CHAPTER-I

INTRODUCTION

Salmonella enterica subspecies *enterica* serovar Typhi is the etiological agent of typhoid fever. According to a recently-revised global estimate, it roughly infects 21.6 million people and kills an estimated 200,000 people every year (Brusch *et al*., 2008). Enteric fever is an important cause of illness and death in the over crowded and unsanitary areas and is the leading cause of morbidity during summer season in Kathmandu. 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). The last outbreak of typhoid fever in Nepal was in Bharatpur during 2002 which was the largest single point source, multi drug resistance typhoid fever outbreak reported in the literature (Lewis *et al.*, 2005).

The major causes of typhoid fever are *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* 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 harboring *S.* Typhi in gallbladder (WHO, 2003). Attack rate are highest in persons younger than 20 years or older than 70 years. The highest rate is found in infants (130 isolates per 100,000) (Owens *et al*., 2008).

Typhoid fever is characterized by the classic prolonged fever, sustained bacteraemia without endocardial or endothelial involvement, and bacterial invasion of and multiplication within the mononuclear cells of liver, spleen, lymph nodes, and payer 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).

The usual case-fatality rate of 10% can be reduced to <1% with prompt antibiotic therapy (Brusch *et al*., 2008). Chloramphenicol, Ampicillin, Tetracycline and Cotrimoxazole were the first line drugs for treatment of typhoid fever (WHO, 2003). Due to emergence of first line drugs, Fluoroquiolone particularly Ciprofloxacin was the most frequently used antibiotic (Ahmed *et al*., 2006).

The antibiotic resistance of *Salmonella* species is a global concern that includes multi-drug resistant strains. Recent outbreak show that a connection may exist between antimicrobial drug treatment and the risk of disease from *Salmonella* species. There are two categories of drug resistance: resistance to antibiotic such as Chloramphenicol, Ampicillin, Cotrimoxazole (MDR strains) and resistance to the Fluroquinolones drug (WHO, 2003). Multidrug resistant *S.* Typhi are still common in many areas of the Asia, although some areas strain that are fully susceptible to first line drug have reemerged (Sood *et al*., 1999).

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). The definitive diagnosis of typhoid fever depends on the isolation of *S.* Typhi from blood and more than 80% of patients have the causative organisms in their blood (WHO, 2003). The organism may be recovered from the bloodstream at any stage of the illness, but are most commonly found during the first 7 to 10 days and during relapses (Chart *et al*., 2006).

The isolation of *S.* Typhi or *S.* Paratyphi from the blood or faeces of patients confirms diagnosis; however, in the absence of a culturable organism, patient's antibody may provide evidence of infection with these serovars of *Salmonella* (Chart *et al*., 2007). The

presence of clinical symptoms characteristics of typhoid fever or the detection of a specific antibody response is suggestive of typhoid fever but not definitive. The result of single Widal test has no diagnostic significance in an endemic region; in part due to difficulty in establishing a steady-state or baseline titre of Widal agglutination, which limits the usefulness of the test as a reliable diagnostic indicator of the disease process (Olopoenia and King, 2000). Widal test can be diagnostic if patients have four-fold or greater increases in O or H agglutinin titres in serum specimens obtained 2-3 weeks apart (Olopoenia and King, 2000; Cheesbrough, 2005). Usually up to 70% of adults show an early rise of antibody titer in the first week of infection and significant titers should be greater than 1:80 for anti-O and greater than 1:160 for anti-H for a presumptive diagnosis of typhoid fever for developing country Nepal (Pokhrel *et al*., 2008).

IDL Tubex test, Typhidot test and IgM dipstick test is the available test for the quick diagnosis of typhoid fever but with different sensitivity and specificity (WHO, 2003). Enterocheck-wb is an immunochromatographic test and detect IgM antibody to *Salmonella* Typhi which develops at early stage of typhoid fever suggesting the current infection (Cheesbrough, 2005). Enterocheck-Wb has the sensitivity and specificity of 79.3% and 90.2% respectively (Cheesbrough, 2005). It is rapid and easy to perform.

The antibiotic sensitivity patterns of *Salmonella* serovars are changing and this need to be revised every year. The present study was conducted to study the prevalence of enteric fever, with changing antibiotic sensitivity pattern of *Salmonella* serovars isolated from blood. Widal test which is one of the most utilized tests was conducted to study the serological response of clinically suspected patients collected during the study period. Enterocheck-Wb is specific for detection of IgM antibody against lipopolysaccharide of *S.* Typhi. It was conducted for early and rapid detection of enteric fever. Our study may help to know the present antibiotic sensitivity pattern of the *Salmonella* isolates with usefulness of Widal test in the diagnosis of the enteric fever.

CHAPTER-II

OBJECTIVES

2.1 General objective

To find the prevalence of enteric fever by conventional blood culture method (gold standard) and perform Widal test of blood samples collected from patients clinically suspected of enteric fever.

2.2 Specific objectives

1. To conform the enteric fever by Widal test.
2. To isolate *Salmonella* serovars from blood samples.
2. To perform antibiotic sensitivity testing of the isolated serovars.

CHAPTER-III

LITERATURE REVIEW

3.1 Morphology of *Salmonella* species

Salmonella species are organisms that conform to the definition of the Enterobacteriaceae (Old and Threlfall, 1996). They are primarily intestinal parasites of man and animals, both domestic and wild. They are frequently found in sewage, river and other waters and soil. They may survive for weeks in water (89 days in tap water and 115 days in pond water) and for years in soil (Abbott and Janda, 2006). They are primarily intestinal parasites of vertebrates. They are pathogenic for many species of animals, giving rise to enteritis and to typhoid-like diseases. G + C content of DNA are 50-53 mol% (Old and Threlfall, 1996).

They are Gram-negative bacilli, 2-4µm x 0.6 µm, non-acid-fast, non-sporing and non-capsulated. Most serotypes are motile with peritrichous flagella, but *S.gallinarum* and *S.pullorum* are non-motile variants (OH-O variation) of other serotypes are ocassionally found. 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).

3.2 Cultural characteristics

On nutrient agar and blood agar after 24hrs, at 37ºC, the colonies of most strains of *Salmonella* species are moderately large (2-3 mm in diameter), grey-white, moist. They are aerobic and facultatively anaerobic. They grow on simple laboratory media in temperature range 15ºC – 41ºC, optimally at 37ºC, producing colonies that are indistinguishable from those of other *Enterobacteriaceae*. On nutrient agar the colonies are circular discs with a smooth convex surface and entire edge. Their size and opacity varies with the serotypes (Janda and Abbott, 2005).

3.3 Virulence factors

There are various virulence factors of *Salmonella* species. Their cell wall contains lipopolysaccharide with an antigenic polysaccharide (O antigen) endotoxin. The surface antigen (Vi antigen) of *S.* Typhi has anti-phagocytic properties. They produce and excrete a protein known as "invasion" that allows non-phagocytic cells to take up the bacterium, where it is able to live intracellularly. It is able to inhibit the oxidative brust of leukocytes, making innate immune response ineffective. Several secreted factor such as catalase, superoxide dismutase, defensins (small cationic proteins) enable the bacteria to survive inside the phagocytic cells. They survive gastric acidity. Invasions are proteins that mediate adherence to and penetration of intestinal epithelial cells. The synthesis of these proteins by the bacterial cell is under the control of invasion "inv" genes. They are protected from stomach acid and acid pH of phagosome by acid tolerance response (ATR) genes of chromosome (Cheesbrough, 2005).

3.4 Nomenclature

The nomenclature for the genus *Salmonella* has evolved from the initial serotype-one species concept proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens. The defining development in *Salmonella* taxonomy occurred in 1973 when Crosa *et al.,* demonstrated by DNA-DNA hybridization that all serotypes and subgenera I, II, and IV of *Salmonella* and all serotypes of "Arizona" were related at the species level; thus, they belonged in a single species. The single exception, subsequently described, is *S. bongori,* previously known as subspecies V, which by DNA-DNA hybridization is a distinct species (Brenner *et al*., 2000).

With the emergence of one species concept, *Salmonella choleraesuis* for the single *Salmonella* species with seven subspecies was defined (Le Minor *et al.,* 1982). Due to confusion caused by using "choleraesuis" as a name for both a species and serovar, Le Minor and Popoff (1987) requested that the name of this species be changed from *Salmonella choleraesuis* to *Salmonella enterica*, an epithet never used before to designate a *Salmonella* serovar. In 2002, the Judicial Commission of the international

committee of Systematic Bacteriology issued an opinion (The Judicial Opinions 80) which finally approved that from January 2005, *Salmonella enterica* would replace *Salmonella choleraesuis* to become the type species of the genus *Salmonella.*

In May 2004, Shelobolina *et al*., proposed the name *Salmonella subterranea* for a strain isolated from a low-pH, nitrate- and U (VI)-contaminated subsurface sediment. Analysis of the 16S rDNA sequence of the isolate indicated that this strain was 96.4% similar to *Salmonella bongori* and 96.3% similar to *Enterobacter cloacae*. The name *Salmonella subterranea* was validly published on 18 March 2005.

3.5 Antigenic structure

The genus *Salmonella* is subdivided in the Kauffmann-White classification into more than 2300 serotypes containing different types of antigenic combinations. The identification of these serotypes depends on detection of the O (somatic) and H (flagellar) antigens by means of agglutination tests with specific antisera. Many different serotypes have one or more of their O or H antigens in common. The antigens of *Salmonella* species are also found in some members of other genera; example *Escherichia* spp., *Shigella* spp., *Citrobacter* spp. and *Proteus* spp.

3.5.1 O (Somatic) Antigens: These antigens represent the side chains of repeating sugar units projecting from the outer lipopolysaccharide layer of the bacterial cell wall. The O-antigens are heat- stable and alcohol-stable.

3.5.2 H (Flagellar) Antigens: These antigens represent the determinant groups in the flagellar protein. They are heat-labile as well as alcohol-labile. The detached flagella remain immunogenic, but not the bacterium. Many *Salmonella* species show diphasic production of flagellar antigens and each strain can spontaneously and reversibly vary between these two phases with different sets of H-antigens.

3.5.3 Vi- Antigen: Almost all strains of *S.* Typhi form the Vi-antigen as a covering layer outside their cell wall. It is an acidic polysaccharide.

3.5.4 M-Antigen: It is an extracellular polysaccharide antigen and is produced by those strains of *S.* Paratyphi B and other serotypes that form mucoid colonies when cultures are held for several days at room temperature after incubation for 1 day at 37ºC.

3.5.5 Fimbrial Antigens: The type-1 fimbriae formed by most strains of *Salmonella* species, bear antigens that determine agglutination by sera containing anti-fimbrial antibodies. Fimbriae are not found in young (6-24 hours old) broth cultures, but can be found in 24-48 hours old broth cultures.

3.6 Kauffman white scheme classification

Serological classification of S*almonella* species is by Kauffman and White scheme which forms the basis of serotyping and depends on the identification by agglutination of the structural formula of O and H antigens of the strains. *Salmonella* species are initially classified into groups based on the presence of distinctive O antigen factors (O antigen factors were formerly designated by Roman numerals – I, II, III, etc - but are now indicated by Arabic numerals- 1, 2, 3 etc). Within each group differentiation is done by identification of phase 1 and phase 2 flagellar antigens (Janda and Abbott and, 2005).

