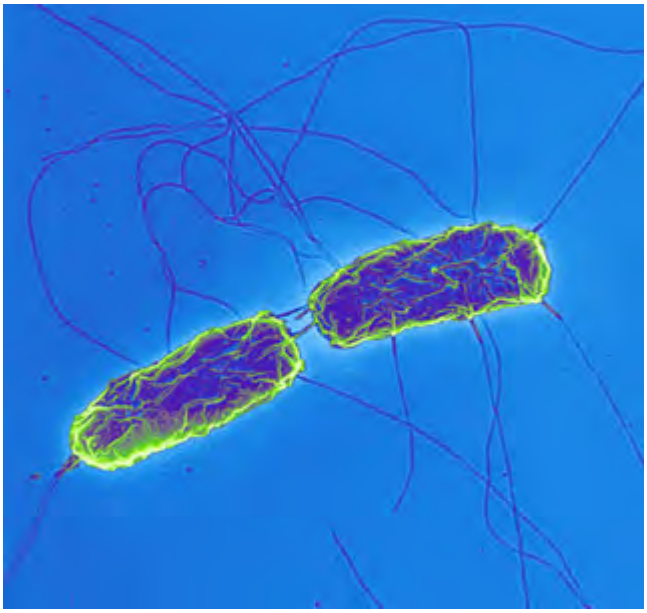
PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS

OF

SALMONELLA SPECIES IN ETHIOPIA

BY

GETENET BEYENE, B. Sc, M. Sc



DEPARTMENT OF MICROBIOLOGY, IMMUNOLOGY

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PHENOTYPIC AND MOLECULAR CHARACTERIZATIONS OF *SALMONELLA* SPECIES IN ETHIOPIA

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BY

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ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrary Primed Polymerase Chain reaction
ATCC	American Type Culture Collection
CDC	Center for Disease Control and Prevention
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DHPS	Dihydropteroate Synthase
ESBL	Extended-Spectrum Beta-Lactamase
ESC	Expanded Spectrum Cephalosporins
HIV	Human Immunodeficiency Virus
Kb	Kilobases
LPS	Lipopolysaccharide
M cells	Micro fold cells
MDR	Multi- drug resistant
MIC	Minimum Inhibitory Concentration
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing
NCCLS	National Committee For Clinical Laboratory Standards
NTS	Nontyphoidal *Salmonella*
PBP	Penicillin Binding Protein
PCR	Polymerase Chain Reaction
PFGE	Pulse Field Gel Electrophoresis
QRDR	Quinolone Resistance Determining Region
RAPD	Random Amplification of Polymorphic DNA
REA	Restriction Enzyme Analysis
RFLP	Restriction Fragment Length Polymorphisms
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species

RSD	Rapid *Salmonella* Diagnostic Antisera
SDS	Sodium dodecyl sulfate
SGI	*Salmonella* Genomic Islands
SNP	Single Nucleotide Polymorphism
SPI	*Salmonella* Pathogenicity Island
ST	Sequence Type
THFR	Tetrahydrofolic acids
TTSS	Type Three Secretion Systems
VNTR	Variable Number of Tandem Repeat Variation
WHO	World Health Organization

ABSTRACT

BACKGROUND: Salmonella infections are very common and an important public health problem in many parts of the world. In sub-Saharan Africa there is very little direct data on strain type or antibiotic resistance. Research to date, as well as unpublished reports from different health institutions in Ethiopia, has indicated that salmonellosis is a common problem and the extensive use of the first line drugs has led to the development of multiple drug resistance at a level which could pose a serious problem in the near future.

OBJECTIVES: The study was aimed at defining the serovars responsible for *Salmonella* infection, estimating the level of antibiotic resistance, investigate molecular basis of resistance and observing molecular polymorphism among *Salmonella* species isolated from children in Ethiopia.

MATERIALS AND METHODS: Pediatric patients (n=1225) presenting with diarrhoea or fever from pediatric out patient department of Tikur Anbessa University Hospital, Addis Ababa (n= 825) and Jimma University Hospital, south west Ethiopia (n= 400) were investigated for enteric pathogens during January 2006 to June 2008. Forty eight *Salmonella* isolates collected in Tikur Anbessa hospital between January, 2004 and December, 2005 from similar age group were analyzed together. Stool specimens were collected for microscopic examination and culture. In addition blood specimens were obtained for culture. Brain Heart Infusion broth was used for blood culture and Selenite F broth enrichment followed by plating on Deoxycholate agar (DCA) and Xylose lysin deoxycholate agar (XLD) were used for stool culture. Identification of *Salmonella* species was carried out using API-20E, serology with antisera and strain were characterized using multilocus sequence typing (MLST). Based on resistance pattern, site of collection and type of specimen in which the isolate was detected, *S.* Concord isolates were selected and investigated for plasmid profile, incompatibility grouping, pulsed field gel electrophoresis (PFGE), MLST profile and *fliC* gene sequencing using standard procedures. In addition, 48 previously collected *Salmonella* isolates before the commencement of the present study were sero-grouped and serotyped.

RESULTS: A total of 463 entropathogens were isolated from 1225 pediatric patients**.** The isolates were: 65 *Salmonella* species, 61 *Shigella* species and 337 parasites. Among the 113

Salmonella isolates (65 + 48 previously collected), serogroup C, B, D and E were isolated at a frequency of 78.8%, 11.5%, 8% and 1.8% respectively. Most of the *Salmonella* isolates were from stool (68%) and the rest were from blood (32%). No isolate was detected from both blood and stool of the same patient. A total of 12 serotypes were identified namely; *S.* Concord (82), S. Colindale (1), S. Gatow (3), *S.* Laronchelle (1), *S.* Garoli (1), *S.* Colorado (1), *S.* Typhimurium (7) *S.* Paratyphi B(2), *S.* Haifa (1) *S.* Typhi (2) *S.* Enteritidis (4), and *S.* Butantan (2). Eighty nine percent of the group C isolates were *S.* Concord. *S.* Concord isolates were highly resistant to ampicillin, trimethoprim-sulfamethoxazole, ceftriaxone, amoxicillin, chloramphenicol, gentamicin, and tetracycline. Low resistance rate was observed for nalidixic acid and ofloxacin and there was no resistance to ciprofloxacin by disk diffusion test. However, E-test result indicated the presence of one resistant, one intermediate and twenty two (26.8%) Concord isolates which showed reduced susceptibility to ciprofloxacin. The extended spectrum beta lactamase (ESBL) screening test result showed that 98.8% of *S.* Concord were positive for ESBL production. Plasmid analysis showed that all characterized *S.* Concord isolates harbored multiple copies of small and large plasmids. The molecular weight of plasmids varied from less than five to 170 kb with 120, 118 and 95 kb being the most prevalent. Plasmid replicons A/C, I1 and incFI were found in the majority of the isolates. Different plasmid studies indicated that A/C and incFI replicons are associated with multi drug resistance (MDR) *Salmonella* isolates. A total of 16 pulse field gel electrophoresis (PFGE) profiles were seen among the 23 Concord group and 5 non Concord isolates. Every sequence type (ST) had a unique PFGE profile which indicates that *S.* Concord in Ethiopia is in an endemic situation, rather than a spread of a clonal type (has many different point sources/reservoirs). The same ST and PFGE types were found in Addis Ababa and in Jimma suggesting movement of infected people/reservoirs between the two cities. Multilocus sequence typing (MLST) analysis showed the presence of a total of seven STs among 58 isolates. ST533, ST534 and ST599 are single locus variants. Because they differ in only one of the seven loci, they are closely related genetically and make a single Concord group. Distantly related serotypes like Gatow and Colindale had sequence types that differ by more than one allele from Concord group. Molecular serotyping using sequencing of the *fliC* gene was able to differentiate

between *S*. Haifa and the *S*. Concord group of isolates indicating the possibility of its role in molecular serology. For our strain collection, it seems therefore that *fliC* sequencing can complement MLST for classification and strain differentiation.

CONCLUSION AND RECOMMENDATIONS: Salmonellosis in children in these two regions of Ethiopia is mainly due to non-typhoidal salmonellae particularly with *S*. Concord. This is different from other countries where *S*. Enteritidis and *S*. Typhimurium accounted for the majority of salmonellosis. Both phenotypic and genotypic characterization indicated that *S*. Concord is highly resistant and present in endemic situation in Ethiopia. The presence of many multi-drug resistant strains containing genes for ESBL production and the emergence of reduced susceptibility to ciprofloxacin in this study posed a major concern in the search for efficient antimicrobial therapy of *Salmonella* infections in the near future. Therefore more comprehensive studies should be designed to trace its source and distribution within the country and to monitor antibiotic resistance pattern over time.

CHAPTER I

INTRODUCTION

CHAPTER 1. INTRODUCTION

1.1. GENERAL INTRODUCTION

Salmonellosis causes significant morbidity and mortality in both humans and animals and has a substantial global socioeconomic impact. *Salmonella* infections in humans can range from a self-limited gastro-enteritis usually associated with non-typhoidal *Salmonella* (NTS) to typhoidal fever with complications such as a fatal intestinal perforation. Non-typhoidal *Salmonella* is one of the principal causes of food poisoning worldwide with an estimated annual incidence of 1.3 billion cases and 3 million deaths each year (Tassios *et al*., 1997).

Typhoid fever, which is caused mainly by *Salmonella* Typhi (*S.* Typhi), continues to be a major problem in developing countries. A recent study estimated that globally there are more than 22 million cases of typhoid fever each year with more than 200,000 deaths, however, the true magnitude is difficult to quantify because the clinical picture is confused with many other febrile illnesses and most typhoid endemic areas lack facilities to confirm the diagnosis (Crump *et al*., 2004).

Antibiotic treatment is not required for *Salmonella* gastroenteritis but is essential for enteric fever, invasive salmonellosis and in patients at risk of extra-intestinal disease. For many years chloramphenicol, ampicillin, and trimethoprim-sulphamethoxazole (cotrimoxazole) were the drugs of choice. In recent years, increasing resistance of *Salmonella* species to commonly used antimicrobials has become a matter of concern. Of particular concern are those strains that have acquired multiple drug resistance (MDR) against two or more therapeutic agents. Although fluoroquinolones, such as ciprofloxacin and ofloxacin, and extended spectrum cephalosporins, such as ceftriaxone and cefotaxime, have proved to be effective alternatives, resistance to these agents has emerged (Parry, 2003).

To track *Salmonella* infections and disrupt epidemic spread, many nations have established extensive surveillance systems. Typing to the strain level has been an important tool in surveillance and out break investigation of *Salmonella* infections. Most of these surveillance projects rely on traditional (phenotypic) methods like serotyping, phage and biotyping, which provide a limited means of distinguishing epidemic from endemic or

sporadic isolates. Nowadays, phenotypic methods are either replaced or complemented by more sensitive and discriminative molecular techniques. Typing schemes based on variation in particular DNA sequences are digital and the same results could be achieved where ever the test is performed. Sequence based typing schemes can also be considered as genetic classification schemes (Liebana, 2002; Winokur, 2003).

Research to date, as well as unpublished reports from different health institutions in Ethiopia has indicated that salmonellosis is a common problem and also showed the presence of a number of serogroups/ serotypes in humans, animals, animal food products and other foods (Asrat, 2008; Erku and Ashenafi, 1998; Gebre-Yohannes, 1985; Gedebou and Tassew, 1979; Gedebou and Tassew, 1981; Mache *et al*., 1997; Molla *et al*., 1999; Molla *et al*., 2003; Muleta and Ashenafi, 2001; Nyeleti *et al*., 2000; Tibaijuka *et al*., 2003). An increase in the resistance of *Salmonella* to commonly used antimicrobials has been also noted in both public health and veterinary sectors in Ethiopia (Ashenafi and Gedebou, 1985; Asrat, 2008; Gebre-Yohannes, 1985; Gedebou and Tassew, 1979; Mache *et al.*, 1997; Molla *et al.*, 1999; Molla *et al.*, 2003;)

In order to establish systems for controlling of *Salmonella* infection and to develop national and local guidelines for antibiotic treatment, beside doing continued epidemiological surveillance and timely monitoring for the emergence and re-emergence of multi-drug resistance (MDR), identification of the different strain circulating within the country, compare and contrast with strains elsewhere in other countries and investigating genes/ plasmids involved in drug resistance are equally important.

Even though *Salmonella* populations in different geographical areas or different hosts and environmental niche may under go different evolutionary change, due to centralization of food production and distribution and population movement, *Salmonella* strains found in different countries of the world are believed to be clonally related (Winokur, 2003). *Salmonella* isolates in Ethiopia may have similar phenotypic and genotypic characteristics with isolates elsewhere in the world and non-typhoidal *Salmonella enterica* infection in children in Ethiopia is a major health problem and is caused by similar serovars to these reported from elsewhere in Africa: *S.* Typhimurium and *S.* Enteritidis. Therefore, to prove or disprove this hypothesis and to address the aforementioned issues, phenotypic and

genotypic characterizations were performed on *Salmonella* strains isolated from children clinically presented with suspected case of typhoid fever and diarrhoeal illnesses in Tikur Anbessa and Jimma University Hospitals, Ethiopia.

1.2. REVIEW OF LITERATURE

1.2.1. Historical Prospective

The first person who was able to isolate the *S. enterica* serovar Typhi was Gaffky in Germany in 1884 from the spleen of infected patients (Edelman and Levine, 1986). Later in 1885, two American veterinarians, Salmon and Smith isolated the bacterium causing hog cholera from infected pigs (Salmon and Smith, 1886). The name *Salmonella* was subsequently adopted in honor of Dr. Salmon. Over the decades following the pioneering work of Salmon and Smith, many other *Salmonella* were isolated from both animals and humans. Widal and others demonstrated that convalescent sera from typhoid fever patients caused the organism to stick together in large balls and lose their motility (Widal, 1896). The antigenic classification or serotyping of *Salmonella* used today is a result of years of study of antibody interactions with bacterial surface antigens by Kauffman and White in the 1920s to 1940s (Kauffmann, 1950; Miller *et al*., 2000). According to this Kauffmann-White scheme, each *Salmonella* serotype is recognized by its possession of a particular lipopolysaccharide (LPS) or O antigen and a flagellar or H antigen. This led to the description of more than 2500 serotypes at present (Brenner *et al*., 2000; Popoff *et al*., 1998; Popoff *et al*., 2004).

1.2.2. The Genus *Salmonella*

1.2.2.1. General characteristics

Members of the genus *Salmonella* are ubiquitous pathogens found in humans and livestock, wild animals, reptiles, birds, and insects. Salmonellae are gram-negative, non-spore forming, facultative anaerobic bacilli, 2 to 3 by 0.4 to 0.6 μm in size. Like other members of the family *Enterobacteriaceae*, they produce acid on glucose fermentation; reduce nitrates to nitrite, and don't produce cytochrome oxidase (Farmer, 1995). Most organisms except *S. gallinarum–pullorum* are motile by peritichous flagella. The differential

metabolism of sugars can be used to distinguish some *Salmonella* serotypes, e.g., most don't ferment lactose. *S.* Typhi is the only organism that does not produce gas in sugar fermentation. *Salmonella* are non- capsulated except *S.* Typhi, *S.* Paratyphi C and some strain of *S*. Dublin (WHO, 2003).

1.2.2.2. Antigenic structures

The typical *Salmonella* are defined mainly by two sets of antigens, O and H that are readily demonstrable by serological reactions in the laboratory. In addition, other bacterial antigens are also available. These include: an exopolysaccharide (Vi, or virulence antigen), the mucus (M), and the fimbrial (F) antigens (Huckstep, 1962; Old, 1996).

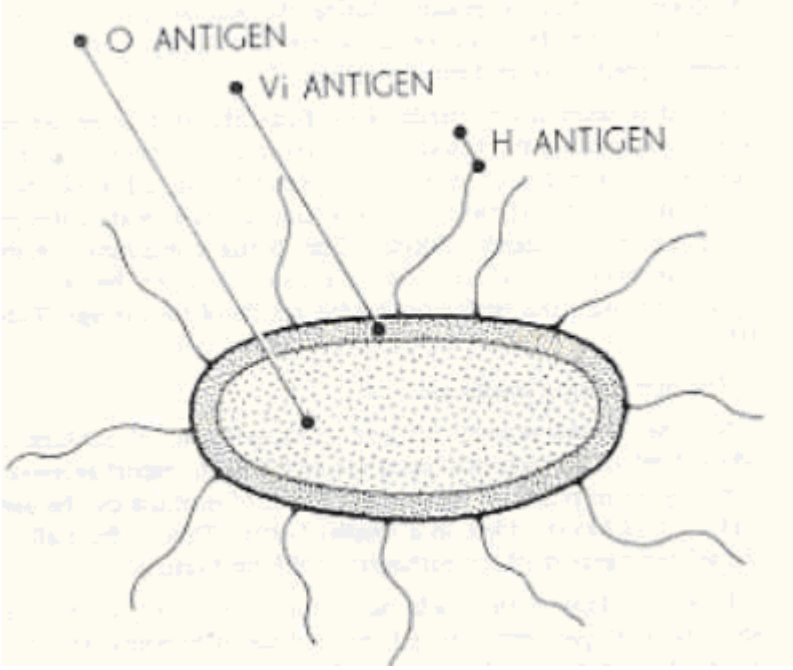

Figure 1.1: Antigenic structure of *Salmonella* species (Adapted from Huckstep, 1962)

a. O (Somatic) antigen

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. Typing the O antigen denotes the serogroup. Over 60 different O antigens have been recognized and they are designated by Arabic numerals. The O antigens are heat stable, being unaffected by

heating for 2.5 hours at 100^{o}C, and alcohol stable withstanding treatment in 96% ethanol at 37^{o}C (Lewis, 1998; Old, 1996).

b. H (Flagellar) antigen

Flagellar (H) antigens are formed from structural proteins, which make up the flagella that endow the organism with motility. They are heat labile. Heating at 60^{o}C and above detaches the flagella from the bacteria. *Salmonella* is unique among the *Enterobacteriaceae* in that it commonly has two distinct H antigens, the phase 1 (protein product of the *fli*C gene) and phase 2 (protein product of the *fljB* gene) flagellar antigens, that are coordinately regulated such that only one flagellar antigen is expressed at a time in a single cell (Smith and Selander, 1991). Some H antigens are composed of multiple antigens, termed factors; for example, H: e,n,x is the designation for a flagellar antigen that consists of three separate factors, e, n, and x, that occur together in one flagellum. These factors represent different epitopes on the flagellar protein.

