25.00

Two (4.17%) *S.* Typhi isolates were MDR strains (i.e. resistant to Chloramphenicol, Amoxycillin and Cotrimoxazole), and also resistant to Nalidixic acid. These two isolates were also intermediately susceptible to Ciprofloxacin. One (2.08%) *S.* Typhi isolate was resistant to Amoxycillin and Cotrimoxazole, including Nalidixic acid and one (2.08%)

isolate was resistant to Cotrimoxazole, including Nalidixic acid. Antibiotic sensitivity pattern of each *Salmonella* Typhi isolate is shown in the table 8.

Table 8 Antibiotic susceptibility pattern of each *Salmonella* Typhi isolate

Isolate Code	Antibiotics Tested						
	Ceftriaxone (30mcg)	Ofloxacin (mcg)	Ciprofloxacin (5mcg)	Amoxycillin (30mcg)	Chloramphenicol (30mcg)	Cotrimoxazole (25mcg)	Nalidixic acid (30mcg)
S1	S	S	S	S	S	S	R
S2	S	S	S	S	S	R	R
S3	S	S	S	S	S	S	S
S4	S	S	S	S	S	S	R
S5	S	S	S	S	S	S	R
S6	S	S	S	S	S	S	R
S7	S	S	S	R	S	R	R
S8	S	S	S	S	S	S	S
S9	S	S	S	S	S	S	R
S10	S	S	S	S	S	S	R
S11	S	S	S	S	S	S	R
S12	S	S	I	R	R	R	R
S13	S	S	S	S	S	S	S
S14	S	S	S	S	S	S	S
S15	S	S	S	S	S	S	R
S16	S	S	S	S	S	S	R
S17	S	S	S	S	S	S	R
S18	S	S	S	S	S	S	R
S19	S	S	S	S	S	S	R
S20	S	S	S	S	S	S	R
S21	S	S	S	S	S	S	S
S22	S	S	S	S	S	S	S
S23	S	S	S	S	S	S	R
S24	S	S	S	S	S	S	R
S25	S	S	S	S	S	S	R
S26	S	S	S	S	S	S	R

S27	S	S	S	S	S	S	R
S28	S	S	S	S	S	S	S
S29	S	S	S	S	S	S	R
S30	S	S	I	R	R	R	R
S31	S	S	S	S	S	S	S
S32	S	S	S	S	S	S	S
S33	S	S	S	S	S	S	R
S34	S	S	S	S	S	S	S
S35	S	S	S	S	S	S	R
S36	S	S	S	S	S	S	R
S37	S	S	S	S	S	S	R
S38	S	S	S	S	S	S	R
S39	S	S	S	S	S	S	R
S40	S	S	S	S	S	S	S
S41	S	S	S	S	S	S	R
S42	S	S	S	S	S	S	R
S43	S	S	S	S	S	S	R
S44	S	S	S	S	S	S	R
S45	S	S	S	S	S	S	R
S46	S	S	S	S	S	S	S
S47	S	S	S	S	S	S	R
S48	S	S	S	S	S	S	R

S: Sensitive R: Resistant I: Intermediate

4.9 ANTIBIOTIC SUSCEPTIBILITY PATTERN OF *SALMONELLA* PARATYPHI A

Out of 39 *Salmonella* Paratyphi A isolates, Ofloxacin, Ceftriaxone and Chloramphenicol were 100% sensitive to all the isolates followed by Ciprofloxacin (94.87%), Cotrimoxazole (94.87%) and Amoxycillin (92.31%). Nalidixic acid resistance was found in74.36% of the isolates. The results are shown in table 9.

Table 9 Antibiotic susceptibility pattern of *Salmonella* Paratyphi A (N= 39)

Anitibiotics used	Antibiotic Susceptibility Pattern					
	Resistant		Intermediate		Susceptible	
	N	%	N	%	N	%
Ceftriaxone	0	0	0	0	39	100.00
Ofloxacin	0	0	0	0	39	100.00
Ciprofloxacin	0	0	2	5.13	37	94.87
Amoxycillin	3	7.69	0	0	36	92.31
Chloramphenicol	0	0	0	0	39	100.00
Cotrimoxazole	2	5.13	0	0	37	94.87
Nalidixic acid	29	74.36	0	0	10	25.64

Two (5.13%) isolates of *S.* Paratyphi A were resistant to Amoxycillin and Cotrimoxazole, including Nalidixic acid and intermediately sensitive to Ciprofloxacin. One (2.56%) isolate was resistant to Amoxycillin, including Nalidixic acid. Antibiotic susceptibility pattern of each *Salmonella* Paratyphi A isolate is shown in table 10

Table 10 Antibiotic susceptibility pattern of each *Salmonella* Paratyphi A isolate

Isolate Code	Antibiotics Tested						
	Ceftriaxone (30mcg)	Ofloxacin (mcg)	Ciprofloxacin (5mcg)	Amoxycillin (30mcg)	Chloramphenicol (30mcg)	Cotrimoxazole (25mcg)	Nalidixic acid (30mcg)
A1	S	S	S	S	S	S	S
A2	S	S	S	S	S	S	R
A3	S	S	S	S	S	S	R
A4	S	S	S	S	S	S	R
A5	S	S	I	R	S	R	R
A6	S	S	S	S	S	S	R
A7	S	S	S	S	S	S	R
A8	S	S	S	S	S	S	R
A9	S	S	S	S	S	S	R
A10	S	S	S	S	S	S	S
A11	S	S	S	S	S	S	S

A12	S	S	S	S	S	S	R
A13	S	S	S	S	S	S	R
A14	S	S	I	R	S	R	R
A15	S	S	S	S	S	S	S
A16	S	S	S	S	S	S	R
A17	S	S	S	S	S	S	S
A18	S	S	S	S	S	S	R
A19	S	S	S	S	S	S	R
A20	S	S	S	S	S	S	R
A21	S	S	S	S	S	S	S
A22	S	S	S	S	S	S	R
A23	S	S	S	R	S	S	R
A24	S	S	S	S	S	S	R
A25	S	S	S	S	S	S	R
A26	S	S	S	S	S	S	R
A27	S	S	S	S	S	S	S
A28	S	S	S	S	S	S	R
A29	S	S	S	S	S	S	R
A30	S	S	S	S	S	S	R
A31	S	S	S	S	S	S	R
A32	S	S	S	S	S	S	S
A33	S	S	S	S	S	S	R
A34	S	S	S	S	S	S	R
A35	S	S	S	S	S	S	R
A36	S	S	S	S	S	S	S
A37	S	S	S	S	S	S	R
A38	S	S	S	S	S	S	R
A39	S	S	S	S	S	S	S

S: Sensitive R: Resistant I: Intermediate

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

Out of 48 *Salmonella* Typhi isolates, 36 isolates were Nalidixic acid resistant. Similarly, out of 39 *Salmonella* Paratyphi A isolates, 29 were Nalidixic acid resistant. Ciprofloxacin sensitivity was found in 94.44% of Nalidixic acid resistant *Salmonella* Typhi and 93.10% of Nalidixic acid resistant *Salmonella* Paratyphi A.