Table 3.1: Illustrations of the Kauffman white scheme of serological classification

O serogroup	Serotype	O antigens	H antigens	
			Phase 1	Phase 2
2 (A)	Paratyphi A	1, 2, 12	a	[1,5]
4 (B)	Paratyphi B	1, 4, [5], 12	b	1, 2
	Stanley	1, 4, [5], 12, 27	d	1, 2
	Schwarzengrund	1, 4, 12, 27	d	1, 7
	Saintpaul	1, 4, [5], 12	e, h	1, 2
	Derby	1, 4, [5], 12	f, g	[1, 2]
	Agona	1, 4, 12	f, g, s	[1, 2]
	Typhimurium	1, 4, [5], 12	i	1,2
	Bredeney	1, 4, 12, 27	1, v	1, 7
	Brandenburg	1, 4, [5], 12, 27	1, v	e, n, z_{15}
	Heidelberg	1, 4, [5], 12	r	1, 2
7 ($C_{1)}$	Choreraesuis	6, 7	c	1, 5
	Paratyphi C	6, 7[Vi]	c	1, 5
	Livingstone	6, 7, 14	d	1, w
	Montevideo	6, 7, 14	g, m, [p], s	[1, 2, 7]
	Thompson	6, 7, 14	k	1, 5
	Virchow	6, 7	r	1, 2
	Infantis	6, 7, 14	r	1, 5
	Mbandaka	6, 7, 14	z_{10}	e, n, z_{15}
8 (C_2-C_3)	Muenchen	6, 8	d	1, 2
	Newport	6, 8, 20	e, h	1, 2
	Hadar	6, 8	z_{10}	e, n, x
9 (D_1)	Typhi	9, 12 [Vi]	d	-
	Enteritidis	1, 9, 12	g, m	[1, 7]
	Dublin	1, 9, 12[Vi]	g, p	-
	Panama	1, 9, 12	1,v	1, 5

	Gallinarum	1, 9, 12		-
3, 10 (E_1)	Anatum	3, 10, (15), [15,34]	e, h	1, 6
1, 3, 19 (E_4)	Senftenberg	1, 3, 19	g, [s], t	-
11 (F)	Rubislaw	11	r	e, n, x
13 (G)	Kedougou	1, 13, 23	i	1, w
6, 14 (H)	VI	1, 6, 14, 25	a	e, n, x
16 (I)	II	16	g, [m], [s], t	[e, n, x]
18 (K)	IIIa	18	z_4, z_{32}	-
21 (L)	IIIb	21	i	1, 5, 7
43 (U)	IV	43	z_4, z_{23}	-
48 (Y)	V	48	z_{35}	-
60	IIIb	60	r	e, n, x, z_{15}
61	IIIb	61	k	1, 5, 7

Source: (Old and Threlfall, 1996)

3.7 Enteric fever

Typhoid fever, also known as enteric fever, is a potentially fatal multi systemic illness caused primarily by *S.* Typhi. It has been a major human pathogen for thousands of years, thriving in conditions of poor sanitation, crowding, and social chaos. The name *S.* Typhi is derived from the ancient Greek typhos, an ethereal smoke or cloud that was believed to cause disease and madness (Brusch *et al.*, 2008). Although antibiotics have markedly reduced the frequency of typhoid fever in the developed world, it remains endemic in developing countries (Christie, 1987; Chau *et al*., 2007).

3.7.1 Transmission of Enteric fever

S. Typhi has no nonhuman vectors. They are transmitted orally from food and beverages handled by an individual who chronically shed the bacteria through stool or less commonly from urine and also from sewage-contaminated water or shellfish especially

in the developing world (Ram *et al*., 2007), hand to mouth transmission after using a contaminated toilets and neglecting hand hygiene (WHO, 2003). Bhatta *et al.*, 2007 reported that urban water supply was poor in Kathmandu and might cause fatal outbreaks.

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)

3.7.2 Risk factors

S. Typhi is able to survive a stomach pH as low as 1.5. Antacids, histamine-2 receptor antagonists (H2 blockers), proton pump inhibitors, gastrectomy, and achlorhydria decrease stomach acidity and facilitate *S*. Typhi infection (Parry *et al*., 2002).

HIV/AIDS is clearly associated with an increased risk of nontyphoidal as well as typhoidal *Salmonella* species (Gotuzzo *et al.*, 1991; Gordon *et al.*, 2008; Monack *et al.*, 2004). Other risk factors for clinical *S.* Typhi infection include various genetic polymorphisms and often predispose to other intracellular pathogens (Ali *et al*., 2006).

On the other hand, protective host mutations also exist. The fimbriae of *S.* Typhi bind in vitro to cystic fibrosis trans-membrane conductance receptor (CFTR), which is expressed on the gut membrane. The homozygous F508del mutation in CFTR is associated with cystic fibrosis. Thus, typhoid fever may contribute to evolutionary pressure that maintains a steady occurrence of cystic fibrosis (Van *et al*., 2005; Poolman and Galvani, 2007).

Environmental and behavioral risk factors that are independently associated with typhoid fever include eating food from street vendors, living in the same household with someone who has new case of typhoid fever, washing the hands inadequately, sharing

food from the same plate, drinking unpurified water, and living in a household that does not have a toilet (Ali *et al*., 2006; Ram *et al*., 2007).

3.7.3 Frequency

Typhoid fever occurs worldwide, primarily in developing nations whose sanitary conditions are poor. Bangladesh, China, India, Indonesia, Laos, Nepal, Pakistan, and central Vietnam these eight countries are home to more than 80% of the world's typhoid fever cases (Chau *et al*., 2007).

3.7.4 Mortality/Morbidity

With prompt and appropriate antibiotic therapy, typhoid fever is typically a short-term febrile illness with a negligible risk of mortality. Untreated typhoid fever is a life-threatening illness with long-term morbidity (Crump *et al*., 2008).

3.7.5 Age

Most documented typhoid fever cases involve school-aged children and young adults (older than 70 years). However, the true incidence among very young children and infants is thought to be higher (Dutta *et al.,* 2001).

3.7.6 Patho-physiology of Enteric fever

All pathogenic *Salmonella* species are engulfed by phagocytic cells, which pass them through the mucosa and present them to the macrophages in the lamina propria. Non typhoidal *Salmonella* species are phagocytized throughout the distal ilium and colon. With toll-like receptor (TLR)–5 and TLR-4/MD2/CD-14 complex, macrophages recognize pathogen-associated molecular patterns (PAMPs) such as flagella and lipopolysaccharides. Macrophages and intestinal epithelial cells then attract T cells and neutrophils with interleukin 8 (IL-8), causing inflammation and suppressing the infection (Raffatellu, 2006; Parry *et al.,* 2002).

In contrast to the nontyphoidal *Salmonella* species, *S.* Typhi enters the host's system

primarily through the distal ilium. S. Typhi has specialized fimbriae that adhere to the epithelium over clusters of lymphoid tissue in the ilium (Peyer patches), the main relay point for macrophages traveling from the gut into the lymphatic system. *S.* Typhi has a Vi capsular antigen that masks PAMPs, avoiding neutrophil-based inflammation. The bacteria then induce their host macrophages to attract more macrophages (Raffatellu, 2006).

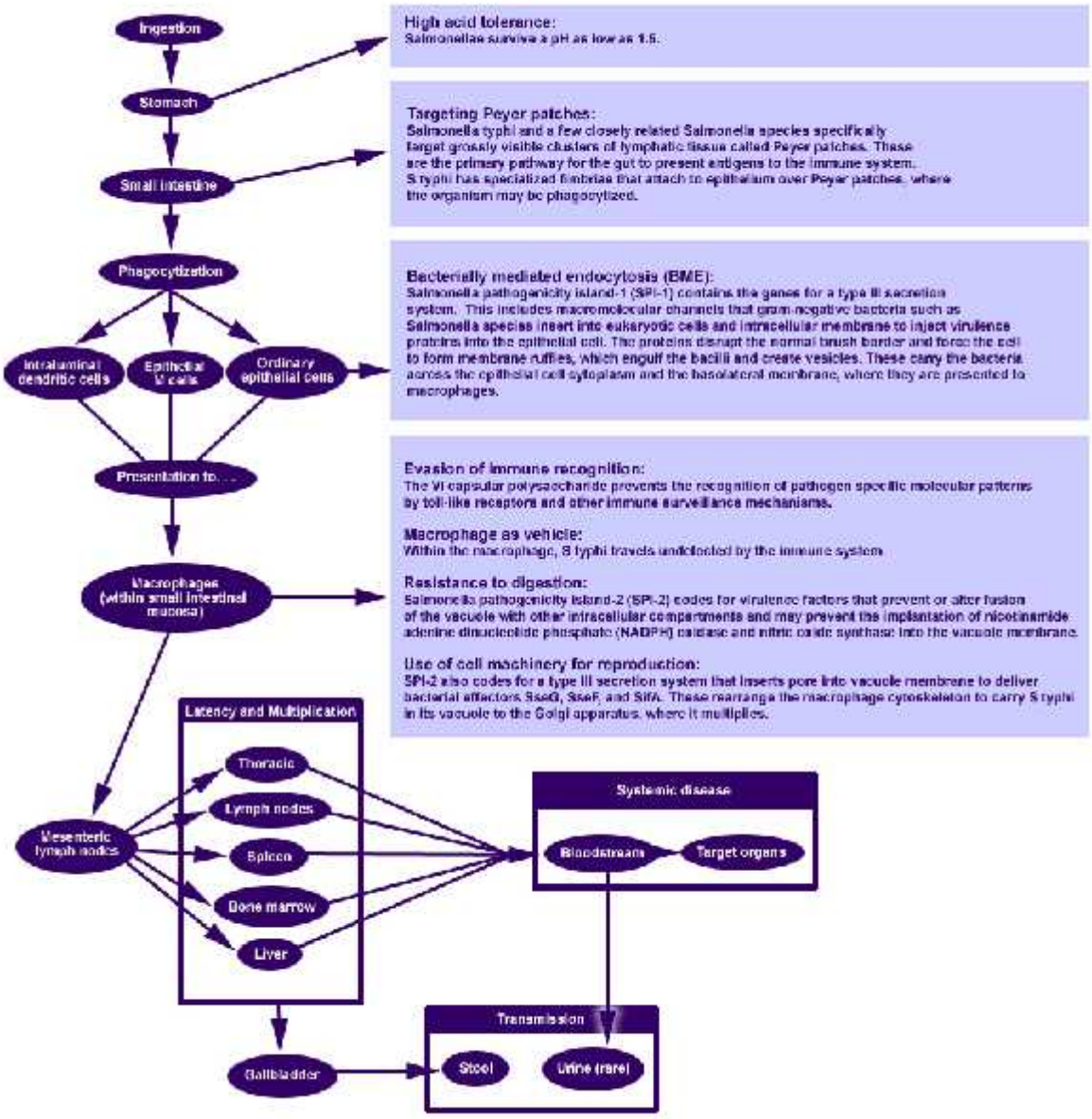


Figure 1: Life cycle of *Salmonella* Typhi (Source: Brusch *et al*., 2008)

It co-opts the macrophages' cellular machinery for their own reproduction as it is carried through the mesenteric lymph nodes to the thoracic duct and the lymphatics and then through to the reticulo-endothelial tissues of the liver, spleen, bone marrow, and lymph nodes. Once there, the *S.* Typhi bacteria pause and continue to multiply until some critical density is reached. Afterward, the bacteria induce macrophage apoptosis, breaking out into the bloodstream to invade the rest of the body (Parry *et al*., 2002). The gallbladder is then infected via either bacteremia or direct extension of *S.* Typhi infected bile. The result is that the organism re-enters the gastrointestinal tract in the bile and re infects Peyer patches. Bacteria that do not re infect the host are typically shed in the stool and are then available to infect other hosts (Parry *et al*., 2002).

3.8 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 the 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 patches and narrows the bowel lumen, causing constipation that lasts the duration of the illness. The individual then develops a dry cough, dull frontal headache, delirium, and an increasingly stuporous malaise (Christie, 1987; Brusch *et al*., 2008).

At approximately the end of the 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, maculopapules usually (1-4) cm wide and fewer than 5 in number; these generally resolve within 2-5 days (Christie, 1987; Brusch *et al*., 2008). During the second week of illness, the abdomen becomes distended, and soft splenomegaly is common. Relative bradycardia and dicrotic pulse may develop (Brusch *et al*., 2008).
In the third week, the still febrile individual grows more toxic and anorexic with significant weight loss. Abdominal distension is severe. Some patients experience foul,

green-yellow, liquid diarrhea (pea soup diarrhea). Necrotic Peyer patches may cause bowel perforation and peritonitis (Rahaman *et al.*, 1977; Cunha *et al.*, 2005).

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

3.8.1 Laboratory diagnosis of enteric fever

3.8.1 Specific serologic test

These assays identify antigen or antibody against *Salmonella* species in the suspected patients and support the diagnosis of typhoid fever, but the results should be confirmed with cultures or DNA evidence.

a. Widal test

This test was the mainstay for diagnosis of typhoid fever from decades. It was developed by Fernand Widal (1862-1929 AD) in 1896. It is based on demonstrating the presence of agglutinin (antibody) in the serum of an infected patient, against the H (flagella) and O (somatic) antigens of *S.* Typhi. The role of the test had been utilized to increase the index of suspicion for the presence of typhoid fever by demonstrating a positive agglutination during the acute and convalescent period of infection with evidence of a four-fold rise of antibody titer (WHO, 2003).

