NALIDIXIC ACID RESISTANT *SALMONELLA* WITH DECREASED CIPROFLOXACIN SUSCEPTIBILITY

A

Dissertation

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In Partial Fulfillment of the Requirements for the Award of the Degree

of

Master of Science in Microbiology

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By

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RECOMMENDATION

This is to certify that Mr. Rup Bahadur Kunwor has completed this dissertation work entitled "**Nalidixic Acid Resistant *Salmonella* with decreased Ciprofloxacin Susceptibility**" as a partial fulfillment of M.Sc. degree in Microbiology. To the best of our knowledge, this is his original research work and has not been submitted for award of any other degree.

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ABSTRACT

A total of 534 patients suspected of enteric fever were studied in Kathmandu Model Hospital from May 2006 to August 2006. Among them 19.28 percent patients were culture positive. The prevalence of culture positive was highest among the age group of 0-10 years (31.42%) and the result was statistically significant ($p > 0.05$). Among 103 culture positive cases, the incidence rate was more in female (23.15) than male (17.15%) and it was statistically insignificant ($p<0.05$). The culture positive rate was found maximum from out patients (19.76%) than inpatients (12.12%).

Among 103 isolates, 67(65.04%) were *Salmonella typhi* and 36(34.95%) were *Salmonella paratyphi A*.

The most sensitive drugs for *Salmonella typhi* were found to be Amoxycillin, Ceftriaxone, Chloramphenicol and Cotrimoxazole and least sensitive being Ofloxacin. The same pattern of susceptibility was shown by *Salmonella paratyphi A*.

Among 73 Nalidixic acid resistant isolates 45(61.64%) were *Salmonella typhi* and 28(38.35%) were *Salmonella paratyphi A*. In contrast, among 30 Nalidixic and susceptible isolates 22(73.33%) were *Salmonella typhi* and 8(26.66%) were *Salmonella paratyphi A.*

In this study, none of the nalidixic acid resistance isolates were found Ciprofloxacin resistance, but all Nalidixic acid susceptible isolates were also susceptible to Ciprofloxacin.

Among 72 bacterial isolates with increased susceptibility to Ciprofloxacin (≥ 0.125 μg/ml), 67 isolates showed Nalidixic acid resistance in disc diffusion method. And among 31 isolates with MIC value < 0.125μg/ml (Ciprofloxacin) 25 isolates were Nalidixic acid susceptible in disc diffusion method.

The sensitivity and specificity of Nalidixic acid resistance test by disc diffusion method to screen for isolates having MICs of Ciprofloxacin ≥ 0.125 μg/ml were 91.98% and 83.33% respectively.

Key words : Enteric fever, *Salmonella*, Nalidixic acid resistant, MIC value.

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LIST OF ABBREVIATIONS

%	:	Percentage
≥	:	Greater than equal to
μg	:	Microgram
<	:	Less than
>	:	Greater Than
ACCo	:	Ampicillin, Chloramphenicol and Cotrimoxazole
BA	:	Blood Agar
BHI	:	Brain Heart Infusion
CFR	:	Case Fatality Rate
CFU	:	Colony Forming Unit
D/W	:	Distilled Water
ESBL	:	Extended Spectrum of Beta Lactamase
LPS	:	Lipopolysaccharide
MA	:	Mac-Conkey Agar
MDR	:	Multi-Drug Resistant
MDRST	:	Multi-Drug Resistant *Salmonella typhi*
MHA	:	Mueller Hinton Agar
MHB	:	Mueller Hinton Broth
MIC	:	Minimum Inhibitory Concentration
NA	:	Nutrient Agar
NARST	:	Nalidixic Acid Resistant *Salmonella typhi*
NCCLS	:	National Committee for Clinical Laboratory Standards
SPS	:	Sodium Plyanethol Sulphonate
TSI	:	Triple Sugar Iron
WHO	:	World Health Organization

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CHAPTER - I

1. INTRODUCTION

Enteric fever continues to be one of the most common infectious diseases in Nepal as in other developing countries where it affects about 12.5 million persons each year (Edman *et al*., 1986). The term enteric fever encompasses both typhoid and paratyphoid fevers (Lesser and Miller, 2003).

These infections (i.e. typhoid and paratyphoid fevers) are caused by a Gram negative bacteria of genus *Salmonella*. Typhoid fever is a life threatening illness caused by *Salmonella typhi*. In the United States about 400 cases occur each year and 70% of these are acquired while traveling abroad. Paratyphoid fever is a similar illness but is usually much milder and is caused by the organism *Salmonella paratyphi* (*A, B or C*) (Lesser and Miller, 2003 and Medline plus medical encyclopedia: typhoid fever, 2004).

Salmonellosis in human is generally contracted through the consumption of contaminated food of animal origin (mainly meat, poultry, eggs and milk). Although many other foods including green vegetables contaminated from manure have been implicated in its transmission. The causative organisms pass through the food chain from primary production to households or food-service establishment and institutions (Ananthanarayan, 2000 and Chakraborty, 2000).

The clinical course of human salmonellosis is usually characterized by acute onset of fever, abdominal pain, diarrhoea, nausea and sometimes vomiting. In some cases, particularly in the very young and in the elderly the associated dehydration can become severe and life threatening (Brooks *et al.*, 2002 and Lesser and Miller, 2003).

Salmonella spp can usually be isolated from blood in 70-90% of patients during the first ten days of infection and about 30% of the patients during the third week. The organisms can be isolated from 40-50% of patients during the second week of infection and from about 80% of the patients during the third

week in faeces. In 25% of patients, *Salmonella* spp can be isolated in the urine in second week but usually urine culture is done on fourth week of infection for the isolation of *Salmonella spp* (Cheesbrough, 1984).

For patients with enteric fever, administration of an effective antibiotic should begin as soon as clinical diagnosis is made, without recourse to results of antimicrobial sensitivity tests. In 1948, Chloramphenicol was introduced and a severe debilitating and fatal disease was transformed to a readily treatable condition. Resistance started to develop within two years of the drug introduction and until 1972 Chloramphenicol resistant *Salmonella typhi* became a major problem. Outbreaks of Chloramphenicol resistant *Salmonella spp* occurred in Mexico, India, Vietnam, Thailand, Korea and Peru. These strains were also found to be resistant to Sulfonamide, Tetracycline and Streptomycin-Amoxycillin and Trimethoprim-sulphamethoxazole were effective alternatives till the end of 1990's when strains resistant to all the first line anti-Salmonella drugs used at that time were reported (Thong *et al.,* 2000, Connerton *et al.,* 2000 and Mirza *et al.,* 2000).

Threllfall and Ward have reported that *Salmonella typhi* with decreased sensitivity to Ciprofloxacin is endemic in several Asian countries, and incidence of such strains has increased in travellers from the Indian subcontinent. They suggested 3rd generation Cephalosporins such as Ceftriaxone or Cefotaxime as possible alternatives and in their study it was assured that all strains were sensitive to these drugs (Threllfall and Ward, 2001).

Reduced susceptibility to fluoroquinolones has become a major problem mostly in Asia (Brown *et al.,*1996, and Threllfall *et al.,* 2001). Outbreak with such strains affected eight thousand people and killed 150 people in Tajikistan in 1997. Isolates responsible for this outbreak were found to have MIC of fluoroquinolone ten times to that of those fully susceptible to the drug. This decreased susceptibility is resulting in treatment failure (Wain *et al.,* 1997 and Threllfall *et al.,* 1999).

While resistance to the fluoroquinolones often emerges as a result of mutations in the bacterial genome (DNA), resistance to other antimicrobials often spread by transfer of DNA between bacterial strains. In some cases multi-drug-resistance is transferred through plasmid (Wain *et al.,* 1997 and Ackers *et al.,* 2000).

Clinical non-response to Ciprofloxacin therapy in enteric fever is increasing being encountered in endemic areas possibly due to the increase in the levels of resistance to Ciprofloxacin in *Salmonella typhi*. The antimicrobial susceptibility tests for *Salmonella typhi* performed by the disc diffusion method using NCCLS breakpoints fail to detect the increasing MIC of Ciprofloxacin, leading to the inappropriate treatment of enteric fever with Ciprofloxacin. The possibility of testing *Salmonella typhi* strains for their susceptibility to Nalidixic acid by disc diffusion method can be used as a marker for high MIC to Ciprofloxacin (Kapil *et al.,* 2002).

Fluoroquinolones have good in vitro and clinical activity against isolates of the *Salmonella sps* and are often the treatment of choice in cases of life threatening salmonellosis due to multidrug resistant strains (Miller *et al.,*1995). In recent years, several treatment failures with flouoroquinolones have been reported due to decreased susceptibility to Ciprofloxacin (Lostec *et al.,*1997 and Asna *et al.,* 2003). Isolates with decreased susceptibility to Ciprofloxacin may appear susceptible with routine disc diffusion tests, identification of the strains with decreased susceptibility to Ciprofloxacin requires susceptibility tests, which give actual MICs. Routine application of these tests for each strain on the other hand is not convenient, and literature reports suggest that resistance to Nalidixic acid may be an indicator of decreased susceptibility to Ciprofloxacin (Ercis *et al.,* 2005).

CHAPTER - II

2. OBJECTIVES

General Objective

To evaluate the value of Nalidixic acid resistance as an indicator of decreased susceptibility to Ciprofloxacin in *Salmonella* strains isolated from patient visiting Kathmandu Model Hospital, Kathmandu.

Specific objectives

1. To isolate and identify *Salmonella* strains from blood samples collected from patients visiting Kathmandu Model Hospital, Kathmandu.
2. To perform the antibiotic susceptibility test of the isolated *Salmonella sps*.
3. To determine the MICs value of Ciprofloxacin among the isolates.
4. Comparative evaluation of Nalidixic acid and Ciprofloxacin resistance to isolated *Salmonella* sps.

CHAPTER - III

3. LITERATURE REVIEW

3.1 Definition

Enteric fever is most usually caused by *Salmonella typhi* or *Salmonella paratyphi A, B* or *C* but can be caused by any *Salmonella* serotypes (Greenwood, 2000). Enteric fever is potentially life threatening systemic illness characterized by high fever and abdominal complaints. The term enteric fever encompasses both typhoid and paratyphiod fever (Kumari and Ichhpujani, 2000).

Enteric fever is a generalized acute infection characterized by cyclic course, definitive temperature curve, general intoxication, bacteraemia and affection of the lymphatic apparatus of the small intestine through which the infection implants itself in the host upon entrance of the causative agent into gastrointestinal tract (Bunin, 1980).

Typhoid and Paratyphoid fever are clinically and pathologically similar, the distinction between them being a bacteriological one. However, paratyphoid fever is clinically milder and of short duration than typhoid (Lesser and Miller, 2003).

Certain other *Salmonella* serotypes that cause septicaemia also frequently give rise to pyemic lesions in the internal organs .According to Bronstein in 1943, the distinction between *Salmonella* septicaemia i.e. enteric fever and *Salmonella* pyemia is useful but by no means absolute and some of the common effects of typhoid fever arise from localization of bacteria in individual organs (Parker and Collier, 1990).

Although no age is exempt, typhoid is a disease of older children and young adults. The sexes are equally affected (Weatherall *et al.*, 1987).

3.2 History

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

Typhoid fever was first observed by Eberth (1880) in the mesenteric lymph nodes and spleen of fatal cases and was isolated by Gaffky. It come to be known as Eberth Gaffky bacilli or Eberthella typhi. Its causative role was confirmed by Metchinicoff and Besredka in 1900 (Old, 1990).

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

Widal in 1896 and Widal and Sicard in 1896 described the Widal reaction, Pfeiffer and Kolle in 1896 and Wright and Semple in 1897 introduced the first prophylatic inoculation against typhoid fever. *Salmon* and *Smith* in 1885 together isolated the American hog cholera bacillus (*Salmonella cholerasuis*). Hence the term *Salmonella* is derived from first author *Salmon* (Old, 1990).

Woodward *et al.,* in 1948 published the first report on the use of Chloramphenicol in typhoid fever. This drug drastically cut the duration of pyrexia from about 35 days to an average of 3-5 days, with corresponding diminution of toxaemia, morbidity and mortality (Ananthanarayan, 2000 and Abhyakar, 2005).

3.3 Epidemiology of Enteric Fever

Enteric fever remains major health related problems in those underdeveloped countries where sanitary conditions are poor. It affects local inhabitants as well as travelers to the endemic areas such as Indian Subcontinent, South East and Far East Asia, The Middle East Africa, Central America and South America (Corales, 2004).

Typhoid fever is a major cause of illness, the global incidence in 2000 was an estimated 21650974 cases with 216510 deaths (Crung, 2004). According to WHO 1994 census, approximately 17 million cases occur per year worldwide. Of these cases 7 million occur in Asia, 4 million in Africa and 0.5 million in Latin America with 600,000 fatalities (WHO, 1994).

Case fatality rates of 10-50% have been reported from endemic countries when diagnosis is delayed or in cases of severe typhoid fever not treated with high dose of corticosteroid therapy and antibiotics (Corales, 2004).

Annual report 2003/2004 by Department of Health Services in Nepal show that the total morbidity of enteric fever was 8326 and rate of enteric fever was highest among male of age group 20-49 years. Total death by enteric fever was 37 and CFR was 0.4%.

In India the annual incidence rate was as high as 760/100000; 980/100000 in Delhi alone (Sinha *et al.,* 1995). Enteric fever is a major health problem in India accounting for more than 300000 cases per year and *Salmonella typhi* is the most common etiological agent (Nair and Sudarsana, 2004).

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

In Korea, the incidence of culture proven typhoid fever for recent 9 years was 0.41 per 100000 people per year (Yoo *et al.,* 2004).

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

3.4 Causative agents of Enteric fever

Salmonella are primarily intestinal parasites of man and animals, both domestic and wild. They are also isolated from the blood and internal organs of vertebrates. They are frequently found in sewage, river and other water and soil in which they do not multiply significantly (Old, 1990).

Salmonella are Gram negative, non acid fast, non-capsulated, non-sporing bacilli, most serotypes are motile with peritrichous flagella, but *Salmonella gallinarum* and *Salmonella pullorum* are non-motile variants (OH-O variation) are occasionally found in other serotypes, most strains of most serotypes form type 1 (mannose sensitive, haemagglutinating) fimbrial. Gallinarum-pullorum and a few strains in other serotypes either form type 2 (non haemagglutinating) fimbrial or are non-fimbriae, most strains of *Salmonella paratyphi A* are non-fimbriate (Old, 1996).

Salmonella typhi, *Salmonella paratyphi A* and *B* and *Salmonella cholerasuis* are primarily infective for humans and infections with these organisms implies acquisition from a human source (Brooks *et al.,* 2002).

Studies performed in human volunteers suggest that in healthy previously unvaccinated men, ingestion of 10^5 organisms led to clinical disease in 25% of volunteers, ingestion of 10^7 organisms caused disease in 50%, and 10^9 organisms caused disease in 95%. As the number of organisms increased, the incubation period decreased. The clinical syndrome was unchanged. A gastric pH of less than 1.5 kills most *Salmonellae*. Patients who continually ingest antacids, have had a gastrectomy, or have achlorhydria due to aging or other factors require lower numbers of organisms to produce clinical disease.