The 114 H antigens are composed of combinations of 99 distinct antigenic factors. Flagellar antigens that are immunologically related are known as complexes. For example, the G complex includes all flagellar antigen types that contain antigenic factor g (e.g., g,m; f,g; g,z_{51}), plus flagellar antigen m,t. Flagellar antigen types that include antigen H:z_4 are considered the Z4 complex (Smith and Selander, 1991). The flagellar antigens of phase I are labeled with lower cases letters (a to z and z_1 z_2 z_3 etc.) and phase 2 with a mixture of lower case letters and Arabic numerals.

The antigenic structure of any *Salmonella* is expressed as an antigenic formula which has three parts, describing the O antigens, the phase I and II flagellar antigens respectively (Lewis, 1998). Phase II used to be termed “the group” or non-specific antigens because numerous serotypes of salmonellae share the same antigens when it is this phase. The presumptive identification of the serovars, therefore mainly depends on the identification of the H antigens in phase I, which are relatively ‘specific’ (Old, 1996). As an example, the antigenic formula for the serovar Typhimurium – **1, 4, 5, 12 : i : 1, 2** where 1, 4, 5 and 12 are O antigens, i is phase 1 H antigen and 1 and 2 are phase 2 H antigens (Kauffmann, 1950)

c. Vi (Capsular antigen)

The Vi antigen is a homopolymer of N-acetyl galactosaminouronic acid. Almost all strains of *S.* Typhi form Vi antigen as a covering layer outside the cell wall. Identical antigens have been also found in *S.* Paratyphi *C* and some strains *S.* Dublin (WHO, 2003).

1.2.2.3. Classification of Salmonella

The classification of *Salmonella* is complex because the organisms are a continuum rather than a defined species. The current classification of the genus *Salmonella* is based on DNA-DNA hybridization studies (Crosa *et al*., 1973). This work suggested that the genus *Salmonella* is divided into two species known as *Salmonella bongori* and *Salmonella enterica. S. enterica* can be further divided into 6 distinct subspecies (*enterica, salamae, arizonae, diarizonae, houtenae,* and *indica*), based on different biochemical profiles, in addition to genetic relatedness (Brenner *et al*., 2000). The majority of the 2500 known serovars that causes disease in warm-blooded animals are found in *Salmonella enterica* subspecies *enterica* as shown in Figure 1.2 (Langridge *et al*., 2008).

The Kauffmann-White classification scheme classified the *Salmonella* into different O groups and serovars based on the expression of somatic lipopolysaccharide 'O' antigen and flagellar 'H' antigens respectively (Kauffmann, 1950). The O groups first defined were designated by capital letters A to Z and those discovered later by the number (51-67) of the characteristics O antigen (Old, 1996). It is now considered more correct to designate each O group by its characteristic O factor, i.e. to abandon the letters A-Z used to designate early O groups. Hence, O groups become: O2 (A), O4 (B), O7 (C_1), O8 (C_2), O9, 12(D_1) etc. (Old, 1996). Groups O2 to 03, 10 (A-E) contain nearly all the salmonellae that are important pathogens in man and animals (Brenner *et al*., 2000). Newly described serovars are listed in regular updates of the Kauffman and White scheme.

1.2.2.4. Salmonella nomenclature

Salmonella nomenclature is complex, since different scientists use different systems to refer and communicate about this genus. Some individuals prefer Kauffman's initial 'one serovar-one species' concept, while others favor schemes based on clinical presentation, biochemical characteristics or genetic relatedness (Brenner *et al*., 2000).

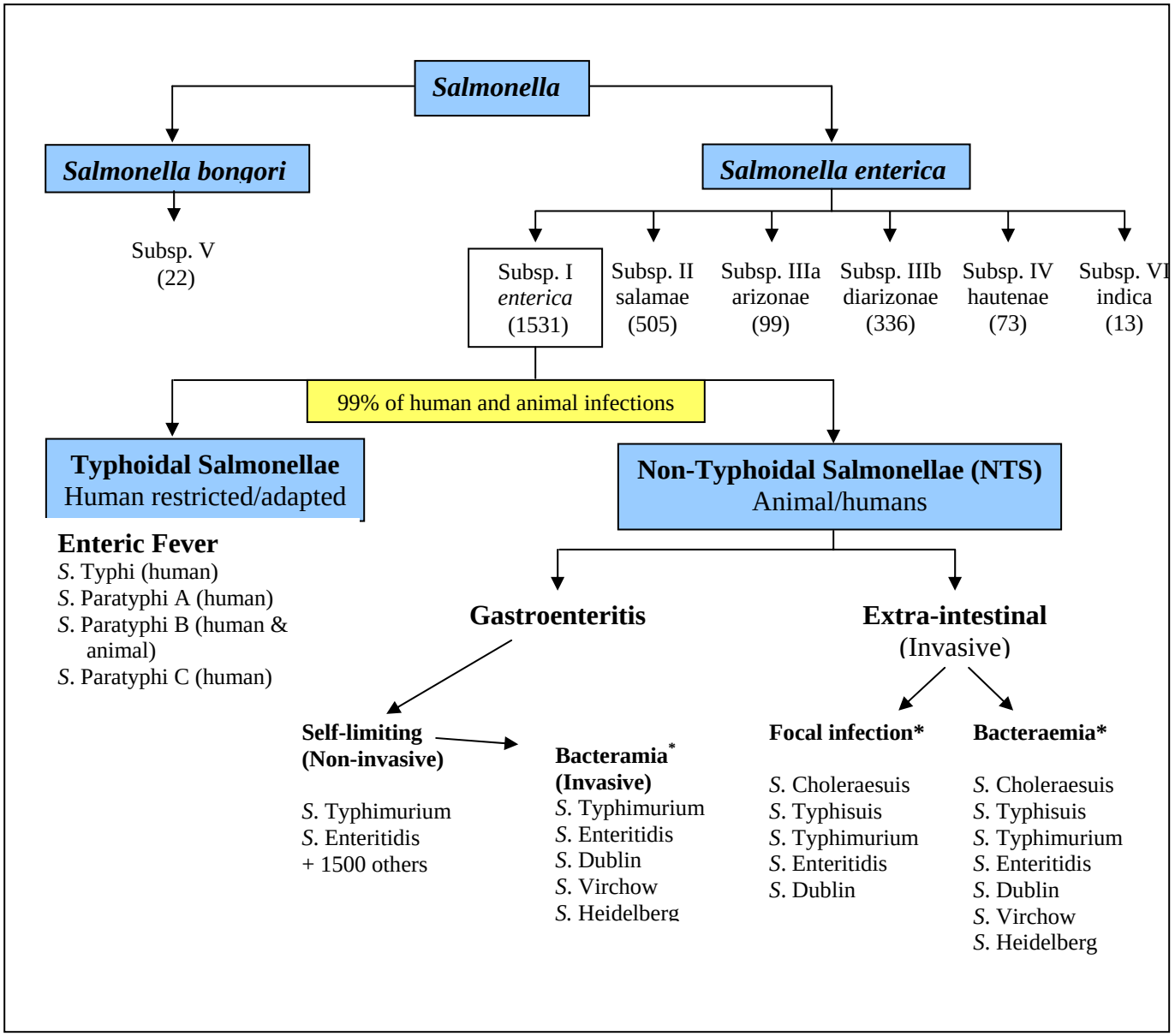

Figure 1.2: Classification of the genus *Salmonella*. (Adapted from Langridge *et al*. 2008)

Numbers in brackets indicate the total number of serotypes included in each subspecies.
* Common serotypes are listed but other serotypes may cause bacteraemia or focal infection; subsp., subspecies.

According to the Communicable Disease and Control (CDC) system, the genus *Salmonella* contains two species, each of which contains multiple serotypes. CDC uses a name for serovars in subspecies I (for example. serovar Enteritidis, Typhimurium, Typhi and Choleraesuis) and uses antigenic formulas for unnamed serotypes (Table 1.1) (Brenner *et al*., 2000). At first citation of a serotype the genus name is given followed by the word 'serovar' or the abbreviation 'ser' and then the serotype. For example, *Salmonella* serotype or ser Typhimurium, subsequently the name may be written with the genus followed directly by the serotype (for example, *Salmonella* Typhimurium or *S.* Typhimurium (Popoff *et al*., 1998).

Table 1.1: Examples of *Salmonella* nomenclature currently seen in the literature (Adapted from Brenner *et al*., 2000)

Complete name	**CDC designation**	**Other designation**
S. enterica subsp.*enterica* ser Typhi	*Salmonella* ser.Typhi	*Salmonella typhi*
S. enterica subsp. *enterica* ser Typhimurium	*S.* ser Typhimurium	*Salmonella typhimurium*
S. enterica subsp. *Arizonae* ser 18: z_4,z_{23}	*S.*IIIa 18: z_4,z_{23}	" *Arizona hinshawii*"ser.7a 7b:1,2,5:-

From clinical perspective, the serovars of *Salmonella* which are pathogenic for humans are traditionally divided into two groups. The typhoidal group included the serovars which cause enteric fever (Typhi, Paratyphi A, Paratyphi B, Paratyphi C and Sendi). The second group usually referred to as the non-typhoidal *Salmonella* (NTS) contains all remaining serovars of subspecies I (Figure 1.2). Recently the International Judicial Commission rejected this naming proposal because the name was not well known to clinicians and its use might cause accidents endangering health or life. Thus, the original rules of the conventional serotypes as species names continue to be used (WHO, 2003).

However, many but not all academic journals, such as those published by the American Society for Microbiology, require the use of the CDC system. In this thesis, the CDC system will be used.

1.2.3. Genome of *Salmonella*

Because of the presence of large number of serovars in *Salmonella*, the genome sequence projects concentrated on serovars that are either of importance to human disease or a representative of a particular branch of the *Salmonella* (Mastroeni, 2006). Genome information can be used to gain insights into the evolution of the *Salmonella* genus, to identify stable regions conserved between different *Salmonella* species and serovars, and to identify regions that appear to be specific for individual serovars (Mastroeni, 2006).

DNA sequence comparison between the genome of *S.* Typhimurium LT2 and *S.* Typhi CT8 show a median homology of 98% (McClelland *et al*., 2001). Comparison of the genes required for DNA replication, transcription, translation and central metabolism ('housekeeping' genes) of the *Salmonella* serovars indicates that they are extremely similar at the DNA level. Pair wise comparisons between any of the sequenced *Salmonella* genomes indicate that the similarity between housekeeping genes ranges from 97.6% to 99.5% at the DNA level (Table 1.2) (Edwards *et al*., 2002).

Table1.2: Average percentage (%) identity of the regions conserved between the five sequenced *Salmonella* serotypes (Adapted from Edwards *et al*., 2002)

	Dublin	Enteritidis	Paratyphi	Typhimurium	Typhi
Dublin	-	99.5%	98.1%	98.7%	98.5%
Enteritidis		-	98.0%	98.7%	98.5%
Paratyphi				98.5%	98.5%
Typhimurium				-	97.6%
Typhi					-

Despite their overall similarity, pair wise comparisons of the *Salmonella* genomes reveal that each serovar has many insertions ('islands') and deletions ('atolls') relative to the other serovars. Approximately 500–600 kb of chromosomal DNA seems to be unique to each serovar, representing 10% to 12% of their 5 Mb genomes. The unique regions are distributed to many sites on the chromosome and range in size from <1 to >50 kb. These unique regions of the genome are likely to encode gene products responsible for the different abilities of the serovars to infect a variety of hosts, to cause a range of symptoms and to survive a diversity of immune responses (Mastroeni, 2006).

The complete genome sequence was determined for a multidrug-resistant strain of *S. enterica* serotype Typhi (CT18). The CT18 genome harbors 4,809,037 base pairs with an estimated 4599 coding sequences. Significant homology has been seen among genomes of *S.* Typhi-CT18, *S.* Typhimurium LT2 (McClelland *et al*., 2001) and *E. coli* K12 (Blattner *et al*., 1997) genomes, indicating a common evolutionary origin.

S. Typhimurium is also a member of sub-species I and is the leading cause of gastroenteritis in humans and unlike *S.* Typhi can infect mice and cause a typhoid-like disease. The genome size of *S.* Typhimurium LT2 is 4,857 Kbp (McClelland *et al*., 2001) and sequence comparison between these two organisms revealed 89% of *S.* Typhimurium LT2 CDSs were homologous to *S.* Typhi CT18 at the nucleotide level (McClelland *et al*., 2001). Although genetically similar to the *E. coli* K12 genome, both *S.* Typhi and *S.* Typhimurium have acquired large regions of extraneous DNA by horizontal transfer known as pathogenicity islands, which offer a selective pathogenic advantage to the organisms (Marcus *et al*., 2000).

Salmonella enterica strains contain plasmids which vary in size from 2 to more than 200 kb. There are different types of plasmids in *Salmonella* and the best described groups of plasmids are the virulence plasmids (50–100 kb in size) present in serovars Enteritidis, Typhimurium, Dublin, Cholerae-suis, Gallinarum, Pullorum and Abortus-ovis. The virulence plasmids have a common 8kb DNA region encoding the *spv (Salmonella* plasmid virulence) gene involved in intracellular macrophage survival of *Salmonella*

(Gulig *et al*., 1993). Depending on serovar these plasmids code for additional virulence-associated genes such as *rck* (resistance to complement killing), *pef* (plasmid encoded fimbriae), *srgA* (SdiA-regulated gene, putative disulphide bond oxidoreductase) or *mig-5* (macrophage-inducible) gene coding for putative carbonic anhydrase) (Rychlik *et al*., 2006).

Another group of high molecular weight plasmids are responsible for antibiotic resistance. The antibiotic resistance genes are often located within transposons which can transpose from plasmids to chromosome, and vice versa (Figure1.3). Resistance genes can be also found in a form of gene cassettes incorporated into integrons (Hall and Collis, 1995). Small ColE1-type plasmids (pC) of 3-5.6kb have been found in *S. enterica serovar* Enteritidis and one of these plasmids carries an active restriction modification system, which could explain the high resistance of pC-carrying *S. enterica* serovar Enteritidis strains to phage infections (Gregorova *et al*., 2002).

Salmonella genomes also contain a variable set of transposable elements and many phage genome sequences (Edwards *et al*., 2002).

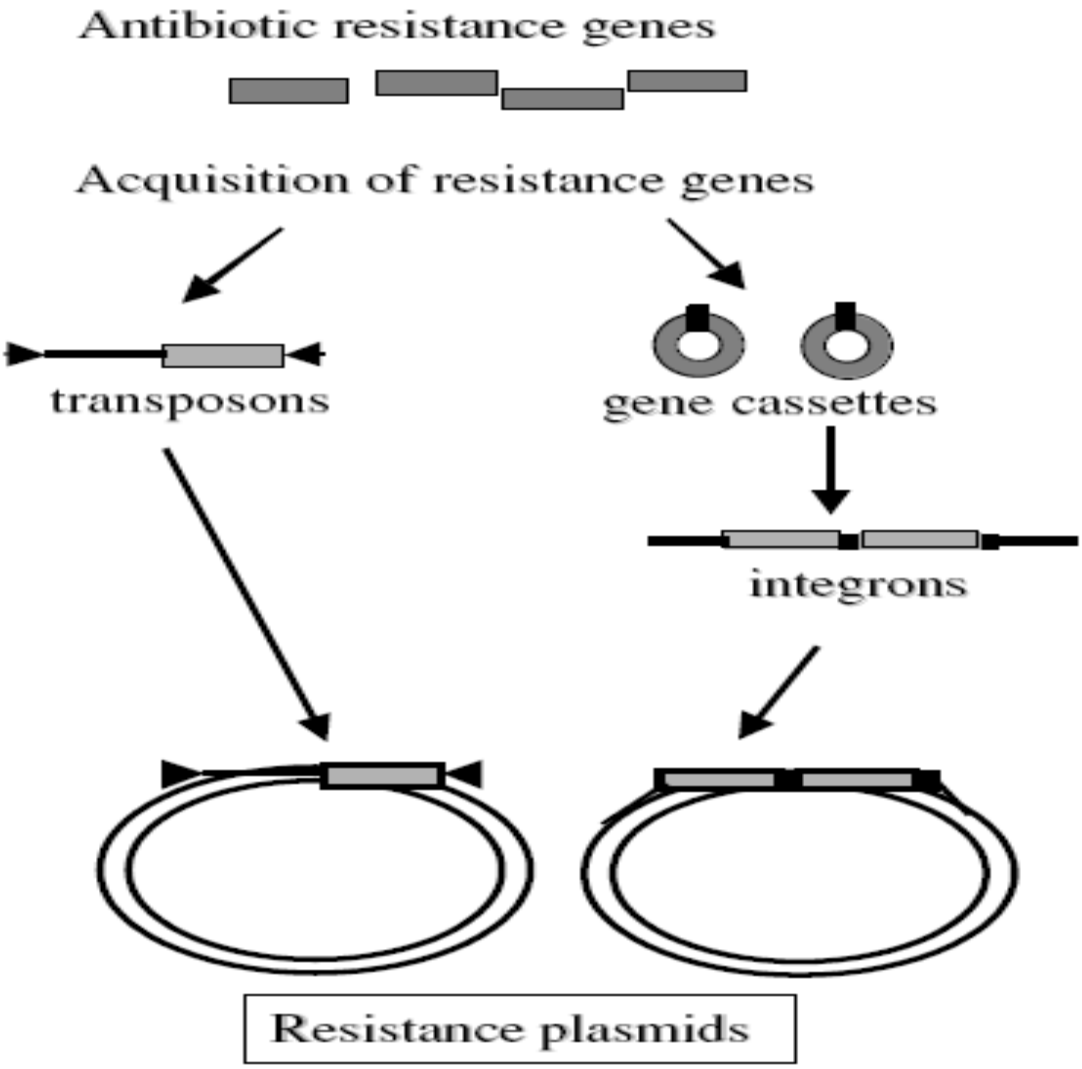

Figure 1.3: Schematic representation of plasmid-mediated horizontal transfer of antibiotic resistance. Resistance genes are indicated by shadowy boxes. Resistance genes are often found located within transposons and can be transposed into the bacterial chromosome or incorporated within plasmids, indicated by white circles. Resistance genes can also be located as gene cassettes within integrons. A gene cassette consists of one complete open reading frame followed by a recombination site (black box). Gene cassettes exist free as covalently closed circular molecules generated by the integrase and the circular intermediate participates in the integrase-mediated process of insertion. As many as seven different gene cassettes have been described within a single integron (Adapted from Carattoli, 2003).