Principle

It provides a simple way of qualitatively and semi-qualitatively estimating the antibodies to *S.* Typhi (TO and TH) and *S.* Paratyphi (AH and BH). It is based on the principle of direct agglutination. When patient's serum (containing antibodies to *S.* Typhi and S Paratyphi) is mixed with the respective antigens, visible agglutination indicated the presence of antibodies to the particular antigens (O, H, AH and BH). A

rising titer of antibodies is indicative of enteric fever. No agglutination is a negative test result indicating absence of anti-*Salmonella* antibodies (Cheesbrough, 2005).

In Widal test the patient's serum is tested for TO and TH antibodies against the following antigen suspensions (usually stained suspensions) (Cheesbrough, 2005).
S. Typhi O 9, 12 suspensions
S. Typhi H d suspension
If testing for paratyphoid, the following antigen suspensions are required
S. Paratyphi A O 1, 2, 12 and S. Paratyphi A H, a [for Paratyphoid A]
S. Paratyphi B O 1, 4, 5, 12 and *S.* Paratyphi B H, b, phase I [for Paratyphoid B]

S. Typhi share O and H antigens with other *Salmonella* serotypes and has cross-reacting epitopes with other *Enterobacteriaceae,* and this can lead to false-positive results. Such results may also occur in other clinical conditions, e.g. malaria, typhus, dengue, miliary tuberculosis, endocarditic condition, brucellosis, bacteraemia caused by other organisms, and chronic liver diseases (WHO, 2003; Olopoenia and King, 2000; Cheesbrough, 2005)

Particularly in endemic areas Widal test continues to be plagued with controversies involving the quality of the antigen used and the interpretation of the result (Olopoenia and King, 2000). Determining an appropriate cut-off titer for a positive result can be difficult since it varies between areas and between times in given areas (WHO, 2003). Two types of agglutination techniques are available (Olopoenia and King, 2000).

The slide test: It is rapid test and is used as a screening procedure. Using commercially available antigens of *S*. Typhi, a drop of the suspended antigen is added to an equal amount of previously prepared serum. Agglutinations are visualized as clumps.
The tube test: It is a macroscopic test. It serves as a means of confirming the results of the slide test. A mixture of suspended antigen and antibody is incubated for up to

20 hours at 37°C in a water bath. Agglutinations are visualized in the form of pellets, clumped together at the bottom of the test tube.

Limitations

There are various limitations of Widal test. Firstly the result of a single test has no diagnostic significance in an endemic region because of difficulty in establishing a steady-state or baseline titre of Widal agglutination; it should be limited to a situation in which there is no other confirmatory supportive test, such as positive culture; the inherent variability of the test as well as the cross-reaction of *Salmonella* species with other non-*Salmonella* species and due to the lack of reproducibility of the test result.

Onuigbo, 1990 reported that the diagnosis of typhoid fever by the widal test alone is prone to error and reported that the misuse of the widal test and subsequently misuse of the antibiotic Chloramphenicol should be very strongly recommended.

Parry, 1999 reported that the earliest serological response in acute typhoid fever is a rise in the titer of the TO antibody with an elevation of the TH antibody titer developing more slowly but persisting longer than that of the antibody cut-off titer and elevated level of both TO and TH antibody as measured in the widal test can be helpful in making a presumptive diagnosis of typhoid fever if interpreted with care.

Bhutta, 2006 reported that the sensitivity of the widal test varies between 47 to 77 where as the specificity varies between 50 to 92. Ochiai *et al*., 2005 and Thelma *et al*., 1991 reported that although Widal test was robust and simple to perform but the test lacks sensitivity and specificity, and reliance on it alone in areas where typhoid is endemic may lead to over diagnosis of the enteric fever. Thelma *et al*., 1991 reported that a single Widal test in an endemic area is of no diagnostic value and this test alone cannot be taken as the basis of deciding the duration of antimicrobial therapy.

b. Enterocheck-wb

Accurate diagnosis of typhoid fever at an early stage is not only important for etiological diagnosis but to identify and treat the potential carriers and prevent acute typhoid fever outbreaks (Gopalakrishnan, 2002). The conventional Widal test usually detects antibodies to S. Typhi in the patient serum from the second week of onset of symptoms (Parry *et al.,* 1999). Early rising antibodies to lypopolysaccharide (LPS) O are predominantly IgM in nature (Olopoenia and King, 2000). Detectable levels of IgM antibodies against S. Typhi appear and persist for four months where as IgG antibodies are detected there after and remain in blood for two years (Gopalakrishnan, 2002). Detection of *S.* Typhi specific IgM antibodies instead of IgG or both IgG & IgM (as measured by the Widal test) would serve as a marker for recent infection (Sippel *et al.,* 1989; Cheesbrough, 2005). Enterocheck-wb is a rapid, qualitative, sandwich immunoassay for the detection of IgM antibodies to *S.* Typhi in human serum/plasma or whole blood specimen.

Principle

Enterocheck-Wb utilizes the principle of immunochromatography, a unique two-site immunoassay on a nitrocellulose membrane. The conjugate pad contains two components Anti-human IgM antibody conjugated to colloidal gold and rabbit IgG conjugated to colloidal gold. As the test specimen flows through the membrane test assembly, the highly specific anti-human IgM antibody-colloidal gold conjugate complexes with the *S.* Typhi specific IgM antibodies in the specimen and travels on the membrane due to capillary action along with the rabbit IgG-colloidal gold conjugate. This complex moves further on the membrane to the test region (T) where it is immobilized by the *S.* Typhi specific LPS antigen coated on the membrane leading to formation of a pink to pink- purple colored band. The absence of this colored band in the test region indicates a negative test result. The unreacted conjugate and unbound complex, if any, move further on the membrane and are subsequently immobilized by the anti-rabbit antibodies coated on the membrane at the control region (C), forming a

pink to pink-purple colored band. This control band acts as a procedural control and serves to validate the results.

Sippel *et al.*, 1989 demonstrated that IgM antibodies to *Salmonella* antigen might be of more diagnostic significance in an endemic population than detection of IgG. Bhutta and Mansurali, 1999 reported that despite improved methods of bacteriologic isolation, rapid serologic diagnostic tests for typhoid fever were needed. There is, however, considerable interest in newer methods of diagnosis of typhoid fever such as latex agglutination, coagglutination, and the polymerase chain reaction. The dot-enzyme immunoassay (EIA) is a relatively newer serologic test based upon the presence of specific IgG and IgM antibodies to a specific 50-kD outer membrane protein (OMP) antigen on *S.* Typhi strains 9 and has been commercially marketed as a dot-EIA. The test incorporates nitrocellulose strips impregnated with the OMP antigen and separately identifies IgM and IgG antibodies.

Hatta *et al.*, 2002 reported that the application of a dipstick assay for the detection of *S.* Typhi specific IgM antibodies on samples collected from *S.* Typhi or *S.* Paratyphi A culture-positive patients at the day of admission to the hospital revealed the presence of specific IgM antibodies in 43.5%, 92.9%, and 100% for samples collected 4–6 days, 6–9 days, and > 9 days after the onset of fever, respectively.

c. Others

Indirect hemagglutination, indirect fluorescent Vi antibody, and indirect enzyme-linked immunosorbent assay (ELISA) for immunoglobulin M (IgM) and IgG antibodies to *S.* Typhi polysaccharide, as well as monoclonal antibodies against *S.* Typhi flagellin, are promising, but the success rates of these assays vary greatly in the literature (Sadallah *et al.*, 1990).

3.8.2 Microbiological process

The definitive diagnosis of the typhoid fever depends on the isolation of *S.* Typhi from

blood, bone marrow or specific anatomical lesions. Identification of any organisms isolated from the clinical specimen is based on the biochemical and serological characterization. In addition culture gives the facility of antibiotic sensitivity testing (Cheesbrough, 2005).

a. Culture

The criterion standard for diagnosis of typhoid fever has long been culture for the isolation of the organism. Cultures are widely considered 100% specific. Blood, intestinal secretions (vomits or duodenal aspirate), and stool culture results are positive for *S*. Typhi in approximately 85-90% of patients with typhoid fever who present within the first week of onset. They decline to 20-30% later in the disease course (Brusch *et al*., 2008).

i. **Bone marrow culture**: Culture of bone marrow aspirate is 90% sensitive until at least 5 days after commencement of antibiotics. However, this technique is extremely painful, which may outweigh its benefit. Bone marrow aspiration and blood are cultured in a selective medium (e.g. 10% aqueous oxgall) or a nutritious medium (e.g. tryptic soy broth) and are incubated at 37°C for at least 7 days. Subcultures are made daily to one selective medium (e.g., MacConkey agar) and one inhibitory medium (e.g., *Salmonella*-Shigella agar). Identification of the organism with these conventional culture techniques usually takes 48-72 hours from acquisition (Brusch *et al*., 2008).

ii. Blood culture: More than 80% of patient with typhoid fever have the causative organism in their blood. Blood culture is the mainstay of the diagnosis of enteric fever (WHO, 2003). Multiple blood cultures (>3) yield a sensitivity of 73-97% (Escamilla *et al.*, 1986; Brusch *et al*., 2008). Large-volume (10-30 ml) of blood culture and clot culture may increase the likelihood of detection of the typhoid causing bacteria.

Blood collection: Blood should be collected before antimicrobial treatment has been started and at the time the patient's temperature begins to rise. To increase the chance of

isolating a pathogen, it is usually recommended that at least two specimen (multiple blood collected at different times) should be cultured. Unless proper precautions have been taken, venous blood gets contaminated with normal flora present on the surface of the skin at the site of the vein puncture. Proper skin antisepsis is extremely important at the time of collection of sample. Tinture of iodine (1 to 2%), provided iodine (10%) and chlorhexidine (0.5% in 70% alcohol) are ideal agents. However, some individuals may be hypersensitive to iodine present in some of these (Cheesbrough, 2005).

Volume of blood collected: Generally small children have higher number of bacteria in their blood as compared to adults and hence less quantity (2 to 4 ml) of blood is sufficient for children. 10 to 15 ml of blood should be taken from school children and adults in order to achieve optimal isolation rates (WHO, 2003).

Traditional method of blood culture: The blood should be directly inoculated into 1% bile broth (ox bile medium is recommended for enteric fever pathogens). The optimum ratio of the volume of blood to traditional culture broth should be 1:10 or more (e.g. 1:12). The blood culture bottles should be transported to the main laboratory at ambient temperature (15 to 40 ^{o}C). In the laboratory, blood culture bottles should be incubated at 37^{o}C and checked for turbidity, gas formation and other evidence of growth after 1, 2, 3 and 7 days on blood agar and MacConkey agar plate at 37^{o}C (WHO, 2003).

iii. Stool culture: In particular, stool culture may be positive for *S.* Typhi several days after ingestion of the bacteria secondary to inflammation of the intraluminal dendritic cells. Later in the illness, stool culture results are positive because of bacteria shed through the gallbladder. Stool culture alone yields a sensitivity of less than 50%. A single rectal swab culture upon hospital admission can be expected to detect *S.* Typhi in 30-40% of patients (WHO, 2003).

iv. Culture of rose spot: Cultures of punch-biopsy samples of rose spots reportedly yield a sensitivity of 63% and may show positive results even after administration of

antibiotics (Brusch *et al*., 2008). *S.* Typhi has also been isolated from the urine culture, cerebrospinal fluid, peritoneal fluid, mesenteric lymph nodes, resected intestine, pharynx, tonsils, abscess, and bone (Cheesbrough, 2005).

Table 2: Sensitivities of Cultures

	Incubation	Week 1	Week 2	Week 3	Week 4
Bone marrow aspirate (0.5-1 mL)		90% (may decrease after 5 days of antibiotics)			
Blood (10-30 mL), stool, or duodenal aspirate culture	40-80%		~20%	Variable (20-60%)	
Urine		25-30%, timing unpredictable			

Source: (Brusch *et al*., 2008)

3.8.3 Molecular techniques

PCR has been used for the diagnosis of typhoid fever with varying success. Nested PCR, which involves two rounds of PCR using two primers with different sequences within the H1-d flagellin gene of *S.* Typhi, offers the best sensitivity and specificity. However, no type of PCR is widely available for the clinical diagnosis of typhoid fever (Ambati *et al*., 2007; Parry *et al.*, 2002).