Possession of VI antigen by *Salmonella typhi* is linked with increased pathogenicity (Corales, 2004).

Taxonomy of *Salmonella*

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

Antigenic Structure of Salmonella sps

a) O antigens: These somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide layer on the surface of the bacterial cell wall. They are hydrophilic and enable the bacteria to form stable, homogenous suspensions in saline (0.85% NaCl) solution. Over 60 different O antigens have been being unaffected by heating for 2.5 hours at 100°C and alcohol-stable withstanding treatment with 96% ethanol at 37°C for 4 hours. The O antigens are unaffected by suspension of the bacteria in 0.2% formaldehyde but if flagella are present their fixation by the formaldehyde renders the bacteria inagglutinable by O antibodies. The O antigens are liable to be changed in character by form variation and

lysogenic conversion and to be lost from the bacteria in S→R mutation (Old, 1996).

b) H antigens: These antigens represent determinant groups on the flagellar protein. They are heat-labile and alcohol-labile but are well-preserved in 0.04-0.2% formaldehyde. Heating at temperature above 60^0C detaches the flagella from the bacteria and detachments of flagella are achieved by heating for 30 minutes at 100^0C. The deflagellated bacteria are inagglutinable by H antibodies but the detached flagella remain immunogenic and suspensions of bacteria to be used for production of O anti-sera should be freed from detached flagella by centrifugation and washing or by inactivation by heating for 2.5 hours at 100^0C (Old, 1996).

In many *Salmonellae* i.e. other than serotypes of subspecies IIIa, IV and V, the production of flagellar antigens is diphasic, each strain varying spontaneously and reversibly between two phases with different sets of H antigens. In phase 1, the bacteria from flagella with one or more antigens form a set of over 70 antigen designated by the small letters of the alphabet 'a to z', a series that is complete except for 'j' then z1,z2, z3, etc. In phase 2, the bacterial form flagella with one or more antigens from a mainly different set of antigens. The first discovered of these were designated by Arabic numerals (not implying any relationship with the similarly numbered O antigens) but later certain phase-1 antigens, especially e,n,x,z, 1 and w were found in the phase 2 of some serotypes. Phase 2 used to be termed the 'group' or 'non-specific' phase because numerous serotypes of salmonellae share the same antigens when in this phase. The presumptive identification of serotypes therefore mainly depends on the identification of the H antigens in phase 1 which are relatively specific (Old, 1996).

The definitive identification of diphasic *Salmonella* always requires the identification of H antigen of both phases. It is therefore necessary to obtain a culture in different phase from that first isolated from the patient. The

alternative phase may be obtained by selective cultivation of the isolate in semisolid agar containing monophasic antiserum to the original phase antigen e.g. by Modified Cragie Tube method.

Other Surface Antigen

Although the serotype of an enterobacterium surface that determines agglutination with homologous antibodies. These include the capsular or K antigens including the Vi antigen the slime (mucus) or M antigen and the fimbrial or F antigens. Such antigens may cause difficulty in the serological identification of bacteria either by masking the O antigens so that the bacteria are inagglutinable by O antibodies or by causing non specific cross-reaction due to their presence in unrelated bacteria (Old, 1996).

- Vi antigen: The Vi antigen is a surface polysaccharide of beta (1-4) linked N-acetyl-D-galactosaminuronic acid, the C3 groups of which are variably acetylated. Almost all recently isolated strains of *Salmonella typhi* form Vi antigen as a covering layer outside their cell wall. This heat-labile antigen is an acidic polysaccharide. When fully developed, it renders the strains of *Salmonella typhi* rich in Vi antigen (V forms) produce more opaque colonies than strains lacking Vi antigens (W forms). Vi rich strains maintained by subculture on conventional media re-rapidly replaced by spontaneously originating Vi deficient mutans (V→W variation) (Old, 1996).
- M antigen: This antigen is a loose extra-cellular polysaccharide slime consisting of colanic acid. It occurs in a serologically similar form in various unrelated enterobacteria including serotypes of *Salmonella* and many strains of *Escherichia coli* and resembles the Vi antigen in preventing agglutination by O antiserum (Old, 1996).
- Fimbrial antigen: The antigenic determinants of the type -1 fimbriae of salmonellae are unrelated to those of most other Enterobacteriaceae. Five type-1 fimbrial antigens were described by Duguid and Campbell (1969). The type-1 fimbriae of *Salmonella* serotypes from different

subspecies shared common fimbrial antigen (Type-1). Strains of the same serotype possess the same type-1 fimbrial antigens but that from different serotypes possesses one or more different additional fimbrial components (2-5). Thus type-1 fimbrial determinants of some representative serotypes are: Montevideo (1 only), Senftenberg (1,2), Paratyphi B and Typhimurium (1,2,3), Cholerasius(1,2,4), Wnteritidis (1,2,5), Typhi (1,5) and Newport (1,2,4,5). There was some sharing of type-1 fimbrial antigens between salmonellae and *Citrobacter freundii* but not with *Citrobacter amalonaticus* or other type-1 fimbriae Enterobactyeriaceae. Type-1 fimbrial antigens is synonymous with X-antigen of earlier workers (Old, 1990).

- R antigen: In S→R mutation, the O antigens are lost and new 'R' antigens are revealed at the bacterial surface. Mutational loss of an enzyme required for the formation of one of the links in the polysaccharide core or side chains of the cell-wall lipopolysaccharide leads to an absence of the hydrophilic side chains that determine O antigen specificity. The exposed incomplete (R_I) or complete (R_{II}) core polysaccharide constitutes the R antigen. The R antigens are the same in the R variants from different *Salmonella* serotypes though different from the R antigens of other enterobacteria (Old, 1996).

Antigenic Variation

- Variation in the O-antigen

a) Rough mutant Strains with defects in the biosynthesis of the O polysaccharide lack the specific side chains responsible for the O specificity and some of them have additional abnormalities of the core structure. They can be classified in a series from Ra, which lacks the side chains only, through Rb to Re which show a progressive loss of sugar constituents from the core. The term 'semi rough' has been applied to organisms that form less than the normal number of

repeating units. Rough mutant strains are not agglutinated by homologous O antiserum (Old, 1990).

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

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

- Variation of the H-antigen

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

e) Diphase variation: Most serotypes of salmonella of subspecies other than IIIa, IV and V express alternately 2 kinds of H flagellar specificity. This phenomenon of diphasic variation was first demonstrated by Andrews in 1922. The genera possess two set of gene coding for flagellar antigen *viz*. H_1 coding for phase 1 flagellar antigen and H_2 encoding for phase 2 flagellar antigens. The transcription of H_2 results in a coordinate expression of gene "rhl" which encodes for a repressor that prevents expression of H_1. About every 10^3-10^5 generation, a 900 base pair region, containing promoter for the H_2

gene, undergoes a site specific inversion, stopping transcription of both H_2 and "rhl". In absence of "rhl" gene product, H_1 gene is then transcribed until the 900 base pair region in H_2 promoter is again inverted, resulting expression of H_2 and "rhl" (Old, 1996).

The Kauffmann-White Classification of *Salmnellae*

This system of classification scheme, first developed in 1934, classifies the Salmonellae into different O groups, O serogroups, each of which contains a numbers of serotypes possessing a common O antigen not found in other O groups. The O groups first defined were designated by capital letters A to Z and those discovered later by the number (51-67) of the characteristic O antigen. It is now considered more correct to designate each O groups by its characteristic O factor i.e. to abandon the letters A-Z used to designate early O groups. Hence, O groups become: O2 (A), O4 (B), O7 (C_1), O8 (C_2-C_3), O9, 12 (D_1), O9, 46 (D_2), O3, 10 (E_1) etc. Groups O2 to O3, 10 (A-E_1) contain nearly all the salmonellae that are important pathogens in man and animals (Popoff and Le Minor, 1992). Within each O group the different serotypes are distinguished by their particular H antigen or combination of H antigens (Old, 1996).

3.5 Pathogenesis of Enteric Fever

3.5.1 Sources of Enteric Infection

Human are the only natural reservoirs of *Salmonella typhi.* Ingestion of food or water contaminated with faeces is the most common mode of transmission. These bacilli are essentially parasitic and cannot exist for long time outside the human body, but they can survive long enough to be transferred by polluted water, milk, ice-cream, shellfish and watercress etc.

According to Corales and DO (2004), transmission of enteric fever occurs most by one of the following mechanisms:

- Ingestion of contaminated food (poultry, meat, eggs, dairy products) or water.

- Contact with an acute case of typhoid fever or faecal oral spread.
- Contact with animal reservoir such as poultry cow, pig, birds, sheep, pets etc.
- Contact with a chronic asymptomatic carrier e.g. Day care center.
- Iatrogenic contamination such as blood transfusion, endoscopy etc.

3.5.2 Carriers of Enteric Infection

The patients suffering from *Salmonella* infection may continue to excrete the organism in their faeces for days or weeks after complete clinical recovery, but eventual clearance of the bacteria from the body is usual. A less number of patients continue to excrete the *Salmonella* for prolonged periods. Age and sex are important determinants of the frequency of carriage, at least *Salmonella typhi*. After enteric fever, less than 1% of patients under 20 years old become carriers, but this proportion rises to more than 10% in the patients over 50 years of age. At all ages woman become carriers twice as often as men (Lewis, 1982).

Carriers are extremely important in the spread of all enteric infection. Carriers seem to be perfectly healthy. In 1890, Harton-Smith had recorded the case of a urinary carrier of typhoid bacillus. Later on, Robert Koch and co-workers established that chronic carriers were main sources of persistence of enteric infection in the community. In 1904, To Von Drigaski showed with clear demonstration that the typhoid bacilli may lead a prolonged existence in human body and be excreted in the faeces (Frubisher *et al.,* 1971).

There are two types of carriers:-

A. Chronic carriers

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

B. Temporary or Transient carrier

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

a) Convalescent excreter

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

b) Asymtomatic carrier

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

3.5.3 Pathogenesis

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

Pathogenesis of enteric infection can be described under three headings:

1. Incubation stage

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

The M cell, epithelial cell that overlie the Peyer's Patches are the potential sites where the bacilli is internalized and transported to underlying lymphoid tissue. Once phagocytosed, the bacteria are protected from polymorphonuclear leucocytes, the complement system and the acquired immune response. *Salmonellae* have evolved mechanisms to avoid or delay killing by macrophages. Upon phagocytosis, the bacteria form a "spacious phagosomes"

and alter the regulation of ~200 bacteria proteins. The best characterized regulatory system is pho P/pho Q, a two component regulon that sense changes in bacteria location and alters bacterial protein expression. The alterations mediated by phoP/phoQ include modification in LPS and in the synthesis of outer membrane protein, these changes presumably remodel the bacteria's outer surface such that the organisms can resist microbicidal activities and possibly alter host cell signalling. phoP/phoQ also mediates the synthesis of divalent cationic transporters that scavenges magnesium (Lesser and Miller, 2003). From the submucosa, the organism travels the mesenteric lymph node, multiply and then enter the blood stream through the thoracic duct (transient primary bacteraemia) to seed other tissues (Chakraborty, 2000).

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

2. Septicaemic stage

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

3. Stage of localization

Some organisms localize in organs from the blood stream example : gall bladder, liver, spleen, bone etc. Some bacilli are discharged from the gall bladder into the intestine which cause inflammation of payer's patches of

intestine and lymphoid follicles producing necrosis and sloughing of the affected follicles with resultant typhoid ulcers which may lead to haemorrhage and perforation (Chakraborty, 2000).

3.6 Symptoms

Symptoms include severe headache, fever, loss of appetite, general discomfort uneasiness, rash during second week of fever, abdominal tenderness, constipation, then diarrhoeal bloody stool, slow sluggish, fatigue, lethargic, weakness, nose bleeding, chills, delirium, confusion, agitation, fluctuating mood, attention, deficit, hallucination etc. (Medline plus medical encyclopedia : Typhoid fever).

3.7 Complication (Corales, 2004)

A. Intestinal manifestation

- The two most common complications of enteric fever are intestinal haemorrhage and perforation.

B. Hepatobiliary manifestation

- Mild elevation of transaminases without symptoms is common in person with typhoid fever.
- Jaundice may occur in person with enteric fever and may be due to hepatitis, cholangitis, cholecystitis, or haemolysis.
- Pancreatitis and simultaneous acute renal failure and hepatitis with hepatomegaly.

C. Cardiopulmonary manifestations

- Non-specific electrocardiographic changes occur in 10-15% of patients with typhoid. Toxic myocarditis occurs in 1-5% of persons with typhoid and is a significant cause of death in disease-endemic countries.

- Toxic myocarditis occurs in patients who are severely ill and toxaemic and is characterized by tachycardia, weak pulse and heart sounds, hypotension, and electrocardiography abnormalities.
- Pericarditis rarely occurs but peripheral vascular collapse without other cardiac finding is described increasingly. Pulmonary manifestations have also been reported in patients with typhoid fever.

D. Neuropsychiatric manifestations

- A toxic confusion state, characterized by disorientation, delirium and restlessness, is characteristic of late-stage typhoid. Occasinally, these and other neuropsychiatric features may dominate the clinical picture at an early stage.
- Facial twitching or convulsions may be the presenting feature; sometimes, paranoid psychosis or catatonia may develop during convalescence. Meningismus is not uncommon, but frank meningitis is rare. Encephalomyelitis may develop and the underlying pathology may be that of demyelinating leukoencephalopathy. Rarely, transverse myelitis, polyneuropathy or cranial mononueuropathy may develop.
- Other less commonly reported events are spastic paraplegia, peripheral or cranial neuritis, Guillain-Barre syndrome, schizophrenia like illness, mania, and depression.

E. Hematologic manifestations

- Sub clinical disseminated intravascular coagulation occurs commonly in persons with typhoid fever.
- Hemolytic-uremic syndrome is rare.
- Haemolysis may also be associated with glucose-6-phosphate dehydrogenase deficiency.

F. Genitourinary manifestations

- Approximately 25% of patients excrete *Salmonella typhi* in their urine at some point during their illness.
- Immune complex glomerulitis and proteinuria have been reported and IgM, C3 antigen and *Salmonella typhi* antigen can be demonstrated in the glomerular capillary wall.
- Nephritic syndrome may complicate chronic *Salmonella typhi* bacteraemia associate with urinary schistosomiasis.

G. Musculoskeletal manifestations

- Skeletal muscle characteristically shows Zenker degeneration, particularly affecting the abdominal wall and thigh muscles.
- Clinically evident polymyositis may occur.

H. CNS manifestations

- Focal intracranial infections are uncommon.
- Recently, multiple brain abscesses have been reported.

3.8 Nalidixic acid Resistance Salmonellae

3.8.1 Definition

Since last 50 years, drug resistance in *Salmonella typhi* has been known to the world. In India, chloramphenical resistance in *Salmonella typhi* was first reported from Kerala in 1972 (Das and Bhattacharya, 2000).

Multi Drug Resistant (MDR) strains (resistant to chloramphenicol, ampicillin and cotrimoxazole) of *Salmonella enterica serovar typhi (Salmonella typhi)* are increasingly being reported from India and world wide (Ackers *et al.*, 2000).