1.2.4. Salmonellosis (The Disease)

1.2.4.1. Pathogenesis

Salmonellosis in the human host is generally associated with *Salmonella enterica* subspecies *enterica* (also termed subspecies I) and acute infections can present in one of four ways: enteric fever, gastro-enteritis, bacteraemia, and extraintestinal (EI) focal infection. As with other infectious diseases the course and outcome of the infection are dependent upon a variety of factors including inoculating dose, immune status of the host and genetic background of both host and infecting organism (Cammie and Miller, 2000).

Broadly speaking the *Salmonella enterica* from human infections can be subdivided in to two groups: the enteric fever (typhoidal) group and non-typhoidal *Salmonella* (NTS), which typically cause gastro-enteritis but can cause invasive disease under certain conditions. There are five serotypes of *Salmonella* associated with enteric fever: *Salmonella enterica* subspecies *enterica* serovar Typhi (*S.* Typhi), *S.* Paratyphi A, *S.* Paratyphi B, *S.* Paratyphi C and *S.* Sendai. *S.* Typhi forms a genetically homogenous group as do and *S.* Paratyphi A and Sendai together, whereas *S.* Paratyphi B and C are heterogeneous (Selander *et al*., 1990).

All *Salmonella* infections begin with the ingestion of organisms in contaminated food or water. After leaving the stomach, *Salmonella* must traverse the mucosal layer overlaying the epithelium of the small intestine. After crossing the mucosal layer overlaying the intestinal epithelium, *Salmonella* interacts with both enterocytes and Microfolds cells (M cells) (Francis *et al*., 1992). The organisms are rapidly internalized and transported into submucosal lymphoid tissue where they may enter into systemic circulation. *Salmonella* have also the ability to induce non phagocytic epithelial cells by a process known as bacterial mediated endocytosis. This process involves the formation of large membrane ruffles around the organism and cytoskeleton rearrangement (Francis *et al*., 1992). *Salmonella* is then internalized within bound vacuoles through which organisms' trancytose from the apical to the basolateral surface (Rathman *et al*., 1997). Once it crosses the intestinal epithelium, *Salmonella* serotypes that cause systemic infections enter macrophages, and migration of infected macrophages to other organs of reticuloendothelial systems probably facilitates the dissemination of bacteria in the host (Figure 1.4).

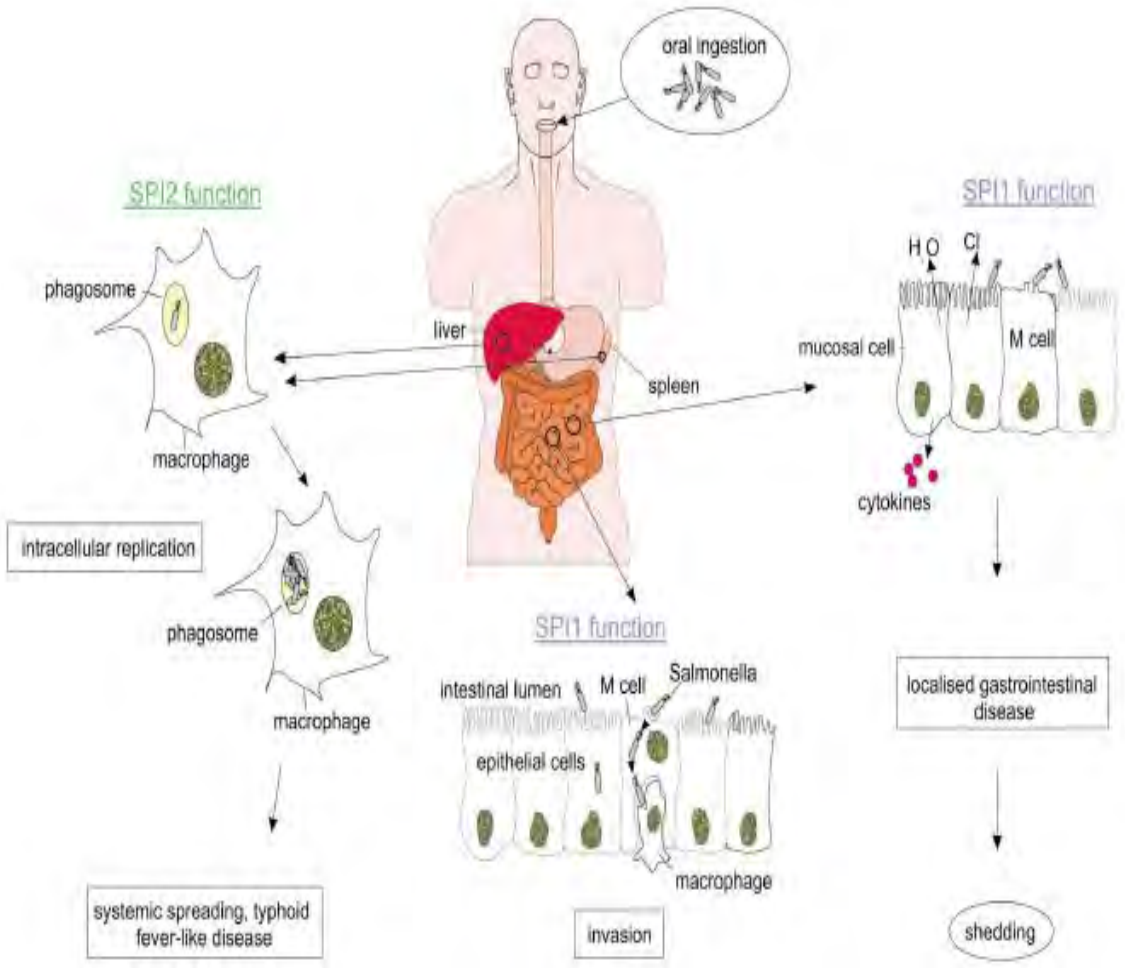

Figure 1.4.: Schematic representation of host–pathogen interactions during pathogenesis of *Salmonella* infections. *Salmonella* Pathogenicity Island (SPI1) function is required for the initial stages of salmonellosis, i.e. the entry of *Salmonella* into non-phagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium. Furthermore, SPI1 function is required for the onset of diarrhoeal symptoms during localized gastrointestinal infections. The function of SPI2 is required for later stages of the infection, i.e. systemic spread and the colonization of host organs. The role of SPI2 for survival and replication in host phagocytes appears to be essential for this phase of pathogenesis (Adapted from Hansen-Wester and Hensel, 2001).

Gastroenteritis due to NTS may persist with fever, nausea, vomiting, abdominal pain and symptoms may continue for over a week. In contrast, the early symptoms of enteric fever are often vague, and may include a dry cough, sever headache, anorexia, fever and a tendency to constipation rather than diarrhoea (Parry *et al*., 2002). If enteric fever is not treated on time, serious complication like hemorrhage from ulcers can occur during the third week of illness or perforation of the Peyer's patches (PP) can cause generalized peritonitis and septicemias; these are the commonest cause of death in typhoid fever. With the introduction of early and appropriate antibiotic therapy, the average case fatality rates for typhoid are less than 1% (Everest *et al*., 2001).

1.2.4.2. Virulence factors

The outcome of a *Salmonella* infection is determined by the status of the host and status of the bacterium (Figure 1.4). The status of the bacterium is determined by the so-called virulence factors (van Asten and van Dijk, 2005).

a. *Salmonella* Pathogenisity Islands (SPIs)

The majority of virulence genes of *Salmonella* are clustered in regions distributed over the chromosome called *Salmonella* pathogenicity islands (McClelland *et al*., 2001). The SPIs are of major importance for the virulence of *S. enterica*. Hallmarks of *Salmonella* virulence, such as cell invasion, intracellular survival and the production of Vi antigens capsule are encoded by SPIs. Until recently more than 10 SPIs have been identified on the *Salmonella* chromosome, but SPI-1 and SPI-2 is the central for pathogenesis of *Salmonella* infections (Hansen-Wester and Hensel, 2001).

All types of *S. enterica* have two large clusters of genes known as *Salmonella* Pathogenicity Island 1 and 2. *Salmonella* Pathogenicity Island 1 encodes genes necessary for invasion of intestinal epithelial cells and induction of intestinal secretory and inflammatory response (Galyov *et al*., 1997). *Salmonella* lacking a functional SPI-1 Type III secretion system are unable to invade epithelia cells and induce cytokine synthesis (Hobbie *et al*., 1997). During invasion of the gut, the SPI-1 encoded SipB protein triggers the activation of

intracellular Caspase-1 within resident macrophages that induces apoptosis in the infected macrophages resulting in escape of *Salmonella* from these cells (Hersh *et al*., 1999). SPI-1 also encodes an effector protein SopB which is an inositol phosphate phosphatase and its enzymatic activity results in activation of chloride channel in the membrane of epithelial target cells leading to the secretion of chloride and loss of fluid into the intestinal lumen (Norris *et al*., 1998).

Salmonella Pathogenicity Island 2 encodes genes essential for intracellular replication and necessary for establishment of systemic infection beyond the intestinal epithelium (Hensel, 2006). The function of the SPI-2 encoded Type III secretion system is required to protect the pathogens within the *Salmonella* containing vacuole (SCV) against the effectors functions of innate immunity. It has been reported that SPI-2 prevents co-localization of the phagocyte oxidase (Vazquez-Torres *et al*., 2000) and the inducible nitric oxide synthtases to the SCV (Chakravortty *et al*., 2002). As a consequence, intracellular *Salmonella* are protected against damage by reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Boonmar *et al*., 1998) and against a potent antimicrobial activity of peroxynitrite, which is generated by reaction of reactive nitrogen species and reactive oxygen species (Chakravortty *et al*., 2002). SPI-2 genes enabled *S. enterica* lineages to establish a new niche as an intracellular pathogen in the intestinal mucosal and systemic tissue.

b. Type III secretion systems

Central to the pathogenesis of *S. enterica* is the function of specialized protein secretion systems, known as Type III secretion system (TTSS). TTSS are specialized virulence devices that have evolved indirect translocation of bacterial virulence proteins into the host cell cytoplasm (Figure 1.5). Type III secretion systems are composed of several proteins that form a remarkable needle-like organelle in the bacterial envelope (Galan, 1998). So far the presence of two SPIs (SPI-1 and SPI-2) each encoding a TTSS, have been described for *Salmonella* species and may reflect the flexibility of this highly successful pathogen in causing different forms of diseases. SPI-1 is not present in *E. coli* isolates, this

suggest that the acquisition of SPI-1 by *Salmonella* was a fundamental step in the divergence of these two genera (Fierer and Guiney, 2001).

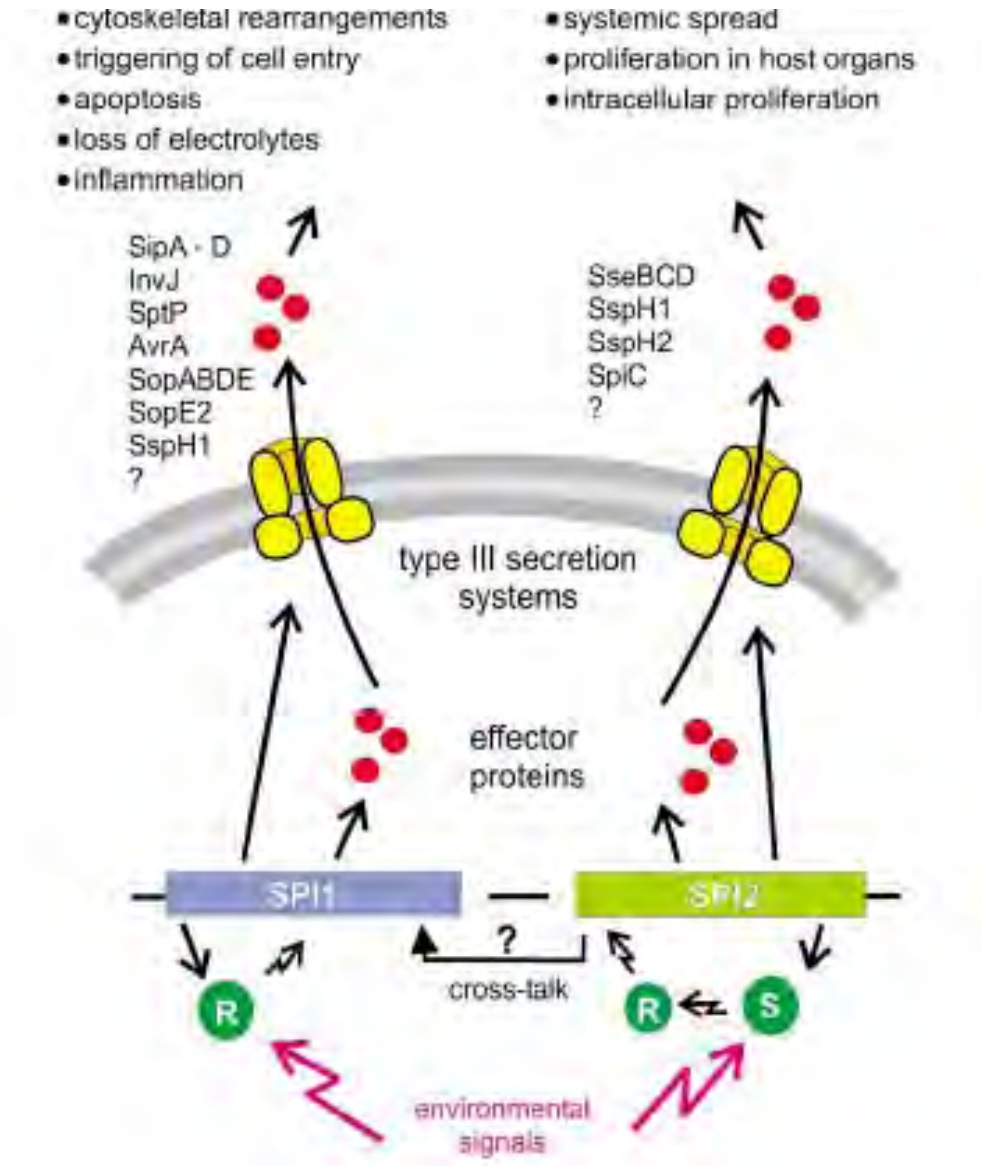

Figure1.5: Model for the functions of two TTSSs involved in the interaction of *Salmonella* spp. with host cells. SPI1 and SPI2 both encode TTSSs and each TTSS has a distinct set of secreted substrate proteins. Furthermore, both SPIs encode specific regulators (R, DNA-binding regulator; S, sensor) that control gene expression. The function of each system is required for different forms of interaction with host cells at different stages of pathogenesis of salmonellosis (Adapted from Hansen-Wester and Hensel, 2001).

c. Regulatory proteins, toxins, plasmids and Vi antigens

Regulatory proteins that control the synthesis of multiple proteins at the level of gene transcription are also essential to *Salmonella* pathogenesis. The best studied example is PhoP/PhoQ, which regulates genes important for survival within macrophages, resistance to cationic antimicrobial proteins and acid pH, and invasion of epithelial cells (Behlau and Miller, 1993). PhoP/PhoQ regulated genes encode an acid phosphatase, cation transporters, outer membrane proteins, and genes important for the modification of lipopolysaccharide; these modifications promote resistance to antimicrobial cationic peptides and alter the ability of lipid A to stimulate tumor necrosis factor-α secretion by macrophage (Guo *et al*., 1997).

Other regulatory genes implicated in pathogenesis include *ompR/envZ*, the regulator of porin gene transcription; *katF*, an alternative bacterial sigma-factor that regulates catalase production; and *ssrAB*, which regulates genes in SPI-2 that are important for systemic pathogenesis (Lindberg, 1980). Non-typhoidal *Salmonella* also carry a variety of virulence plasmids which might play a role in multiplication inside the cell, destabilizing the cytoskeleton of the eukaryotic cell and also might be involved in resistance of *Salmonella* species to the bacteriolytic activity of serum. Enterotoxin may also play a role in *Salmonella* gastroenteritis. An enterotoxin antigenically similar to Cholera toxin also has been identified (Aguero *et al*., 1991). Flagella phase variation that is exploited by the majority of flagellated *Salmonella* might be related to escaping the host defense system (van Asten and van Dijk, 2005).

The Vi antigen of *S.* Typhi prevents antibody mediated opsonization, increases resistance to peroxide, and confers resistance to complement activation by the alternative pathway and to complement mediated lysis (Looney and Steigbigel, 1986).

1.2.4.3. Epidemiology of Salmonellosis

a. Typhoidal Salmonellosis

Typhoid fever is a global problem and its real impact is difficult to estimate because the clinical picture is similar with those of many other febrile infections, a recent study estimated that globally there are more than 22 million cases of typhoid fever each year with more than 200,000 deaths (WHO, 2003).

S. Typhi have high host specificity for humans and most often, acquisition of the organisms occurs by ingestion of food or water contaminated with human excreta and associated with poor sanitation and hygiene (Miller *et al*., 2000). Laboratory accidents have also resulted in typhoid fever transmission to laboratory workers (Blaser *et al*., 1980).

The disease is endemic in many developing countries, particularly in Asia, Africa, Latin America and Caribbean regions (Figure 1.6) (Crump *et al*., 2004). These countries share several characteristics including rapid population growth, increased urbanization, inadequate human waste treatment, limited water supply, and over-burdened health care systems. In developed countries, typhoid fever is predominantly related to traveling to the previously mentioned regions.

Figure 1.6: Geographical distribution of typhoid fever (Adapted from Crump *et al.* 2004).

The epidemiology of paratyphoid fever is less well described than typhoid fever. Nevertheless, estimates suggest that as much as 25% of enteric fevers may be caused by *S.* Paratyphi A (Crump *et al*., 2004). Furthermore, *S.* Paratyphi A is on the rise in South Asia (Ochiai *et al*., 2005) and may cause as severe infection as *S.* Typhi. The disease patterns associated with *S.* Paratyphi A in developing countries are probably similar to that of *S.* Typhi (Maskey *et al*., 2006).

b. Non-typhoidal Salmonellosis

Unlike *S.* Typhi and *S.* Paratyphi, whose only reservoir is humans; non-typhoid salmonellosis is acquired from multiple animal reservoirs. The main mode of transmission is from food products contaminated with animal products or wastes, most commonly eggs and

poultry products (Miller *et al*., 2000). Data concerning NTS serovars is notoriously difficult to obtain, as most patients have a mild and usually self-limiting illness, rather than systemic infections, so do not need to consult the health services. Estimates from over a decade ago suggested that there were 1.3 billion NTS infections and 3 million deaths each year (Tassios *et al*., 1997).