3.8.4 Other nonspecific laboratory studies (Brusch *et al*., 2008)

- Most patients are moderately anemic, have an elevated erythrocyte sedimentation rate (ESR), thrombocytopenia, and relative lymphopenia.
- Most have a slightly elevated prothrombin time (PT) and activated partial thromboplastin time (aPTT) and decreased fibrinogen levels.
- Circulating fibrin degradation products commonly rise to levels seen in subclinical disseminated intravascular coagulation (DIC).
- Liver transaminase and serum bilirubin values usually rise to twice the reference range.

- Mild hyponatremia, hypokalemia and leukopenia are common.

3.8.5 Imaging Studies (Brusch *et al*., 2008)

- Radiography: Radiography of the kidneys, ureters, and bladder (KUB) is useful if bowel perforation (symptomatic or asymptomatic) is suspected.
- CT scanning and MRI: These studies may be warranted to investigate for abscesses in the liver or bones, among other sites.

3.8.6 Treated typhoid Fever

If appropriate treatment is initiated within the first few days of full-blown illness, the disease begins to remit after about 2 days, and the patient's condition markedly improves within 4-5 days. Any delay in treatment increases the likelihood of complications and recovery time (Brusch *et al*., 2008).

3.9 Prevention and control

3.9.1 Antibiotics

Definitive treatment of typhoid fever (enteric fever) is based on susceptibility. As a general principle of antimicrobial treatment, intermediate susceptibility should be regarded as equivalent to resistance. The sensitivity profiles of *S.* Typhi vary geographically (WHO, 2003). The initial antibiotic choice should be based on the sensitivity data of the area in which the infection was acquired. Antibiotic susceptibility varies widely among *S.* Typhi and *S.* Paratyphi strains, depending chiefly on geography. Nalidixic acid is a nontherapeutic drug that is used outside of the United States as a stand-in for fluoroquinolones in sensitivity assays. It is no longer useful clinically (Capoor *et al*., 2007; Brusch *et al*., 2008).

3.9.2 Emerging drug resistance problem

Chloramphenicol was used universally to treat typhoid fever from 1948 until the 1970s, when widespread resistance occurred. Ampicillin and trimethoprim-sulfamethoxazole

(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 of these agents (WHO, 2003). Fluoroquinolones and third-generation Cephalosporins are currently the first-line treatments and yield a better cure rate than Cephalosporins. However, resistance to Fluoroquinolones is widespread in some areas, and sporadic resistance to Cephalosporins has been reported (Brusch *et al*., 2008).

The genes for antibiotic resistance in *S.* Typhi and *S.* Paratyphi A are acquired from *Escherichia coli* and other gram-negative bacteria via plasmids. The plasmids contain cassettes of resistance genes that are incorporated into a region of the *Salmonella* genome called an integron. Some plasmids carry multiple cassettes and immediately confer resistance to multiple classes of antibiotics. The initial strains of antibiotic-resistant *S.* Typhi and *S.* Paratyphi A carried chloramphenicol acetyltransferase type I, which encodes an enzyme that inactivates Chloramphenicol via acetylation. MDR strains may carry dihydrofolate reductase type VII, which confers resistance to trimethoprim (Brusch *et al*., 2008). MDR is associated with a transferable plasmid, while reduced susceptibility to the Fluoroquinolones in serovar Typhi is usually associated with point mutations in the bacterial target genes encoding DNA gyrase and/or DNA topoisomerase IV (Chau *et al.,* 2007). The use of Nalidixic acid as an in vitro stand-in for Fluoroquinolones is unreliable. Mutations in gyr A are the most common form of Fluoroquinolone resistance (Brusch *et al*., 2008).

Roumagnac *et al.,* 2006 suggested that Fluoroquinolone use has driven the clonal expansion of a Nalidixic acid-resistant serovar Typhi haplotype, 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 be the prelude to a worsening drug resistance problem in Asia (Chau *et al*., 2007). The rate of Fluoroquinolone resistance in Southeast Asia is generally high.

Susceptibility to chloramphenicol, TMP-SMZ, and Ampicillin in these areas is rebounding. In Southeast Asia, MDR strains remain predominant (Chau *et al*., 2007).
The most recent professional guideline for the treatment of typhoid fever in south Asia was issued by the Indian Association of Pediatrics (IAP) in October 2006.

Table 3: Antibiotic Recommendations by Origin and Severity

Location	Severity	First-Line Antibiotics	Second-Line Antibiotics
South Asia, East Asia	Uncomplicated	Cefixime PO	Azithromycin PO
	Complicated	Ceftriaxone IV or Cefotaxime IV	Aztreonam IV or Imipenem IV
Eastern Europe, Middle East, sub-Saharan Africa, South America	Uncomplicated	Ciprofloxacin PO or Ofloxacin PO	Cefixime PO or Amoxicillin PO or TMP-SMZ PO or Azithromycin PO
	Complicated	Ciprofloxacin IV or Ofloxacin IV	Ceftriaxone IV or Cefotaxime IV or Ampicillin IV or TMP-SMZ IV
Unknown geographic origin or Southeast Asia	Uncomplicated	Cefixime PO plus Ciprofloxacin PO or Ofloxacin PO	Azithromycin PO*
	Complicated	Ceftriaxone IV or Cefotaxime IV, plus Ciprofloxacin IV or Ofloxacin IV	Aztreonam IV or Imipenem IV, plus Ciprofloxacin IV or Ofloxacin IV

*Note that the combination of azithromycin and fluoroquinolones is not recommended because it may cause QT prolongation and is relatively contraindicated.

3.9.3 Methods of Antimicrobial Susceptibility Testing

Antimicrobial susceptibility testing methods are divided into types based on the principle applied in each system (Lalitha, 2004). They include:

Diffusion	Dilution	Diffusion & Dilution
Stokes method	Minimum Inhibitory Concentration	E Test method
Kirby-Bauer method	i) Broth dilution ii) Agar Dilution	

However, Kirby-Bauer NCCLS modified disc diffusion technique is most commonly applied method in hospitals and other institutions for antimicrobial susceptibility testing of bacteria against the tested antibiotics.

3.9.4 Sanitation

Proper sanitation contributes to reducing the risk of transmission of all diarrheal pathogens including *Salmonella* Typhi. The following measures must be taken for reducing the risk of transmission of all diarrheal pathogens:

1. Appropriate facilities for human waste disposal must be available for all the community.
2. Collection and treatment of sewage, especially during the rainy season, must be implemented.
3. In area where typhoid fever is known to be present, the use of human excreta as fertilizer must be discouraged (WHO, 2003).

3.9.5 Health education: Health education is paramount to raise public awareness on all the above mentioned preventive measures. Health education messages for the vulnerable communities need to be adapted to local conditions and translated into local languages. In order to reach communities, all possible means of communication (e.g. media, schools, women's groups, religious groups) must be applied. Prevention is based on ensuring access to safe water and by promoting safe food handling practices (WHO, 2003).

3.9.6 Vaccination

Vaccination is the cost effective weapon for disease prevention. The WHO estimates that million of infant deaths in the world are due to disease that could be prevented by existing vaccines. For typhoid fever live attenuated vaccines (Ty 21 vaccine), a heat inactivated whole cell vaccine, Vi Polysaccharide vaccine are available. Trial in Nepal for Vi polysaccharide vaccine people involving age 5-44 years showed 75% protection during the 20 months of active surveillance. In a recent study in South Africa, 55% efficacy was demonstrated three years after immunization of children 5-16 years old (WHO, 2003).

3.9.7 Recommendation in vaccine use

The occurrence of *S.* Typhi strain that are resistant to fluoroquinolones emphasizes the need to use safe and effective vaccines to prevent typhoid fever. WHO recommends vaccination for people travelling in high-risk areas where the disease is endemic. People living in such areas, people in refugee camps, microbiologist, sewage workers and children should be the target groups for vaccination (WHO, 2003).

CHAPTER-IV

4. MATERIALS AND METHODS

4.1 Materials

Different materials required for present study are mentioned in Appendix II

4.2 Methods

4.2.1 Study design

The study was a cross sectional and carried out at from June to August 2008 at Bir Hospital.

4.2.2 Collection of sample

Blood samples were collected by using standard aseptic techniques (CDC, 2007). For adults 8 to 10 ml of blood sample was collected by vein puncture using sterile syringe aseptically. For children 5 ml of blood was collected from vein puncture.

4.2.3 Processing of sample

4.2.3.1 Serological test

About 5 ml of blood was transferred into a dry and clean khan's tube, centrifuged and serum was separated for serological test.

Widal test: Widal test in 1:1 ratio of serum and Widal antigen (Tulip Co.) were tested and the titer was determined as less than 80, equal to 80, 160 and 320.

Enterocheck-Wb: About 5µl of serum sample were loaded in the sample loading well, appearance of a line in the test region was observed which was positive for Enterocheck-Wb (Zephyr Co.) after 15 minutes.

4.2.3.2 Blood culture

About 5 ml of blood sample was transferred directly into blood culture bottles (45 ml of 1% bile broth). Immediately after the blood culture bottles were received, they were

given laboratory number and further incubated at 37^{o}C for 24 hours incubation was continued till 7 days and then the samples were discarded.

The culture bottles were examined for the turbidity, gas formation, and other evidence of growth after 1, 2, 3, and 7 days (WHO, 2003) to make presumptive diagnosis of positive culture. The sub culture was done on MacConkey agar plate and blood agar plate (WHO, 2003) after 24 hours of incubation on 1% bile broth. Blind subculture was done on 48 hours, 96 hours and 7 days respectively. The sub cultured plated were examined after over night incubation period aerobically, for non-lactose fermenting colony on MacConkey agar.

4.2.4 Identification of isolates

Identification of significant isolate was done by microbiological technique as described in the Bergey's manual which involves morphological character of the colonies, staining reactions and the biochemical properties (Cheesbrough, 2005).

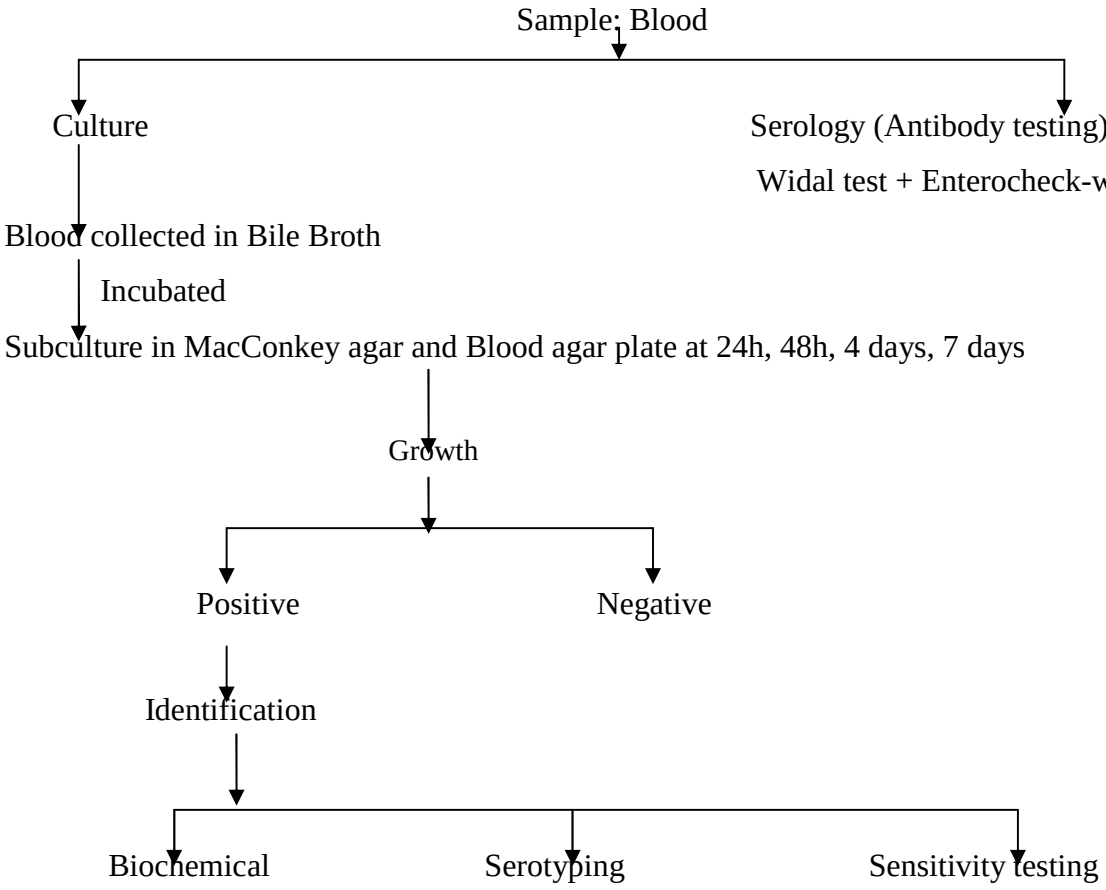
4.2.4.1 Serotyping

Type-specific *Salmonella* antisera (DENKA SEIKEN Co. Ltd. Tokyo Japan) were used for serotyping of the isolates.