Table 11 Ciprofloxacin susceptibility pattern of Nalidixic acid resistant *Salmonella* isolates

Nalidixic acid resistant *Salmonella* isolates	**Sensitivity to Ciprofloxacin**						**Total**
	Sensitive		**Intermediate**		**Resistance**		
	Number	**%**	**Number**	**%**	**Number**	**%**	
S. Typhi	34	94.44	2	5.56	0	0	36
S. Paratyphi A	27	93.10	2	6.90	0	0	29

CHAPTER V

5. DISCUSSION

Altogether 1542 blood samples were collected from clinically suspected enteric fever cases attending Bir Hospital, Kathmandu during June 2011 to September 2011.

The number of male patients was higher (64.20%) than the female patients (35.80%). The load of outdoor patients for blood culture was high (78.60%) than the indoor patients (21.40%). The majority of the patients belonged to age group 21-30 years and represented 35.41% of the total population studied.

Out of 1542 blood samples processed during the study period, 126 (8.17%) samples showed positive culture result**.** Ghimire (1995) found an incidence rate of 13.95%; Tibrewal (1999) found an incidence rate of 17%; Shakya (2001) showed an incidence rate of 15.4% and Wagley (2004) showed an incidence rate of 21.47%.

Shrestha (2007) showed an incidence rate of 10.71%. Ali and Kebede (2008) reported culture positivity rate of 24.2% from febrile patients. Prajapati (2009) showed culture positivity result of 12.29%. Recent study conducted by Thriemer *et al*. (2012) showed 8% culture positivity result.

The patients between the age group of 11-20 years showed the maximum culture positivity and it covered 50.58%of the total *Salmonella* isolates.

Gupta *et al*. (1985) stated that while no age group seems to be immune, typhoid fever was common (77.6%) in young adults. Akinyemi *et al*. (2000) showed that most isolates of *Salmonella* Typhi were found among severely-ill young adults between age group of 16-30 years. Shakya (2001) found that age group of 15-30 years showed maximum positive growth which is also comparable to the result of our study. Amatya (2005) reported maximum culture positivity in patients between age group 20-30 years and it covered 51.22%. Aryal (2008) reported higher percentage of culture positivity (14.8%) in age group 11-20 years.

Out of 990 male patients, 86 (8.69%) cases showed positive blood culture result whereas in case of females 40 out of 552 (7.25%) showed culture positive result. This indicates higher culture positivity in male patients compared to females and this was statistically

insignificant. Amatya (2005) also reported higher incidence in male patients (82/123) compared to female patients (41/123).

Out of 1212 outdoor patients, 86 (8.69%) cases showed culture positivity. Similarly, out of 330 indoor patients, 23 (6.97%) cases showed culture positivity. Shrestha (2007) also reported higher incidence in outdoor patients (162/179 isolates) than indoor patients (17/179 isolates).

Out of 126 culture positive cases, 87 (69.05%) were *Salmonella* positive cases. Of 87 *Salmonella* isolates, 48 (55.17%) isolates were of Salmonella Typhi and 39 (44.83%) isolates were of *Salmonella* Paratyphi A. *Salmonella* Typhi was found to be 38.37% in males and 17.24% in females. Similarly, *Salmonella* Paratyphi A was found to be 28.74% in males and 16.09% in females. *Salmonella* isolates were found to be 96.55% in outdoor patients and only 3.45% in indoor patients. There were no isolates of *S.* Paratyphi A from indoor patients. This indicates higher incidence of typhoid fever than paratyphoid fever. WHO estimates the ratio of disease caused by *S.* Typhi to that caused by *S.* Paratyphi A is about 10 to 1 in most of the countries where this matter has been studied. This ratio was not seen in present study.

Thapa (1991) showed that the incidence rate of *Salmonella* Typhi and *Salmonella* Paratyphi A were 69.89% and 29.03% respectively from clinically suspected patients with enteric fever. Ghimire *et al.* (1995) reported that 71% of the isolates were infected with *S.* Typhi and 29% with *S.* Paratyphi A whereas Shrestha (1996) observed that 63.63% patients were infected with *S.* Typhi, 35.06% with *S.* Paratyphi A and 1.29% with *S.* Paratyphi B. Akinyemi *et al.* (2000) reported 67.3% of *S.* Typhi, 16.8% of *S.* Paratyphi A and 15.8% of *S. arizonae* among clinically diagnosed typhoid fever patients. Vollard *et al.* (2004) reported 9% of the isolates to be *S.* Typhi and 3% *S.* Paratyphi A. However, Pokharel *et al.* (2006) reported 47% of *S.* Typhi and 53% *S.* Paratyphi A isolates in a study done at Tribhuvan University Teaching Hospital, Nepal.

Sharma *et al.* (2006) reported 41% of *Salmonella* Typhi and 10.7% of *Salmonella* Paratyphi A isolates from blood of febrile patients. Manchanda *et al.* (2006) reported a total of 56 *S.* Typhi and five *S.* Paratyphi A isolates among 673 blood cultures performed. Shrestha (2007) reported that 71% of the isolates were infected with *S.* Typhi and 29% were infected with *S.* Paratyphi A. In a study done by Khatiwada (2006) 29 (76.3%) isolates were *S.* Typhi and 9 (23.7%) isolates were *S.* Paratyphi A.

Similarly, Amatya *et al*. (2007) reported that the most frequent isolated bacteria was *Salmonella* Typhi (63.4%) followed by *Salmonella* Paratyphi A (35.8%) and *E. coli* (0.8%). Aryal (2008) reported 52 (50%) *S*. Typhi isolates and 45 (43%) *S*. Paratyphi A isolates among 104 blood culture positive cases. Raveendran *et al.* (2008) reported 431 *Salmonella enterica* serotype Typhi and 198 serotype Paratyphi A isolates from a total of 25,953 samples of blood culture. Prajapati (2009) reported 69.21% *S*. Typhi, 30.67% *S*. Paratyphi A and 0.06% each of *S*. Paratyphi B and C. Deen *et al.* (2012) also reported highest incidence of *Salmonella* Typhi in bloodstream infections accounting for 30% isolates in adults and 25% in children. Thriemer *et al*. (2012) reported that most frequent pathogenic bacteria isolated was *S*. Typhi (58%), followed by *Streptococcus pneumoniae* (15%).