In United States according to CDC estimates in 2001, there were 2 million cases annually with 500 to 2,000 deaths per year and the majority of the reported cases were caused by *S.* Typhimurium or *S.* Enteritidis (Cammie and Miller, 2000). In developing countries NTS is also an important cause of invasive disease, particularly in tropical regions of Africa, where *Salmonella enterica* serovar Typhimurium (*S. typhimurium*) and *Salmonella enterica* serovar Enteritidis (*S. enteritidis*) are consistently the most common causes of childhood bacteraemia, as well as important causes of meningitis, septic arthritis and pneumonia (Kariuki *et al*., 2006).

According to the 2002 WHO Global *Salmonella* Surveillance report on the distribution of *Salmonella* serotypes from 2000 to 2002, during the 3-year period, *Salmonella enterica* serovar Enteritidis was by far the most common serotype reported from human isolates globally (Galanis *et al*., 2006).

In 2002, it accounted for 65% of all isolates, followed by *S.* Typhimurium at 12% and *S.* Newport at 4%. Among nonhuman isolates, *S.* Typhimurium was the most commonly reported serotype in all 3 years, accounting for 17% of isolates in 2002. It was followed by *S.* Heidelberg (11%) and *S.* Enteritidis (9%). In Africa in 2002, *S.* Enteritidis and *S.* Typhimurium were each reported from approximately one fourth of isolates from humans (Figure1.7) (Galanis *et al*., 2006).

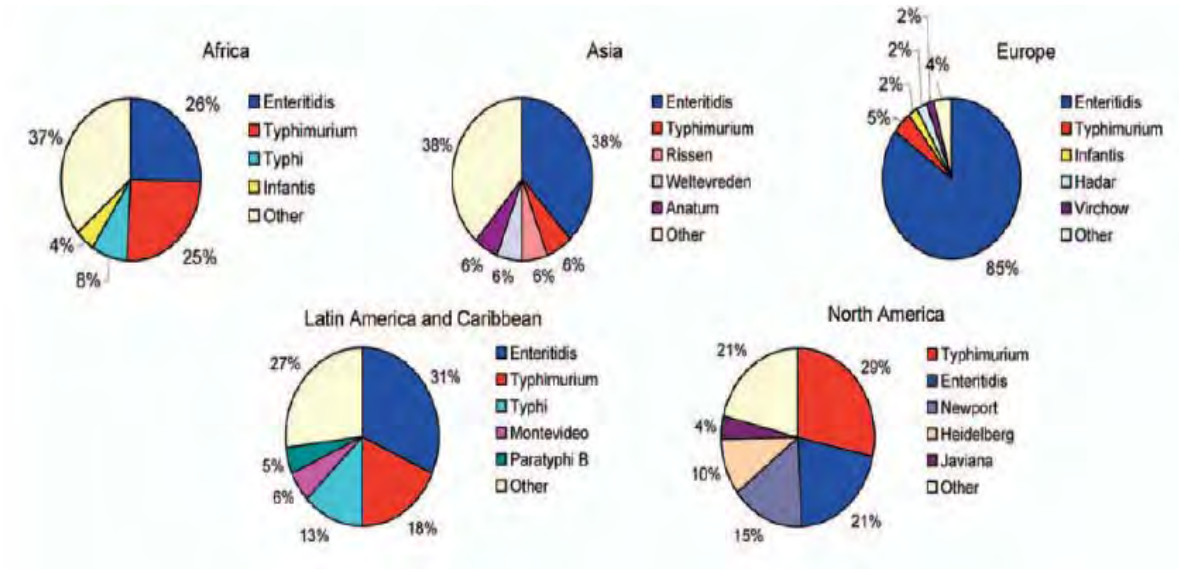

Figure 1.7: Proportion of most common serotypes of reported human *Salmonella* isolates by region, 2002 (Adapted from Galanis *et al.*, 2006)

Nontyphoidal *Salmonella* has a particularly profound impact in populations of high HIV/AIDS prevalence, since mortality in those with co-infections has been reported to be high. However, very little is known regarding the source and transmission of NTS in developing countries. It is likely that animal-human transmission via the food chain is less responsible than is human-human transmission, with contaminated water an important vehicle in communities with poor hygiene, sanitation and overcrowding (Graham, 2002; Kariuki *et al*., 2006). A major and worrying development that has global significance is the increasing and often rapid emergence of multidrug resistant (MDR) strains for all the above serovars (Graham, 2002).

Studies from tropical Africa have consistently shown that NTS bacteremia is more common during the rainy season. This may be the result of a seasonal increase of intestinal salmonellosis which has been reported from other (non-malarious) tropical regions. It may also reflect the seasonal pattern of diseases such as malnutrition and malaria, which may confer an increased risk of *Salmonella* infection (Graham, 2002).

1.2.5. Immunity

The immune response in *Salmonella* includes innate and adaptive immunity. The intestinal epithelium, neutrophil, macrophage, dendritic cell, NK cell, and γδT cells take important part in innate immunity and antigen specific T and B cells take part in the adaptive immunity (Mizuno, 2004).

The following are some early defense mechanisms in the gut: a) gastric acid may directly kill the bacteria or activate the proteolytic activity of pepsin which is required for the cleavage of Histone 2A, into antibacterial peptide (Mastroeni, 2006); b) phagocyte and innate immunity: usually phagocytic cells control the growth of *S. enterica* in the first few days of the infection using reactive oxygen species generated via the phagocyte NADPH-oxidase (Mastroeni *et al*., 2000), while RNS, that are produced following the activation of the inducible nitric oxide synthase, play a role in resistance in the later stage of infection of *S. enterica* (Mastroeni *et al*., 2000); c) Cytokines are key regulators of the host responses in intracellular pathogenesis and various bacterial products from *Salmonella* are potent inducers of cytokine expression by immune cells (Lalmanach and Lantier, 1999); d) antibody response to *S. enterica* infections induce early IgM antibody responses followed by IgG and IgA production (Mastroeni, 2006).

A large number of antigens including LPS determinants (O-polysaccharide and core regions), Vi antigen, porins, outer membrane proteins, lipoproteins, heat shock proteins, flagella and fimbriae are recognized by *Salmonella*-specific antibodies (Mastroeni, 2002); e), T cell response: *S. enterica* infections induce proliferation of CD4+ T cells which plays a pivotal role in activation of macrophage (Mastroeni, 2002). CD8+ T cells is also involved in producing IFN-γ and lysing target cells infected with *S. enterica* (Salerno-Goncalves *et al*., 2003). Suppression of the growth of *S. enterica* is followed by the elimination of the bacteria from the tissue. If the bacteria are not cleared, a late resurgence of bacterial growth can occur (relapse), or a chronic carrier state can develop, which can be a serious problem since it constitutes a reservoir of infection (Mastroeni, 2006).

1.2.6. Laboratory Diagnosis of Salmonellosis

In developing countries, salmonellosis is frequently diagnosed solely on clinical grounds. However, isolation of the causative organism is necessary for a definitive diagnosis, for performing antimicrobial susceptibility testing, and for further characterization.

1.2.6.1. Culture

A summary of guidance for identification of *Salmonella* species in a diagnostic laboratory is shown in Figure 1.8 (BSOP, 2007). Various enrichment and selective media are used to isolate salmonellae from different clinical specimens. Non-typhoidal *Salmonella* gastroenteritis is commonly diagnosed from stool culture. In cases where there is concern about bacteremia, blood culture is indicated (WHO, 2003).

S. Typhi and other typhoidal *Salmonella* are frequently isolated from blood during the first weeks of illness and usually positive stool cultures occur during the second and third weeks of disease (Cheesbrough, 2000). *S.* Typhi can also be isolated from bone marrow, rose spots and, infrequently, from urine cultures (Khan *et al*., 1998). Occasionally *Salmonella* may be cultured from other samples such as joint aspirates, cerebrospinal fluid or endocarditic heart valves. Specialist environmental laboratories may look for *Salmonella* in food or water samples, either as routine or in outbreak situations. The best recovery of *Salmonella* species from fecal samples can be achieved by the use of direct plating and inoculating on standard enrichment broths. Many selective agar plates are available for *Salmonella*. Most laboratories use one medium with low selectivity, such as MacConkey, Deoxycholate agar (DCA) or Cystine Lactose Electrolyte-deficient (CLED) agar, and one with higher selectivity, such as Xylose Lysin Deoxycholate agar Agar (XLD) (Cheesbrough, 2000). *Salmonella* enrichment broths (e.g. selenite broth and tetrathionat broth) may help to recover low numbers of organisms.

1.2.6.2. Biochemical tests

Salmonella species are motile (with a few exceptions), facultative anaerobic, produce acid from glucose usually with the production of gas, and are oxidase negative. Most

produce hydrogen sulphide except *Salmonella* Paratyphi A and *Salmonella* Typhi, which is a weak producer. Most *Salmonella* species don't ferment lactose. How ever, approximately 1% of the organism is able to ferment this sugar and thus may not be detected by clinical laboratories that use MacConkey agar and most laboratories use XLD agar or similar selection media to detect lactose fermenter *Salmonella* isolates (Miller *et al*., 2000). Urease production and indole production are negative, and most NTS produce citrate and hydrogen sulfide (Cheessbrough, 2000).

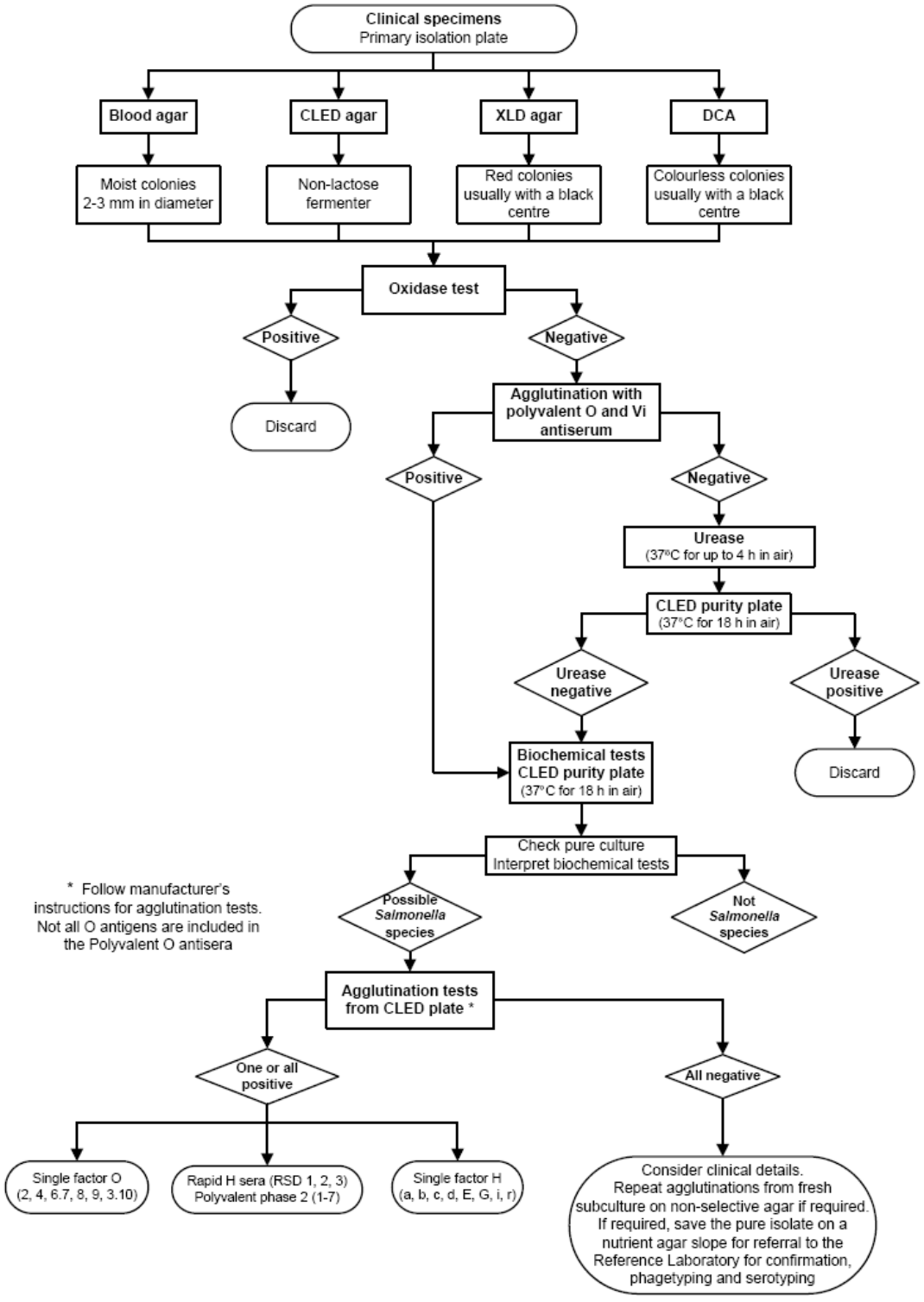

Figure 1.8: Flow chart for identification of *Salmonella* species (Adapted from BSOP, 2007)

1.2.6.3. Serogrouping/Serotyping

Salmonellae can be characterized by their somatic (O) and flagellar (H) antigens, the latter existing in some serotypes of phases 1 and 2. Some salmonellae also have an envelope antigen called Vi (virulence). The O antigen is usually determined by means of slide agglutination test with group-specific antiserum followed by agglutination with factor antiserum. H antigen is usually determined by means of the tube agglutination test. Partial serotyping is often sufficient for diagnosing purpose. Although serotyping seems convenient and easy to perform, there is a delay of three days or more to generate result. Complete serotyping is limited to specialist laboratories, because it requires highly trained personnel, and tube agglutination and phase-conversion plates are labor intensive. A wide range of antisera is required; production of antisera to rare antigens is expensive. A further limitation is that 5-8% of isolates are only partially typed or untyped (Kim *et al*., 2006). These include mucoid strains, in which the capsular polysaccharides block the exposure of O antigens. Non motile isolates cannot be fully typed to serovar level. Prolonged sub-culturing may theoretically affect the antigenic properties of a strain.

A few of the *Salmonella* serovars that commonly causes enteric fever, gastroenteritis or other infections have the following antigenic compositions (Table 1.3).

Table 1.3: O and H antigens commonly used for serogrouping and serotyping of typhoidal and non-typhoidal *Salmonella* species (Adapted from WHO, 2003)

Organism	O antigen	H antigen Phase 1 and 2	Serogroup
S. Typhi	9,12,[Vi]	d:-	D
S. Choleraesuis	6,7	c:1,5	C_1
S. Paratyphi B	1,4,5,12	b:1,4,5,12	B
S. Concord	6,7	l,v:1,2	C_1
S. Typhimurium	1,4,5,12	i:1,2	B
S. Enteritidis	1,9,12	g,m,:-	D
S. Butantan	3,10	b:1,2	E

1.2.6.4. Serology

The Widal test is used for serological diagnosis of typhoid fever and measures agglutinating antibody levels against O and H antigens. The levels are measured by using double dilutions of sera. Usually, O antibodies appear on days 6-8 and H antibodies on days 10-12 after the onset of the disease (WHO, 2003). The test has only moderate sensitivity and specificity. It can be negative in up to 30% of culture proven cases of typhoid fever. This may be because of prior antibiotic therapy that has blunted the antibody response. On the other hand, *S.* Typhi shares O and H antigens with other *Enterobacteriacae,* and this can lead to a false positive result (Pang and Pothocheary, 1989). A study conducted in Vietnam on the evaluation of Widal test, showed that the antibody responses to both antigens were highly variable among individuals infected with serotype Typhi, and elevated antibody titers were also detected in a high proportion of serum samples from healthy subjects from the community (House *et al*., 2001).

In a study done to evaluate the significance of Widal test on 242 typhoid suspected patients, the Widal qualitative test was positive in 92 (38%), 10 (43.5%), and 55 (31.3%) in typhoid suspected patients, febrile non-typhoidal patients, and healthy blood donors, respectively(Awol, 2004). It is therefore important to determine the antibody level in normal population in a particular locality in order to determine a threshold above which the

antibody titer is significant. This is important, if as usually happens, a single acute sample is available for testing. If paired sera are available, a fourfold rise in the antibody titer between acute sera and convalescent is diagnostic. Despite these limitations, this test could be of use for the diagnosis of typhoid fever in patients who have clinical typhoid fever but are culture negative or in regions where bacterial culturing facilities are not available**.**

1.2.6.5. Molecular technique

The rapid detection of microbial pathogens is critical since people's lives may depend on it. Thus, there is a need for more reliable and faster methods .*Salmonella* cultures take 4–7 days for isolation and identification, a problem for diagnosis and treatment. In addition, sensitivity of cultures can be affected by antibiotic treatment, inadequate sampling, variations of bacteraemia and a small number of viable organisms in faeces (Jordan *et al*., 2009).

DNA probes and polymerase chain reaction (PCR) protocols have been developed to detect *S*. Typhi directly from the blood (Haque *et al*., 1999). PCR has proven an invaluable tool for detection and it should be implemented to obtain a rapid yes/no answer on-site. It is possible, using molecular methods, to identify and distinguish between different *Salmonella* serovars within 4 h if a whole cell PCR is performed or 7 h if genomic DNA is to be extracted first. With gene specific PCR, it is also possible to specifically detect a pathogenic organism from a mixed bacterial culture (Chaudhry *et al*., 1997).

It was also proven that RFLP analysis of 16S rRNA PCR amplicons could be used as a first step fingerprint in the molecular based approach for distinction between different *Salmonella* serovars. With Multiplex PCR, multiple gene products can be amplified in a single PCR reaction and in this study it is clearly shown as a rapid method to distinguish between *Salmonella* Typhimurium and *Salmonella* Typhi, taking 4 h to make that distinction (Nathisuwan et al.; Prakash et al., 2005). Haque and his colloquies (1999) tried to compare polymerase chain reaction-based technique with blood culture and Widal test during the first week of illness of 82 suspected cases of typhoid and found that the respective figures of positivity for PCR, blood culture and Widal test were 71.95%, 34.1%, and 36.5%.