4.2.5 Antibiotic sensitivity test

The isolated *Salmonella* species were subjected to in vitro susceptibility testing by modified Kirby-Bauer disc diffusion method for antibiotic test performed on Muller-Hinton agar plates against Ceftriaxone (30µg), Ofloxacin (5µg), Chloramphenicol (30µg), Ciprofloxacin (5µg), Cotrimoxazole (25µg), Ampicillin (30µg), Nalidixic acid (30µg) (Hi Media Laboratory Ltd., Mumbai, 400086 India). Procedures were followed as described by CLSI, 2005 mentioned in Appendix IV.

FLOW CHART OF METHODS



CHAPTER-V

RESULTS

In this study 970 (631 male and 339 female) blood samples from enteric fever suspected patients visiting Bir Hospital were collected from June 2008 to August 2008. Of 970 blood samples, only 503 blood samples were tested for Widal test and 48 samples were tested for Enterocheck-Wb.

5.1. *Salmonella* antibody titer among patients clinically suspected of enteric fever

Of 503 serum samples clinically suspected of enteric fever processed for Widal test for TO, more than half samples had titer less than 1:80, 127 (25.2%) of samples had titer of 1:80, 59 (11.7%) samples had titer of 1:160, and only 8 (1.7%) samples had titer of 1:320. For Widal test of TH, more than half of the samples (63.8%) had titer of less than 1:80, 75 (15%) samples had titer of 1:80, 82 (16.3%) samples had titer of 1:160, and only 25 (5%) samples had titer of 1:320. For Widal test of AH, 451 (89.6%) samples had titer less than 1:80, 21 (4%) samples had titer of 1:80, 24 (4.7%) samples had titer of 1:160, and only 7(1.7%) samples had titer of 1:320. For widal test of BH, maximum (99.4%) sample had titer less than 1:80 and only 3 (0.6%) samples had titer of 1:160.

Table 4: *Salmonella* antibody titer against different antigens

Salmonella agglutinin	TO antigen	TH antigen	AH antigen	BH antigen
≤ 80 titer	309 (61.4%)	321 (63.8%)	451 (89.6%)	500 (99.4%)
80 titer	127 (25.2%)	75 (15%)	21 (4%)	0
160 titer	59 (11.7%)	82 (16.3%)	24 (4.7%)	3 (0.6%)
320 titer	8 (1.7%)	25 (5%)	7 (1.7%)	0
Total	503	503	503	503

Titer of 1:80 for TO and 1:160 for TH was considered suggestive of typhoid fever. From the mentioned criteria, 38.6% (194/503) had titer of 1:80 for TO and 21.3% (107/503) had titer of 1:160 for TH.

5.2. Comparison of different antibody with sample culture positive for *S.* Typhi

Of 43 blood samples positive for *S.* Typhi, 4 (9.45%) samples had titer greater than 1:80 in TO only; 2(4.6%) samples had titer greater than 1:80 in TH only; 10 (23.25%) samples had titer greater than 1:80 in TO and TH both; 2 (4.6%) samples had titer greater than 1:80 in TO, TH, AH; 1(2.3%) samples had titer greater than 1:80 in TO and AH; 1 (2.3%) samples had titer greater than 1:80 in TH and AH; 23 (53.5%) samples had titer less than 1:80 in TO, TH, AH and BH.

Table 5: Comparison of different antibody with sample culture positive for *S.* Typhi

Isolate code	*Salmonella* Agglutinin			
	TO Titer	TH Titer	AH Titer	BH Titer
S_1	<80	<80	<80	<80
S_2	<80	<80	<80	<80
S_3	<80	<80	<80	<80
S_4	<80	160	<80	<80
S_5	<80	<80	<80	<80
S_6	<80	<80	<80	<80
S_7	80	<80	<80	<80
S_8	80	<80	<80	<80
S_9	80	<80	80	<80
S_{10}	<80	<80	<80	<80
S_{11}	<80	<80	<80	<80
S_{12}	<80	<80	<80	<80
S_{14}	<80	<80	<80	<80
S_{16}	160	80	<80	<80
S_{17}	80	<80	<80	<80
S_{18}	160	160	<80	<80
S_{19}	320	320	80	<80

S_{20}	<80	<80	<80	<80
S_{21}	<80	<80	<80	<80
S_{22}	<80	160	160	<80
S_{23}	<80	<80	<80	<80
S_{24}	160	320	80	<80
S_{25}	80	80	<80	<80
S_{26}	80	320	<80	<80
S_{28}	<80	<80	<80	<80
S_{29}	<80	<80	<80	<80
S_{30}	80	80	<80	<80
S_{31}	<80	<80	<80	<80
S_{32}	<80	<80	<80	<80
S_{33}	<80	<80	<80	<80
S_{34}	80	<80	<80	<80
S_{35}	80	80	<80	<80
S_{37}	<80	<80	<80	<80
S_{38}	80	80	<80	<80
S_{39}	<80	80	<80	<80
S_{41}	<80	<80	<80	<80
S_{44}	80	160	<80	<80
S_{45}	160	160	<80	<80
S_{47}	80	<80	<80	<80
S_{48}	<80	<80	<80	<80
S_{49}	<80	<80	<80	<80
S_{50}	<80	<80	<80	<80
S_{51}	<80	<80	<80	<80
S_{52}	<80	<80	<80	<80

Rise in titer of TO and TH antibody is considered suggestive of typhoid fever. 23.25% of sample had titer greater than 1:80 in TO and TH.

5.3. Comparison of different antibody with sample culture positive for *S.* Paratyphi A

Of 39 blood samples positive for *S.* Paratyphi A, 1 (2.5%) sample had titer greater than 1:80 in AH; 5 (12.5%) samples had titer greater than 1:80 in TO and AH; 5 (12.5%) samples had titer greater than 1:80 in TO, TH and AH; 4 (10%) samples had titer greater than 1:80 in TO; 4 (10%) samples had titer greater than 1:80 in TO and TH; 1 (2.5%) sample had titer greater than 1:80 in TH; where as 18 (47.5%) samples had titer of less than 1:80 in TO, TH, AH and BH.

Table 6: Comparison of different antibody with sample culture positive for *S*. Paratyphi A

Isolate code	*Salmonella* Agglutinins			
	TO Titer	TH Titer	AH Titer	BH Titer
B_1	<80	<80	<80	<80
B_2	160	160	160	<80
B_3	<80	<80	<80	<80
B_4	<80	<80	<80	<80
B_5	<80	<80	<80	<80
B_6	<80	<80	<80	<80
B_7	<80	<80	<80	<80
B_8	<80	<80	<80	<80
B_9	80	<80	80	<80
B_{11}	<80	<80	<80	<80
B_{12}	<80	<80	<80	<80
B_{13}	<80	<80	<80	<80
B_{14}	<80	<80	<80	<80

B_{15}	160	<80	<80	<80
B_{16}	80	160	80	<80
B_{17}	80	<80	<80	<80
B_{19}	80	<80	<80	<80
B_{21}	<80	<80	<80	<80
B_{22}	<80	<80	<80	<80
B_{23}	160	80	<80	<80
B_{24}	<80	<80	<80	<80
B_{26}	<80	<80	<80	<80
B_{27}	80	<80	80	<80
B_{28}	160	<80	<80	<80
B_{29}	160	320	320	160
B_{30}	160	80	<80	<80
B_{31}	160	80	<80	<80
B_{32}	<80	<80	160	<80
B_{33}	<80	80	<80	<80
B_{35}	<80	<80	<80	<80
B_{36}	<80	<80	<80	<80
B_{37}	<80	<80	<80	<80
B_{38}	160	320	<80	<80
B_{39}	160	80	160	<80
B_{40}	160	80	80	<80
B_{41}	<80	<80	<80	<80
B_{43}	80	<80	80	<80
B_{44}	80	<80	320	<80
B_{45}	80	80	<80	<80

Rise in titer of TO and AH antibody is considered suggestive of typhoid fever. 10 (25%) of sample had titer greater than 1:80 in TO and TH.

5.4. Comparison of Enterocheck-Wb with culture

Of 48 serum samples from patients clinically suspected of enteric fever, 28 (58%) samples were Enterocheck-Wb positive. In all 48 samples, 14 (29.16%) were culture positive, 5 (10.41%) were *S.* Typhi, 7 (14.3%) were *S.* Paratyphi A. No samples were culture positive for *S.* Typhi but negative on Enterocheck-Wb. Of 20 samples negative for Enterocheck-Wb 2 (4.5%) were culture positive for *S.* Paratyphi A.16 (33.3%) samples were culture negative but positive on Enterocheck-Wb. 18 (37.5%) were negative on culture as well as Enterocheck-Wb.

Table 7: Comparison of Enterocheck-Wb with culture

Result of Culture		Result of Enterocheck-Wb		Total
		Positive	Negative	
S. Typhi	Positive	5 (10.41%)	0	5 (10.41%)
	Negative	16 (33.3%)	18 (37.5%)	34 (70.8%)
S. Paratyphi A	Positive	7 (14.3%)	2 (4.5%)	9 (18.75%)
	Negative	0	0	0
Total		28 (58%)	20 (42%)	48 (100%)

The appearance of colored bands in the test (T) region and control region (c) is diagnostic of enteric fever. 28 (58%) of the tested samples were positive for IgM antibody for lipopolysaccharide of *S.* Typhi.

5.5 Age and gender wise distribution with culture positive cases of enteric fever

The majority of patients 378 (35%) belonged to age group of 20-29 years. Higher percentage of 34 (14.8%) of blood culture positive was observed in the age group of 11-20 years. There was only one case among age group greater than 92 years. The median age of patients was 24 (95% confidence level) years. The male to female ratio was 1.26. There was no significant association of presence of enteric fever with gender of patients (p=0.27). Of total male patients, only 10.8% (68/631) positive blood cultures were

obtained and 8.6% (29/339) of total female patient positive blood cultures were obtained.

Table 8: Age and gender wise distribution with culture positive cases of enteric fever

	Male		Female		Total	
Age Group	Total	Culture positive	Total	Culture positive	Total	Culture positive
2-11	6	0	6	0	12	0
11-20	164	25 (15.24%)	65	9 (13.84%)	229	34 (14.84%)
20-29	257	29 (11.3%)	121	12 (10%)	378	41 (10.8%)
29-38	100	12 (12%)	57	7 (12.28%)	157	19 (12.1%)
38-47	37	2 (5.4%)	37	1 (2.7%)	74	3 (4.1%)
47-56	23	0	24	0	47	0
56-65	18	0	7	0	25	0
65-74	13	0	17	0	30	0
74-83	11	0	3	0	14	0
83-92	2	0	1	0	3	0
92 above	0	0	1	0	1	0
Total	631 (65%)	68 (10.8%)	339 (35%)	29 (8.6%)	970 (100%)	97 (10%)

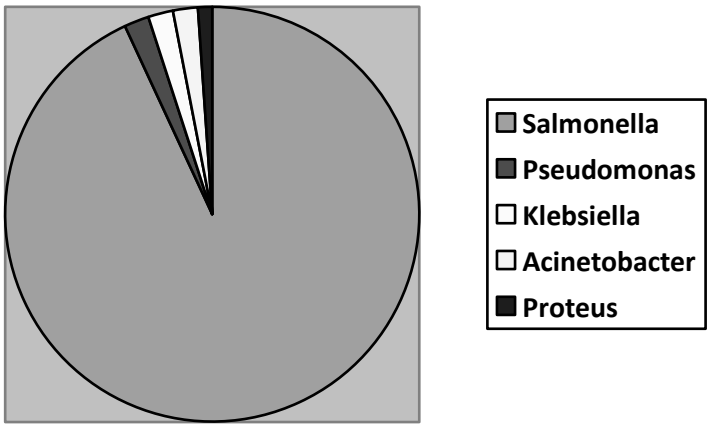


Figure 2: Pattern of bacterial isolates from total positive culture cases

As shown in figure 2, out of 970 blood samples processed for culture, 104 (10.72%) positive growth were obtained. Among total isolates 97 (93.26%) were *Salmonella* spp., 2(2%) *Pseudomonas* spp., 2 (2%) *Klebsiella* spp., 2 (2%) *Acinetobacter* spp., and 1 (1%) *Proteus* spp. Of 97 (10%) *Salmonella* spp. isolated from blood culture, 87 (8.9%) were isolated after 24 hours, 93 (9.5%) after 48 hours, 95 (9.8%) after 96 hours and 97 (10%) after the seventh day of incubation period. 10 (9.6%) of *Salmonella* would have been missed if only 24 hours of incubation was considered.