In present study Ofloxacin and Ceftriaxone remain the principal drugs based on their effectiveness on blood culture isolates.

The isolates of *Salmonella enterica* serovar Typhi showed 100% susceptibility towards Ofloxacin and Ceftriaxone. This was followed by Ciprofloxacin (95.83%), Chloramphenciol (95.83%) and Amoxycillin (93.75%). Cotrimoxazole was found to be least effective among the tested antibiotics and its efficacy rate was found to be 91.67%.

Study conducted by Ackers *et al*., 2000 showed that Ciprofloxacin and Ceftriaxone as appropriate empirical therapy for suspected typhoid fever. Study conducted by Abucejo *et al.* (2001) showed that all *S*. Typhi isolates were found to be sensitive to Chloramphenciol, Cotrimoxazole, Ampicillin, Ceftriaxone, Ciprofloxacin and Ofloxacin. According to Wagley (2004) Ceftazidime was 100% effective towards *Salmonella* Typhi, Chloramphenicol and Ceftriaxone were the second choices with 95.31% effectiveness, Ciprofloxacin and Cephalexin were found to be 90.63%, Ofloxacin and Ampicillin showed 87.52% and 85.94% susceptibility pattern respectively.

Similarly, study conducted by Rahman *et al*. (2006) showed that all strains of *Salmonella enterica* serovar Typhi were susceptible to Ceftriaxone. Srikantiah *et al*. (2007) found that 15% of the isolates of *Salmonella* Typhi were resistant to Ampicillin, Chloramphenicol and Trimethoprim-sulphamethoxazole. Amatya *et al*. (2007) reported that *S*. Typhi isolates were 100% susceptible to Ceftriaxone, followed by Cephotaxime (98.7%), Chloramphenicol (97.4%), Cotrimoxazole and Ampicillin (96.1%), Cefixime and Ofloxacin (94.9%). Aryal

(2008) reported *S.* Typhi isolates to be 98% sensitive to Ceftriaxone, followed by Ofloxacin (96%), Ciprofloxacin (94.2%), Cotrimoxazole (88.5%) and Ampicillin (5.7%). Gurung (2008) reported that isolates of *S.* Typhi were 100% susceptible to Chloramphenicol, Amoxycillin, Cotrimoxazole and Ceftriaxone. Thriemer *et al.* (2012) reported that 51%, 49% and 49% of the *S.* Typhi isolates were resistant towards Ampicillin, Chloramphenicol and Cotrimoxazole, respectively.

In the present study, *Salmonella enterica* serovar Paratyphi A isolates were found to be 100% sensitive to Ceftriaxone, Ofloxacin and Chloramphenicol. Ciprofloxacin and Cotrimoxazole each showed 94.87% susceptibility rate. Least effective drug for *S.* Paratyphi A was found to be Amoxycillin with susceptibility rate of 92.31%.

This result is supported by Shakya (2001) who revealed that Chloramphenicol and Ceftriaxone were the most effective antibiotics against *S.* Paratyphi A. Study conducted by Sharma *et al.* (2006) showed that all *S.* Typhi and *S.* Paratyphi A isolates were susceptible to Ceftriaxone, while susceptibility to Ciprofloxacin and Chloramphenicol was recorded in 94.8% and 94.5% of cases, respectively. Cephalexin and Amoxycillin had lowest rates of susceptibility (64.2% and 54.1%, respectively). Khatiwada (2006) reported that *S.* Paratyphi A isolates were susceptible to Tetracycline, Ciprofloxacin, Ceftriaxone and Cotrimoxazole. Amatya *et al.* (2007) reported that *S.* Paratyphi A was 100% susceptible to three antibiotics, Chloramphenicol, Amoxycillin and Cotrimoxazole. Aryal (2008) reported that isolates of *S.* Paratyphi A were 100% susceptible to Ceftriaxone, Ofloxacin, Chloramphenicol and Ciprofloxacin followed by Cotrimoxazole (95.5%). KhattriChhetri (2008) reported that *S.* Paratyphi A isolates were susceptible to Ceftriaxone only.

Typhoid fever is the most common clinical diagnosis among febrile patients presenting to hospital in Kathmandu. *Salmonella enterica* serovar Typhi and *Salmonella enterica* serovar Paratyphi A with decreased susceptibility to Fluoroquinolones and resistance to Nalidixic acid are common in recent years (Shirakawaa *et al*., 2005).

Nalidixic acid resistance is a marker for predicting low-level resistance to Ciprofloxacin among *S.* Typhi and also an indicator of treatment failure to Ciprofloxacin. Hence, it is suggested that all *S.* Typhi isolates should be screened for Nalidixic acid resistance along with Ciprofloxacin (Kapil *et al.*, 2002). Any isolate that shows resistance to Nalidixic acid should be reported as intermediately susceptible to Ciprofloxacin. The clinician should be

advised to change the antibiotic. Such strains have been found to be endemic in different parts of the world. All patients with Nalidixic acid resistant strains should be treated with higher doses of Ciprofloxacin or Ofloxacin. There are reports that when the sensitivity pattern indicates resistance to Nalidixic acid, which is marker for delayed clinical response to Fluoroquinolones, it is necessary to increase the oral dose of Ciprofloxacin or treat the patients with third generation cephalosporin like Ceftriaxone (Rodrigues *et al.*, 1998). Kumar *et al*. (2009) reported that 96% of the isolates of *Salmonella* Typhi were found to be Nalidixic acid resistant (NARST) while all isolates were found to be Ciprofloxacin sensitive.

In our study, 75% of isolated *Salmonella* Typhi and 74.36% of isolated *Salmonella* Paratyphi A showed resistance to Nalidixic acid. Out of 65 Nalidixic acid resistant *Salmonella* isolates, 5.56% of *Salmonella* Typhi and 6.90% of *Salmonella* Paratyphi A showed decreased susceptibility to Ciprofloxacin though there was not a single isolate which was resistant to that antibiotic.

Amatya (2005) reported that 62.5% of *S*. Typhi was resistant to Nalidixic acid but none of the isolate was resistant to Ciprofloxacin. Khatiwada (2006) reported that isolates of *Salmonella* Typhi were 58.62% resistance to Nalidixic acid and 100% susceptible to Ciprofloxacin. Chau *et al.* (2007) reported that in Nepal, 18.1% of *S.* Typhi isolates were resistant to Chloramphenicol and Nalidixic acid. Prajapati (2009) reported that 68.19% *Salmonella* Typhi and 94.83% of *Salmonella* Paratyphi A were resistant to Nalidixic acid.