A study in Jordan on *Salmonella* isolates from poultry and meat products showed that out of 212 total samples, *Salmonella* was detected in 185 samples (87%) by PCR technique, while 172 (81%) samples were detected *Salmonella* positive by conventional microbiological methods (Malkawi and Gharaibeh, 2003). PCR assay proved to be an effective method for *Salmonella* detection in meat and poultry products with less time-consuming procedure. Using PCR, *Salmonella* spp. detection could be achieved within 24–36 h compared to 3–8 days for the conventional microbiological methods(Malkawi and Gharaibeh, 2003).

Pradyot Prakash *et al.* compared nested PCR using *H1-d* primers, which is specific for *Salmonella enterica* serovar Typhi, with blood culture and the single-tube Widal test and found out that nested PCR can be used as a gold standard to determine the cut off titer of the Widal test for diagnosis of typhoid fever. (Prakash *et al.*, 2005.

1.2.7. Treatment

1.2.7.1. Typhoidal salmonellosis

In areas of endemic disease, more than 60 to 90 percent of cases of typhoid fever are managed at home with antibiotics and bed rest. For the hospitalized patient, effective antibiotics, good nursing care, adequate nutrition, careful attention to fluid and electrolyte balance, and prompt recognition and treatment of complications are necessary to avert death (Parry *et al*., 2002). The fluoroquinolones are widely regarded as optimal for the treatment of typhoid fever. They are well tolerated and more rapidly and reliably effective than the former first-line drugs, viz. chloramphenicol, ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole (WHO, 2003).

The fluoroquinolones attain excellent tissue penetration, kill *S.* Typhi in its intracellular attaining stage in monocytes/macrophages, and achieve higher active drug levels in the gall bladder than other drugs. They produce a rapid therapeutic response, i.e. clearance of fever and symptoms in three to five days and very low rates of post-treatment carriage (Cristiano *et al*., 1995). Concern has been expressed about three main issues regarding the use of fluroquinolones in the treatment of typhoid fever: the potential for toxic effects in children, the cost, and the potential emergence of resistance (Bethell *et al*., 1996).

Chloramphenicol, amoxicillin, and trimethoprim-sulfamethoxazole remain appropriate for the treatment of typhoid fever in areas of the world where the bacterium is still fully susceptible to these drugs and where the fluoroquinolones are not available or affordable (Cammie and Miller, 2000). The disadvantage of using chloramphenicol includes a relatively high rate of relapse (5-7%), long treatment courses (14 days) may cause bone marrow depression, and the frequent development of a carrier state in adults (Bhutta *et al*., 1991). The third generation cephalosporins (ceftriaxone, cefixime, cefotaxime and cefoperazone) and azithromycin are also effective drugs for typhoid. In general, in areas with high prevalence of multi-drug resistant *Salmonella* infection, all patients suspected of having typhoid fever should be treated with a quinolone or third-generation cephalosporin until the results of culture sensitivity studies become available (Miller *et al*., 2000).

1.2.7.2. Non-typhoidal salmonellosis

Gastroenteritis caused by *Salmonella* is usually a self-limiting disease and therapy should be directed primarily to the replacement of fluid and electrolyte losses. Therefore, antimicrobials should not be used routinely to treat uncomplicated non-typhoidal *Salmonella* gastroenteritis or to reduce convalescent stool excretion (Richards *et al*., 1993). However, antimicrobial therapy should be considered for any systemic infection (Parry *et al*., 2002). For susceptible organisms, treatment with an oral quinolone, trimethoprim-sulfamethoxazole, or amoxicillin is adequate.

Because of the increasing prevalence of antimicrobial resistance, empirical therapy for life threatening bacteremia or local infection suspected to be caused by non-typhoidal *Salmonella* should include a third generation cephalosporin and a quinolone until susceptibility patterns are known. Amoxicillin and trimethoprim-sulfamethoxazole are effective in eradication of long-term carriage. The high concentration of amoxicillin and quinolone in bile and the superior intracellular penetration of quinolone are theoretical advantages over trimethoprim-sulfamethoxazole (WHO, 2003).

1.2.8. Mechanisms of Drug Resistance

Efforts aimed at identifying new antibiotics were once a top research and development priority among pharmaceutical companies. The potent broad spectrum drugs that emerged from these endeavors provided extraordinary clinical efficacy. Success, however, has been compromised. We are now faced with a long list of microbes that have found ways to circumvent different structural classes of drugs and are no longer susceptible to most, if not all, therapeutic regimens (Alekshun and Levy, 2007).

Resistance to various classes of antimicrobial agents has been encountered in many bacteria of medical and veterinary relevance. Over the years, various studies have reported the presence of genes and mutations conferring resistance to antimicrobial agents in zoonotic bacteria such as *Salmonella* (Michael *et al*., 2006).

There are three major mechanisms by which bacteria have become resistant to antimicrobial agents: enzymatic inactivation; reduced intracellular accumulation of the antimicrobials; protection, alteration or replacement of the cellular target sites (Schwarz and Chaslus-Dancla, 2001).

a. Chloramphenicol

Chloramphenicol (CAF) is a broad-spectrum bacteriostatic agent and the antibacterial action of chloramphenicol is mediated by inhibiting protein synthesis after binding to the 50S subunit of the bacterial ribosome and thus preventing the transfer of the new amino acid from its tRNA to the growing peptide chain. This binding is achieved by molecular mimicry of the peptidyl adenyl terminus of the tRNA molecule (Schwarz *et al*., 2004). The first and still most frequently encountered mechanism of bacterial resistance to CAF is enzymatic inactivation by acetylation of the drug via different types of chloramphenicol acetyltransferases (CATs) (Murray and Shaw, 1997). However, there are also reports on other mechanisms of CAF resistance, such as efflux systems, inactivation by phosphotransferases, mutations of the target site and permeability barriers (Murray and Shaw, 1997).

There are two defined types of CATs which distinctly differ in their structure: CatA and CatB enzymes. In *Salmonella,* enzymatic inactivation by type A or type B chloramphenicol acetyltransferases (Cat) as well as the export of chloramphenicol or

chloramphenicol/florfenicol by specific efflux proteins is the dominant resistance mechanisms (Michael *et al*., 2006). Two different types of CatA proteins, encoded by the genes *catA1* and *catA2*, have so far been detected in *Salmonella* isolates. While the Tn9-borne resistance gene catA1 has been found in several serovars, including Typhimurium (Chen *et al*., 2004), the gene *catA2* was detected on a multiresistance plasmid from *S*. Enteritidis, and *S*. Typhimurium (Randall *et al*., 2004)).

Three different types of *catB* genes, *catB2*, *catB3* and *catB8*, are known to occur in *Salmonella*. All these *catB* genes are located on gene cassettes and have mainly been identified in class 1 multi-resistance integrons in *S*. Typhimurium (Nogrady *et al*., 2005). The chloramphenicol exporter gene *cmlA* is also a cassette borne gene which has been found in plasmid-located class 1 integrons in *S*. Typhimurium (Nogrady *et al*., 2005).

b. Co-trimoxazole

A combination of trimethoprim and sulphamethoxazole, known as co-trimoxazole has been successfully used to treat *Salmonella* infections. Since these two antimicrobials inhibit sequential stages in tetrahydrofolic acid (THFA) synthesis it was believed that administration of a combination therapy would have a selective advantage over a single administration (Nogrady *et al*., 2005).

Sulfonamides and trimethoprim block different enzymatic steps in tetrahydrofolate biosynthesis (Figure 1.9). Sulfonamides are structural analogs of *p*-aminobenzoic acid and competitively inhibit the enzyme dihydropteroic acid synthase (DHPS) while trimethoprim competitively inhibits the enzyme dihydrofolate reductase (DHFR) (Sköld, 2001).

Chromosomal, plasmid and transposon mediated resistance have all been reported for this antimicrobial. Chromosomal mutations involve the over production of the dihydrofolate reductase (DHFR), which leads to trimethoprim resistance involving the need for a higher inhibitor concentration of drug inside the cell to decrease the residual enzyme activity. The commonest mechanism of trimethoprim resistance is associated with the production of an additional non-susceptible form of DHFR encoded by genes located on self-transmissible or mobile plasmids and transposons (Huang *et al*., 2004).

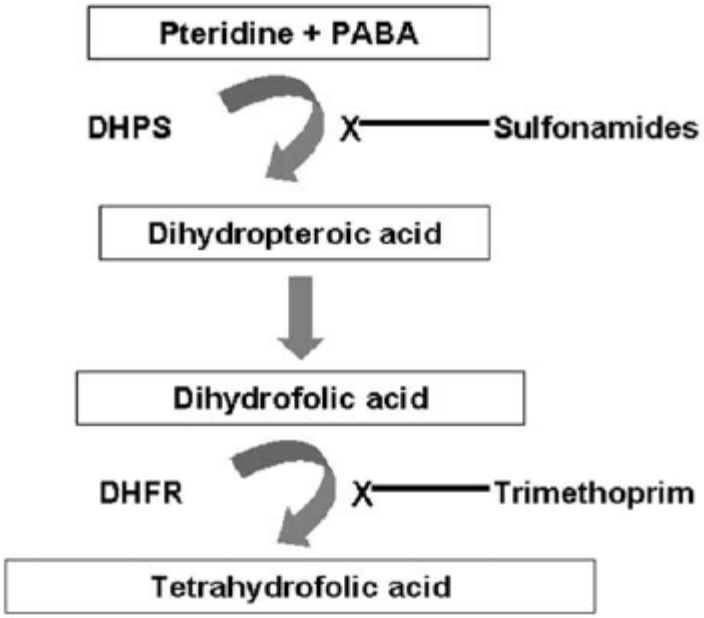

Figure 1.9: Inhibition of folate synthesis by sulfonamides and trimethoprim. PABA, paraaminobenzoic acid; DHPS, dihydropteroate synthase; DHFR, dihydrofolate reductase (Adapted from Huang *et al*., 2004)

So far, more than 30 different trimethoprim resistance mediating dihydrofolate reductase (*dfr)* genes have been identified. These are subdivided on the basis of their structure into two major types 1 and 2, which nowadays are referred to as *dfrA* and *dfrB.* While *dfrB* genes have not yet been identified in *Salmonella*, a total of 13 different *dfrA* genes-most of which are cassette-borne genes located in class 1 or class 2 integrons have been sequenced from various *Salmonella enterica* serovars (Michael *et al*., 2006).

c. Fluoroquinolones

Fluoroquinolones (FQ) represent the second, third and fourth generation of generation of quinolone development. The first generation of this group of compounds is represented by agents such as nalidixic acid and pipemidic acid and are characterized by limited activity against Gram-negative bacteria (Bager and Helmuth, 2001). The second-generation quinolones (namely ciprofloxin, norfloxacin, ofloxacin) have increased potency and antibacterial spectrum by modifying the original two-ring quinolone nucleus with different side chain substitutions and introducing fluorine at the 6th position. Their use now

accounts for about 11% of overall prescriptions of antimicrobials in human medicine and one of them, ciprofloxacin, is the most used antibiotic in the world (Acar and Goldstein, 1997).

Fluoroquinolones mechanism of action in *Salmonella* include inhibition of tertiary super coiling of bacterial DNA, primarily by inhibiting the action of DNA gyrase, a Type II topoisomerase, which consists of two GyrA and two GyrB subunits encoded by *gyrA* and *gyrB*, respectively (Figure 1.10) (Anderson, 2007). A single point mutation in *gyrA* between amino acids 67 and 106 (known as the quinolone resistance-determining region or QRDR) can give rise to nalidixic acid (a first-generation quinolone) resistance among isolates of *Salmonella*. This resistance is usually accompanied by a reduction in the susceptibility (MIC 0.125-1.0mg/L) of these isolates to ciprofloxacin (Murray *et al*., 2005).

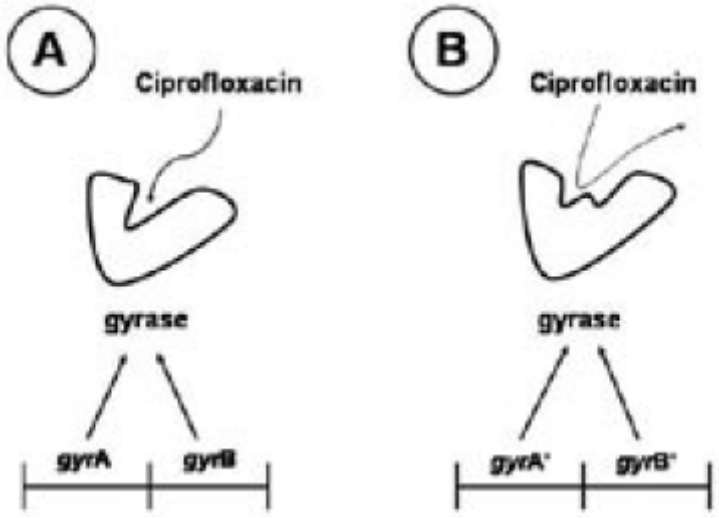

Figure 1.10: Mechanism of ciprofloxacin resistance. (A) Ciprofloxacin interacts with gyrase, inhibiting its enzymatic activity. (B) A mutation in either of the genes, gyrA or gyrB, can change the conformational structure of gyrase, and reduce the binding affinity of the enzyme for ciprofloxacin (Adapted from Andrson, 2007).

A study of >1000 stored *Salmonella* isolates from Finland has confirmed that show that resistance to nalidixic acid by means of disk diffusion is a sensitive and specific way of screening *Salmonella* isolates for reduced susceptibility to fluoroquinolones

(Hakanen *et al*., 1999). Resistance to nalidixic acid appears to be a predictor of clinical "quinolone hyporesponsiveness," and it is a harbinger of bona fide resistance to the clinically useful fluoroquinolones.

Mutation in *gry*B, which encodes the B subunit of gyrase, was identified at a much lower frequency than *gyr*A mutations. As in *E. coli* and other Gram-negative bacteria, topoisomerase IV, whose *parC* and *parE* genes are, respectively, homologous to *gyrA* and *gyrB*, is considered a secondary target for quinolones in *Salmonella*. This means that mutational modifications of this enzyme are expected to occur only in strains which already possess a mutated gyrase. Quinolone-resistance mutations in parC generally occur at codons Ser80 or less frequently at codon Glu84, which are homologous, respectively, to the Ser83 and Asp87 codons of gyrase (Casin *et al*., 2003). It is assumed therefore that the mode of action of quinolones against both enzymes is similar. In Gram negative bacteria, for many fluoroquinolones, DNA gyrase is the primary target and topoisomerase IV may be a secondary target associated with high level resistance. However, for many fluoroquinolones in Gram positive bacteria, such as *Staphylococcus aureus*, topoisomerase IV is the primary target (Wain, 2000).

Fluoroquinolones resistance mechanisms also include decreased accumulation due to active efflux and possibly decreased outer membrane permeability. Very recently, the presence of a plasmid-borne *qnr*-like gene conferring low-level quinolone resistance has been reported in clinical isolates of *S. enterica* serovar Enteritidis (Cheung *et al*., 2005). Thus, FQ resistance as observed in *Salmonella* isolates is the endpoint result of the accumulation of several, sometimes cooperating, and biochemical mechanisms, themselves resulting from various genetic events.

d. β-lactam antibiotics

The mode of action of beta lactam antibiotics is via penicillin binding proteins (PBPs) and inactivation of transpeptidases. The final stage in the synthesis of bacterial cell wall peptidoglycan is the transpeptidation of the free peptide strands of the cell wall peptide-

glycan sub-units. This is achieved by acylation of the terminal amine group of one peptide chain onto the D-alanine residue of the next peptide chain (Wain, 2000).

Resistance to β-lactam antibiotics is mainly mediated by a large number of β-lactamases which differ in their abilities to hydrolyse the various beta-lactam antibiotics (Livermore, 1995). These enzymes can bind β-lactam molecules thus protecting the penicillin binding proteins in bacterial cell walls. Once bound, the acylation of a serine residue at the active site of the β-lactamase results in cleavage of the β-lactam ring of the antibiotic and regeneration of the β-lactamase. Other resistance mechanisms include the acquisition of penicillin binding proteins with reduced affinity to β-lactams, mutations in the PBPs (Schwarz *et al*., 2004). Reduced β-lactam uptake due to alterations in the outer membrane of gram negative bacteria or export by multi-drug transporters have been also reported (Paulsen *et al*., 1996).

Resistance against β-lactam antibiotics (penicillin and cephalosporins) in *Salmonella* is mainly mediated by β-lactamase enzymes, which inactivate the antibiotics (Bush *et al*., 1995). The β-lactamases so far detected in *Salmonella* constitute a diverse group of enzymes encoded by a considerable number of genes. At least 10 different subgroups of b-lactamase genes (*bla*) coding for TEM-, SHV-, PSE-, OXA-, PER-, CTX-M-, CMY-, ACC-, DHA-, or KPC-type β-lactamases have been identified (Michael *et al*., 2006).

e. Ampicillin

Bacterial resistance to ampicillin is mediated most commonly by a β-lactamase enzyme and the main family of β-lactamases responsible for ampicillin resistance is the temoxicillinase (TEM) β-lactamases. The gene for this enzyme has been shown to be present in resistant isolates of *S.* Typhi from Mexico and South East Asia (Taylor and Brose, 1985).

f. Cephalosporins

The cephalosporin nucleus is synthesized with a β-lactam ring attached to a six membered dihydrothiazine ring and based on cephalosporin C. Their mode of action is

similar to the pencillins and unlike the penicillin nucleus; the cephalosporin nucleus is much more resistant to β-lactamase.

Cephalosporins are classified by four groups or subdivided into four generations based on the spectrum of their activity (Livermoore and Williams, 1996). First-generation cephalosporins (cefalotin, cefaloridin, cephalexin, cephapirin, cefazolin, cefadroxil, cephradine, and others) possess high biological activity with respect to *Staphylococci, Streptococci, Pneumococci*, and many types of enteric bacteria.

Second-generation cephalosporins (cefuroxime, cefamandole, cefoxitin, cefotetan, cefaclor, and others) are characterized by high activity with respect to Gram-positive microorganisms that are resistant to β-lactamase action. They do not have a noticeable effect on enterococci (Parry *et al*., 2002).