Since emergence of resistance on these classic first lines of drug, quinolones became the alternative drug of choice to treat *Salmonella typhi* infection. But over the past decade, there have been increasing reports of treatment failure

using fluoroquinolones for patient whose isolates are susceptible to fluoroquinolones and resistance to Nalidixic acid, the prototypic quinolone in vitro (Crump *et al.,* 2003, Threlfall *et al.,* 1999). These strains are called Nalidixic acid resistant *Salmonella typhi* (NARST). In 1990, resistant to nalidix acid resistant was reported in southeast Asia (Parry *et al.,* 2002).

Isolates of *Salmonella typhi* with reduced susceptibility to fluoroquinolnes (as indicated in the laboratory by resistance to Nalidixic acid) have now appeared in the Indian subcontinent and other regions (Wain *et al.,* 1997). These Nalidixic acid resistant but Ciprofloxacin sensitive strains have increased minimum inhibitory concentrations (MICs) for Ciprofloxacin, although they are still within the current NCCLS range for susceptibility (0.12-0.5μg/ml). The current NCLLS range for Salmonella spp for Ciprofloxacin are ≥1μg/ml (Resistant) and ≤1μg/ml (Sensitive) (Crump *et al.,* 2003).

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

3.8.2 Mechanisms of Nalidixic Acid Resistance

Quinolone antibiotics, which include the fluoroquinolones, act by inhibiting the topoisomerase enzymes, DNA gyrase and topoisomerase IV, which maintain the level of supercoiling in the bacterial DNA. Both of these enzymes are tetrameric, being composed of two A subunits and two B subunits encoded by the gyrA and gyrB genes for DNA gyrase and the parC and parE genes for topoisomerase IV (Arthur *et al.,* 2003).

Bacteria most commonly developed resistance to quinolones by non transmissible, spontaneously occurring point mutations in chromosomal genes (gyrA, gyrB, parC and parE). These point mutations alter the enzymes (DNAgyrase and topoisomerase IV) that are targets for quinolone drugs. Although altered permeability of bacteria cell membranes and effux pumps are not well understood, these mechanisms also play a role in quinolone resistance for some isolates and are not known to be transmissible. More recently, a multi drug resistance plasmid was discovered that encodes transferable resistance to quinolones via the qnv gene. The qnv gene product has been demonstrated to directly protect DNA gyrase from quinolone inhibition (Crump, 2003).

Chromosomal point mutations resulting in alterations of the A subunit of DNA gyrase that lead to quinolone resistance have been defined in a substantial number of clinical and laboratory isolates of Enterobacteriaceae, including *Escherichia coli*. These alterations of the target enzyme are clustered between amino acids 67 and 106 in the amino terminus of the A protein known as the quinolone resistance-determining region (Crump, 2003). Similar chromosomal mutation and changes in the A subunit have been documented for isolates of *Salmonella enterica* (Nakaya *et al.*, 2003). Single chromosomal point mutations have been demonstrated to be sufficient to cause an amino acid change and to result in Nalidixic acid resistance. Two or more chromosomal point mutations are usually necessary to result in Ciprofloxacin resistance, on the basis of current NCCLS interpretive criteria (Crump, 2003). For the high levels all resistance to fluoroquinolones, the presence of additional mutations in gyrA and/or in another target such as parC is required. Thus, it has been proposed that the MIC of Nalidixic acid could be used as a generic marker of resistance for the quinolone family in Gram-negative bacteria (Ruiz, 2003).

Quinolones may cross the outer membrane in two different ways: through specific porins or by diffusion through the phospholipid bilayer. The degree of

diffusion of a quinolone is greatly associated with and dependent on its level of hydrophobicity. All quinolones may cross the outer membrane through the porins, but only those with a greater level of hydrophobicity may diffuse through the phospholipid bilayer. Thus alterations in the composition of porins and/or in the lipo-polysaccharides may alter susceptibility profiles. In lipo-polysaccharide defective mutants, increased susceptibility to hydrophobic quinolones has been described, without alteration in the level of resistance to the hydrophilic quinolones (Ruiz, 2003).

3.9 Laboratory Diagnosis

Definitive diagnosis of enteric fever requires the isolation of *Salmonella typhi* and *Salmonella paratyphi (A, B or C).* Cultures of blood, stool, urine, aspirate from rose spots, bone marrow and gastric or intestinal secretions may each be useful in establishing the diagnosis (Pillay *et al.*, 1975).

3.9.1 Microbiological Procedure

Blood culture system comprise (i) aseptic collection of blood (ii) culture of this in a liquid medium to allow organisms present in small number to multiply (iii) a system or combination of systems to detect them and (iv) a final phase of subcultures so that the bacteria can be identified and their antibiotic susceptibility is determined (Philips and Eykyn, 1990).

3.9.1.1 Specimens used for detection

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

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

b) Faeces : Organisms can usually be isolated from 40-50% of patients during the second week of infection and from about 80% of patients during the third week from faeces.

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

3.9.1.2 Blood collection

3.9.1.2.1 Timing of blood collection

Whenever possible, blood should be taken before antibiotics are administered. The best time is when the patient is expected to have chills or a temperature spike. It is recommended that two or preferably three blood cultures be obtained, separated by intervals of approximately 1 hours (or less if treatment cannot be delayed). More than three blood cultures are rarely indicated (Vandepitte *et al.,* 2004)

3.9.1.2.2 Quantity of blood

Because the number of bacteria per millilitre of blood is usually low, it is important to take a reasonable quantity of blood. 10ml per venepuncture for adults, 2-5ml may suffice for children, who usually have higher levels of bacteraemia for infants and neonates, 1-2ml is often the most that can be obtained (Vandepitte *et al.,* 2004).

3.9.1.3 Types of Broth Media

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

3.9.1.4 Blood Dilution

The blood sample should be diluted between 1 in 5 and 1 in 10 in the culture medium in order to reduce the concentration of natural antibacterial constituents such as serum complement, immunoglobulin, lysozyme, monocytes and PMNs to a sub-effective level. The dilution also reduces the

concentration of any therapeutically administered antibiotic (Collee *et al.,* 1996). Clotting of blood is also prevented by dilution (Strokes *et al.,* 1993).

3.9.1.5 Anticoagulant

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

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

3.9.1.6 Subculture from Blood Cultures

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

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

3.9.1.7 Identification

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

3.10 Antimicrobial Susceptibility Test

Antibiotic susceptibility test measures the ability of an antibiotic or other antimicrobial agents to inhibit the growth of microorganisms. Sensitivity test must never be performed on commensal organism or contaminants because this would mislead the clinician and could result in the patient in receiving ineffective and unnecessary antimicrobial therapy, causing possible side effect and resistance to other potentially pathogenic organisms. (Hugo and Russel, 1981 and Vandepitte *et al.,* 2004).

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

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

An organism is called susceptible to the drug when the infection caused by it is likely to respond to treatment with this drug at recommended disc. Antibiotics that are moderately sensitive to an organism can be used for treatment at a higher doses because of its low toxicity as antibiotic is concentrated in the focus of infection. Resistance can be defined as the temporary or permanent ability of an organism and its progeny to remain

viable or multiply under environmental condition that would destroy or inhibit other cells. The organisms that are resistant to three or more than three groups of antibiotics is called multi drug resistant organism (Hugo and Russel, 1993).

3.11 Minimum Inhibitory Concentration

Minimum inhibitory concentration (MIC) is the lowest concentration of antibacterial agent that will inhibit visible growth of an organism after overnight incubation (WHO, 1991). There are different methods to determine the MIC of an antibiotic. Among these methods, two fold serial dilution method is commonly used.

Dilution susceptibility testing methods are used to determine the minimal concentration, usually expressed in units or micrograms per millilitre, of an antimicrobial agent required to inhibit or kill microorganism. Antimicrobial agents are usually tested at log2 (two fold) serial dilutions, and the lowest concentration that inhibits visible growth of an organism is recorded as the minimum inhibitory concentration (MIC) (Balows *et al.,* 1991).

For broth dilution methods, decreasing concentrations of the antimicrobial agents (s) to be tested, usually prepared in two fold serial dilutions, one placed in tubes of a broth medium that will support the growth of the test organism (Baron and Finegold, 1990). This technique is recommended by WHO in 1991 for the qualitative estimation of antibiotic activity. The broth dilution methods are :

a) Macro-broth dilution method

The general approaches for broth methods include macro-broth dilution, in which the broth volume for each antimicrobial concentration is 1.0ml contained in 13 by 100 tubes.

b) Micro-broth dilution method

In this micro-broth dilution method, antimicrobial dilutions are in 0.05 to 0.1ml volumes contained in well of microtitre trays.

3.12 Treatment

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

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

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

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

3.13 Control

This is possible only by:

- Observing proper sanitation
- Ensuring a safe water supply
- Public Health legislation designed to ensure uncontaminated food materials (Agrawal, *et al.,* 2004).

3.14 Vacination

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

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

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

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

3.15 Antibiotic susceptibility pattern of the *Salmonellae*

Antibiotic therapy is essential and should begin empirically if clinical evidence is strong. When the pathogenic microorganism has been isolated from a patient its sensitivity to different antimicrobial agent is checked. With results of such test physician can choose the drug best suited for the patient. Sensitivity tests measure antimicrobial activity against bacteria under laboratory condition (Cheesbrough, 1984)

The antibiotics may act by destroying the bacteria or by inhibiting the growth of bacteria. The mechanism of action of antibacterial drugs can be divided into 5 groups (Chakraborty, 2000). They are :

1) Drugs interfering with cell wall synthesis
2) Drugs affecting cytoplasmic membrane
3) Drugs inhibiting protein synthesis and impairment of function of the ribosomes.
4) Drugs inhibiting synthesis of nucleic acid.
5) Drugs with antimetabolite action

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

There are four mechanisms which are responsible for the ability of microorganism to resist the action of antimicrobials (Boyd and Marr, 1980).

1) Interference with transport across the cell wall or cytoplasmic membrane.
2) Enzymatic modification of the drug.
3) Alteration of the antimicrobial target in the microorganism
4) Synthesis of resistant metabolic pathways.

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

Malaysia demonstrated that transferable resistant plasmids play a major role in antibiotic resistance in Enterobacteriacae.

Most strains of *Salmonalle* spp are sensitive to Chloramphenicol (MIC 2mg/lit), Ampicillin (MIC 1-8mg/lit) Gentamycin (MIC 0.25mg/lit), Tetracycline, Cotrimoxazole and some other antibiotics. Chloramphenicol has remained the gold standard for the treatment of typhoid fever for several decades and the alternative drug is Cotrimoxazole. Ampicillin has been disappointing in treatment of fever but is used in elimination of the carrier state to some extent (Collee *et al.*, 1996). Until about 1960 nearly all *Salmonellae* were sensitive to a wide range of antimicrobial agents, but since 1962 plasmid mediated resistance has appeared in them world wide, the relative importance of antibiotic resistance and the serotypes in which it develops differs from country to country (Parker and Collier, 1990).

Since its introduction in 1948, Chloramphenicol has been the treatment of choice for typhoid fever and remained the standard against which newer antimicrobials must be compared. Treatment with Chloramphenicol reduces mortality due to typhoid fever from approximately 20 percent to 1 percent and duration of fever from 14-28 days to 3-5 days (Mandal *et al.*, 2004). However Chloramphenicol therapy has been associated with the emergence of resistance, a high relapse rate, a high rate of continued and chronic carriage, bone marrow toxicity and high mortality rates in some recent series reported from the development world (Miller, 2000). Widespread plasmid mediated Chloramphenicol resistance emerged in *Salmonella typhi* in the early 1970s with outbreaks in Mexico, South India, Vietnam, Korea and Thailand (Srivastava and Agrawal, 1994).

Malla *et al.*, in 1984 found that Chloramphenicol or combination of Chlorompheniсol and Ampicillin can be used successfully to treat enteric fever.

Faried *et al.*, 1987 in USA had reported the successful treatment of typhoid fever in children with parenteral ceftriaxone.

Tanphichitra *et al.,* 1988 in Thailand showed the clinical evaluation of ofloxacin 0.123gm/lit b.i.d. dosage in enteric fever.

Stanely *et al.,* 1985 in Britian showed that Ciprofloxacin was cent percent effective for treat of enteric fever.

Watson and Pettibone, 1991 in Nepal found 80 percent resistance cases of chloramphenicol and Ampicillin whereas Sharma *et al.,* 1992 in Nepal reported about the clinical improvement of chloramphenicol, ceftriaxone and Ciprofloxacin were 34 percent, 100 percent and 93 percent respectively for the treatment of enteric fever in Nepalese children.

Chloramphenicol, Ampicillin, Tetracycline, Sulphonamide are the first line of drug of choice for *Salmonella typhi.* Almpicillin, Amoxycillin being Beta-lactam drugs are widely used in treatment of typhi infection. Alarming reports have been pointed out the rapid development of resistant to these agents. Clinical strains of *Salmonella* producing large spectrum Beta-lactamase which are resistant to penicillin (Vatopoulos *et al.,* 1994) or *Salmonella* producing extended spectrum of Beta-lactamase which are resistant to Cephalosporin such as Cephotaxime, Ceftazidime or Ceftriaxone have been isolated from large outbreaks (Llanes *et al.,* 1999).

In the recent past, Cephalosporins have gained importance for the treatment of enteric infections (Gatuam *et al.,* 2002). Parentarally administered third generation Cephalosporins are effective in the treatment of typhoid fever. Ceftriaxone, administered either intravenously or intramuscularly for 10-14 days is equivalent to oral or intravenous Chloramphenicol administered for treatment of susceptible *Salmonella typhi* strains. It is now considered to be the drug of choice for the treatment of enteric fever unless the *in vitro* susceptibility tests prove otherwise. First and second generation Cephalosporins are ineffective and should not be used to treat typhoid fever (Gatuam, *et al.,* 2002).

The high cost and need for parenteral administration are further disadvantages of cephalosporin therapy (Parry, 2004). Also some authors

reported treatment failure with these Cephalosporins in the recent years (Mandal *et al.,* 2004)

Ciprofloxacin was considered the drug of choice for the treatment of multidrug resistant typhoid, replacing Chloramphenicol (Mandal *et al.,* 2004). Hemalatha *et al.,* from Hyderabad, India, reported a sensitivity of 95 percent top Ciprofloxacin in 1999 (Rodrigues *et al.,* 2003). This was similar to studies from other parts of India. All these reports were about the time when the drug was introduced into the market i.e. during the early part of 1990s. Consequent to the widespread use of Ciprofloxacin especially in the community, resistance and treatment failures were increasingly observed and reported.

The minimum inhibitory concentration (MIC) to the drug was reported to steadily increase from 0.025 to 0.5μg/ml (Mandal *et al.,* 2004 and Parry, 2004). Gautam *et al.,* from Haryana reported a decrease in sensitivity from 89 percent to 81 percent (1997 to 2001 (Gautam *et al.,* 2002). Lakshmi *et al.,* 2006 reported that, the sensitivity of Ciprofloxacin was decreased from 96 percent in 2001 to 83 percent by 2004 (p. 0.005).