Resistance to Nalidixic acid may be an indicator of low-level resistance to Ciprofloxacin (Launay *et al*., 1997; Murdoch *et al.*, 1998; Vasallo *et al*., 1998). A single point mutation in the Quinolone resistance-determining region of the topoisomerase gene gyrA in *Salmonella* usually leads simultaneously to resistance against Nalidixic acid and decreased Ciprofloxacin susceptibility (Nair and Sudarsana, 2004).

Identification of Nalidixic acid resistance by the disc diffusion method has been reported to provide a sensitivity of 100% and a specificity of 87.3% as a tool to screen for isolates having MICs of Ciprofloxacin 0.125 g/ml (Hakanen *et al.*, 1999). Typhoid fever caused by NARST infection is associated with poor clinical outcomes, probably due to delay in initiating appropriate antibiotic therapy. Fluoroquinolone breakpoints for *S.* Typhi need to be redefined and Fluoroquinolones should no longer be used as first-line therapy, if the prevalence of NARST is high (Kadhiravan *et al.*, (2005).

Ackers *et al.* (2000) suggested Ciprofloxacin and Ceftriaxone as appropriate empirical therapy for suspected typhoid fever; however, resistance may be anticipated. Continued monitoring of antimicrobial resistance among *Salmonella* Typhi strains will help determine vaccination and treatment policies. However, Raveendran *et al.* (2008) suggested that Ciprofloxacin can no longer be considered as the drug of choice in treating *Salmonella* infection. While first-line antimicrobials may still have a role to play in the treatment of enteric fever, Ceftriaxone remains the sole defense against Ciprofloxacin-resistant *Salmonella* infection.

Antimicrobial resistance is a global problem. It is now generally accepted as major public health issue and has significant implication on health and patient care. Resistance to antimicrobial drugs is associated with high morbidity and mortality, high health-care cost and prolonged hospitalization. The problem of the drug resistance to antimicrobial drug is more troublesome to developing countries. The World Health Organization (WHO) and the European Commission (EC) have recognized the importance of studying the emergence and determinants of resistance and the need for strategies for its control.

Multiple drug resistance (MDR) was defined as resistance to three or more of the antimicrobial agents evaluated in the study (Sunenshine *et al.*, 2007). Two (4.17%) *S.* Typhi isolates were multidrug resistant strains i.e. resistant to Chloramphenicol, Amoxycillin and Cotrimoxazole, and also resistant to Nalidixic acid. One (2.08%) *S.* Typhi isolate was resistant to two antibiotics i.e. Amoxycillin and Cotrimoxazole, including Nalidixic acid and one (2.08%) isolate was resistant to Cotrimoxazole, including Nalidixic acid.

Similarly, two (5.13%) isolates of *S.* Paratyphi A were resistant to Amoxycillin and Cotrimoxazole, including Nalidixic acid. One (2.56%) isolate was resistant to Amoxycillin, including Nalidixic acid. No MDR strain of *S.* Paratyphi A was isolated during the research.

Ackers *et al.* (2000) reported that 25% of *Salmonella* Typhi isolates were resistant to one or more antimicrobial agent, 17% were resistant to 5 or more agents, including Ampicillin, Chloramphenicol and Trimethoprim-sulfamethoxazole. Kabra *et al*. (2000) reported that 80% of *Salmonella* isolates were resistant to Amoxycillin, Chloramphenicol and Cotrimoxazole, but all were sensitive to Ciprofloxacin and Ceftriaxone.

Rahman *et al.* (2006) reported that 32% of the isolates of *Salmonella enterica* serotype Typhi were multidrug-resistant (MDR, resistant to Chloramphenicol, Ampicillin and Trimethoprim-sulfamethoxazole). The emergence of MDR *S.* Typhi with decreased Ciprofloxacin susceptibility will further complicate the therapy of typhoid fever because of the lack of optimum treatment guidelines. Amatya *et al*. (2007) reported 3.84% (3/78) S. Typhi was resistant to more than two antibiotics (resistant to Chloramphenicol, Amoxycillin, Ofloxacin, Cefixime, Cotrimoxazole).

Chau *et al.* (2007) reported that the proportion of MDR *S.* Typhi was 63.2% in 1993 and increased to more than 80% in the late 90s and early 2000. Since 1998, a high proportion of strains show the combination of MDR and Nalidixic acid. The study also showed that the combination of MDR and Nalidixic acid resistance was found in 4.3% (2/47) of *S.* Typhi isolates from Central Vietnam, 8.7% (2/23) of isolates from India and 23.5% (8/140) of isolates from Pakistan. The combination of MDR and Nalidixic acid resistance might be particular problem because it severely restricts the therapeutic options for patients with typhoid fever.

Aryal (2008) reported 5 (9.6%) *S.* Typhi isolates to be resistant to more than two classes of antibiotics. KhattriChettri (2008) reported that 8.9% (4/45) isolates were resistant to 3 or more antibiotics. Similarly, Gurung (2008) reported that 5.6% (1/18) of *S.* Typhi was resistant to more than two antibiotics. Thriemer *et al*. (2012) reported that MDR of *Salmonella* Typhi isolates against three antimicrobials (Ampicillin, Chloramphenicol and Cotrimoxazole) was detected in 42% of the isolates.

Khatiwada (2006) and Aryal (2008) did not isolate any MDR strain of *S.* Paratyphi A. Amatya *et al.* (2007) also did not isolate any *S.* Paratyphi A resistant to more than two antibiotics. Gurung (2008) isolated 2 (8.7%) *S.* Paratyphi A resistant to 3 or more antibiotics. KhattriChhetri (2008) isolated 4 (8.8%) *S.* Paratyphi A resistant to 3 or more drugs.

The emergence of MDR is clearly related to the quantity of antibiotics and how they are being used (Levy, 1991). Resistant strains are now reported against all available classes of antibiotics (Kunin, 1983 and Levy, 1991). The recent increase of MDR strains in hospital has started to pose great difficulty in selecting antimicrobial agents for the management of the infection they caused. Some factors responsible for the emergence of resistant strains in hospital include the indiscriminate use of antibiotics, the prolonged hospitalization, the

increase in uses of insertion devices etc. Moreover, results obtained from these surveillance systems must be used to implement preventive programs and policy decisions to prevent the emergence and spread of antimicrobial resistance.