Third-generation cephalosporins (cefotaxime, ceftizoxime, ceftriaxone, ceftazidime, cefoperazone, and many others) differ in the highly antimicrobial activity against *Enterobacteriaceae* and are effective in treating typhoid fever. The fourth generation cephalosporin (Cefepime) is effective against Gram-positive (including methicillin-susceptible *S. aureus*, α hemolytic streptococci, and some coagulase negative staphylococci) and Gram-negative organisms, including *P. aeruginosa* (Nathisuwan *et al*., 2001).

Resistance to the broad spectrum cephalosporin is mainly due to the production of an enzyme called the extended spectrum β –lactamase (ESBL). The ESBL enzymes are plasmid - mediated enzymes capable of hydrolyzing and inactivating a wide variety of β lactams, including third generation cephalosporins, penicillins and aztreonam. These enzymes are the result of mutations of TEM-1 and TEM-2 and SHV-I. All of these β-lactamase enzymes are commonly found in the *Enterobacteriaceae* family.

Widespread use of third generation cephalosporins and aztreonam is believed to be the major cause of the mutations in these enzymes that has led to the emergence of the ESBLs (Paterson and Bonomo, 2005). These enzymes mediate resistance to cefotaxime, ceftazidime and other broad spectrum cephalosporins and to monobactams such as aztreonam, but have no detectable activity against cephamycins and imipenem. Because, of their greatly extended substrate range these enzymes were called extended spectrum β-lactamases (Cheung *et al*., 2005).

Widespread fluoroquinolone use in children has been discouraged because of the potential adverse effects on cartilage development. Therefore, extended-spectrum cephalosporins (especially cefotaxime or ceftriaxone) are the mainstay of treatment of serious infections due to *Salmonella* species in children. The production of ESBLs or AmpC β-lactamases consequently has considerable implications for clinical microbiology laboratories and physicians in areas in which infections with *Salmonella* species are common (Kruger *et al*., 2004).

Although reports of ESBLs associated with *Salmonella* spp. are relatively rare compared to those for other species in the family *Enterobacteriaceae*, the number of reported cases in this organism has been increasing in recent years. *Salmonella* have been found to express a wide variety of ESBL types, including TEM, SHV, PER, OXA, and CTX-M enzymes (Winokur *et al*., 2001).

The genes encoding these extended-spectrum cephalosporinases are carried by conjugative plasmids, transposons, or integrons. These mobile genetic elements could spread horizontally between enteric organisms. Thus, not only can antimicrobial resistance in salmonellae emerge because of the selection pressure derived from inappropriate antimicrobial use in food animals, but drug-susceptible salmonellae can also become resistant via the in vivo acquisition of drug resistance plasmids from other enteric pathogens in the intestinal tract of patients (Su *et al*., 2003).

ESBLs are typically encoded on large, 80-kb to 300-kb plasmids that can be exchanged between bacterial species. In many cases, these plasmids also encode other antimicrobial resistance genes. Therefore, it is common for organisms expressing an ESBL to express co-resistances to aminoglycosides, trimethoprim-sulfamethoxazole, and tetracyclines (Jacoby and Medeiros, 1991). *Salmonella* isolates that demonstrate reduced susceptibility to one or more of ceftazidime, cefuroxime, cefotaxime, ceftriaxone, cefpodoxime or aztreonam but remain susceptible to cefoxitin or cefotetan are considered as potential producers of ESBLs (Sturenburg and Mack, 2003).

Expanded-spectrum cephalosporins (ESCs) such as ceftriaxone, together with fluorinated quinolones, are the choice of antibiotics in the treatment of invasive *Salmonella* infections. Resistance to ESCs among non-typhoid *Salmonella* has been recognized since

the late 1980s. Currently, ESC-resistant *Salmonella* strains are reported world-wide and in some areas their incidence is significant. Resistance is mainly due to acquisition of multi-resistant plasmids encoding a variety of extended-spectrum and AmpC-type -lactamases (Miriagou *et al*., 2004).

1.2.9. Trends of Drug Resistance in *Salmonella*

Since the beginning of the 1990s, strains of *Salmonella* which are resistant to a range of antimicrobials, including first-choice agents for the treatment of humans, have emerged and are threatening to become a serious public health problem. This resistance results from the use of antimicrobials both in humans and animal husbandry. Multi-drug resistances to critically important antimicrobials are compounding the problems (Parry *et al*., 2003).

Surveillance data demonstrated an obvious increase in overall antimicrobial resistance among *Salmonella* from 20%-30% in the early 1990s to as high as 70% in some countries at the turn of the century. The resistance rate, however, varies with different serotypes, antibiotics and in different geographical area of the world (Su *et al*., 2004).

1.2.9.1. Salmonella enterica serovar Typhi

The presence of multi-drug resistance (MDR) *S.* Typhi, the casual agent of typhoid fever, now poses a major threat to the effective treatment of the disease (Mirza *et al*., 2000). In developing countries, the antibiotics most readily available for treatment of typhoid are: chloramphenicol, ampicillin, and co-trimoxazole. Since 1948, chloramphenicol became the standard antibiotic for treating typhoid, but plasmid encoded chloramphenicol resistance emerged first in the early 1970's (Mirza *et al*., 2000). Although slightly less effective than chloramphenicol, ampicillin was used both for therapy and for elimination of the carrier state, again plasmid–encoded resistance soon developed (Wain and Kidgell, 2004). Finally, co-trimoxazole was introduced in 1980, and plasmid encoded resistance to trimethoprim and sulfonamides were observed shortly afterwards (Datta *et al*., 1981).

Towards the end of 1980s and the 1990s, *S.* Typhi developed resistance simultaneously to all the drugs that were then used as first line treatment (chloramphenicol, trimethoprim, sulfamethoxazole and ampicillin) (Parry *et al*., 2002). Outbreaks of infections

with these strains occurred in India, Pakistan, Bangladesh, Vietnam, the Middle East, and Africa (Pang *et al*., 1995). These multi-drug resistant strains also carried the 100,000 to 120,000 KD IncHI self-transmissible plasmids that encoded the resistance genes.

Multi-drug resistance *S. enterica* serovar Typhi are less prevalent in Africa compared to Asia, but have been present in South Africa, in Ghana (Mills-Rovertson *et al*., 2002) and in Kenya (Kariuki *et al*., 2002). In recent years, however, there have been reports of declining resistance levels. At a major hospital in Mumbai, India, for example, the proportion of MDR strains declined from 46% in 2000 to 17% in 2002 (Rodrigues *et al*., 2003). In an infectious diseases hospital in Cairo, the isolation of multiple drug resistant isolates of *S*. Typhi declined from 100% in 1993 to 5% in 2000 (Wafsy *et al*., 2002). The authors of both reports suggest that this decline in the levels of resistance may allow drugs such as chloramphenicol to be used again as first line therapy for enteric fever.

Since the appearance of MDR to the commonly used drugs, ciprofloxacin and the third generation of cephalosporin (namely ceftriaxone) has became the first line of treatment for typhoid fever. Since 1993 however, there has been a global epidemic of nalidixic acid resistant *S. enterica* serovar Typhi. These strains exhibit decreased response to fluoroquinolones compared to those infected with nalidixic acid susceptible strains (Wain *et al*., 1997). In 1999 ceftriaxone resistant *S.* Typhi was detected in Bangladesh (Asna *et al*., 2003). Although it is threatening that resistance to ciprofloxacin is now emerging, ciprofloxacin remains the drug of choice for the treatment of MDR typhoid fever (Rowe *et al*., 1995).

1.2.9.2. Non-typhoidal Salmonella enterica serovars

In the last two decades, the emergence and spread of antimicrobial-resistant pathogens, among the non-typhoidal *Salmonella*, has become a serious health hazard worldwide. The routine practice of giving antimicrobial agents to domestic livestock as a means of preventing and treating diseases, as well as promoting growth, is an important factor in the emergence of antibiotic-resistant bacteria that are subsequently transferred to humans by the food chain (Angulo *et al*., 2004a).

Multi-drug resistance in the NTS is defined as resistance to four or more drugs (Cooke and Wain, 2006). In the developed world, the most common serotypes isolated from humans are *S.* Typhimurium, *S.* Enteritidis, *S.* Virchow and *S.* Hadar, which are zoonotic in origin and are often resistant to many commonly used antibiotics. The situation is different in the developing world, where the above serotypes, together with *S.* Dublin and *S.* Choleraesuis, tend to cause more invasive disease, particularly in the immuno-suppressed, neonates, children and the elderly (Gordon *et al*., 2002).

In the 1980s, nontyphoidal *Salmonella* species were fairly "sensitive" organisms. A remarkable bacterial "success story" of the l990s was the emergence and worldwide spread of *S.* Typhimurium definitive phage type 104 (DT104), having resistance to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, and tetracycline (ACSSuT R-type) was a major concern worldwide (Threlfall, 2002). This penta-resistance was found to be mediated by the antibiotic resistance gene cluster in *Salmonella* genomic island 1 (SGI1), and SGI1 has been identified in other *Salmonella* Typhimurium phage types and several other serotypes (Doublet *et al*., 2005).

Multiple drug resistance is a problem in other Typhimurium phage types and *Salmonella* serotypes. A strain of Typhimurium DT204b resistant to ampicilin, chloramphenicol, gentamycin, kanamycin, streptomycin, sulphonamides and tetracyclines and with low-level ciprofloxacin resistance caused an outbreak in five European countries in 2000 with more than 350 confirmed cases (Lindsay *et al*., 2002). In a European surveillance study in 2000, 36% of Virchow and 37% of Hadar isolates were resistant to four or more antimicrobials. Antimicrobial resistance has generally been less of a problem in *S.* Enteritidis. In Europe in 2000, 2% of isolates were resistant to four or more antimicrobials (Threlfall *et al*., 2003).

In some Africans countries NTS, in particular MDR *Salmonella enterica* serotype Typhimurium is the causative agent of serous out breaks. For example in Zaire (Cheesbrough *et al*., 1997), Rwanda (Lepage *et al*., 1990) and Kenya (Kariuki *et al*., 2000) multidrug-resistant *S.* Typhimurium was the predominant cause of bacteraemic illness. A ten years (1994-2003) retrospective study in Kenya indicated a steady increase in the proportion of multidrug resistance among NTS isolated from adult patients with

bacteraemia. The prevalence of NTS multiple resistant to all commonly available drugs including ampicillin, streptomycin, co-trimoxazole, chloramphenicol and tetracycline rose from 31% in 1994 to 42% at 2003, with concomitantly higher minimum inhibition concentrations (MICs) of each drug. Resistance was found to be encoded on large self-transferable 100–110 kb plasmids (Kariuki *et al*., 2005).

Because of the increased resistance to conventional antibiotics, extended-spectrum cephalosporins and fluoroquinolones have become the drugs of choice for the treatment of infections caused by multi-drug resistant *Salmonella* serotypes. However, since 1991, many countries have been reporting outbreaks or cases of infections due to *Salmonella*e that were resistant to extended-spectrum cephalosporins (Dunne *et al*., 2000).

Resistance to other antibiotics notably, resistance to quinolones (e.g., nalidixic acid) and their derivatives, such as fluoroquinolones (e.g., ciprofloxacin) has also begun to emerge. Of particular concern is an outbreak of infection with quinolone-resistant *S.* Typhimurium DT104 that has spread from animals to humans and has caused mortality (Molbak *et al*., 1999). It is now known that nalidixic acid the prototypic quinolone is a good predictor for the reduced susceptibility to fluoroquinolones in salmonellae (Hakanen *et al*., 1999). The increase in nalidixic acid resistance, in some way, may reflect the emergence of fluoroquinolone resistance.

Perhaps the most alarming report of resistance from a clinical view point is the combined resistance to fluoroquinolones, associated with double mutations in topoisomerases, and broad spectrum cephalosporins caused by β-lactamase in *Salmonella enterica* serovars Choleraesuis. This serovar is observed in Taiwan and it was found that it is highly invasive and simultaneously resistant to ceftriaxone and ciprofloxacin in 2002 (Chiu *et al*., 2004). The emergence of such a resistance trait in *S.* Choleraesuis poses a serious threat to human health and should be monitored closely.

1.2.10. Prevention and Control

Theoretically, it is possible to eliminate salmonellae that cause enteric fever since the bacteria survive only in human hosts and are spread by contaminated food and water. The control and near elimination of typhoid fever in developed countries has been achieved

largely because of improved sanitation, surveillance, contact tracing and successful therapy; this is also supported with vaccination. In developing countries reducing the number of cases in the general population requires the provision of safe drinking water, effective sewage disposal and hygienic food preparation (Mastroeni, 2006). In areas where the epidemic is high, mass immunization has been used successfully. Currently three vaccine alternatives are available: 1) a heat–killed, phenol extracted, whole cell vaccine, 2) Ty21a, an attenuated *S.* Typhi vaccine, 3) Vi vaccine, consisting of purified Vi polysaccharide from the bacterial capsule (Cammie and Miller, 2000). In developed countries, most cases are the result of travel to endemic areas. Travelers in such areas need to take particular care with water and food (Parry *et al*., 2002).

Non-typhoidal *S. enterica* infections are a major public health problem world-wide and reduction of these diseases presents a serious and challenging problem. These diseases have several animal reservoirs. In additions, the fact that a large number of different *S. enterica* serovars cause gastroenteritis in humans probably makes vaccines very difficult to realize and/or use commercially (Strugnell and Wijburg, 2006). The incidence of non-typhoidal salmonellosis continues to rise along with rates of emergence of antibiotic-resistant strains and increased centralization of food production. Thus, it is important to monitor every step of food production, from handling of raw products to preparation of finished foods. In particular, with the increasing prevalence of *S.* Enteritidis in egg-laying hens, it is recommended that pasteurized eggs should be substituted for bulk-pooled eggs. The prudent use of antimicrobial agents in both humans and animals is necessary to minimize the further emergence of antibiotic resistant strains (Cammie and Miller, 2000).

1.2.11. Typing of *Salmonella*

Typing is splitting organisms in to useful groupings; if those grouping are based on genetic differences then the typing scheme is also a classification. Such information may be of clinical or epidemiological value.

Clonally related organisms are members of the same species that share virulence factors, biochemical traits, and genomic characteristics. However, there is sufficient diversity in the species level that organisms isolated from different sources at different times

and in different geographical regions may be differentiated or classified into subtypes or strains (Olive and Bean, 1999). Typing is necessary when trying to determine the distribution of the widespread organism, association of type with disease, exclusion of sources, identification of carriers, determination of route of infection, and assessment of the efficiency of preventative measures (Struelens, 1998). Methods for *Salmonella* species typing fall into three broad categories, phenotyping, genotypic and sequence-based typing techniques (Maslow *et al*., 1993).

1.2.11.1. Phenotyping

Phenotypic methods are those that characterize the product of gene expression in order to differentiate strains. Properties such as biochemical profiles, bacteriophage types, and antigen or protein types present on cell surface, and antimicrobial susceptibility profiles are examples of phenotypic properties that can be determined in the laboratory. Because they involve gene expression, these properties all have a tendency to vary, based on changes in growth condition, growth phase, and spontaneous mutation (Arbeit, 2000). Phenotypic methods are associated with problems such as low discriminatory power, poor reproducibility and less typeability properties (Maslow *et al*., 1993).

a. Serotyping

Salmonella serotyping is a surveillance tool that detects widespread outbreaks, identifies outbreak sources, monitors trends over time, and attributes human disease to various foods and animals sources. Serological analysis usually remains the first step in an epidemiological investigation of *Salmonella* and may be sufficient for epidemiological investigations associated with uncommon serotypes (Winokur, 2003). Serotyping by traditional methods has several drawbacks (section 1.2.6.3).

b. Phage typing

In this technique *Salmonella i*solates are characterized by their susceptibility or resistance to lysis by each member of a panel of bacteriophage (Maslow *et al*., 1993). The power of resolution is limited, for example several distinct strains of *S*. Typhimurium can

belong to the same phage type and the technique is largely empirical and doesn't reflect true evolutionary classification (Schmieger, 1999). Phage susceptibility may be relatively plastic and susceptibility changes can occur rapidly. Within six weeks, *S.* Enteritidis isolated from one patient changed from DT4 to DT7 and DT9a (Powell *et al*., 1995). Phage typing is often an important early step in an investigation, but needs to be supplemented with other techniques. Phage typing is technically demanding and requires the maintenance of stocks of biologically active phage and control strains are conditions that relegate this technique to reference laboratories (Maslow *et al*., 1993).

c. Biotyping

Biotyping refers to analysis of cellular metabolic enzymes. Some diseases often clusters among a few serotypes, so other methods like biotyping have been used to further subdivide a particular serotype (Winokur, 2003). Biotyping like most phenotyping methods has only modest reproducibility because the organism can alter unpredictable expression of many cellular products (Tenover *et al*., 1995).

d. Antimicrobial susceptibility pattern

This technique is used to group *Salmonella* serotypes according to resistance profiles or R-types (Tenover *et al*., 1997). Antibiotic resistance in many *Salmonella* serotypes is increasing with a number of isolates showing multi-drug resistance (Winokur, 2003). Therefore recognition of new or unusual antibiotic resistance isolates has often instigated epidemiological investigations. Antimicrobial susceptibility patterns also have relatively poor discriminatory power, because antimicrobial resistance is under tremendous selective pressure in healthcare institution and often is associated with mobile genetic elements (e.g., transposons and plasmids) which can be lost or acquired over short periods of time (Winokur, 2003). Thus, antimicrobial resistance is not one of the most stable epidemiological markers for outbreak analyst.

1.2.11.2. Genotyping

Molecular genotyping of *Salmonella* strains is fundamental in tracking disease-associated and drug-resistant strains in various populations. Genotypic methods are those that are based on analysis of the genetic structure of an organism and include polymorphism in DNA restriction patterns based on cleavage of the chromosomes (DNA) into hundreds of fragments (frequent cutters), or into 10 to 30 fragments (infrequent cutters), and the presence and absence of extra-chromosomal DNA. They are less subjected to natural variation, although DNA changes (insertions, deletions, and random mutations) can have an effect on resulting fingerprinting (Arbeit, 2000).