Nalidixic acid resistance is a marker for predicting low-level resistance to Ciprofloxacin among *Salmonella typhi* and also an indicator of treatment failure to Ciprofloxacin (Mandal *et al.,* 2004). When the sensitivity pattern indicates resistance to Nalidixic acid, which is marker for delayed clinical response to fluoro-quinolones, it is necessary to increase the oral dose of Ciprofloxacin or treat the patient with third generation Cephalosporins like Ceftriaxone (Miller *et al.,* 1995 and Rodrigues *et al.,* 1998). Hence it is suggested that all *Salmonella typhi* isolates should be screened for Nalidixic acid resistance along with Ciprofloxacin. Any isolate that shows resistance Nalidixic acid should be reported as intermediately susceptible to Ciprofloxacin. The clinician should be advised to change the antibiotic (Mandal *et al.,* 2004 and Madhulika *et al.,* 2004).

Identification of Nalidixic acid resistance by the disc diffusion method has been reported to provide a sensitivity of 100 percent and a specificity of 87.3 percent as a tool to screen for isolates having MICS of Ciprofloxacin ≥ 0.125μg/ml (Hakanen *et al.,* 1999). Rodrigues *et al.,* 2003 reported 82-88 percent resistance to Nalidixic acid among their isolates with an associated increase in MIC to Ciprofloxacin from 0.125 to 1.5μg/ml. Lakshmi *et al.,* 2006 found 92-96 percent isolates were resistant to Nalidixic acid. Madhulika *et al.,* who also found a high resistance to Nalidixic acid (92%), documents high MICs (> 0.5μg/ml) to Ciprofloxacin in majority of their isolates. (Shrivastava and Agrawal, 1994).

The sensitivity of *Salmonella paratyphi A* to Ciprofloxacin was decreased from 93 percent to 83 percent (Gautam *et al.,* 2002). Lakshmi *et al.,* 2006, observed that the sensitivity of Ciprofloxacin was reduced from 86 percent to 70 percent.

CHAPTER - IV

4. MATERIALS AND METHODS

4.1 Materials

The media, equipments, apparatus, chemicals and reagents and antibiotic discs used in this study are listed below:

4.1.1 Equipment

a) Autoclave

b) Centrifuge

c) Freeze

d) Hot Air Oven

e) Incubator

f) Microscope

4.1.2 Media used (Hi-Media,India)

4.1.2.1 Culture media

i. Mac-Conkey agar

ii. Nutrient Agar

iii. Brain Heart infusion broth

iv. Nutrient broth

4.1.2.2 Biochemical media

a) Methyl Red Voges Proskauer Medium (oxoid)

b) Sulphide Indole Motility Agar (oxoid)

c) Hugh Leifson's Agar

d) Simmon's Citrate Agar (oxoid)

e) Triple Sugar Iron Agar (oxoid)

f) Urea Agar (oxoid)

4.1.2.3 Antibiotic Sensitivity testing media

Mueller-Hinton agar (oxoid)

4.1.2.4 MIC media

Mueller-Hinton broth

4.1.3 Antibiotics discs (Hi Media, India)

a) Amoxycillin (30mcg)

b) Ceftriaxone (30mcg)

c) Cefixime (5mcg)

d) Chloramphenicol (30mcg)

e) Ciprofloxacin (5mcg)

f) Cotrimoxazole (1.25mcg)

g) Cephotaxime (30mcg)

h) Ofloxacin (5mcg)

i) Nalidixic acid (30mcg)

4.1.4 Antibiotics powder

i) Ciprofloxacin

4.1.5 Chemicals used

i. Sodium chloride

ii. Crystal violet

iii. Gram's Iodine

iv. 95% Alcohol

v. Safranin

vi. Paraffin

4.1.6 Test reagent

i. 3% Hydrogen peroxide

ii. Tetra methyl paraphenylene diamine dihydrochloride

iii. Iso-amyl alochol

iv. p-dimethyl aminobenzaldehyde

v. Concentrated hydrochloric acid

vi. Methyl red

vii. Potassium hydroxide

viii. 1-napthalamine

4.2 Methods

4.2.1 Collection of Sample

Blood samples were collected at Kathmandu Model Hospital, Exhibition Road, Kathmandu using standard aseptic techniques. (Parker and Finegold, 1990)

4.2.1.1 For Adult

Culture bottle containing about 45 ml of BHI broth was provided and 5ml of patient's blood was collected and dispensed in the culture bottle.

4.2.1.2 For Children

Culture bottle containing 20ml of Brain Heart infusion broth was provided for sample collection. 2ml of blood was drawn from the patient and dispensed in the culture bottle.

4.2.2 Processing of Sample

Immediately after the blood culture bottles were received, they were given laboratory numbers and further processed.

4.2.2.1 Incubation of Sample

The culture bottles were incubated at 37°C. Incubation was continued for 7 days unless the visible growth was obtained. Terminal subculture for visually negative culture was done after 7 days of incubation. The day of collection of sample was defined as the first day in this study.

4.2.2.2 Macroscopic examination of broth culture

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

4.2.2.3 Sub culture

The subculture was done on Mac-Conkey agar and Blood agar. Repeated subcultures of the culture bottles were made at different times during their incubation from 24 hours to 7 days.

4.2.2.4 Incubation of Subculture plate

The Mac-Conkey agar and Blood agar plates were incubated at 37°c for 24 hrs.

4.2.2.5 Examination of subculture plate

The subculture plates were examined after overnight incubation. Mac-Conkey agar plates was examined for growth of non lactose fermenters.

4.2.2.6 Identification of Bacterial Pathogen

Identification of bacteria from positive culture plates were done with the use of standard microbiological technique which included colony morphology, Gram stain, biochemical reaction and serotyping (Cheesbrough 1984, Collee *et al.*, 1996).

4.2.2.7 Purity Plate

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

4.2.2.8 Biochemical tests used for identification of bacteria

Appropriate biochemical tests were done for the identification of the bacteria. A single colony of suspected pathogen was inoculated in Nutrient Broth and incubated at 37°C for 4 hrs. After incubation Gram stain and hanging drop preparation were performed. Various biochemical media were inoculated and the results were observed on following day.

Table No. 1: Biochemical test performed for identification of bacteria

S.N.	Biochemical Medium	Tests
1	Triple sugar iron (TSI) agar	Glucose/sucrose/lactose fermentation H_2S production, Gas production
2	Simmons citrate agar	Utilization of citrate
3	Sulphide Indole Motility (SIM) agar	Motility, Indole ring production, Hydrogen sulphide production
4	Glucose phosphate (MR-VP) broth	Mixed acid fermentation, Acetoin production
5	Urea agar	Urease (urea hydrolysis)
6	Hydrogen peroxide	Catalase
7	1% Tetramethyl paraphenylene diamine dihydrochloride	Oxidase

4.2.2.9 Serotyping of *Salmonella* spp

Isolated *Salmonella* were subjected for serotyping using kit (Denka Seiken Co. Ltd. Tokyo, Japan) as per kit's instruction.

4.2.2.10 Antibiotic sensitivity test

All isolates were tested for susceptibility to antimicrobial agents on Mueller-Hinton agar by the standard disc method (NCCLS, 2003).

The media was prepared and sterilized as per manufacturer instruction. The pH of the media was maintained at 7.2-7.4. The freshly prepared and cooled media was poured into glass petridish to give uniform depth of approximately 4mm. The well-isolated colonies were transferred into test-tube containing 5ml of peptone broth. The broth culture was incubated at 37°C until it achieves or exceeds the turbidity of the 0.5 McFarland standard usually for 2-5 hours. A sterile cotton swab was dipped into the adjusted suspension and the dried surface of Muller-Hinton Agar plate was inoculated by spreading the swab over the entire sterile plate. The plates were incubated at 37°C for 24hrs. After overnight incubation, the plates were examined for confluent growth. The diameters of the zone of inhibition were measured including the diameter of disc. The size of the zone of inhibition was interpreted by referring to the zone size interpretative chart provided by Hi-Media Laboratories Limited.

4.2.2.11 Quality control

For the best interpretation of results and recognition of any source of error in disc diffusion sensitivity methods the correct use of control was taken.

Thus control strains *Escherichia coli* (ATCC, 25922), *Staphylococcus aureus* (ATCC 25923) and *Pseudomonas aerugenosa* (27855) were used for the standardization of the Kirby-Bauer test by correct interpretation of the zone diameters.

4.2.2.12 Determination of MIC

The MIC of Ciprofloxacin was determined by two fold serial dilution method of Finegold and Baron (1990). Media used was Mueller Hinton broth.

a) Preparation of working solution

The working solution was prepared as :

1. 0.128gm Ciprofloxacin powder was dissolved in 10ml of distilled water.
2. 0.1ml of this solution was diluted with 9.9ml D/W giving 128µg/ml concentration of working solution.

b) Preparation of standard culture inoculum

Standard culture inoculum was prepared as :

Three to five colonies of similar appearance of the organism to be tested were touched with the inoculating loop. It was then transferred to a tube containing (5ml) MHB. The inoculated tube was incubated at 37°C for 4hrs. The tube was then compared with turbidity standard, McFarland 0.5 standard as recommended by WHO (1991).

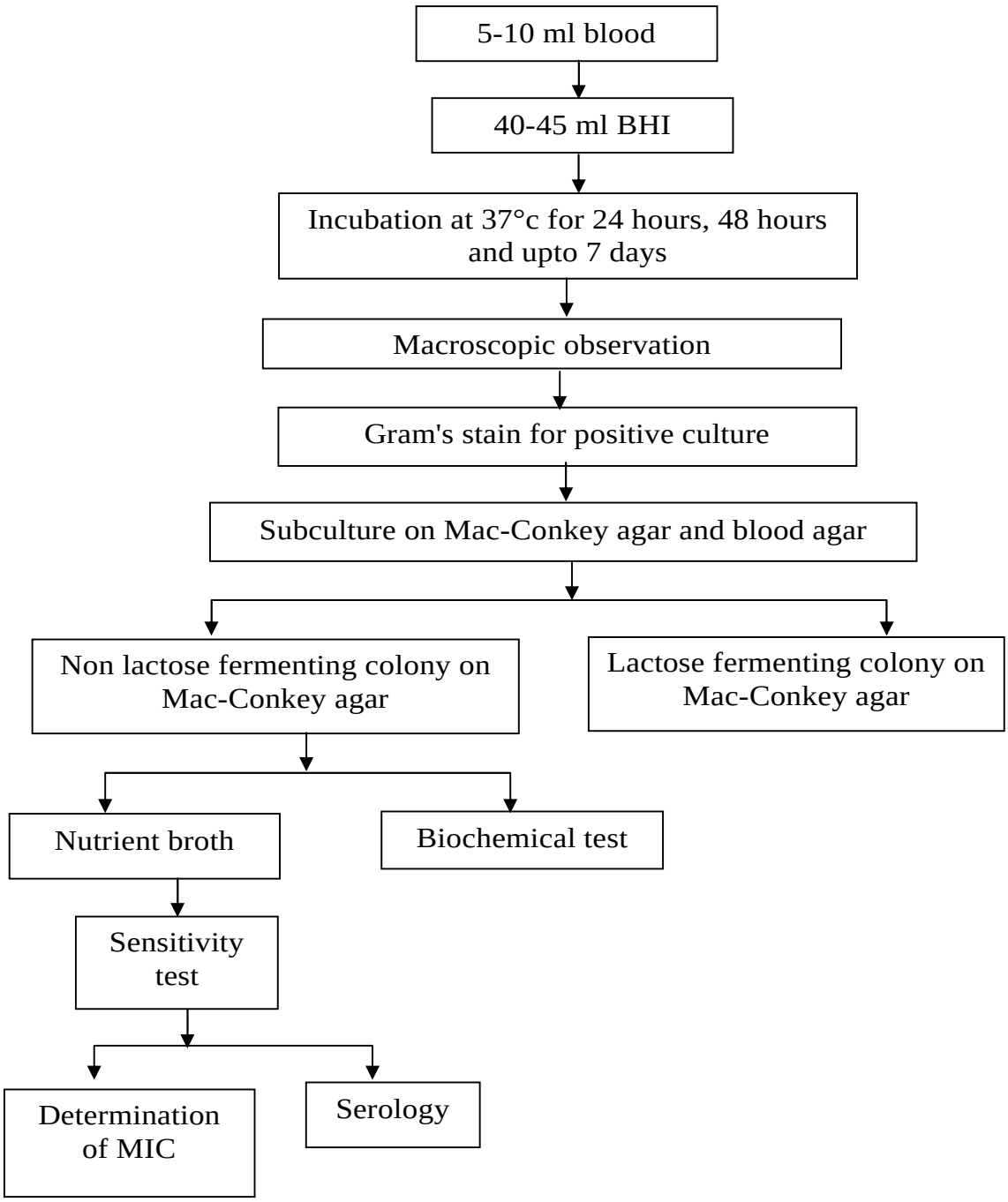
For each bacterial isolate, a set of 12 screw capped test tubes containing 1ml MHB was required. The test tubes were labeled as positive growth control, negative growth control and no. 1 to 10. In case of negative control the MHB was discarded. Then 1ml of working solution of Ciprofloxacin was added

aseptically to each tube labeled as negative growth control and no. 1 labeled test tube.

1ml of broth and 1ml of working solution of Ciprofloxacin were contained in 1st tube. After complete homogenization, 1ml of its content was transferred aseptically to 2nd tube. Similarly the second tube was homogenized and 1ml of its content was transferred to 3rd tube. This process of homogenization followed by transfer was carried upto the 10th tube. Finally after homogenization 1ml of the content from the 10th tube was discarded. In this way, two fold serial dilution of the Ciprofloxacin was prepared upto the 10th tube having concentration (μg/ml) 64, 32, 16, 8, 4, 2, 1, 0.5,m 0.125, 0.0625 respectively.

No antibiotic solution was added to the tube labeled as positive control. Now with the help of micropipette, 20μl of culture inoculum of test bacteria was added to each tube except the one which was labeled as negative control.

All the tubes were incubated at 37°C for 18-24hrs and observed for turbidity by comparing with +ve and -ve controls. The results were interpreted on the basis of the fact that growth occurs in the positive control and any other tube in which the concentration of the extract is not sufficient to inhibit growth and the lowest concentration of the antibiotic that inhibits growth of the organism as detected by lack of visible turbidity is designed as minimum inhibitory concentration (MIC).



Flow chart for the isolation and identification of *Salmonella* from blood culture (Cheesbrough, 2000)

CHAPTER - V

5. RESULTS

A total of 534 patients suspected with enteric fever were studied during May 2006 to August 2006 at Kathmandu Model Hospital, Kathmandu. Among the 534 blood samples collected for culture, 344 (64.41%) were from male and 190 (35.58%) were from female. The young adults having age 20-30 years were most frequently suspected to enteric fever and requested for blood culture (41.94%) than any other age groups. The age group 10-20 was second with 25.65% requests. Only one i.e. 0.18% blood culture was requested for age group 80-90 years.