5.6 Antibiotic sensitivity pattern of each *Salmonella* Typhi

Out of 52 *S.* Typhi isolates, they showed variable antibiotic susceptibility pattern with 98%, 96%, 94.2%, and 88.5% sensitive with Ceftriaxone, Ofloxacin and Chloramphenicol, Ciprofloxacin, and Cotrimoxazole respectively. 90.38% were resistance to Ampicillin. 79% were resistance to Nalidixic acid.

2 (3.82%) of *S.* Typhi were MDR strains (i.e. resistance to Chloramphenicol, Ampicillin, Trimethoprim-Sulfamethoxazole), and also resistant to Nalidixic acid. 5

(9.6%) *S.* Typhi was resistant to Ampicillin and Cotrimoxazole. 3 (5.76%) *S.* Typhi was resistant to Ciprofloxacin as well as Nalidixic acid.

5 (9.6%) of *S.* Typhi were resistant to more than 2 classes of antibiotics.

Table 9: Antibiotic sensitivity pattern of each *Salmonella* Typhi

	Antibiotics tested						
Isolate code	Ampicillin (30µg)	Chloramphenicol (30µg)	Cotrimoxazole (25µg)	Ciprofloxacin (5µg)	Ofloxacin (5µg)	Ceftriaxone (30µg)	Nalidixic acid (30µg)
S_1	R	S	S	R	R	S	R
S_2	R	S	S	S	S	S	R
S_3	R	S	R	R	S	S	R
S_4	R	S	S	S	S	S	S
S_5	R	S	R	R	S	S	R
S_6	R	S	S	S	S	R	S
S_7	R	S	S	S	S	S	R
S_8	R	S	S	S	S	S	R
S_9	I	S	S	S	S	S	S
S_{10}	R	S	R	S	S	S	S
S_{11}	R	S	S	S	S	S	R
S_{12}	R	S	S	S	S	S	R
S_{13}	R	S	S	S	S	S	R
S_{14}	R	S	S	S	S	S	R
S_{15}	S	S	R	S	S	S	R
S_{16}	R	S	S	S	S	S	R
S_{17}	R	S	S	S	S	S	S

S_{18}	R	S	S	S	S	S	R
S_{19}	R	S	S	S	S	S	S
S_{20}	R	S	S	S	S	S	R
S_{21}	R	S	S	S	S	S	S
S_{22}	R	S	S	S	S	S	R
S_{23}	I	S	S	S	S	S	R
S_{24}	R	R	R	S	S	S	R
S_{25}	R	S	S	S	S	S	R
S_{26}	R	S	S	S	S	S	R
S_{27}	R	S	S	S	S	S	R
S_{28}	R	S	S	S	S	S	R
S_{29}	R	S	S	S	S	S	S
S_{30}	R	S	S	S	S	S	R
S_{31}	R	S	S	S	S	S	R
S_{32}	R	S	S	S	S	S	R
S_{33}	R	S	S	S	S	S	S
S_{34}	R	S	S	S	S	S	R
S_{35}	R	S	S	S	S	S	R
S_{36}	R	R	R	S	R	S	R
S_{37}	R	S	S	S	S	S	S
S_{38}	R	S	S	S	S	S	R
S_{39}	R	S	S	S	S	S	R
S_{40}	R	S	S	S	S	S	R
S_{41}	R	S	S	S	S	S	S
S_{42}	R	S	S	S	S	S	R
S_{43}	R	S	S	S	S	S	R
S_{44}	R	S	S	S	S	S	R

S_{45}	R	S	S	S	S	S	R
S_{46}	R	S	S	S	S	S	R
S_{47}	R	S	S	S	S	S	R
S_{48}	R	S	S	S	S	S	R
S_{49}	R	S	S	S	S	S	R
S_{50}	S	S	S	S	S	S	R
S_{51}	R	S	S	S	S	S	R
S_{52}	R	S	S	S	S	S	R

R: Resistant S: Sensitive I: Intermediate

Each strain were considered multidrug resistant when they were resistant to Ampicillin, Cotrimoxazole and Chloramphenicol.

5.7 Antibiotic sensitivity pattern of each *Salmonella* Paratyphi A

Of 45 isolates, *S.* Paratyphi A were 100% susceptibility to Ceftriaxone, Ofloxacin, Chloramphenicol, Ciprofloxacin followed by Cotrimoxazole (95.5%). 95.5% were resistance to Ampicillin. 82.2% were resistance to Nalidixic acid. 2 (4.4%) of *S.* Paratyphi A were resistant to Ampicillin and Cotrimoxazole.

Table 10: Antibiotic sensitivity pattern of each *Salmonella* Paratyphi A

	Antibiotics tested						
Isolate code	Ampicillin (30µg)	Chloramphenicol (30µg)	Cotrimoxazole (25µg)	Ciprofloxacin (5µg)	Ofloxacin (5µg)	Ceftriaxone (30µg)	Nalidixic acid (30µg)
B_1	R	S	S	S	S	S	R
B_2	R	S	S	S	S	S	R
B_3	R	S	S	S	S	S	S
B_4	R	S	S	S	S	S	R

B_5	R	S	S	S	S	S	R
B_6	R	S	S	S	S	S	S
B_7	R	S	S	S	S	S	R
B_8	R	S	S	S	S	S	S
B_9	R	S	S	S	S	S	R
B_{10}	R	S	S	S	S	S	R
B_{11}	R	S	S	S	S	S	R
B_{12}	R	S	S	S	S	S	S
B_{13}	R	S	S	S	S	S	R
B_{14}	R	S	S	S	S	S	R
B_{15}	R	S	S	S	S	S	S
B_{16}	R	S	S	S	S	S	R
B_{17}	R	S	S	S	S	S	R
B_{18}	R	S	S	S	S	S	R
B_{19}	R	S	S	S	S	S	R
B_{20}	R	S	S	S	S	S	S
B_{21}	R	S	S	S	S	S	R
B_{22}	R	S	S	S	S	S	R
B_{23}	R	S	S	S	S	S	R
B_{24}	R	S	S	S	S	S	R
B_{25}	R	S	S	S	S	S	R
B_{26}	R	S	S	S	S	S	R
B_{27}	R	S	S	S	S	S	R
B_{28}	R	S	S	S	S	S	S
B_{29}	R	S	R	S	S	S	R
B_{30}	R	S	S	S	S	S	R
B_{31}	R	S	S	S	S	S	R

B_{32}	R	S	S	S	S	S	R
B_{33}	R	S	S	S	S	S	R
B_{34}	R	S	S	S	S	S	R
B_{35}	R	S	S	S	S	S	R
B_{36}	R	S	S	S	S	S	R
B_{37}	R	S	S	S	S	S	R
B_{38}	R	S	S	S	S	S	R
B_{39}	R	S	S	S	S	S	R
B_{40}	R	S	R	S	S	S	R
B_{41}	R	S	S	S	S	S	S
B_{42}	R	S	S	S	S	S	R
B_{43}	R	S	S	S	S	S	R
B_{44}	S	S	S	S	S	S	R
B_{45}	S	S	S	S	S	S	R

R: Resistant S: Sensitive

CHAPTER-VI

6. DISCUSSION AND CONCLUSION

6.1 DISCUSSION

The present study was carried out from July 2008 to August 2008. In it 970 enteric fever suspected patients, visiting Bir hospital, were enrolled, 503 blood samples were processed for Widal test and 48 blood samples were tested for Enterocheck-wb.

In this study male patients were higher in number 631 (65%) than female patients 339 (35%). The majority of patients belonging to age group 20-29 years were requested for blood culture. They represent 378 (39%) of the total population. This result is consistent with other studies. Khatiwada, 2006 reported that the majority of patients belonging to age group 20-29 years were requested for blood culture representing 35.73% in male and 0-9 years in female representing 18.56%. Gurung, 2008 reported that the majority of patients belonging to age group 21-30 years were requested for blood culture representing 210 (40%) in male and less than 10 years in female representing 33 (6.42%).

Of 503 blood samples processed for Widal test, about 50% of samples had titer of less than 80 for typhoid fever. Even for 43 blood samples culture positive for *S.* Typhi, 23 (53.54%) had titer of less than 1:80 in TO, TH, AH and BH. WHO 2003, recommended that Widal test could be negative in up to 30% of culture proven cases of typhoid fever. This result is consistent with study done by Hosoglu *et al.,* 2008 in which even in culture proven cases only 75 cases (45%) were positive. 12 (27.85%) samples showed titer greater than 1:80 in TO and TH. Rise in titer of TO and TH was strongly suggestive for typhoid fever. But this result is not consistent with other results. Khatiwada, 2006 reported that in culture positive cases rise in TO and TH antibody titer >= 1:80 was 70% (14/20) which was suggestive of typhoid fever. Similarly KC, 2008 reported that 77.3% (22/30) in culture positive cases rise in TO and TH antibody titer >= 1:80 was strongly suggestive for active typhoid fever.

Brodie in his study of the 1964 Aberdeen outbreak involving 403 cases of bacteriological proven typhoid cases reported that TH agglutinins did not develop in 15% of patients tested and TO antibodies did not develop in as many as 41%. Wicks *et al.,* 1971 in Rhodesian reported that widal test to be completely negative in 21% (26/123) of patients with positive blood cultures.

Phang and Puthucheary in 1983 reported that 93.1% of typhoid fever cases had a significant widal test result. 6.4% of these sera had a significant TH titer only, 18.7% had a significant TO titer only and 79.9 % had significant titers for both antigens. In areas of endemicity there was often a low background level of antibodies in the normal population. Determining an appropriate cut-off titer for a positive result can be difficult since it varies between areas and between times in given areas. They concluded that in an endemic areas the TH and TO agglutinins could appear earlier in the course of illness which was most probably attributable to a hyperimmune or immunologically sensitized population which were continually exposed to *S.* Typhi and other *Salmonella* species. In endemic area Widal test has significant diagnostic value provided judicious interpretation of the test was made against a background of pertinent information.

Duthie and French in 1990 concluded that Widal test was useful in selected patients only. Over the time period, 2258 assessable single slide agglutination test 68 was positive (0.03%) of which 23 were false positive. There were 13 culture positive case of typhoid fever in whom the Widal test was falsely negative.

Tupasi *et al.,* 1991 reported that a positive widal test may be seen in non-typhoid patients and healthy controls from endemic areas and found that 55% healthy Filipinos have detectable TO and TH agglutinins. Isolation of bacteria from blood and bone marrow was diagnostic and a single widal test in an endemic area was of no diagnostic value.

Kulkarmi and Rego in 1994 in Karnataka India suggested that when the 2 titer were considered together either ‘TO’ or ‘TH’ of 1:160 or more were suggestive of typhoid fever. TO agglutination titer of 1:160 had a specificity of 97%, a sensitivity of 70% and an accuracy of 90%. TH agglutination titer of 1:160 had a specificity of 97%, a sensitivity of 30% and accuracy of 83%. Based on his analysis ‘TO’ and ‘TH’ titers of 1:160 or more were indicative of typhoid fever.