Molecular techniques which are commonly used in typing bacteria include: pulse field gel electrophoresis (PFGE), ribotyping, plasmid profile analysis, amplified fragment polymorphism, arbitrary primed PCR (AP-PCR), and repetitive sequence PCR (Winokur, 2003). For many bacterial species, combination of different methods or selection of the most discriminative methods is usually required in identifying a particular strain. For typing *Salmonella* species including *S.* Typhi, *S.* Typhimurium and *S.* Enteritidis, a combination of PFGE, ribotyping, and plasmid analysis are used by most researchers/laboratories (Tsen *et al*., 2002).

a) Plasmid profile

Plasmid profile was among the earliest DNA based techniques applied to epidemiologic studies (Tenover *et al*., 1997). In the most basic system, plasmids are isolated from each isolate and then separated electrophoretically in agarose gel to determine their number and size. Additional information can be obtained by digesting the plasmid with a restriction endonuclease and then comparing the number and size of the resulting restriction fragments (Maslow *et al*., 1993). This procedure often referred as restriction enzyme analysis of plasmids, it is now the method of choice for plasmid studies; it is technically simple, requires only modest specialized equipment, and can be performed relatively quickly (Arbeit, 2000).

Typing systems based on plasmid analysis suffer from significant limitations inherent in the fact that plasmids are mobile extra chromosomal genotypes that define the

host strain. Plasmids can be spontaneously lost from or readily acquired by a host strain; consequently, epidemiologically related isolates can exhibit different plasmid profiles**.** Some studies regard the presence of a single, identical plasmid as sufficient proof that isolates are identical and therefore epidemiologically related (Liebana, 2002). Other studies suggest that numerous plasmids must be present, and regard the presence of a single plasmid as insufficient representative of a clone (Maslow *et al*., 1993). Open circular or linear plasmid forms display different electrophoretic migration patterns to confuse the interpretation of banding patterns (Liebana, 2002).

Despite this limitation, plasmid pattern determination has proven to be a useful epidemiological tool in out-breaks of *Salmonella* serotypes (Winokur, 2003). Plasmid profiles are most useful when they are combined with other methods for screening or typing (Arbeit, 2000).

b) Plasmid incompatibility

Plasmids contain genes that are essential for plasmids maintenance functions, such as the initiation and control of replication. Some contain genes that control traits ensuring stable inheritance, such as equi-partitioning during cell division or conjugal transfer. Many plasmids contain genes that are useful not only to themselves, but also to their host. Examples are genes controlling drug resistance, degradation of organic compounds, and virulence factors, including the production of toxins (Couturier *et al*., 1988).

Identification and classification of plasmids are especially important in medicine, because genes for clinically important traits, such as drug resistance and virulence factors, are frequently present in plasmids. The recognition of the type of virulence plasmid or resistance (R) plasmid present in a pathogen can be instrumental in tracing the source and spread of an infection and it may also serve in establishing a diagnosis. Besides these practical uses, there is another, more basic, use, the tracing of genetic relatedness and of evolutionary origins (Couturier *et al*., 1988).

A formal scheme of plasmid classification is based on incompatibility (Inc) groups. The procedure for incompatibility grouping is based on the introduction, by conjugation or transformation, of a plasmid of an unknown Inc group into a strain carrying a plasmid of a

known Inc group. If the resident plasmid is eliminated in the progeny, the incoming plasmid is assigned to the same Inc group. Plasmids with the same replication control are incompatible, whereas plasmids with different replication controls are compatible (Carattoli, 2003).

Couturier and coworkers (1988) developed a hybridization method for the comprehensive typing of bacterial plasmids according to replicon type. From this study, a bank of Rep probes corresponding to 19 different Inc groups in the *Enterobacteriaceae* was developed. Although this procedure represented a significant advance in plasmid typing, the method was time-consuming, labor-intensive, and incompatible with current high-throughput approaches. Currently a PCR-based replicon typing protocol is used to detect 18 plasmid replicons frequently found among the *Enterobacteriaceae.* In this method, 18 pairs of primers were designed to perform 5 multiplex- and 3 simplex-PCRs, recognizing the FIA, FIB, FIC, HI1, HI2, I1-Ig, L/M, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA replicons, representative of the major plasmid (Johnson *et al*., 2007).

c) Pulsed field gel electrophoresis (PFGE) of whole chromosomal DNA

PFGE is often considered the “gold standard” of molecular typing methods. PFGE is an agarose gel electrophoresis that permits analysis of bacterial DNA fragments over an order of magnitude larger than that of conventional restriction enzyme analysis (REA). Chromosomal DNA is digested with restriction enzymes that have few restriction sites, yielding 5-50 fragments ranging from 10kb to 800kb in length (Olive and Bean, 1999; Struelens, 1998). A major limitation of REA with enzymes with relatively frequent recognition sites is the difficulty of analyzing the resulting patterns composed of large numbers of overlapping, poorly resolved restriction fragments (Arbeit, 2000).

PFGE is a variation of agarose gel electrophoresis in which the orientation of the electric field across the gel is changed periodically (pulsed) rather than being kept constant as in conventional agarose gel electrophoresis used for the REA and southern blot (Tenover *et al*., 1995; Thong *et al*., 1996). Theoretically, all bacterial isolates are typable by PFGE. PFGE has proved to be one of the most discriminative methods for the sub-typing of *Salmonella* strains (Rivera *et al*., 1991). Thong *et al*. (1995) used the PFGE method for the

sub typing of *S.* Typhi strains isolated from several Southeast Asian countries and found that PFGE was a powerful technique for the analysis of *S.* Typhi strains. Thong and his colleagues (1996) also found that although considerable genetic diversity existed among *S.* Typhi strains, some PFGE patterns might be shared between isolates obtained from different countries, for example, Malaysia, Indonesia, and Thailand.

A study in Vietnam also showed that PFGE is both reproducible and discriminatory and can be used to analyze multiple drug-resistant *S.* Typhi strains in regions where typhoid is endemic (Wain *et al*., 1999). In Malaysia, analysis of *S*. Typhi using PFGE indicated that an individual outbreak was associated with closely related strains, whereas isolates of *S.* Typhi from sporadic cases were very diverse (Thong *et al*., 1994). In South East Asia, PFGE studies revealed that multiple genetic variants of *S.* Typhi were present simultaneously and are associated with sporadic cases of typhoid fever and occasional outbreaks (Thong *et al*., 1995). PFGE uses expensive equipment and software is needed for comparative results, so the technique is limited to reference and research laboratories. Over all it samples a small proportion of sequence variation in *Salmonella* genome. Many authors have concluded that when, PFGE is used in combination with other methods (e.g. R-typing, plasmid profiling) seems to give the best discrimination for epidemiological purpose.

d) Integron analysis

Horizontal gene transfer increases genetic diversity in prokaryotes to a degree not allowed by the limitations of reproduction by binary fission (Michael *et al*., 2004). The integron/cassette is one of the most recently characterized examples of a system that facilitates horizontal gene transfer (Michael *et al*., 2004).

An efficient route of acquisition and vertical and horizontal dissemination of resistance determinants is through mobile elements including plasmids, transposons, and gene cassettes in integrons (Guerra *et al*., 2000). There are at least three classes of integrons (based upon the type of integrase gene they possess) and class 1 integrons are the most frequent in clinical strains, being found in many different organisms (Collis *et al*., 1998).

Integrons are mobile DNA elements with the ability to capture genes, notably those encoding antibiotic resistance, by site-specific recombination. Integrons have an intergase gene *(int),* a nearby recombination site (attI), and a promoter (Michael *et al*., 2004).

Class 1 integrons have been examined most extensively and found that they consist of a variable region bordered by 5' and 3' conserved regions. The 5' region is made up of the *int* gene, *attI*, and the promoter which drives transcription of genes within the variable region. The 3' region consists of an ethidium bromide resistance locus (qacED1), a sulfonamide resistance gene (*sulI*), and an open reading frame containing a gene of unknown function (Collis *et al*., 1998).

Class 1 integrons are mobile DNA elements that often encode one or more antimicrobial resistance genes (Winokur, 2003). At the present time, about 60 different cassettes associated with resistance genes have been identified, and the same cassettes can be found in different classes of integrons (Guerra *et al*., 2000). Primers have been designed that amplify the variable cassette regions where these genes are inserted (Winokur, 2003). Integron PCR has been used for identification and to complement other typing techniques in studies of *S.* Virchow, the variability of the inserted integrons cassette was used to differentiate strains. These locus specific PCR technique show good reproducibility within and between laboratories, but care must be taken with integron analysis performed over time since these mobile DNA elements (Martin MC, 2001).

e) Arbitrary Primed Polymerase Chain Reaction (AP-PCR)

Arbitrary Primed PCR (AP PCR), also referred to as the randomly amplified polymorphic DNA assay, is a DNA fingerprint technique that uses short (typically 9-15 bp) random sequence primers that hybridize at multiple random chromosomal sites (Winokur, 2003). It is a variation of the PCR technique employing a single short primer that is not targeted to amplify any specific bacterial DNA sequence. Rather, at low annealing temperatures, the primer will hybridize at multiple random chromosomal locations and initiate DNA synthesis (Tenover *et al*., 1997). If one copy of the primers binds to one strand of DNA, and another copy of the primer binds on the opposite strand of DNA but in

proximity of the first primer, a DNA fragment will be synthesized and amplification of that fragment will occur (Fadl *et al*., 1995; Lin *et al*., 1996; Tenover *et al*., 1995).

The number and location of these random primer sites vary for different strains of a bacterial species. Thus, following separation of the amplification products by agarose gel electrophoresis, patterns of bands, which in theory, are characteristic of the particular bacterial strain results (Olive and Bean, 1999). All isolates are typable and no prior knowledge of target genome sequences is necessary and the material costs and labor are low. However, identification of appropriate primers is arbitrary and requires significant test development time.

Lin and his colleagues (1996) screened 65 primers before selecting six primers that resulted in multiple AP PCR banding patterns from *S.* Enteritidis isolates. After this laborious primer selection process, AP PCR did perform better than phage typing, ribotyping and PFGE. In a study conducted in Brazil, to type 30 strain of *S*. Typhi, it was found conventional phenotyping methods as well as the DNA plasmid analysis, presented non-significant discriminatory power, however, RAPD-PCR analysis showed discriminatory power, reproducibility, easy interpretation and performance over phage types and plasmid DNA analysis, three phage types, four plasmids profile and 8 genotypic pattern were detected (Quintaes *et al*., 2002).

Fadal and his colleague (1995) proved that the superiority of RAPD over phage in typing *S.* Enteritidis isolates (three phages types and AP-PCR generated seven distinct random amplified DNA pattern were detected), while in certain serotypes, such as *S*. Dublin, ribotyping or other techniques have been superior (Fadl *et al*., 1995; Keriuanton *et al*., 1999). Even though AP-PCR is rapid and relatively inexpensive, the reproducibility and discriminatory power of this technique is a subject of active discussion and investigation and has several draw back. Banding patterns can vary with pH, magnesium and DNA concentrations and the source of DNA polymerase (Tenover *et al*., 1997).The banding patterns, too, are typically comprised of several dominant and several less intense bands. The intensity of these faint bands can vary from gel to gel and can complicate computer based gel analysis. This technique has poor inter-laboratory reproducibility and is best used

to evaluate a set of isolates analyzed in a single amplification reaction separated on a single gel (Tenover *et al*., 1997).

1.2.11.3. Sequence-based typing

Typing schemes based on variation in particular DNA sequences have the advantage of being digital in nature. This means that the same results should be achieved wherever the test is performed and any comparison of results is simple, quantitative and absolute in nature. Sequence-based typing schemes can also be considered as classification schemes and so genetic and evolutionary inferences can be made (Cooke *et al*., 2006).

Current DNA-sequence-based typing methods include the detection of DNA repeats and single nucleotide polymorphisms (SNPs). Variable Number of Tandem Repeat variation (VNTR) does not penetrate into the actual DNA sequence but produces data on the copy number of short repetitive sequences of individual isolates, by determining the size of the PCR product (Lindstedt, 2005). To date, VNTR seems to differentiate reliably between isolates of *S.* Typhimurium, but requires modification to be applied successfully to *S.* Enteritidis isolates.

The availability of a large amount of genomic sequence from different strains of *Salmonella* in combination with microarray technology opens yet another perspective for typing of *Salmonella*. Microarrays containing all known sequences from *S. enterica* serotypes have already been prepared and used to interrogate the gene content of different serotypes. Although not currently suitable for diagnostic laboratories these methods may find utility in reference labs for defining some aspects of genetic diversity such as antibiotic resistance. Other sequence-based methods that have been used include analysis of gene profiles coding for antibiotic resistance and pathogenicity markers (e.g. use of real-time PCR to distinguish mutations in the *sopE* virulence and *fliC* gene in *S.* Typhi (Mortimer *et al*., 2004).

a) Multilocus Sequence Typing

Nucleotide sequence-based methods for bacterial typing (multilocus sequence typing; MLST) allow rapid and global comparisons between results from different

laboratories. Combining this advantage with the reduced cost of high throughput sequencing, increasing automation and the amenability of sequence data for evolutionary analysis, it seems inevitable that sequence based typing will eventually predominate over phenotypic methods like serotyping (Ikumapayi *et al*., 2007; Kidgell *et al*., 2002).

Multilocus sequence typing (MLST) was developed by Maiden *et al*. (1998), for the naturally transformable Gram-negative pathogen *Neisserria meningitidis* but has since been applied to many pathogenic species and, recently, Ikumapayi and his colleague (2007) used MLST to characterize 62 invasive NTS isolates among children aged 2-29 months in rural Gambia and got ten different sequence types (STs). For example, in the case of *S.* Typhi for MLST analysis seven housekeeping genes were selected from the genome of *S.* Typhi CT18 on the basis that they are scattered around the chromosome, are flanked by genes of known function and that neither the gene chosen for sequencing nor the flanking genes are likely to be under diversifying selection (Kidgell *et al*., 2002).

The genes used were *aroC* (chorismate synthase), *dnaN* (DNA polymerase III beta subunit), *hemD* (uroporphyrinogen III cosynthase), *hisD* (histidinol dehydrogenase), *purE (*phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase) and *thrA* (aspartokinase+homoserine dehydrogenase) (Kidgell *et al*., 2002). The procedure is essentially an updated version of multilocus enzyme electrophoresis (MLEE), except that allelic types are determined by the sequence of house keeping genes rather than by the electrophoretic mobilities of the enzyme they encode (Maiden, 2006). A serious drawback of MLEE, and other gel-based methods such as pulsed-field gelectrophoresis (PFGE), is that it is often difficult to compare results between laboratories (Cooper and Feil, 2004).

MLST utilizes variability in the sequences of particular genes, due to mutation or recombination events, to determine the relatedness of bacteria. With MLST, multiple genes with conserved sequences are compared for nucleotide base changes. Housekeeping genes (genes required for basic cellular functions) are most often sequenced because they are present in all isolates and are not subject to strong selective pressures that can lead to relatively rapid sequence changes (Foley *et al*., 2006). MLST is used for long-term epidemiology and for the identification of lineages that have an increased propensity to cause disease (Urwin and Maiden, 2003).

Compared to other molecular techniques MLST has more discriminatory power among *Salmonella* isolates (Foley *et al*., 2006). Among the 128 *Salmonella* isolates tested, Foley and his colleagues (2006) were able to observe 84 Rep-PCR profiles, 86 PFGE patterns, 89 MLST patterns, 36 plasmid profiles, and 38 susceptibility profiles.

Even though MLST originally was defined as a sequencing-based subtyping approach that includes the sequencing of 450 to 600 nucleotide fragments for six to seven housekeeping genes (Foley *et al*., 2006; Sukhnanand *et al*., 2005; Urwin and Maiden, 2003), *Salmonella* MLST schemes described in the literature have sometimes used sequencing of three to four genes (Sukhnanand *et al*., 2005). Some of these MLST schemes also included the sequencing of virulence or virulence-associated genes, e.g., *spaM*, fimA, *manB and mdh* which were shown to provide discriminatory power similar to that of a seven-gene MLST (Sukhnanand *et al*., 2005).

In a study conducted in USA on population based comparisons of human and cow associated *Salmonella* subtypes and to evaluate the potential for cow-associated subtypes to be transmitted to humans, a collection of 179 human and 156 bovine clinical *Salmonella* isolates obtained from across New York state over the course of 1 year was characterized using serotyping and a multilocus sequence typing scheme based on the sequencing of three genes (*fimA*, *manB*, and *mdh*). The 335 isolates were differentiated into 52 serotypes and 72 sequence types (Alcaine *et al*., 2006). Therefore, the above experimental results indicate that MLST may be a good molecular epidemiological option to replace serotyping.

b) Sequences of the gene coding for phase 1 flagellin (*fliC*)

Salmonella is unique among the members of the family *Enterobacteriaceae* , as it commonly has two distinct flagellar antigens phase 1 and phase 2, that are coordinately regulated, so that only one flagellar antigen is expressed at any time (Kilger and Grimont, 1993). Serotyping by the traditional Kauffmann-White scheme is time consuming, requires well-trained technicians, and uses large amounts of high-quality sera. For these reasons, the use of DNA methods, such as the multiplex PCR for H-antigen identification, is an attractive alternative to the more traditional techniques (Herrera-Leon *et al*., 2004).

The *fliC* and *fljB* genes encode the phase-1 and phase-2 flagellins, respectively. These genes are coordinately expressed by a phase-variation mechanism. *fliC* is located in one of the flagellar biosynthesis operons, is present in all *Salmonella,* and has a homologue in *Escherichia coli*. *fljB* is located in a region of the genome that is unique to *Salmonella* and is present in four of the six subspecies. Isolates of *S. bongori* have been reported to have a gene homologous to *fljB,* although this species is typically monophasic. A triphasic isolate that was genetically described possessed the third flagellin gene, *flpA*, on a plasmid (Jacob and Jenabian, 2005; McQuiston *et al*., 2004).

The *fliC* and *fljB* genes are found at two different locations on the chromosome. Comparison of the amino acid sequences of *Salmonella* flagellins has led to the definition of eight variable regions. The amino- and carboxy-terminal sequences (regions I and II and region VIII, respectively) are conserved particularly at the 5' and 3', across most bacterial species and are thought to be important for polymerization and transportation. The central region, which comprises regions IV, V, and VI, is highly variable in both sequence and length between flagellar antigen genes and, encodes the surface-exposed and antigenically variable portion of the filament. The central region corresponds approximately to amino acids 181 to 390 (Herrera-Leon *et al*., 2004). *Salmonella* exhibits 70 serologically distinct flagellins, used internationally to diagnose and track infections. The terminal sequences of flagellin protein subunits are conserved in a range of bacteria and are here used as evolutionary markers to reveal how new serotypes arise (Mortimer *et al*., 2007). The remarkable feature of *fliC* alleles is the sequence conservation of distal parts of the gene, thus making the gene of any serotype suitable for easy amplification.