Table No. 2 : Age and gender wise distribution of patients

Age group	Gender						Total	%
	Male			Female				
	Out patient	In patient	Total	Out patient	In patient	Total		
0-10	19	0	19	16	0	16	35	6.55
10-20	78	4	82	55	0	55	137	25.65
20-30	155	6	161	59	4	63	224	41.94
30-40	36	2	38	18	2	20	58	10.86
40-50	21	2	23	10	3	13	36	6.74
50-60	4	1	5	13	3	16	21	3.93
60-70	7	1	8	1	2	3	11	2.05
70-80	7	1	8	1	2	3	11	2.05
80-90	0	0	0	1	0	1	1	0.18
Total	327	17	344	174	16	190	534	100
%	61.23	3.18	64.41	32.58	2.99	35.58	100	

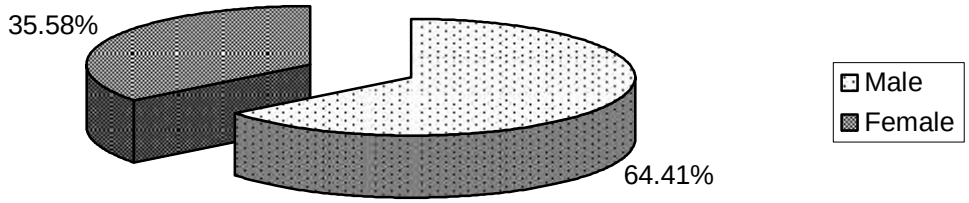


Figure No.1 : Gender wise distribution of the patients for blood culture suspected of enteric fever

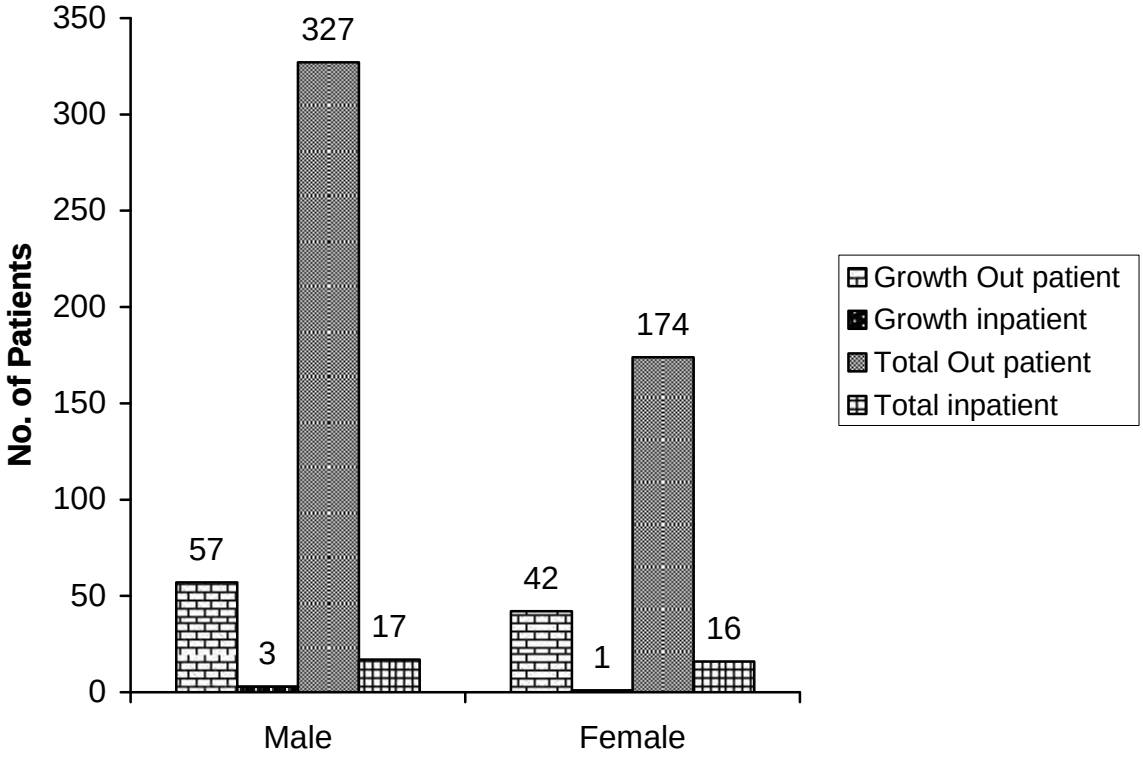


Figure No. 2 : Origin wise distribution of the patients requesting for blood culture

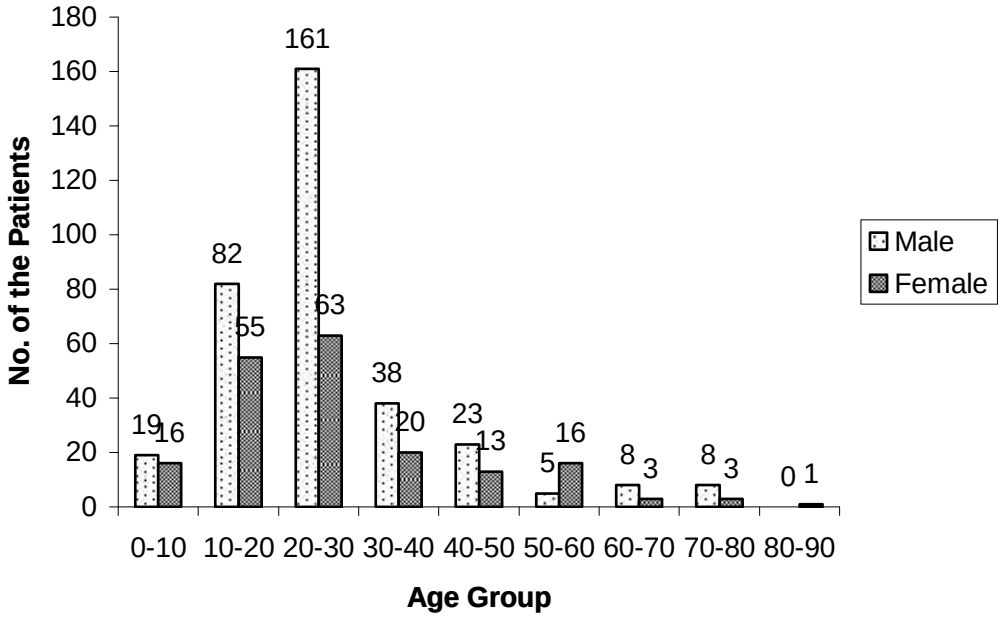


Figure No. 3 : Age and sex wise distribution of the patients requesting for blood culture

Table No. 3 : Growth pattern in blood culture with relation to age and sex

Age	Male	Female	Culture positive male	Culture positive female	Total positive culture
0-10	19	16	5 (26.31%)	6 (37.5%)	11 (31.42%)
10-20	82	55	19 (23.17%)	19 (34.54%)	38 (27.73%)
20-30	161	63	29 (18.01%)	14 (22.22%)	43 (19.19%)
30-40	38	20	4 (10.52%)	4 (20%)	8 (13.79)
40-50	23	13	-	-	-
50-60	5	16	1(20%)	1 (6.25%)	2 (9.52%)
60-70	8	3	1 (12.5%)	-	1 (9.09%)
70-80	8	3	-	-	-
80-90	-	1	-	-	-
Total	344	190	59	44	103

As given in the table 3, out of 103 culture positive samples, 43 (19.19%) were found in age group 20-30 years. The next was age group 10-20 years in which 38 culture positive samples were found. The highest percentage of positive

culture was found in age group 0-10 years i.e. 31.42 percent. The total culture positive males were 59 (17.15%) and females were 44 (23.15%). The age group 40-50 years and patient with > 70 years age were not found positive growth results.

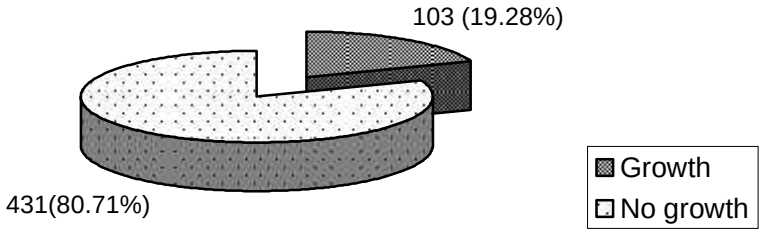


Figure No. 4 : Growth Pattern in blood culture

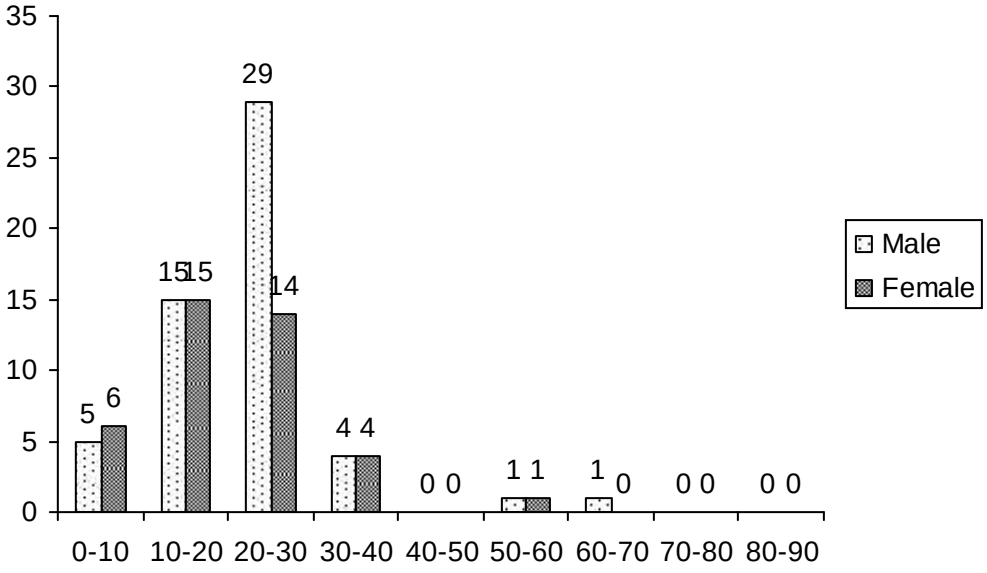


Figure No. 5 : Age and gender wise distribution of culture positive cases

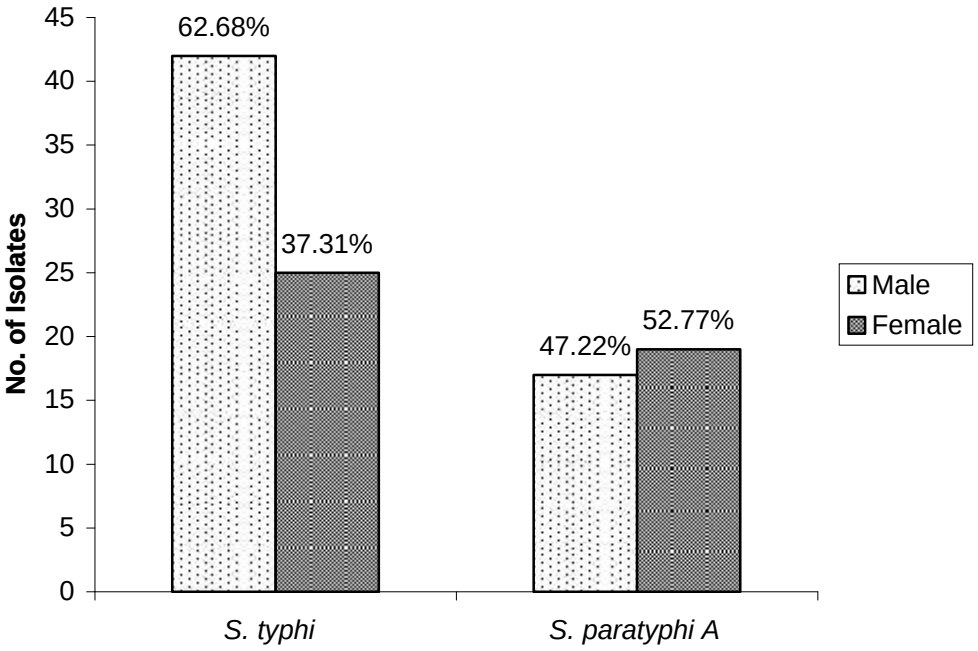


Figure No.6 : Sex wise distribution of bacterial isolates

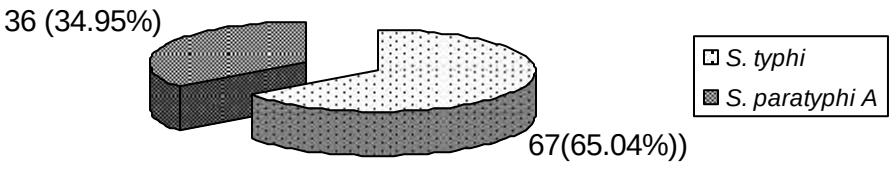


Figure No. 7 : Pattern of bacteria isolated from culture positive samples

Table No. 4 : Distribution of the isolates in inpatients and out patients

Origin	No. of patients	Bacterial Isolates		Total
		Salmonella typhi	*S. paratyphi A*	
Outpatient	501	66 (98.50%)	33 (91.66%)	99 (96.11%)
Inpatient	33	1 (1.49%)	3 (8.33%)	4 (3.89%))
Total	534	67 (65%)	36 (35%)	103

Table 4 showed that among 103 growth 99 were from out patient and 4 were from inpatient. In outpatient out of the total growth 99, 66 were *S. typhi* and 33 were *S. paratyphi A*. In inpatient the number of *S. paratyphi A* was 3 and *S. typhi* was 1. Among 103 isolates from blood culture positive cases 67(65%) isolates were identified as *Salmonella typhi* and 36(35%) isolates were identified as *Salmonella paratyphiA*.