CHAPTER VI

6. CONCLUSION AND RECOMMENDATIONS

6.1 CONCLUSION

From this study, it was concluded that typhoid fever was more prevalent than paratyphoid fever in patients suspected of enteric fever. The culture positivity was highest among the patients of age group 11-20 years and the incidence was higher in males than in females, and this was statistically insignificant ($p>0.05$). The highest sensitivity was shown to Ofloxacin and Ceftriaxone by both *Salmonella* Typhi and *Salmonella* Paratyphi A. Isolates of *S.* paratyphi A were also 100% sensitive to Chloramphenicol. Two (4.17%) *S.* Typhi isolates were MDR with Nalidixic acid resistance.

Ciprofloxacin sensitivity was found in 94.44% of Nalidixic acid resistant *Salmonella* Typhi and 93.10% of Nalidixic acid resistant *Salmonella* Paratyphi A. Increased Nalidixic acid resistance in *Salmonella* spp. acts as a marker for decreased Fluoroquinolone susceptibility. Continued monitoring of antimicrobial susceptibility patterns could inform empirical treatment regimens and prevention strategies.

6.2 RECOMMENDATIONS

1. As this study was confined to Bir Hospital, it does not necessarily reveal the picture of the country, thus systematic prospective surveillance should be carried out covering wide geographical region in order to obtain information on seasonal, geographical and ethnic variation of pathogens and their antibiotic susceptibility profile.

2. In this study, *Salmonella* spp. from blood culture was considered. Surveillance of *Salmonella* from stool, urine, lesions from rose spot may be considered for further study.

3. Genotypic characterization of MDR strains and Nalidixic acid resistance stains should be carried out in order to ascertain the location of drug resistance genes and to characterize the mechanism of drug resistance.

4. The MIC of Ciprofloxacin should be done for Nalidixic acid resistant *Salmonella* spp.

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APPENDIX A

Questionnaire

CLINICAL AND MICROBIOLOGICAL PROFILE OF THE PATIENTS

A) Clinical profile

Name: …… Lab No: ……..

Age / Gender: …… Date: ……...

Address: …… Patient: OPD /Emergency

Ward: ……..

Bed No.: ……..

Culture request for: ……..

Brief Clinical History:

Current antibiotic treatment: Yes No

If yes, Antibiotic(s) taken: 1) ……. 2) ……

Duration of treatment: ……

B) Microbiological profile

I) Evidence of microbial growth within: 24hrs / 48 hrs/ 72 hrs

II) Microbial growth on MA (after subculture):

III) Colony characteristics on different media

Media	Size	Shape	Color	Margin	Elevation	Opacity	Consistency

IV) Gram's reaction: …….

V) Biochemical characterization

Catalase: …… Oxidase: …… O/F: ……..

Others:

Indole	Motiity	Sulphide	MR	VP	Citrate	Urease	TSI

Serological identification of microorganisms:

Antisera used: 1) … … … … … … 2) … … … … … … 3) … … … … …

VI) Microorganism identified as: ……..

VII) Antibiotic susceptibility test

Media used: Muller Hinton Agar

Antibiotics used	Zone of inhibition (mm)	Interpretation

……. ……

Performed By Checked By

APPENDIX B

LIST OF EQUIPMENTS AND MATERIALS USED DURING THE STUDY

A.EQUIPMENTS

Hot air oven	Advantec (Japan)
Incubator	Yamato (Japan)
Autoclave	Ravi (India)
Refrigerator	Sanyo (Japan)
Microscope	Olympus (Japan)
Centrifuge	Remi (India)
Weighing Machine	Chyo MP (Japan)

B. MICROBIOLOGICAL MEDIA

Bile broth	Mueller Hinton agar
Blood agar base	Triple Sugar Iron agar
Mueller Hinton broth	Nutrient agar
Mac Conkey agar	Urea broth
Simmon’s Citrate agar	Sulphur Indole Motility
MR-VP medium	

C. CHEMICALS AND REAGENTS

3%Hydrogen peroxide	Barritt’s reagent
Crystal violet	Kovac’s reagent
Gram’s iodine	Barium chloride
Absolute (95%) alcohol	Sulphuric acid
Safranine	Normal saline

D. ANTIBIOTIC DISCS

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

Amoxycillin (30mcg)
Cephotaxime (30mcg)
Chloramphenicol (30mcg)
Ciprofloxacin (5mcg)
Cotrimoxazole (Trimethoprim/Sulphamethoxazole) (1.25/23.75mcg)
Nalidixic acid (30mcg)
Ofloxacin (5mcg)

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 sticks

APPENDIX C

A. COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

The culture media used were from two companies:

a. Hi-Media Laboratories Pvt. Limited, Bombay, India.
b. Oxoid Unipath Ltd. Basingstoke, Hampshire, England

(All compositions are given in grams per liter and at 25^0C temperature)

1. MacConkey Agar (Hi Media Laboratories)

Ingredients	**gm/litre**
Peptic digest of animal tissue	1.5
Casein enzymic hydrolysate	1.5
Pancreatic digest of gelatin	17.0
Lactose	10.0
Bile salts	1.50
Sodium chloride	5.0
Crystal violet	0.001
Neutral Red	0.03
Agar	15.0
Final pH (at 25^0C)	7.1±0.2

Direction: 51.5 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 121^0C (15 lbs pressure) for 15 minutes. The medium was poured into sterile petriplates.

2. Mueller Hinton Agar (Hi Media Laboratories)

Ingredients	**gm/litre**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Agar	17.0
Final pH (at 25^0C)	7.4±0.2

Direction: 38 grams of the medium was suspended in 1000 ml distilled water and the medium was warmed to dissolve completely. It was sterilized by autoclaving at 121ºC (15 lbs pressure) for 15 minutes and poured into sterile petriplates.

3. Nutrient Agar (Hi Media Laboratories)

Ingredients	**gm/litre**
Peptic digest of animal tissue	5.0
Sodium Chloride	5.0
Beef Extract	1.5
Yeast Extract	1.5
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction: 28 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 121^0C (15 lbs pressure) for 15 minutes.

5. Mueller Hinton Broth (Hi Media Laboratories)

Ingredients	**gm/litre**
Beef, Infusion form	300.0
Casein Hydrolysate	17.5
Starch	1.5
Final pH (at 25^0C)	7.4±0.2

Direction: 21 grams of medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers and was sterilized by autoclaving at 121˚C for 15 minutes.

6. Nutrient Broth (Hi Media Laboratories)

Ingredients	**gm/litre**
Peptic digest of animal tissue	5.0
Sodium chloride	5.0

Beef extract	1.50
Yeast extract	1.50
Final pH (at 25^0C)	7.4±0.2

Direction: 13 grams of the medium was dissolved in 1000 ml of distilled water, boiled and dispensed into small containers. It was then sterilized by autoclaving at 121˚C for 15 minutes.