Parry *et al.,* 1999 reported that the test was more sensitive for children than for adults at each cut-off for both TO and TH antigen but was less specific at O- and H-antigen titers of 100. In adults, the sensitivity for both O and H antigens increased with the duration of illness before admission. There was no significant relationship between the Widal test results and a history of prior antibiotic therapy. The earliest serological response in acute typhoid fever was a rise in the titer of the TO antibody, with an elevation of the TH antibody titer which develops slowly but persist for long time than that of the TO antibody cutoff titer. He also reported that the level of stimulation of the immune system by other *Salmonella* species with shared antigens may therefore also be low. Previous typhoid vaccination may contribute to elevated agglutinins in the non infected population.

Olopoenia and King, 2000 reported that the result of single test has no diagnostic significance in an endemic region in part due to difficulty in establishing a steady state or baseline titer of Widal agglutination, which limits the usefulness of the test as reliable diagnostic indicator of the disease process.

Olsen *et al.,* in 2004 reported that in the widal test, the ‘TO’ and ‘TH’ agglutinins usually appear around day 8 and days 10 to 12 respectively. Ideally, the widal test should be run on both acute and convalescent phase sera to detect an increase in agglutination titer. However, to inform treatment decision before convalescent samples can be obtained, it is common for a single acute phase serum sample to be run. The result from a single sample are difficult to interpret because high background rates of

circulating antibodies to serotype Typhi or other *Salmonella* serotypes may produce false positive result. In Vietnam, highly endemic area, a single widal test can lead to many false positive and false negative results. Operator variability also contributes to unreliable results.

House *et al.,* 2005, reported that not all individuals exposed to *S.* Typhi develop a detectable agglutinating antibody response and because elevated Widal TO and TH titers can be common in healthy subjects living in regions where typhoid is endemic and can be raised in patients infected with non typhoidal *Salmonella* species. This test is usually performed on an acute serum. A convalescent serum should preferably also be collected so that paired titrations can be performed. In an endemic region, the value and clinical application has diminished considerably due to antigenic related determinants of both typhoid and non typhoid *Salmonella* species.

Mohanty and Ramana 2007, reported that of 553 blood samples available for blood culture, only 47.7% (264/553) were culture positive having a corresponding widal titer greater than 1/160 for *Salmonella* Typhi "O" antigen and 1/ 320 for *Salmonella* Typhi "H" antigens. They concluded that high titer (>1/160 for *Salmonella* Typhi "O" and >1/ 320 for *Salmonella* Typhi "H" antigens) in Widal test performed on single acute phase sera must be considered as significant and diagnostic. Kariuki *et al*., 2008 reported that Widal test would have missed out 7% of typhoid cases, while at the same time categorize another 18.7% as typhoid cases when they were not.

Of 39 blood samples positive for *S.* Paratyphi A, 18 (47.5%) showed titer of less than 1:80 in TO, TH, AH and BH. 10 (25%) samples showed titer greater than 1:80 in TO and AH. Rise in titer of TO and AH was strongly suggestive for paratyphoid fever.

Duthie and French in 1990 reported that Widal test has the Sensitivity of 78%, specificity 99%, Positive predicative value 66% and Negative predicative value 99%. Only 3% of more than 2000 Widal test gave a positive result. False negative results

occurred in 0.6 % of tests. They emphasize on quality control of Widal test for laboratory which consistently produced poor results in an external quality control programs. False positive results may be due to faulty technique or poor quality of the antigen suspension. There was conflicting evidence as to the relative importance of somatic and flagellar agglutination titer for the diagnosis of typhoid fever.

As Nepal is endemic region. The base line titer of people is determined recently. Most of the healthy patient due to constant exposure to low inoculum of bacteria may have low level of antibody titer. Rise in titer of TO and TH must be considered for interpretation of the result. As rise in TO might be non specific and rise in TH reflects the past infection. The blood sample may have been collected before rise in antibody, or due to antibiotic treatment, even culture proven cases may have low titer in Widal test.

WHO, 2003 reports that widal test has low sensitivity and specificity. Widal test should be interpreted in the light of baseline titers in a healthy local population. But it is still used in places where there is no culture facility and this test should be replaced by other more specific test with higher sensitivity and specificity. Or follow up testing for rise or fall of antibody titer may also give better result.

Culture is gold standard and Widal test itself should be used more selectively. A single Widal test is not reliable for the diagnosis of typhoid fever because false positive and false negative results are common. In a patient strong suspected to have Typhoid fever it may be useful to perform Widal test only if two blood cultures are negative (Duthie and French, 1990). Bacteria can be isolated form blood in 73 to 97% of cases before antibiotic use, (Willke *et al.,* 2002). Blood culture is the only method that can be used routinely for determination of drug resistance in typhoid patients. The results are obtained after nearly one week; therefore the early detection rate is about 30% (Haque *et al.,* 2005). Bhutta, 2006 reported that the sensitivity of blood culture is only 40% to 80%, for bone marrow culture sensitivity is 55 to 67% and specificity is 30%, PCR has

100% sensitivity and specificity and Widal test has the sensitivity of 47 to 77% and specificity of 50 to 92%.

The use of rapid test as well as culture for the diagnosis of typhoid is highly recommended to reduce the morbidity and complications. Enterocheck-Wb can be use for early detection as no blood samples were culture positive for *S.* Typhi but negative on EnterocheckWb. This increases the sensitivity of Enterocheck-Wb. 7 (14.5%) samples were culture positive for *S.* Paratyphi A and also positive for Enterocheck-Wb. 2 (4.16%) sample were positive for culture of *S.* Paratyphi A but negative for Enterocheck-Wb. This might be due to cross-reacting epitops of lipopolysaccharide of both *S.* Typhi and *S.* Paratyphi A. 16 (33.3%) samples were culture negative but positive on Enterocheck-Wb. This might be due to early antibiotic treatment or the patient might be at the early stage of typhoid fever. 18 (37.5%) samples were negative on culture as well as on Enterocheck-Wb. This result clearly reflects that those patients were not suffering from enteric fever.

Ghimire and Uppadhya, 2006 reported that Enterocheck-wb showed its diagnostic sensitivity and specificity of 71% and 57%, respectively which were lower than those of widal test (70 % and 95 %, respectively), but combined culture and *S.* Typhi IgM assay (sensitivity 96% and specificity 98 %) were superior to combined culture and widal test (sensitivity 87% and specificity 95 %). Cheesbrough, 2005 reported that Enterocheck-Wb has the sensitivity and specificity of 79.3%and 90.2% respectively.

The present study revealed high prevalence of typhoid fever 14.8% (34/97) in age group of 11-20 years with 15.24% (25/164) blood culture positive in male patients and 13.84% (9/65) in female patients. Gurung, 2008 reported higher prevalence of typhoid fever 12.85% (9/41) in age group of 11- 20 years with 12% (6/50) blood culture positive in male patients and 15% (3/20) in female patients. The higher prevalence in age group of 11-20 years may be contributed by less health conscious and eating contaminated food and water in school or open places.

Of 970 blood samples processed for culture, 104 (10.72%) showed positive growth, 97(10%) were *Salmonella* species. Lewis, June 2002 during Bharatpur outbreak reported the prevalence rate of 33.3%. Khanal *et al.,* 2007 reported the prevalence rate of 5.14%. Gurung, 2008 reported the prevalence rate of 7.97% for enteric fever. Ahmed *et al.,* 2006 in Bangladesh reported the prevalence rate of 4.43% for enteric fever.

Of 104 (10.72%) blood culture positive cases, 52 (50%) were *Salmonella* Typhi, 45 (43%) *Salmonella* Paratyphi A, 2 (2%) *Pseudomonas* spp., 2 (2%) *Klebsiella* spp., 2 (2%) *Acinetobacer* spp., and 1(1%) *Proteus* spp. This indicates higher prevalence rate 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. In similar study done by Khatiwada, 2006 29 (76.3%) isolates were *S.* Typhi and 9 (23.7%) *S.* Paratyphi A. Similarly Amatya *et al.,* 2007, reported that the most frequent isolated bacteria was *S.* Typhi 78 (63.4%) followed by *S.* Paratyphi A 44 (35.8%) and *E. coli* 1 (0.8%).

Of 97 (10%) *salmonella* isolates, 87 (8.9%) *Salmonella* species were isolated at 24 hours, 93 (9.5%) isolated at 48 hours, 95 (9.8%) isolated at 96 hours and 97 (10%) isolated on the seventh day. WHO, 2003 recommends subculture on 1, 2, 3, and 7 days respectively on non-selective agar as *S.* Typhi is not the only bacterial pathogen found in blood. But in usual practice, reporting is done only on the basis of overnight incubation. For any disease, culture positive is 100% diagnostic. This study indicates the need for follow up incubation reporting up to seven days to cover maximum positive cases. As 10 (9.6%) cases would have been missed if only 24 hour's incubation was considered.

In this study *S.* Typhi was 98% sensitive to Ceftriaxone, followed by Ofloxacin (96%), Ciprofloxacin (94.2%), Cotrimoxazole (88.5%), Ampicillin (5.7%) and Nalidixic acid (21%) respectively. This result is different form Khatiwada, 2006 who reported that *S.*

Typhi were 100% susceptible to Tetracycline, Ceftriaxone and Ciprofloxacin respectively followed by Cotrimoxazole and Chloramphenicol (resistance being 6.9%), Ampicillin (resistance being 20.69%) and least effective being Nalidixic acid (resistance being 58.62%). Amatya *et al.,* 2007 reported that Ceftriaxone was drug of choice (100% susceptible) followed by Cephotaxime (98.7% susceptible), Chloramphenicol (97.4% susceptible), Cotrimoxazole and Ampicillin (96.1% susceptible), Cefixime and Ofloxacin (94.9% susceptible), Ciprofloxacin (93.6% susceptible) and Nalidixic acid (37.5% susceptible). Gurung, 2008 reported that *S.* Typhi were 100% susceptible to Chloramphenicol, Amoxicillin, Cotrimoxazole and Ceftriaxone.

Dutta *et al.,* 1981 reported the acquisition of R-plasmid by *S.* Typhi in the bowel of man from other enteric bacteria (especially MDR) and *E. coli* isolates from Urinary tract infection cases. Furthermore, due to several treatment failures with Ciprofloxacin and Ofloxacin during and after 1995-1996 Chloramphenicol in addition to the third generation Cephalosporins like Ceftriaxone were tried in typhoid fever.

Sood *et al.,* 1999 reported Chloramphenicol sensitivity among 71.9-91.6% isolates during 1994-1998. Bhattacharya and Das 2000, isolated *S.* Typhi strains from Orissa of which 87.6% were Chloramphenicol sensitive. Chande *et al.,* 2002 reported Chloramphenicol sensitive in 74.5% *S.* Typhi isolates from Nagpur with MIC of <+ 4µg/ml.

Tankhiwale *et al.,* 2003 India reported that Chloramphenicol sensitivity was variable, 100% susceptibility was observed in Hubi in 1997, more than 95% susceptible in Hyderabad in 1999, 91.6 % from New Delhi in 1998. Similar variability was observed with Ampicillin sensitivity. As many as 85 - 100 % isolates were reported as resistant in recent reports. Cotrimoxazole sensitivity was observed to be 70 %. Decreasing efficacy to Ciprofloxacin which lately is the drug of choice for the treatment for enteric fever has been reported 13.05 % (4/23) resistant to Ciprofloxacin. All the 23 isolates were

sensitive to a third generation Cephalosporins, Cephotaxime. 7/23 (30.43%) were resistant to Chloramphenicol, Ampicillin, Cotrimoxazole indicating MDR strains.
Mandal *et al*., 2004 reported that Ciprofloxacin replaced Chloramphenicol, the best choice of antibiotic in the treatement of enteric fever When Chloramphenicol resistant enteric fever emerged and caused outbreaks in different parts of the world. Chloramphenicol sensitive *S.* Typhi emerged again due to withdrawal of the antibiotic pressure. The use of Chloramphenicol against infection in many other enteric bacteria, the strains remain resistant to the drug and play role as the reservoir plasmid encoding multiple drug (including Chloramphenicol) resistance. This selective pressure of antibiotic may be the cause of acquisition of R plasmid by *S.* Typhi isolates in the year 2000 and again in 2002 and 2003 which in turn caused Chloramphenicol resistant strains to emerge. The high degree of Chloramphenicol susceptibility to *S.* Typhi isolates has also been reported very recently from many other parts of India. Kumar *et al.,* 1991 reported from Ludhiana that there was an increase of Chloramphenicol susceptibility from 43% (1995) to 96% (1999) among *S.* Typhi.