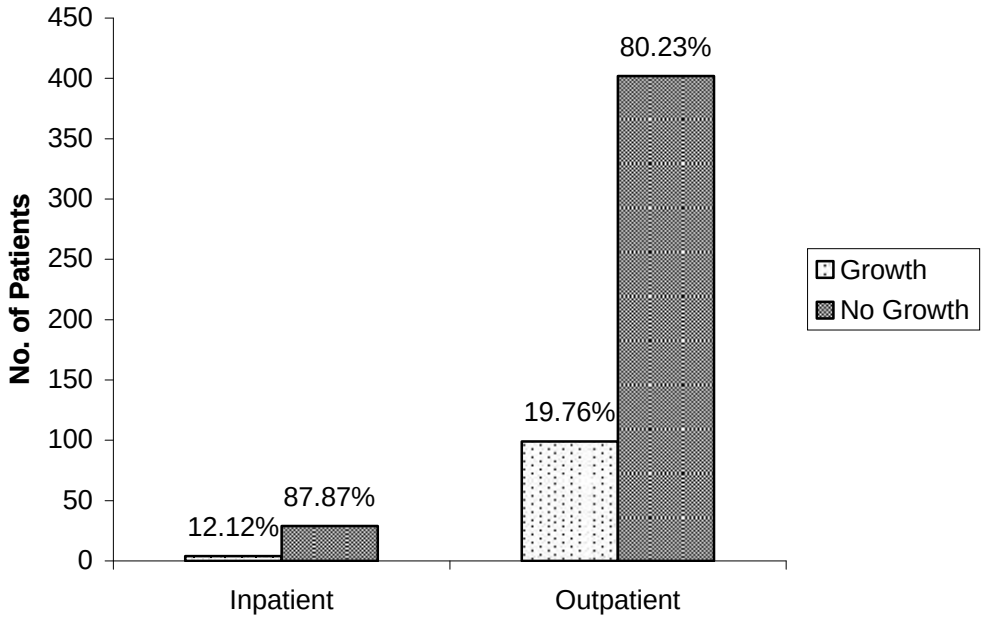


Figure No. 8 : Growth Pattern in blood culture with relation to origin of the sample

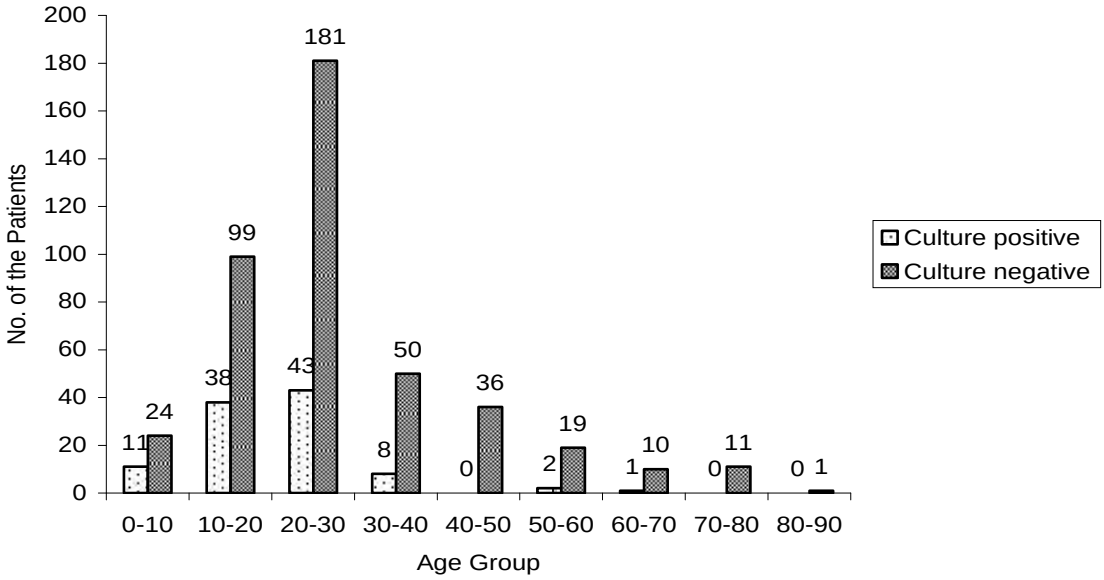


Figure No. 9 : Growth pattern of different age group

Table No. 5 : Antibiotic susceptibility pattern of *Salmonella typhi*

Antibiotic used	Antibiotic susceptibility pattern						Total
	Susceptible		Intermediate		Resistant		
	Number	%	Number	%	Number	%	
Amoxycillin	67	100.00	-	-	-	-	67
Cefixime	60	89.55	4	5.97	3	4.47	67
Ceftriaxone	67	100	0	0	-	-	67
Cephotaxime	65	97.01	2	2.98	-	-	67
Ciprofloxacin	61	91.04	6	8.95	-	-	67
Chloramphenicol	67	100	-	-	-	-	67
Cotrimoxazole	67	100.00	-	-	-	-	67
Nalidixic acid	22	32.83	-	-	45	67.16	67
Ofloxacin	58	86.56	4	5.97	5	7.46	67

As given in table 5, among the isolated *Salmonella typhi*, 100 percent sensitivity was found to the antibiotics Amoxycillin, cettriaxone, chloramphenicol, and cotrimoxazole. 91.04 percent isolates were susceptible

towards Ciprofloxacin and remaining 8.95 percent were intermediate. Altogether 4.47 percent of *Salmonella typhi* were resistant to cefixime, 67.16 percent to Nalidixic acid and 7.46 percent to ofloxacin.

Table No. 6 : Antibiotic susceptibility pattern of *S. paratyphi A*

Antibiotics used	Sensitive		Intermediate		Resistant		Total Isolates
	Number	%	Number	%	Number	%	
Amoxycillin	36	100	-	-	-	-	36
Cefixime	28	77.77	6	16.66	2	5.55	36
Ceftriaxone	36	100	-	-	-	-	36
Cephotaxime	34	94.44	2	5.55	-	-	36
Ciprofloxacin	36	100	-	-	-	-	36
Chloramphenicol	36	100	-	-	-	-	36
Cotrimoxazole	36	100	-	-	-	-	36
Nalidixic acid	8	22.22	-	-	28	77.77	36
Ofloxacin	30	83.33	3	8.33	3	8.33	36

As given in the table 6, among the *Salmonella paratyphi A* isolates, none of the isolates were found to be resistant to the antibiotics Amoxycillin, Ceftriaxone, Ciprofloxacin, Chloramphenicol and Cotrimoxazole, 5.55 percent of them were intermediate sensitive to Cephotaxime. Altogether 5.55 percent Cefixime, 77.77 percent Nalidixic acid and 8.33 percent Ofloxacin resistant *Salmonella paratyphiA* isolates were found.

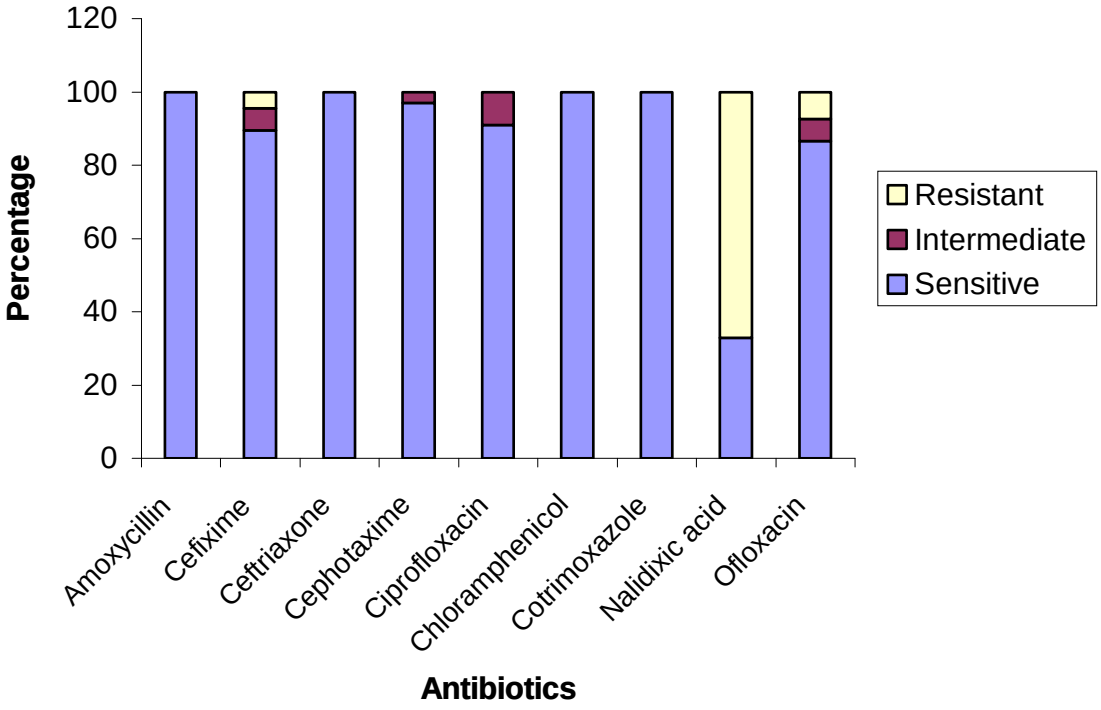


Figure No. 10 : Antibiotic susceptibility pattern of *Salmonella typhi*

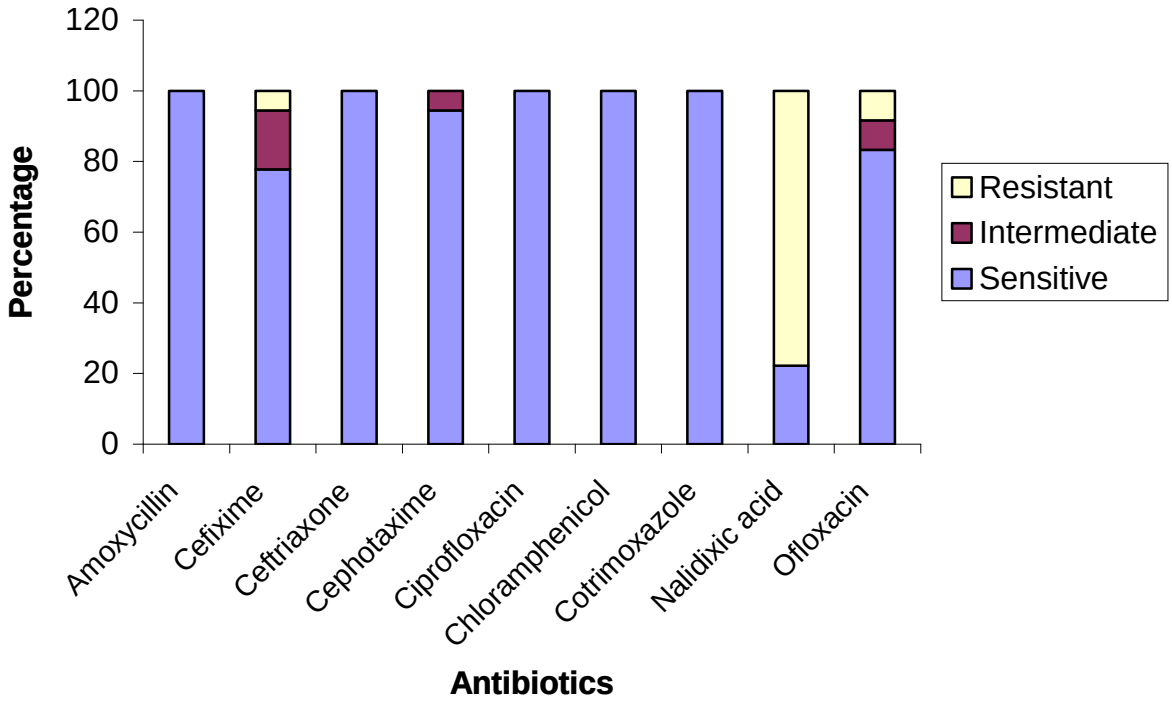


Figure No. 11 : Antibiotic susceptibility pattern of *Salmonella paratyphi A*

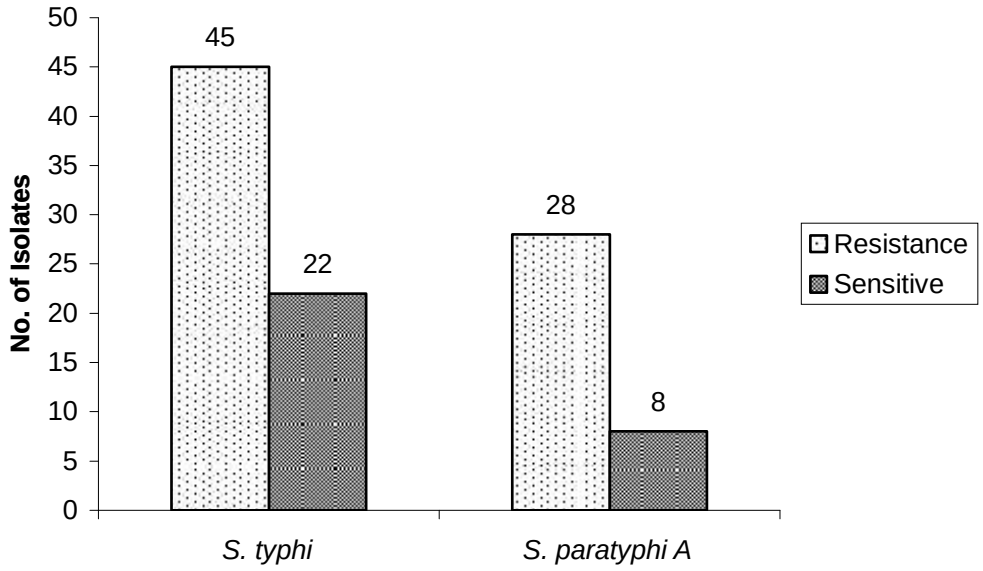


Figure No. 12 : Nalidixic Acid resistance pattern of the isolates

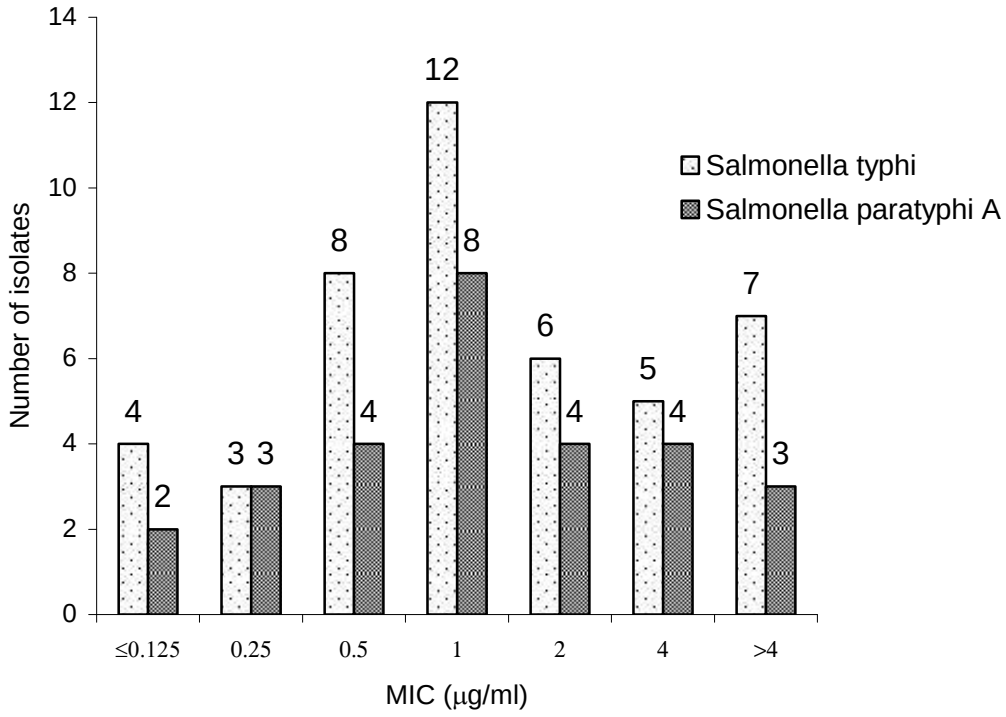


Figure No. 13 : MIC values of Ciprofloxacin in nalidixic acid resistant *Salmonella* (n = 73)