7. Bile broth

Ingredients	**Units**
Bile salt	5.0 gm
Nutrient broth	1 liter

Direction: As directed by manufacturing company, 10 gm of bile salt was added to 1000ml of nutrient broth. 45 ml of media was placed into blood culture bottles. The media was then sterilized by autoclaving at 15lbs pressure (121^0C) for 15 mins.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL TESTS MEDIA

1. MR-VP Medium (Hi-Media laboratories)

Ingredients	**gm/litre**
Peptone	5.0
Dextrose	5.0
Dipotassium Phosphate	5.0
Final pH (at 25^0C)	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 121^0C for 15 minutes.

2. Sulphide Indole Motility (SIM) medium (Hi Media Laboratories)

Ingredients	**gm/litre**
Beef extract	3.0

Peptone	30.0
Peptonized iron	0.2
Sodium Thiosulphate	0.25
Agar	3.0
Final pH (at 25^0C)	7.3±0.2

Direction: 36 grams of the medium was suspended in 1000 ml of distilled water and dissolved completely. Then it was distributed in tubes to a depth of about 3 inches and sterilized by autoclaving at 121ºC for 15 minutes.

3. Simmon's Citrate Agar (Hi Media Laboratories)

Ingredients	**gm/litre**
Magnesium sulfate	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 25^0C)	6.8±0.2

Direction: 24.2 grams of the medium was dissolved in 1000ml of distilled water. 3ml medium was distributed in test tubes and sterilized by autoclaving at 121^0C for 15 minutes. After autoclaving, tubes containing medium were tilted to form slant.

4. Triple Sugar Iron Agar (TSI) (Hi Media Laboratories)

Ingredients	**gm/litre**
Peptone	10.0
Tryptone	10.0
Yeast extract	3.0
Beef extract	3.0
Dextrose	1.0

Lactose	10.0
Sucrose	10.0
Ferrous sulphate	0.2
Sodium chloride	5.0
Sodium thiosulphate	0.3
Phenol red	0.024
Agar	12.0
Final pH (at 25^0C)	7.4±0.2

Direction: 65 grams of the medium was dissolved in 1000ml of distilled water and sterilized by autoclaving at 15 lbs (121^0C) pressure for 15 minutes. The medium was allowed to set in sloped form with a butt about 1 inch of length.

5. Urea Base Agar (Hi Media Laboratories)

Ingredients	**gm/litre**
Peptone	1.0
Dextrose	1.0
Sodium chloride	5.0
Dipotassium phosphate	1.2
Monosodium phosphate	0.8
Phenol red	0.012
Agar	15.0
Final pH (at 25^0C)	7.4±0.2

Direction: 24 grams of the medium was suspended in 950 ml of distilled water and sterilized by autoclaving at 121^0C for 121 minutes. After cooling to about 45^0C, 50 ml of 40% urea solution was added aseptically, mixed well and distributed 5 ml amount in sterile test tubes.

C. COMPOSITIN AND PREPARATION OF DIFFERENT STAINING AND TESTS REAGENTS

1. For Gram's Stain

(a) Crystal Violet solution

Crystal Violet	20.0 g
Ammonium Oxalate	9.0 g
Ethanol or Methanol	95 ml
Distilled Water (D/W) to make	1 litre

Direction: In a clean piece of paper, 20 gm of crystal violet was weighed and transferred to a clean brown bottle. 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. Final 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

Direction: 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. Final volume was made 1 litre by adding D/W.

(c) Acetone-Alcohol Decoloriser

Acetone	500 ml
Ethanol (Absolute)	475 ml
Distilled Water	25 ml

Direction: To 25 ml D/W, 475 ml of absolute alcohol was added, mixed and transferred into a clean bottle. Then immediately, 500 ml acetone was added to the bottle and mixed well.

(d) Safranin (Counter Stain)

Safranin	10.0 g
Distilled Water	1000 ml

Direction: In a clean piece of paper, 10 gm of safranin was weighed and transferred to a clean bottle. Then 1 litre D/W was added to the bottle and mixed well until safranin dissolved 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 premarked 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. Biochemical Test Reagents

(a) Catalase Reagent (For Catalase test)

Hydrogen peroxide	3 ml
Distilled Water	97 ml

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

(b) Oxidase Reagent (impregnated in a Whatman's No. 1 filter paper)
(For Oxidase Test)

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

Direction: 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) Kovac's Indole Reagent (For Indole Test)

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

Direction: 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) Methyl Red Solution (For Methyl Red Test)

Methyl red	0.05 g
Ethyl alcohol (absolute)	28 ml
Distilled Water	22 ml

Direction: To 28 ml ethanol, 0.05 gm of methyl red was dissolved and transferred to a clean brown bottle. Then 22 ml D/W was added to that bottle and mixed well.

(e) Barritt's Reagent (For Voges-Proskauer Test)

Solution A

α-naphthol	5.0 g
Ethyl alcohol (absolute)	100 ml

Direction: To 25 ml ethanol, 5 g of α-naphthol was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

Solution B

Potassium hydroxide	40.0 g
Distilled Water	1000 ml

Direction: To 25 ml D/W, 40 gm of KOH was dissolved and transferred into a clean brown bottle. Then the final volume was made 100 ml by adding D/W.

5. **McFarland tube (No. 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

A. GRAM-STAINING PROCEDURE

First devised by Hans Christian Gram during the late 19th century, the Gram-stain can be used effectively to divide all bacterial species into two large groups: those that take up the basic dye, crystal violet (Gram-positive) and those that allow the crystal dye to was out easily with the decolorizer alcohol or acetone (Gram-negative). The following steps are involved in Gram-stain:

1. A thin film of the material to be examined was prepared and dried.
2. The material on the slide was heat fixed and allowed to cool before staining.
3. The slide was flooded with crystal violet stain and allowed to remain without drying for 1 minute.
4. The slide was rinsed with tap water, shaking off excess.
5. The slide was flooded with iodine solution and allowed to remain on the surface without drying for twice as long as the crystal violet was in contact with the slide surface.
6. The slide was rinsed with tap water, shaking off excess.
7. The slide was flooded with alcohol acetone decolorizer for 10 seconds and rinsed immediately with tap water until no further color flows from the slide with the decolorizer. Thicker smear requires more aggressive decolorizing.
8. The slide was flooded with counter-stain (safranine) for 1 minute and washed off with tap water.
9. The slide was blotted between two clean sheets of bibulous paper and examined microscopically under oil immersion at 100X.