Olsen *et al.,* in 2004 reported that of 58 isolates tested, 14(24%) were pan sensitive. All the remaining 44 isolates were resistant to Nalidixic acid; 33 were also resistant to Chloramphenicol and Tetracycline and 29 of them were also resistant to Ampicillin.

Haque *et at.,* 2005 reported that, 23 MDR isolates of *S.* Typhi tested by disc diffusion method against Ciprofloxacin, Chloramphenicol, Cotrimoxazole, Amoxicillin resistance was observed in 0%, 17(73.9%), 17(73.9%) and 22(95.7%) isolates respectively. This resistant pattern of Ampicillin is similar to this study (resistant 94.3%).

Chau *et al.,* 2007 reported that during early 2000, there was a dramatic increase in Nalidixic acid resistance. In 1993, 3.5% (2/57) *S.* Typhi isolated from patients in Southern Vietnam were Nalidixic acid resistant. Nalidixic acid resistance surged to 88.6% (109/123) in 1998. It has remained at high level since then with 97% (196/202) of isolates in 2004.

In this study, 5 (9.6%) of *S.* Typhi were resistant to more than 2 classes of antibiotics. This result was consistent with K.C 2008 who 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 were resistant to three or more antibiotics. Amatya *et al.,* 2007 reported 3.84% (3/78) *S.* Typhi was resistant to more than 2 antibiotics (resistant to Chloramphenicol, Amoxicillin, Ofloxacin, Cefixime, Cotrimoxazole).

Similarly, among total *S.* Typhi isolates, 2 (3.84%) was found to be MDR strain (resistant to Cotrimoxazole, Chloramphenicol, and Amoxicillin). Both of the strains 3.84% (2/52) were Nalidixic acid resistant (the combination of MDR and Nalidixic acid). Similarly, Chau *et al.,* 2007 reported that the proportion of MDR *S.* Typhi was 63.2% in 1993 and increased two 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 resistance. He also reported 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 form 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.

Of total *S.* Typhi isolates 5.76% (3/52) were Ciprofloxacin as well as Nalidixic acid resistant. Khatiwada, 2006 reported that ciprofloxacin was 100% susceptible and 58.62% of *S.* Typhi were resistant to Nalidixic acid. Amatya *et al.,* 2007 reported that Ciprofloxacin resistant *S.* Typhi was not isolated and 62.5% were resistant to Nalidixic acid. Roumagnac *et al*., 2006 suggested that Fluroquinolones use has driven the cloned expansion of Nalidixic acid resistant haplotypes H58 in South East Asia. Chau *et al.*, 2007 reported that in Nepal, 18.1% (27/149) *S.* Typhi isolates were resistant to Chloramphenicol and Nalidixic acid. The emergence of resistance of *S.* Typhi to Ciprofloxacin 4% (6/149) in Nepal, together with reports of high level Ciprofloxacin resistance in India and Bangladesh might be the prelude to a worsening drug resistance

problem in Asia. Patients infected with Nalidixic acid resistance serovar Typhi show poor clinical response, high failure rates (up to 36 %) and prolonged fecal carriage when treated with the old generation Fluroquinolones such as Ofloxacin.

In this study Fluroquinolones, Ciprofloxacin (94.2% sensitive) and Ofloxacin (96 % sensitive) were the choices of treatment but resistant strains are being reported. 1.92% (1/52) of *S.* Typhi isolates was MDR, Nalidixic acid resistant, Ofloxacin resistant. 1.92% (1/52) of *S.* Typhi isolates was Ciprofloxacin and Ofloxacin resistant. Amatya *et al.,* 2007 reported that 5.7% of *S.* Typhi resistant to Ofloxacin was sensitive to Ciprofloxacin. Lakshmi *et al.,* in Hyderabad, 2006 reported that comparison of cumulative data (2001- 2006) for *S.* Typhi and *S.* Paratyphi A suggest a significant association between MDR and Ofloxacin in *S.* Typhi, 59.8% of MDR *S.* Typhi isolates with resistance to Ofloxacin compared to only 16.5 % of sensitive *S.* Typhi isolates.

Chau *et at.,* 2007 clearly suggested that the choice of Fluroquinolones and the dose used for the treatment of *S.* Typhi may be critical and underline that clearly not all Fluroquinolones are as susceptible to this common mutation. Continued use of older generation Fluroquinolones (Ciprofloxacin and Ofloxacin) may encourage the persistence of resistance isolates and lead to the development of new mutations which might compromise the efficacy of the new generation.

In a developing country like Nepal where antibiotics are available freely in the market and patients take antibiotics as prescribed by the pharmacist of the shop and visit doctor only in case the disease is not cured. Maximum patients before visiting doctors undergo self medication. Insufficient dose of antibiotic as well as irrational use of antibiotic may have led to the isolation of multidrug resistant bacteria.

In this study, *S.* Paratyphi A was 100% susceptible to Ceftriaxone, Ofloxacin, Chloramphenicol, Ciprofloxacin followed by Cotrimoxazole (95.5%), Ampicillin (4.5%) and Nalidixic acid (17.78%). Katiwada 2006 reported that *S.* Paratyphi A was

susceptible to Tetracycline, Ciprofloxacin, Ceftriaxone and Cotrimoxazole. Amatya *et al.,* 2007 reported that *S.* Paratyphi A was 100% susceptible to 3 antibiotics, Chloramphenicol, Amoxicillin and Cotrimoxazole. KhattriChhetri, 2008 reported that the *S.* Paratyphi A susceptible to Ceftriaxone only.

In this study, *S.* Paratyphi A resistance to more than 2 antibiotics as well as MDR strains was not observed. This result was supported by Khatiwada 2006 who did not found any MDR strains. Similarly Amatya *et al*., 2007 did not isolate any *S.* Paratyphi A resistant to more than 2 antibiotics. But this result is not supported by Gurung 2008, who isolated 2 (8.7%) *S.* Paratyphi A resistant to 3 or more antibiotics. KC, 2008 isolated 4 (8.8%) *S.* Paratyphi A resistance to 3 or more drugs.

Hasan *et al.,* 2008 reported that resistance in *S.* Paratyphi A is emerging with a number of reports documenting prevalence of MDR *S.* Paratyphi A in their regions. MDR among *S.* Typhi in the country decreased from 50% in 1995 to 20% in 2001 while, the rate in *S.* Paratyphi A increased from 14% in 1996 to 44% in 2003.

6.2 CONCLUSION

From this study, it was concluded that *S.* Typhi (52%) was more prevalent than *S.* Paratyphi A (47%). Generally this disease is highly prevalent in the age group of 11 to 20 years. The blood culture should not be reported on the basis of over night incubation only. In order to cover maximum positive cases, culture should be reported on 24, 48, 96 and 7 days respectively. None of the antibiotics (Ceftriaxone, Ofloxacin, Chloramphenicol, Ciprofloxacin, Cotromoxazole, Ampicillin) were 100% susceptible to *S.* Typhi. 3.82% (2/52) were MDR with Nalidixic acid resistance. 5.76% (3/52) of *S.* Typhi isolates were Ciprofloxacin as well as Nalidixic acid resistant. Gatifloxacin (and potentially other newer generation fluroquinolones) which would prove a better choice for use in typhoid fever. *S.* Paratyphi A was found to be 100% susceptible to

Ceftriaxone, Ofloxacin, Chloramphenicol, and Ciprofloxacin respectively. Traditional drugs are still effective for *S.* Paratyphi A.

As Nepal is as endemic region and cut off titer of the people leaving in this region has been determined. Cut off titer greater than 1:80 for TO and titer greater than 1:160 for TH is diagnostic of enteric fever. However this test should not be taken as the confirmatory test. Rise or fall in titer of both TO and TH should be taken for the interpretation of typhoid fever. The high titer of TO is considered non specific whereas high titer in TH reflects recent past infection. Similarly, rise or fall in titer of both TO and AH should be taken for the interpretation of paratyphoid fever.

The major advantages of Enterocheck-wb are rapid, easy to use, not require special equipment and special training. However in laboratories where culture is not possible this test can be used for early diagnosis of typhoid fever. Only culture is 100% diagnostic for any disease. Culture is must for any cases and from any places in order to understand the emerging trend of antibiotic for better treatment of the patient and also to tackle the emerging MDR cases.

Antibiotic treatment is not advisable to start only on the basis of the serology. Isolation of bacteria from blood should be followed by the antibiotic susceptibility test which can decrease the emerging MDR cases. The value of culture cannot be replaced by the PCR or serology. Both Enterocheck-wb (for rapid detection) and culture (gold standard) can be used for early diagnosis and treatment in order to decrease the morbidity and complications.

CHAPTER-VII

7. SUMMARY AND RECOMMENDATIONS

7.1 SUMMARY

1. The study was conducted in Bir hospital from June to August 2008 with the objective to study the changing antibiotic sensitivity pattern of *Salmonella* species.

2. Of the 970 clinically suspected patients of typhoid fever, 631 (65%) were male and 339 (35%) were female.

3. Of 503 serum samples processed for the single slide agglutination test, more than 50% of samples had titer less than 1:80 for TO,TH, AH and BH.

4. Of 43 blood samples positive for *S.* Typhi, 53.5% (23/43) showed titer less than 1:80 in TO, TH, AH, BH. 27.9% (12/43) showed titer greater than 1:80 in TO and TH both.

5. Of 39 blood samples positive for *S.* Paratyphi A, 47.5 (18/39) showed titer less than 1:80 in TO, TH, AH and BH. 28.2% (11/39) showed titer greater than 1:80 TO and AH both.

6. Of 48 blood samples processed for Enterocheck-wb 28 (53.8%) were positive. 14 showed culture positive, 5 were *S.* Typhi and 9 were *S.* Paratyphi A. No sample culture positive for *S.* Typhi was negative on Enterocheck-wb. 2 cultures positive cases of *S.* Paratyphi A was negative result on Enterocheck-Wb.

7. For both male and female, higher number of patients for blood culture was in the age group of 20- 29 years. There was no significant association of presence of enteric fever

with gender of patients (p=0.27). The higher percentage of culture positive cases was in the age group of 11- 20 years.

8. Of 970 blood samples processed for culture, 104 (10.72%) was culture positive. Among total isolates, 45(43%) were *S.* Paratyphi A, 52(50%) *S.* Typhi, 2 (2%) *Pseudomonas* spp., 2 (2%) *Klebsiella* spp., 2 (2%) *Acinetobacter* spp., and 1 (1%) *Proteus* spp.

9. Of 97 (10%) *Salmonella* species isolated from culture, 87(8.9%) *Salmonella* species were isolated after overnight, 93 (9.5%) at 48 hours, 95 (9.8%) at 96 hours and 97 (10%) on the seventh day of incubation.

10. None of the antibiotic was found to be 100% effective for *Salmonella* Typhi. Ceftiaxone, Ofloxacin and Chloramphenicol were found to be 98%, 96%, and 94.2% sensitive respectively. Ampicillin was found to be least effective.

11. Of 53 *Salmonella* Typhi isolated, 2 (3.8%) of were MDR as well as Nalidixic acid resistant, 3 (5.76%) were Ciprofloxacin as well as Nalidixic acid resistant and 5 (9.6%) were resistant to more than two antibiotics.

12. Similarly Ceftriaxone, Ofloxacin, Chloramphenicol and Ciprofloxacin were 100% effective for *S.* Paratyphi A and Ampicillin was least effective.

7.2 RECOMMENDATIONS

On the basis of the study carried out, we would like to forward the following recommendations:

1. The present study was confined to Bir Hospital and attendants represent the most visible part of the population. But the surveillance should be carried out throughout the country

covering wide geographical region in order to obtain information regarding variations of pathogen and their antibiotic sensitivity profile.

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

3. Molecular analysis of MDR strains and Nalidixic acid resistance strains of *Salmonella* species should be done in order to ascertain the drug resistance gene and their mechanism.

4. Patients suffering from enteric fever should be prescribed antibiotics only on the basis of antibiotic susceptibility pattern of the isolates in order to reduce the emergence of the MDR strains.

5. Widal test alone should not be considered as the confirmatory test. Acute phase serum as well as convalescent phase serum should be used for confirmation of typhoid fever. Rise in titer of antibody is considered only suggestive of typhoid fever.

6. Culture (gold standard) and Enterocheck-wb (rapid diagnosis) can be used simultaneously to reduce morbidity as well as complications of the disease.

CHAPTER VIII

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