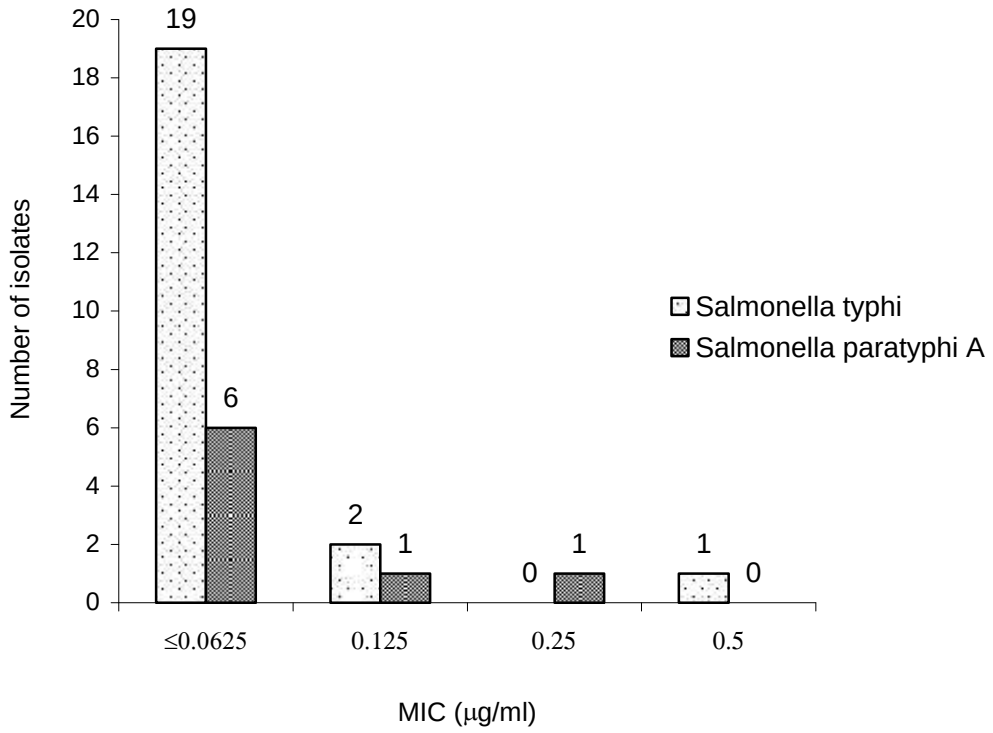


Figure No. 14 : MIC values of Ciprofloxacin in Nalidixic acid susceptible *Salmonella* (n=30)

Table No. 7 Validity of Nalidixic acid resistance test by disc diffusion method to screen for isolates having MICs of Ciprofloxacin ñ 0.125 «g/ml.

Nalidixic Acid susceptibility	MIC value for Ciprofloxacin		Total
	≥ 0.125µg/ml	< 0.125µg/ml	
Resistance	67	6	73
Sensitive	5	25	30
Total	72	31	103

Sensitivity of the test = $\frac{67}{73} \times 100\% = 91.98\%$.

Specificity of the test = $\frac{25}{30} \times 100\% = 83.33\%$.

The current MIC breakpoints for *Salmonella* for Ciprofloxacin and ≥ 4µg/ml (Resistant) and ≤ 1µg/ml (sensitive). Decreased susceptibility of Ciprofloxacin was defined as an MIC of ≥ 0.125µg/ml (Hakanen *et al.*, 1999).

MIC value for Ciprofloxacin was found to be ≥ 0.125 in 91.98% of Nalidixic acid resistant cases, where as only 16.66% having MIC value of ≥ 0.125 were sensitive to Nalidixic acid. This is an indication of decreased susceptibility of Ciprofloxacin in Nalidixic acid disks diffusion tests.

All isolates were not found to be resistant to Ciprofloxacin with the Clinical and Laboratory Standards Institute breakpoints, however 72 isolates (69.9%) had decreased susceptibility. Resistance to Nalidixic acid was observed in 73 (70.87%) isolates in disk diffusion test. Screening with 30μg Nalidixic acid disks had a sensitivity of 91.98% and a specificity of 83.33% for determination of decreased susceptibility to Ciprofloxacin.

CHAPTER - VI

6. DISCUSSION AND CONCLUSION

6.1 Discussion

Blood samples were collected from 534 enteric fever suspected cases visiting Kathmandu Model Hospital (KMH), Kathmandu. Outpatients were higher (93.82%) than hospitalized patients (6.17%). Male patients were high in number 64.41%) than the females (35.58%). The majority of the patients belonged to age group 20-30 years (with 41.94% of the total population studied).

Among the total 534 enteric fever suspected cases, bacterial growth on culture could be observed only in 19.28% (103) cases. Similar results were observed in different studies at different countries by Ako-Nai *et al.,* 1990 (25.3% growth on blood culture) and Leibovici *et al.,* 1991(21% growth on blood culture). Similar culture positivity (13.95 - 21.47%) results were also observed in some of the studies done in Nepal. Shrestha, 1996 observed culture positivity of 14.83%; Shrestha, 2004 reported 16.26% culture positivity whereas Khatiwada, 2006 reported 14.61% culture positivity in suspected enteric fever cases.

In this study, the incidence rate of outpatients was 19.76% whereas the incidence rate of inpatient was 12.12%. The incidence rate of female patients (23.15%) was higher than that of male patients (17.15%). Prevalence of enteric fever among male was 57.28 percent and female was 42.71 percent. In this study, no difference was observed in gender male and female. The total number of male patients were higher than that of female patients, where as the blood culture positive rate was higher in female than male. This may be because of self prescribing attitude of male patients.

In this study, the blood culture positive rate was found to be highest in the age group 0-10 years in both male and female patients. This finding is different than the findings of the study of Shrestha, 1996 indicating higher positivity in the age group of 15-30 years. Chowta and Chowta, 2005 in Kasturba Medical

College Hospital India during the year 1999-2001, with 47.7% male and 52.3% female with average age of 23.9 years. Similarly, Khatiwada , 2006 found typhoid prevalent in the age group of 20-29 years in male and 0-9 years in female.

The higher prevalence in children of 0-10 years of age may be contributed by less health conscious and eating contaminated food and water in school. Hence 0-10 years of age children are highest among the age group to show isolation of typhoid and paratyphoid causing bacteria.

Out of 103 blood culture positive cases, 65.04% (67) were *Salmonella typhi* and remaining 34.95% (36) were *Salmonella paratyphi-A;* indicating higher prevalence of typhoid cases than paratyphoid cases. *Salmonella paratyphi B* and *C* were not isolated during the study period. This finding is similar to other studies done by Schwartz *et al.,* 1990 (*Salmonella typhi* -67% and *Salmonella paratyphi A* - 33%); Thapa ,1991 (*Salmonella typhi* -69.89% and *Salmonella paratyphi A* -29.03%); Ghimire,1995 (*Salmonella typhi- 71%* and *Salmonella paratyphi A*- 29%); Shrestha,1996, *Salmonella typhi-63.63%, Salmonella paratyphi A*- 35.06%, and *Salmonella paratyphi B*-1.29%); Kingemi *et al.,* in Nigeria in 2000 (*Salmonella typhi- 67.3%, Salmonella paratyphi A*-16.8% and *Salmonella arizonae* -15.8%).

The isolated *Salmonella typhi* and *Salmonella paratyphi A* were tested against the nine antibiotics in the laboratory. In our study 100% of the *Salmonella typhi* was found to be sensitive to Amoxycillin, Ceftriaxone, Chloramphenical and Cotrimoxazole. The efficacy rate of Cephotaxime, Ciprofloxacin, Cefixime and Ofloxacin were found as 97.01%, 91.04%, 89.55% and 86.56% respectively. So the last drug of choice among the tested antibiotic was Cefixime.

Similarly, 100% of the *Salmonella paratyphi A* isolated in the study were sensitive to Amoxycillin, Ceftriaxone, Ciprofloxacin, Chloramphenicol and Cotrimoxazole. The efficacy of Cephotaxime, Ofloxacin and Cefixime were found as 94.44%, 83.33% and 77.77% respectively. Cefixime was the least effective antibiotic among the tested antibiotics.

During this study, none of the isolates were multi drug resistant and all the isolates were sensitive to commonly used drug Ciprofloxacin. This finding is not in agreement with the finding of Threlfall *et al.,* 2003, who reported that for *Salmonella paratyphi A,* multi drug resistance increased from 9% in 1995 to 25% in 2001 and decreased Ciprofloxacin susceptibility from 6% to 17%.Thapa, *et al.,* 1991 also reported that Ciprofloxacin, Cotrimoxazole and Ampicillin resistant in *Salmonella paratyphi A* were 3.7%, 33.3% and 51.9% respectively.

In a study done in India from April 1996 to July 1999, during 1996-1997, all the isolated *Salmonella paratyphi A* were uniformly susceptible to all the antibiotics. However, in 1998, incidence of enteric fever caused by *Salmonella paratyphi A* abruptly increased and the number of drug resistant isolates susceptible to Ciprofloxacin were markedly decreased. In first 6 months of 1999, 32% of the isolates were resistant to both Chloramphenicol and Cotrimoxazole and 13% were resistant to more than two drugs (Chandel *et al.,* 1999).

During this study, the isolated *Salmonella paratyphi A* showed resistance against Ofloxacin (8.33%) and Cephotaxime (5.55%), 100% and other isolated were not found resistance towards remaining drugs.

Trenholme *et al.,* 1989 reported that 31 patients with gram-negative bacteracemia had organism susceptible to Cephotaxime and the study supported that Cephotaxime can be used at an 8 or 12 hours intervals in selected patients with gram negative bacteraemia.

Similar results was also found by Ghimire, 1995. She found that Chloramphenicol was the most effective antibiotics followed by Amoxycillin and Cotrimoxcizole against gram-negative rods.

Tibrewal, 1999 found that Ciprofloxacin was the most effective antibiotic (93.26%) whereas Ampicillin was found to be 20.22% effective against all the 89 gram-negative rods.

Study conducted by Shrestha *et al.*, 1996 showed that Chloramphenicol was the most effective antibiotic followed by Ciprofloxacin, Cotrimoxazole, Norfloxacin and Amoxycillin for *Salmonella typhi*. According to Wagley *et al.*, 2004 Ceftazimide was 100% effective towards *Salmonella typhi*, Chloramphenicol and Ceftriaxone were the second choices with 95.31% effectiveness Ciprofloxacin and Cephalexin were found to be 90.63%, Ofloxacin and Ampicillin showed 87.52% and 85.94% susceptibility pattern respectively. Shrestha *et al.*, 2004 found 100% efficacy rate of the antibiotics Ofloxacin, Ciprofloxacin, Chloramphenicol, Ceftriaxone and Cotrimoxazole.

Shakya *et al.*, 2001 revealed that Chloramphenicol and Ceftriaxone were found to be the most effective antibiotics against *Salmonella paratyphi A*. According to Shrestha *et al.*, 2004, Cotrimoxazole and Ceftriaxone were found to be the most effective (100%) antibiotics against *Salmonella paratyphi A*. followed by Chloramphenicol and Ciprofloxacin (95%), Ofloxacin and Ampicillin (90%). Wagley *et al.*, 2004 showed that efficacy rate of drug Ciprofloxacin and Ofloxacin against *Salmonella paratyphi A* were 90.91% and 72.71% respectively.

Amatya *et al.*, 2005, reported the 100% efficacy of Amoxycillin, Chloramphenicol and Cotrimoxazole and efficacy rates of Ceftriaxone, Cephotaxime, Ofloxacin, Ciprofloxacin and Cefixime were 93.18%, 86.36%, 81.82%, 79.54% and 75% respectively in *Salmonella paratyphi A*.

According to Khatiwada, 2006, 100% isolates of *Salmonella paratyphi A* were sensitive towards Tetracycline, Ciprofloxacin, Ceftriaxone and Cotrimoxazole.

In this study the isolates were tested against Nalidixic acid, to understand the level of decreased susceptibility to Ciprofloxacin in Nalidixic acid resistance isolates. 67.16% *Salmonella typhi* and 77.77% *Salmonella paratyphi A* isolates were found resistant to Nalidixic acid.

In our study, all Nalidixic acid susceptible isolates were also sensitive to Ciprofloxacin and none of the Nalidixic acid resistant isolates were resistant to Ciprofloxacin.

Miller *et al.,* 1995 and Rodrigues *et al.,* 1998, reported that when the sensitivity pattern indicates resistance to Nalidixic acid, which is marker for delayed clinical response to fluoroquinolones, it is necessary to increase the oral dose of Ciprofloxacin or treat the patient with third generation Cephalosporins like Ceftriaxone.

In a study conducted at Calicut, India during 1999-2003, 90.22% of the *Salmonella typhi* strains isolated were resistant to Nalidixic acid showing treatment failure with Ciprofloxacin (Nair and Sudarsan, 2004).

In this study, Ciprofloxacin and Cephotaxime resistant *Salmonella* isolates were not detected. But other studies have shown prevalence of Ciprofloxacin resistant among the *Salmonellae* resistant. The epidemic of Ciprofloxacin resistance was first reported from Tajikistan in which 90% of the isolates were multi drug resistant (MDR) in which 82% were resistant to Ciprofloxacin (Murdoch *et al.,* 1998). In this study there was no. multi drug resistant *Salmonella* isolates.

A similar result was reported by Chomal and Deodar, 2000. They found none of the strain showed resistance to Ciprofloxacin in susceptibility pattern, 66% strains were resistant to Nalidixic acid. Similarly, Madhulika *et al.,* 2004 showed that 83.43% strains were Nalidixic acid resistant and 82.16% strains were Ciprofloxacin sensitive.

Joshi *et al.,* 2004 showed that 147 isolates (84 *Salmonella typhi* and 63 *Salmonella A*) of *Salmonella* were resistant to Nalidixic acid out of 246 strains isolates. The Nalidixic acid resistant strains were either sensitive or moderately sensitive to Ciprofloxacin according to the zone size. Ampicillin, Chloramphenicol and Cotrimoxazole resistance was seen in 27% Nalidixic acid resistant *Salmonella typhi* strains and none in *Salmonella paratyphi A.* The MICs of Ciprofloxacin in Nalidixic resistant strains ranged from 0.38 to 3μg/ml.

Clinical and laboratory standard institute (CLSI) guidelines were followed throughout the study (CLSI, 2005). The lowest and highest concentration tested were 0.0625-8μg/m for Ciprofloxacin. *Escherichia coli* ATCC 25922

was used as the control organism. Decreased susceptibility of Ciprofloxacin was defined as an MIC of ≥0.125 μg/ml. (Hakanen *et al.,* 1999)

The current MIC break points for Enterobacteriacea for Ciprofloxacin are ≥ 4μg/ml (resistant) and ≤ 1μg/ml (susceptible) (Crump *et al.,* 2003).

In our study, all isolates were found to be susceptible to Ciprofloxacin with the Clinical and Laboratory Standards Institute breakpoints, however 72 isolates (69.9%) had decreased susceptibility. Resistance to Nalidixic acid was observed in 73(70.87%) isolates in disk diffusion test. Screening with 30μg Nalidixic and disks had a sensitivity of 91.98% and a specificity of 83.33% for determination of decreased susceptibility to Ciprofloxacin.