B. 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 is 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 autoagglutination

APPENDIX E

METHODOLOGY OF BIOCHEMICAL TESTS USED FOR IDENTIFICATION OF BACTERIA

A. Catalase test

This test is performed to demonstrate the presence of catalase, an enzyme that catalyses the release of oxygen from hydrogen peroxide. During aerobic respiration, in the presence of oxygen, microorganisms produces hydrogen peroxide, which is lethal to the cell itself. The enzyme catalase is present in most cytochrome containing aerobic and facultative anaerobic bacteria, the main exception being *Streptococcus* spp.

Procedure: A small amount of a culture from Nutrient Agar plate was taken in a clean glass slide and about 2-3 drops of 3% H_2O_2 was put on the surface of the slide. The positive test is indicated by the formation of active bubbling of the oxygen gas. A false positive reaction may be obtained if the culture medium contains catalase (e.g. Blood Agar) or if an iron wire loop is used.

B. Oxidase test

This test is performed for the detection of cytochrome oxidase in bacteria which catalyzes the transport of electrons between electron donors. In the presence of redox dye Tetramethyl-*p*-phenylene diamine dihydrochloride, the cytochrome oxidase oxidizes it into a deep purple colored end product Indophenol which is detected in the test. The test is used for screening species of *Neisseria*, *Alcaligenes*, *Aeromonas*, *Vibrio*, *Campylobacter* and *Pseudomonas* which give positive reactions and for excluding the Enterobacteriaceae, all species of which give negative reactions.

Procedure: A piece of filter paper was soaked with few drops of oxidase reagent (Whatman's No. 1 filter paper impregnated with 1% tetramethyl-p-phenylene diamine dihydrochloride). Then the colony of the test organism was smeared on the filter paper. The positive test is indicated by the appearance of blue-purple color within 10 seconds.

C. Indole Production test

This test detects the ability of the organism to produce an enzyme: 'tryptophanase' which oxidizes tryptophan to form indole, skatole (methyl indole) and indole acetic acid. The enzyme tryptophanase catalyses the deamination reaction attacking the tryptophan molecule in its side chain and leaving the aromatic ring intact in the form of indole.

Procedure: A smooth bacterial colony was stabbed on SIM (Sulphide Indole Motility) medium by a sterile stab wire and the inoculated media was incubated at 37°C for 24 hours. After 24 hours incubation, 2-3 drops of Kovac's reagent was added. Appearance of red color on the top of media indicates indole positive. Indole if present combines with the aldehyde present in the reagent to give a red color in the alcohol layer. The color reaction is based on the presence of the pyrrole structure present in indole.

D. Methyl Red test

This test is performed to test the ability of an organism to produce and maintain stable acid end product from fermentation of glucose to give a red color with the indicator methyl red and to overcome the buffering capacity of the system. Medium used in the study was Clark and Lubs medium (MR/VP broth, pH 6.9). Methyl red is an indicator which is already acid and will denote changes in degree of acidity by color reactions over a pH range of 4.4- 6.0.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of methyl red reagent was added and mixed well. The positive test was indicated by the development of bright red color, indicating acidity and negative with yellow color.

E. Voges-Proskauer (VP) test

The principle of this test is to determine the ability of some organisms to produce a acetyl methyl carbinol, a neutral end product (acetoin) or its reduction product 2, 3-butanidiol during fermentation of carbohydrates. An organism of the Enterobacteriaceae group is usually either methyl red positive and Voges- proskauer- negative or methyl red negative

and Voges-Proskauer positive. The Voges proskauer test for acetoin is used primarily to separate *E. coli* from *Klebsiella* and *Enterobacter* species.

Procedure: A pure colony of the test organism was inoculated into 2 ml of MRVP medium and was incubated at 37°C for 24 hours. After incubation, about 5 drops of Barritt's reagent was added and shaken well for maximum aeration and kept for 15 minutes, positive test is indicated by the development of pink red color.

F. Citrate Utilization test

This test is performed to detect if an organism utilizes citrate as sole source of carbon for metabolism resulting alkalinity. The medium used for citrate fermentation (Simmon's Citrate medium) also contains inorganic ammonium salts. Organisms capable of utilizing citrate as its sole carbon source also utilizes the ammonium salts present in the medium as its sole nitrogen source, the ammonium salts are broken down to ammonia with resulting alkalinity.

Procedure: A loopful of test organism was streaked on the slant area of Simmon's Citrate Agar medium and incubated at 37°C for 24 hours. A positive test was indicated by the growth of organism and change of media by green to blue, due to alkaline reaction. The pH indicator bromothymol blue has a pH range of 6.0-7.6, i.e. above pH 7.6; a blue color develops due to alkalinity of the medium.

G. Motility test

This test is done to determine if an organism was motile or non-motile. Bacteria are motile by means of flagella. Flagella occur primarily among the bacilli; however a few cocci forms are motile .Motile bacteria may contain a single flagella. The motility media used for motility test are semisolid, making motility interpretations macroscopic.

Procedure: Motility of organism was tested by hanging drop and cultural method. In cultural method, the test organism was stabbed in the SIM medium and incubated at 37ºC for 48 hours. Motile organisms migrate from the stab-line and diffuse into the medium

causing turbidity. Whereas non-motile bacteria show the growth along the stab-line, and the surrounding media remains colorless and clear.

H. Triple Sugar Iron (TSI) Agar Test

The TSI agar is used to determine the ability of an organism to utilize specific carbohydrate incorporated in the medium (glucose, sucrose and lactose in concentrations of 0.1%, 1.0% and 1.0% respectively), with or without the production of gas (indicated by cracks in the media as well as an air gap at the bottom of the tube) along with determination of possible hydrogen sulfide production (detected by production of black color in the medium). A pH indicator (phenol red) included in the medium can detect acid production from fermentation of these carbohydrates and it gives yellow reaction at acidic pH, and red reaction to indicate an alkaline surrounding.

Procedure: The test organism was streaked and stabbed on the surface of TSI and incubated at 37°C for 24 hours .Acid production limited only to the butt region of the tube is indicative of glucose utilization, while acid production in slant and butt indicates sucrose or lactose fermentation. The results are interpreted as follows:

a. Yellow (Acid)/ Yellow (Acid), Gas, H_2S → Lactose/ Sucrose fermenter, H_2S producer.
b. Red (Alkaline) / Yellow (Acid), No Gas, No H_2S → Only Glucose, not lactose/ Sucrose fermenter, not aerogenic, No H_2S production.
c. Red (Alkaline) / No Change → Glucose, Lactose and Sucrose non-fermenter.
d. Yellow (Acid)/ No Change → Glucose- Oxidiser.
e. No Change / No Change →Non-fermenter.

I. Urea Hydrolysis test

This test demonstrates the urease activity present in certain bacteria which decomposes urea, releasing ammonia and carbon dioxide. Ammonia thus produced changes the color of indicator (phenol red) incorporated in the medium.

Procedure: The test organism was inoculated in a medium containing urea and indicator phenol red. The medium was then incubated at 37°C overnight. Positive organism shows

pink red color due to the breakdown of urea to ammonia. With the release of ammonia the medium becomes alkaline as shown by a change in color of the indicator to pink.

APPENDIX F

ZONE SIZE INTERPRETATIVE CHART

Antimicrobial Agents used	Symbol	Disc Content	Resistant (mm or less)	Intermediate (mm)	Susceptible (mm or more)
Amoxycillin/ Clavulanic acid	AMC	20/10 µg 30 µg	19	-	20
Ceftriaxone	CTR	30 µg	13	14-20	21
Chloramphenicol	C	30 µg	12	13-17	18
Ciprofloxacin	CIP	5 µg	15	16-20	21
Cotrimoxazole (Trimethoprim/ Sulphonamide)	COT	1.25/23.75 µg	10	11-15	16
Ofloxacin	OF	5 µg	12	13-15	16

(Source: Product Information Guide, Hi-Media Laboratories Pvt. Limited, Bombay, India)

APPENDIX G

DATA ANALYSIS (CHI-SQUARE TEST)

Association of prevalence of enteric fever between genders

Gender	*Salmonella* positive	*Salmonella* negative	Total
	58	932	**990**
Female	29	523	**552**
Total	**87**	**1455**	**1542**

Test statistic is χ^2

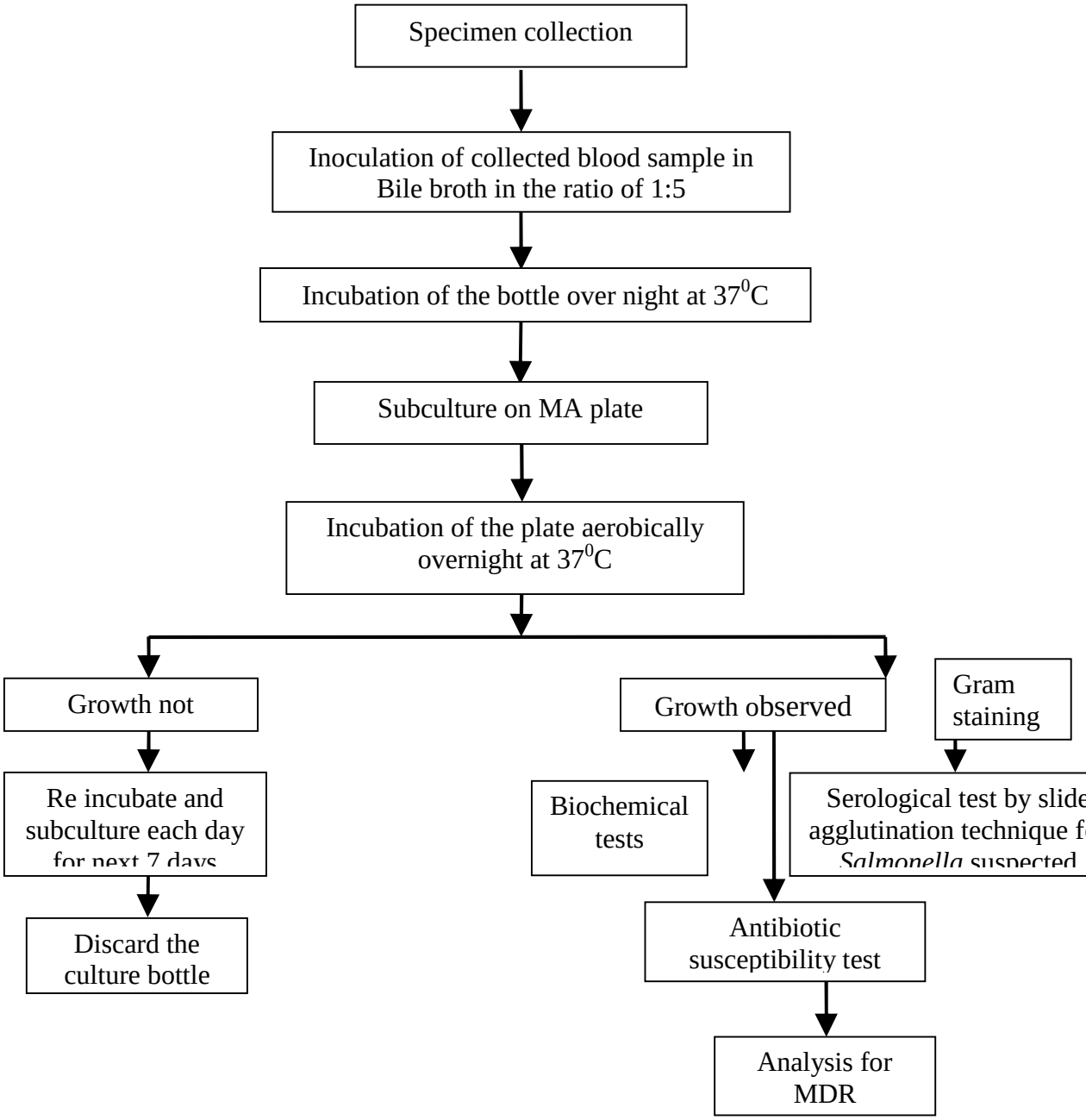
Ho: There is no significant association of prevalence of enteric fever between male and female patients.

H1: There is significant association of enteric fever between male and female patients.

From $\chi^2=\Sigma (0-E)^2/E$ we find $\chi^2=0.2157$

Thus $\chi^2_{cal}(0.2157) < \chi^2_{tab}$ at $\alpha=0.05$ and d.f. = 1 i.e.3.841

Hence, Ho is accepted i.e. there is no significant association of prevalence of enteric fever between male and female patients.


Specimen collection
Inoculation of collected blood sample in Bile broth in the ratio of 1:5
Incubation of the bottle over night at 37^{0}C
Subculture on MA plate
Incubation of the plate aerobically overnight at 37^{0}C
Growth not
Re incubate and subculture each day for next 7 days
Discard the culture bottle
Growth observed
Biochemical tests
Gram staining
Serological test by slide agglutination technique f
Salmonella suspected
Antibiotic susceptibility test
Analysis for MDR