In England, decreased susceptibility to Ciprofloxacin was found to be 23% in 1999 (Threllfall and Ward, 2001). In another study by Hakanen *et al.,* 629 of 1210 Salmonella isolates in 1995-1998 were isolated from the Finnish patients who had returned within 1 month before specimen collection. Between there years, the rates of decreased susceptibility to Ciprofloxacin were reported to have increased to 23.5 from 3.9% (Hakanen *et al.,* 2001). The studies indicates that all the strains with decreased susceptibility to Ciprofloxacin were also resistant to Nalidixic acid. (Threllfall and Ward, 2001 and Hakanen *et al.,* 2001).

In a study by Albayrak *et al.,* 2004, Nalidixic acid resistance in 73 Salmonella strains determined with a disk diffusion test was reported to be highly efficient in detecting the decreased susceptibility to Ciprofloxacin (sensitivity, 100%, specificity 98.4%) which was similar to the study conducted by Ercis *et al.,* 2006 in Turkey.

Hakanen *et al.,* 1999 showed that identification of Nalidixic acid resistance by the disc diffusion method has been reported to provide a sensitivity of 100% and a specificity of 87.3% as a tool to screen for isolates having MICs of Ciprofloxacin ≥ 0.125μg/ml.

Chandel *et al.,* 2000 reported more than 12% of isolates of *Salmonella typhi* to have increased MICs to Ciprofloxacin, most of which included non MDR

strains. Chandel *et al.,* 2000 reported decreased susceptibility in *Salmonella paratyphi* A strains to Ciprofloxacin (MIC > 2.0μg/ml.)

In a recent report from Mumbai, India a decrease in ACCO resistance to 17% and an increase in Nalidixic acid resistance to 88% was observed in *Salmonella typhi* in 2002, the MICs of Ciprofloxacin were between 0.19-1.5μg/ml (Rodrigues *et al.,* 2003).

Hirose *et al.,* 2002 examined 31 clinical isolates of *Salmonella* enterica Serovar Typhi and 13 clinical isolates of *Salmonella* enterica Serovar Paratyphi. Of 25, 6 isolates of Serovar Typhi had decreased susceptibility to Ciprofloxacin and were Ciprofloxacin susceptible respectively and seven and six strains of Serovar Typhi had decreased susceptibility to Ciprofloxacin and were Ciprofloxacin susceptible, respectively.

This uncertain clinical response of flouroquinolone-susceptible, Nalidixic acid resistant strains has prompted debate over changing the established NCCLS break points for the fluroquinolones. Some of the researcher say that moreover, the clinical effectiveness of fluroquinolones for Serovar Typhi isolates, for which MICs of Ciprofloxacin were high, but which were positive for Nalidixic acid susceptibility is unknown (Shakespeare *et al.,* 2005).

Some of the researchers feels that it is important to identify Nalidixic acid resistance in *Salmonella* as a predictor for decreased fluoroquinolone susceptibility. The current NCCLs breakpoints may have to be re-evaluated for *Salmonella* and clinicians may have to revert back to the older options for enteric infections. This implies that quinolones may no longer be the drug of choice in treating enteric fever (Joshi *et al.,* 2004).

The results of this study, is silent on the above comment, as there was none of the isolates were Ciprofloxacin resistant. To agree or dis-agree on the above statement, it requires more investigation not only on antibiotic sensitivity testing but also on clinical outcome evaluation of the patient.

6.2 Conclusion

- In this study, *Salmonella typhi* was found to be prevalent than Salmonella paratyphi A and most of the isolates were resistant to Nalidixic acid (70.87%).

- The sensitivity and specificity of the Nalidixic acid resistance by disc diffusion method to screen for the *Salmonella* isolates having MIC of Ciprofloxacin ≥ 0.125 μg/ml were 91.98% and 83.33% respectively.

- So, Nalidixic acid resistance test can be used as screening test to determine the increase MIC value of Ciprofloxacin in *Salmonella* spp.

CHAPTER - VII

7. SUMMARY AND RECOMMENDATION

7.1 Summary

- This study was conducted at Kathmandu Model Hospital, Kathmandu from May 2006 to August 2006 with the objective to evaluate the Nalidixic acid resistant *Salmonella* isolates with decreased susceptibility to Ciprofloxacin.
- Out of 534 clinically suspected patients of typhoid fever, 344 (64.41%) were male and 190 (35.58%) were female.
- By using conventional system of blood cultures, 103 (19.28%) causative bacteria were isolated from 534 enteric fever suspected patients.
- The growth pattern showed that 99 cases were from out patients and 4 cases were from hospitalized patients.
- Among 103 positive cases, 59 cases were from male patients and 44 cases were from female patients and there was no significant association of presence of enteric fever in male and female patients.
- Among 103 blood culture positive cases, the infection rate was highest in age group 0-10 years in both male and female patients i.e. 26.31% and 37.5% respectively.
- Among 103 blood culture positive cases, 67 (65%) were *Salmonella typhi* and 36(35%) were *Salmonella paratyphi A*.
- Amoxycillin, Ceftriaxone, Chloramphenicol and Cotrimoxazole were found to be most effective drug for *Salmonella typhi*.
- Similarly, Amoxycillin, Ceftriaxone, Chloramphenicol, Ciprofloxacin and Cotrimoxazole were found to be most effective drug for *Salmonella paratyphiA*.
- The Nalidixic acid resistance *Salmonella typhi* and *Salmonella paratyphi A* were 67.16% and 77.77% respectively.
- The sensitivity and specificity of Nalidixic acid resistance test by disk diffusion method to screen for isolates having MICs of Ciprofloxacin ≥ 0.125μg/ml were 91.98% and 83.33% respectively.

7.2 Recommendation

Following recommendation can be put forward for further research.

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

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

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

- Determination of MIC value of Nalidixic acid in comparision with Ciprofloxacin may be more informative.

- Microbiology laboratory should routinely perform Nalidixic acid susceptibility test to screen increased MIC value of Ciprofloxacin for *Salmonellae.*

CHAPTER VIII

8. REFERENCES

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Appendix - I

Clinical and Microbiological profile of the patients

A) Clinical profile

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

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

Time:.....

Patient: OPD/Emergency

Ward: .....

Bed No:.....

Culture request for: .....

Brief Clinical History:.....

Current antibiotic treatment: Yes No

If Yes, Antibiotics (s) taken: 1)..... 2) .....

Duration of treatment : .....

B) Microbiological profile

1. Sample collection (first day)
2. Subculture (second day)
   a. MA
   b. BA
3. Observation:

   Growth : Yes/No 3rd day

4th day	5th day	6th day	7th day

Colony characteristics on different media

Media	size	shape	margin	elevation	opacity	consistency

Gram's rxn: .....

4. Biochemical tests:
   a) Catalase test:
   b) Oxidase test :
   c) Motility test:
   d) Indole test:

e) Methyl red test :

f) Voges prausker test :

g) TSI:

h) Citrate test:

i) Oxidative/fermentative

j) Urease

5. Serological identification of microorganisms:

   Antiseraused: .....

   1) ..... 2) ..... 3) .....

6. Microorganism identified as: .....

7. Antibiotic Susceptibility test: .....

   Media used – MHA

Antibiotics used	Zone of inhibition (mm)	Interpretation
Chloramphenicol (C)		
Cefixime (Cfx)		
Cefotaxime (Ce)		
Cotrimoxozole (Co)		
Amoxycillin (Am)		
Cephotaxime (Ce)		
Ciprofloxacin (Cf)		
Nalidixic acid (Na)		
Ofloxacin (Of)		

8. Determination of MIC:

   Media-MHB

Antibiotics	MIC
Nalidixic acid	
Ciprofloxacin	

_____

Performed by

Rup Bahadur Kunwor

Date:

_____

Checked by

Ms. Basudha Shrestha

Microbiologist

Kathmandu Model Hospital

Date:

APPENDIX - II

A. Gram Staining Procedure (Forbes *et al*., 2002).

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

B. Slide Agglutination Test for Identification of *Salmonalla* spp.

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

APPENDIX - III

A COMPOSITION AND PREPARATION OF DIFFERENT CULTURE MEDIA

1. Nutrient Agar (M, 001)

Composition	gm/lit
Peptic digest of animal issue	5.00
Beef extract	1.50
Yeast extract	1.50
NaCl	5.00
Agar	15.00

Final pH at 25°C 7.4 ± 0.2

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

2. Blood Agar

Sterilized nutrient agar was prepared in conical flask, then allowed to cool the media in 45°C and added 10% sterilized blood well mixed, then poured in sterile petriplate.

3. Macconkey Agar (M, 008)

Composition	gm/lit
Peptic digest of animal tissue	17.00
Proteose peptone	3.00
Lactose	10.00
Bile salt	1.50
NaCl	5.00
Neutral red	0.03
Agar	15.00

Final pH at 25° 7.1 ± 0.2

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

4. Nutrient Broth (M, 002)

Composition	gm/lit
Peptic digest of animal tissue	5.00
NaCl	5.00
Beef extract	1.50
Yeast extract	1.50

Final pH at 25° 7.4 ± 0.2

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

5. Muller Hinton Agar (M, 173)

Composition	gm/lit
Beef infusion form	300.0
Casein acid hydrolysate	17.50
Starch	1.50
Agar	17.00

Final pH at 25° 7.3 ± 0.2

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

6. Brain Heart Infusion Broth (Bhi, M 2101)

Composition	gm/lit
Peptic digest of animal tissue	10.00
Calf brain infusion (solids)	12.50
Brain Heart Infusion (solids)	5.00
Dextrose	2.00
NaCl	5.00
Di-sodium phosphate	2.50

Final pH at 25° 7.4 ± 0.2

Preparation : As directed by manufacturing company, 37gm of the media was dissolved in 1000ml of the distilled water. Also added 0.25gm of sodium polyanethol sulphonate. The media was the dispensed in blood culture bottle in amount of 45ml in each, sterilized by autoclaving at 151bs pressure (121°C) for 15min.

B. COMPOSITION AND PREPARATION OF DIFFERENT BIOCHEMICAL MEDIA

1. Hugh and Leifson Media (M, 826)

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

Final pH at 25° 6.8 ± 0.2

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

2. Sim (Sulphide Indole Motility Agar (M, 181)

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

Final pH 7.3 ± at 25°C

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

3. Glucose Phosphate Broth (M, 070)

Composition	gm/lit
Buffered peptone	7.00
Dextrose	5.00
K_2PO_4	5.00

Final pH 6.9 ± 0.2 at 25°C

Preparation : As directed by manufacturing company, 17gm of the media was dissolved in 1000ml of the distilled water. The media was then dispensed in test tubes and sterilized by autoclaving at 15lbs pressure (121°C) for 15min.

4. Simmon Citrate Media (M, 099)

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

Final pH 6.8 ± 0.5 at 25°C

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

5. Triple Sugar Iron Agar (TSI, M, 021)

Composition	gm/lit
Peptic digest of animal tissue	10.00
Casein enzymatic hydrolysate	10.00
Yeast extract	3.00
Beef extract	3.00
Lactose	10.00
Sucrose	10.00

Dextrose	1.00
NaCl	5.00
Ferrous sulphate	0.20
Sodium thio-sulphate	0.30
Phenol red	0.024
Agar	12.00

Final pH 25°C 7.4 ± 0.2

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

6. Urea Agar Base (Christensen M, 112)

Composition	gm/lit
Peptic digest of animal tissue	1.00
Dextrose	1.00
NaCl	5.00
Na_2PO_4	1.20
KPO_4	0.80
Phenol red	0.012
Agar	15.00

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

C. COMPOSITION AND PREPARATION OF DIFFERENT STAINING REAGENT

1. Gram Staining Reagent

a) Crystal violet stain

Crystal violet	5gm/lit

b) Gram's iodine

Iodine	10gm/lit
Potassium iodide	20gm/lit

c) Propanol

Propanol	950gm/lit

d) Safranine

Safranine O	5gm/lit

(Source : Biolab Diagnostics, India)

2. Catalase Reagent

To make 100ml

Hydrogen peroxide	3ml
Distilled water	97ml

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

3. Oxidase Reagent

To make 100ml

Tetra methyl paraphenylenediamine dihydrochloride	1.00gm
Distilled water	100ml

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

4. Kovac's Reagent

To prepare 40ml reagent

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

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

5. Methyl Red

To make 50ml solution

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

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

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

a) VP Reagent A:

To make 100ml

Alpha-naphthol	5gm
Ethanol (absolute)	100ml

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

b) VP reagent B:

To make 100ml

Potassium hydroxide	40gm
Distilled water	100ml

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

APPENDIX - IV

Different equipments used during the study period were as follows:

Equipments

a) Autoclave	ATCO Industries LTD. India
b) Incubator	Universal and Ambassador
c) Hot air oven	Ambassador
d) Microscope	Olympus
e) Refrigerator	Sanyo
f) Weighing machine	Ohaus Corporation, USA
g) Gas burner	
h) Glass equipments	
i) Inoculating wire and loops	

Different antibiotics used for susceptibility test (HiMedia Laboratories Pvt. Limited)

Antibiotics

a) Amoxycillin (Am, 30mcg)
b) Ceftriaxone (ci, 30mcg)
c) Cefotaxime (ce, 30mcg)
d) Cefixime (Cfx, 5mcg)
e) Chloramphenicol (C, 30mcg)
f) Cotrimoxazole (Co, 1.25/23.75mcg)
h) Ciprofloxacin (Cf, 5mcg)
i) Ofloxacin (Of, 5mcg)
j) Nalidixic acid (Na, 30mcg)

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

a) Groups O Sera
   Polyvalent A-S
b) Group H Sera
   H-a
   H-d

Zone Size Interpretative Chart

Antibiotics used	Symbol	Disc content (mcg)	Diameter of zone of inhibition in mm		
			Resistant	Intermediate	Sensitive
Amoxycillin	Am	30	13	14-16	17
Cephotaxime	Ce	30	14	15-22	23
Ceftriaxone	Ci	30	13	14-20	21
Cefixime	Cfx	5	15	16-18	19
Cotimoxazole	Co	1.25/23.75	10	11-15	16
Chloramphenicol	C	30	12	13-17	18
Ciprofloxacin	Cf	5	15	16-20	21
Ofloxacin	Of	5	12	13-15	16
Nalidixic acid	Na	30	13	14-18	19

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

Appendix - V

Association of Culture positive and culture negative in gender :

	Male	Female
Culture Positive	59	44
Culture Negative	285	146
Total	344	190
Incidence rate	17.15	23.15

Ho : There is no significant association of presence of enteric fever in male and female patients.

H_1 : There is significant association of presence of enteric fever in male and female patents.

From $\chi^2 = \Sigma \frac{(O - E)^2}{E}$

We find calculated $\chi^2 = 0.893$

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

Since the tabulated value of χ^2 is greater than the corresponding calculated χ^2 value, the null hypothesis is accepted i.e. there is no significant association of presence of enteric fever in male and female patients.