Jacob and Jenabian (2005) sequenced the variable region of the flagellin gene, *fliC*, from 96 *Salmonella* strains representing 51 different phase 1 H antigens and they found unique sequences for 45 of the 51 different antigens. This study showed that sequence-based typing of the phase 1 H antigen of *Salmonella* is a good alternative to serotyping when strains are non-typable by serological methods.

Table1.4: Additional Genotyping/sequence based techniques applicable for *Salmonella* species (Adapted from Cooke *et al.*, 2006).

Technique	Brief description	Advantage	Disadvantage
Restriction digest of plasmids	Digestion of plasmid DNA with a restriction enzyme	Useful for describing the spread of resistance in bacterial populations	Can only be used for a strain that contain similar plasmids. Tells you nothing about the bacterial host
PCR for specific genes, or islands	PCR for resistance genes, pathogenicity factors or metabolic markers	Straightforward to apply to isolates with known genetic difference(s)	To identify candidate genes is difficult. Is used to differentiate within serotypes, not yet available to define serotypes
Variable number of tandem repeats (VNTR)	Size of PCR products represents copy number of short repetitive sequences	Robust and reproducible, can be automated. Has been used for Typhi and Typhimurium.	Needs to be defined for each of the current serotypes, may not discriminate as well as PFGE
IS200 typing or ribotyping	Analysis of IS200 multicopy elements or rRNA genes using either restriction digests or Southern blot	IS200 elements remain fairly constant in natural populations of bacteria. Has been used for several serotypes	Discrimination between strains is not very big
Microarrays	DNA–DNA hybridization of the whole genome against known sequences. Measures gene content	The presence or absence of genes can be defined across the whole genome of several isolates. Excellent at describing genetic diversity	High cost; Detection of point mutations difficult. Can only detect features represented on array, cannot recognize novel insertions etc.

Several criteria are proposed for evaluating the performance of typing systems. These criteria include: typability, reproducibility, discriminatory power, and ease of performance (Tenover *et al*., 1997). Beside fulfillment of the above-mentioned criteria, the ideal typing system should be rapid, inexpensive, easy to interpret and technically simple. Because there is no optimal typing system that meets all the above requirements, it is as a rule necessary to use a combination of systems (Arbeit, 2000).

1.2.12. Salmonellosis in Ethiopia

1.2.12.1. Prevalence

In Ethiopia, like other developing countries, it is difficult to evaluate the situation of salmonellosis. This is mainly because of the very limited scope of studies, lack of coordinated epidemiological surveillance system and inadequacy of laboratory facilities for culture. In addition, under reporting of cases and the presence of other diseases considered to be of high priority may have overshadowed the problem of salmonellosis in some countries, including Ethiopia.

Typhoid fever is endemic in Ethiopia, as it is in most developing countries in Asia, Africa, and Latin America (Fekade, 2001). In Ethiopia, even though it is believed that typhoid fever is a public health threat, there are no reliable data to estimate its prevalence. This is mainly due to lack of laboratory facilities to confirm the causative agent and most diagnosis is based on clinical features coupled with Widal test, which has limited value (Awole, 2004). Infections caused by non-typhoidal salmonellae are often reported under gastroenteritis.

To our knowledge, the first published report on salmonellosis in Ethiopia was in 1972 where 5 year statistics 1959 through 1963 of the Anti-epidemic Service showed that an average of 3,469 cases of salmonellosis was reported per annum, i.e. 17.3 cases per 100,000 inhabitants (Schaller, 1972). It was noted that, cases of typhoid fever were almost three times the number of that of the other types of salmonellosis. In 1975, another researcher (Wallace, 1977) reported that of over 700 stool specimens processed, about less than 1% of the cultures grew only *S.* Typhi.

The first comprehensive study on the serogroup of *Salmonella* species in Ethiopia was conducted by Gedebuo and Tassew (1981) in Addis Ababa. In this study, a total of 165 *Salmonella* isolates, collected from various hospitals in Addis Ababa from 1975-80, of which 131 (79.4%) were from blood, 18 (11%) from stool and the rest 16 (9.7%) were from other specimens. Of the 165 isolates, 7 were group A, 16 B, 1 E, 17 C and the remaining 124 were *S.* Typhi. Since most of the specimens available were blood collected from inpatients with suspected typhoid fever, this may not reflect the true isolation rate of non-typhoidal

salmonellae from stool. However it is clear that typhoid fever was a major cause of community acquired bacteraemia at that time.

From 1974 to 1981 Gebre-Yohannes conducted a study to identify the prevalent serotypes and their susceptibility to drugs in Addis Ababa, which serves as a base-line of data for further surveillance of *Salmonella* species in Ethiopia (Gebre-Yohannes, 1985). He studied 216 *Salmonella* strains which were predominantly isolated from adult patients referred from different hospitals to the Central Laboratory and Research Institute, Addis Ababa between January 1974 and October 1981. Serogroups B, C, D, E and A were isolated at frequencies of 20.4%, 19.4%, 6.9%, 2.8% and 1.9% respectively. Out of the 216 *Salmonella* strains, 48.6% were *S.* Typhi. Most of the *Salmonella* isolates were from stool (54.6%), followed by blood (34.7%), pus (5.6%) and urine (5.1%). This base-line study identified the existence of 26 different serotypes among the 216 *Salmonella* isolates. In his findings, *S.* Concord (12.5%) and *S.* Typhimurium (11.1%) predominates the non-Typhi isolates. The high isolation rate of *S.* Concord in Ethiopia was unusual in contrast to high prevalence of *S.* Typhimurium elsewhere (Lepage *et al*., 1984; WHO, 1982) and the author suggested further study should be done to clarify the animal or food source associated with its epidemiology.

Similarly, Ashenafi and Gedebou (1985) conducted a study to determine the frequency and drug sensitivity of *Salmonella* and *Shigella* in the etiology of diarrhoea from adult out-patients in Addis Ababa. For this study, a total of 1000 adult diarrhoeal cases from different hospitals and clinics were investigated. The strains were from patients in various hospitals and clinics isolated from 1982-83. They isolated a total of 45 *Salmonella* isolates in the decreasing order: Group C, B, *S.* Typhi, other Group D, Group A, and Group E. In another study conducted in a rehabilitation camp in Korem, a total of 42 (21.1%) of the camp residents had a positive culture with *Enterobacteriaceae*, with an isolation rate of 15.6% for *E. coli*, 3.5% for *Shigella* spp. and 2% for *Salmonella* species (Desenclos *et al*., 1988).

Salmonella can cause food poisoning in humans, as demonstrated by Aseffa and his colleagues (1994), who isolated, between December and January 1992, nine *S.* Newport from an outbreak of food poisoning among college students in Gondar, believed to be due to

contaminated eggs. In their investigation, *S.* Newport *w*as isolated from the stool of six students and three food handlers, none of the food source remained available for analysis.

S. Newport is a rather infrequent isolate in series reports in Ethiopia as elsewhere, such as 2.3% in previous study in Ethiopia (Gebre-Yohannes, 1985) and 0.14% in Uganda (Lumbwama, 1985) where only five cases of *S.* Newports were isolated in 15 years.

Asrat (2008) reported that among the 37 *Salmonella* strains collected during the period of 1992-1993 in Tikur Anbesa Hospital, the most common serogroup was group B (81.1%), followed by group D (*S.* Typhi) (10.8%) and group C (8.1%).

In a study determining the prevalence of *Shigella* strains and *Salmonella* species among the outpatients of the Gondar College Teaching Hospital (1994-1996) it was found that out of 7,993 miscellaneous specimens cultured, 147 *Shigella* and 80 *Salmonella* isolates were identified (Aseffa *et al*., 1997). In this study, serogroup B was the most frequently isolated at 61%.

Another study conducted in Addis Ababa to determine *Salmonella* serogroups in adult diarrhoeal outpatients in 1995 (Mache *et al.,* 1997) forty five *Salmonella* strains were isolated from 700 stool specimens. Among the isolates, serogroup C comprised 31.1%, B 24.4%, *S.* Typhi 15%, D (other than *S.* Typhi) 13.3%, A 8.9% and E, 6.7%. The 6.4% *Salmonella* isolation rate in this study is not dissimilar to the isolation rate of 4.5 % (Ashenafi and Gedebou, 1985) reported a decade previously. Of all the *Salmonella* isolates in this study, 84.4% belonged to non-Typhi serogroups, which reflects that the majority of *Salmonella* related infections are acute gastroenteritis.

In 1993-1996, Wolday studied *Salmonella* strains isolated from patients hospitalized in the Tikur Anbessa Hospital, Addis Ababa, between 1993 and 1996. He isolated 110 *Salmonella* strains, of which 62 (56.4%) were non-Typhi salmonellae (Wolday, 1998). The high isolation rate of NTS in this study is in agreement with previous studies conducted in Ethiopia (Ashenafi, 1983; Gebre-Yohannes, 1985; Gedebou and Tassew, 1981; Mache *et al*., 1997).

Diarrhoea has become a major clinical problem in HIV-infected patients, which implies that there is a need to monitor the causative agents and their antimicrobial susceptibility patterns in order to ensure appropriate treatment and control of infections. In

this regard, a retrospective study was conducted on cases of *Salmonella* infections occurring between 1991 and 1995 among HIV-infected and uninfected patients. Of the total 49 *Salmonella* infected patients/cases, 28 cases (18 sero-positives and 10 sero-negatives) were due to NTS infection whereas the other 21 cases (9 sero-positives and 12 sero-ngatives) were due to *S.* Typhi infections. Although *S.* Typhi was more frequent among HIV-uninfected than HIV-infected patients (24.5% versus 18.4%), non-typhoidal *Salmonella* infection was more common in the HIV-infected group (36.7% versus 20.4%) (P=0.029) (Wolday and Erge, 1998).

Salmonellosis caused by non-typhoidal *Salmonella* is particularly common in children of developing countries. In a study conducted in Jimma Hospital, South West Ethiopia, from March to July 2000, a total of 59 *Salmonella* strains were isolated from 384 pediatric outpatients with diarrhoeal illness (Mache, 2002). Of these, Serogroup A comprised 5 (8.5%) isolates, B 17 (28.8%), C 13 (22%), D 8 (13.6%, other than *S.* Typhi), E 3 (5.1%) and *S.* Typhi 13 (22%). The most frequently isolated serogroup was B (28.8%) while the least frequent was group E (5.1%). The increased isolation rate *Salmonella* in Jimma diarrhoeal pediatric outpatients, may indicate the poor sanitary condition of the town and endemicity of the isolates in the area.

The research conducted by Faris and Kaba in 1999, indicated that the local practice of sanitation was far from satisfactory and that the personal hygiene status of house mothers responsible for food preparation and child rearing was poor (Faris and Kaba, 1999). Mache (2002) reported that of all his *Salmonella* isolates, 78 % (46/59) belonged to non-Typhi serogroups, which in turn indicates that these serogroups are responsible for the majority of diarrhoea in children. In many developing countries, non-Typhi serogroups have become serious pathogens especially among immuno-suppressed adults, particularly those with HIV/AIDS (Arthur *et al*., 2001).

A cross-sectional study was conducted from Feburary to July 2001 in Jimma Hospital, South West Ethiopia (Awole *et al*., 2002). The aim this study was to isolate and determine the magnitude of potential bacterial pathogens in the stool of HIV-positive and negative patients and their antimicrobial resistance profile. A total of 372 patients (192 HIV-positive and 180 HIV-negative) were selected and stool specimens were collected and

cultured. Among 99 HIV-positive patients with diarrhoea, a total of 8 (8.1%) *Salmonella* strains were isolated. This result is in agreement with studies conducted in Rwanda (11%) and Argentina (5%) (Clerinx *et al*., 1995; Olmos *et al*., 1996) but much lower than the previous study conducted in Ethiopia (55.1%) (Wolday and Erge, 1998).

Another recent study in 2000-2001, compared the prevalence of *Yersinia enterocolitica* isolates with other commonly encountered enteropathogens causing diarrhoea among patients in Addis Ababa. This study showed that among stool samples of 205 patients 3 (1.5%) were positive for *Y. enterocolitica*, 22 (10.7%) for *Salmonella* and 12 (5.8%) for *Shigella* (Andualem and Geyid, 2003). The frequency of isolation of *Salmonella* in their study (10.7%) was much higher than the isolation rate in all age groups (Asrat *et al*., 1999) or from adult outpatients (4.5%) (Ashenafi, 1983). From this previous data, the isolation rate of both *S.* Typhi and NTS appears to be decreasing from time to time. This does not mean that *Salmonella* infection in the country is deceasing rather the reverse seems true. In the early 1970's and 80's there were only two laboratories (Central Laboratory and Research Institute and Black Lion Hospital) with cultural facilities and almost all hospitals in the country were referring *Salmonella* suspected case to these laboratories.

Most of the information on *Salmonella* isolates in these laboratories was published in different journals. But later more laboratories were established in different parts of the country. Despite the presence of more *Salmonella* cases and more *Salmonella* isolates on their daily record books in these laboratories, there is no published data except reports in very few researches conducted in some places of the country.

Compared to the isolation rate of *S.* Typhi, there is more isolation of NTS from time to time; this could be due to the increase of immunosuppressed individuals, particularly with HIV/AIDS which are more prone to NTS infection.

1.2.12.2. Drug resistance profile

The extent of antibiotic resistance in developing countries like Ethiopia is difficult to evaluate. *Salmonella* are not routinely cultured and their resistance to the antibiotics, commonly used in both veterinary and human medicine, is seldom assessed.

The first published report to our knowledge on drug resistance of *Salmonella* species in Ethiopia was in 1981, by Gedebou and Tassew who conducted a research with the aim of determining and assessing the antimicrobial resistance profile and R factor presence in 124 *S.* Typhi and 41 non-Typhi isolates. The disk diffusion method was performed to test isolates against commonly prescribed drugs. Most of the 165 *Salmonella* strains were susceptible to all the antimicrobial agents except streptomycin and sulfadiazine. Seventy one percent, 31%, and 94% of serogroups A, B, and C were resistant to two or more drugs respectively. A higher percentage of single and multiple drug resistance were seen in serogroup C isolates than others. All non-Typhi salmonellae were sensitive to gentamicin, polymixin B, and trimethoprim-sulphamethoxazole, which is comparable to other studies conducted in different parts of the world at that time (Paramasivan *et al*., 1977; Tanaka *et al*., 1976).

In 1985 Gebre-Yohannes isolated 27 *S.* Concord and tested to commonly used drugs and the result showed that all *S.* Concord isolates were MDR and approximately 63% of them were resistant to 8 drugs (cephalothin, tetracycline, chloramphenicol, ampicillin, carbenicillin, kanamycin, streptomycin and sulphadiazine). *S.* Braenderup *S.* Montevideo and *S.* Infantis, belonging to the same sub-group as *S.* Concord, were also multiple drug resistant. The other *Salmonella* serotypes were sensitive to most drugs used in the study.

Gebre-Yohannes (1985) concluded that the high incidence of the multi-resistant *S.* Concord in Ethiopia is difficult to explain. A drug-sensitive strain of this species was identified only once previously, from a bone-processing factory in Addis Ababa (Pegram *et al*., 1981). Gebre-Yohannes (1985) reported that all 27 *S.* Concord isolates were multi drug resistant (5 to 9 drugs) and, there has not been a similar report of drug resistance in this serotype during the time of his findings. However the investigator indicated that multiple drug resistance to at least 6 drugs has been demonstrated in clinical isolates of *S.* Wien, *S.* Oranienburg, *S.* Isangi, *S.* Saintpaul and *S.* Newport which are found in the same serogroup.

Gebre-Yohannes also commented that further surveillance of isolates of multi-resistant *S.* Concord in Ethiopia is needed to define its role in outbreaks. Other members of Subgroup C1 (*S,* Braenderup, *S.* Oranienbur*g* and *S.* Infantis) were MDR; their isolation rate was, however, much lower (usually one). MDR strains, which don't include resistant to chloramphenicol, were noted in 4 out of 24 isolates of *S.* Typhimurium as well.

Ashenafi and Gedebou, (1985) isolated 45 *Salmonella* strains and the susceptibility data showed all isolates were sensitive to gentamicin, polymyxin B and trimethoprim-sulphamethoxazole. Sensitivity to the remaining eight drugs varied between 73% and 83%. The single isolates of Group A and E *Salmonella*, almost all group D *Salmonella* other than *S.* Typhi and most Group B isolates were sensitive to all 11 drugs. Group C strains were sensitive only to gentamicin, polymyxin B and trimethoprim-sulphamethoxazole. About 69% of all isolates were sensitive to the 11 drugs. Multiple drug resistance was detected in 22.2% of *Salmonella* all of which were group C. Of the seven different resistance patterns noted, the most common was ampicillin, carbenicillin, chloramphenicol, kanamycin, streptomycin, sulphadiazine, tetracycline, which was 18%. Asrat (2008) showed that among all antibiotics tested for *Salmonella* species, isolated during the period of 1992-1993, the highest resistance was observed with ampicillin (81.2%), cephalothin (86.4%), chloramphenicol (83.7%), erythromycin (100.0%) gentamicin (75.6), sulfonoamide (81.1%) tetracycline (94.5%) and trimethoprim-sulfamethoxazole (75.7%).

Aseffa *et al.,* (1997) reported multiple drug resistance among the 80 *Salmonella* isolates from the Gondar area, north-west Ethiopia, tested against the 5 commonly used drugs: ampicillin, chloramphenicol, gentamicin, tetracycline and cotrimoxazole. Mache (2002) in a study conducted in South-West Ethiopia, described susceptibility of all serogroups to polymixin B and gentamicin as 100% and 91.1% respectively. Between 28.9 % and 62.2% of this isolates were sensitive to the other antibiotics. Among all the antibiotics tested the highest resistance in each *Salmonella* serogroup was observed to ampicillin, tetracycline and cephalothin. Although the studies were conducted at different time, regions and age groups, this observation was in contrast to with findings of Messele and Alebachew (1981) and of Mogessie (1983) where more than 98.4% and 77.8%, of the isolates in Addis Ababa were susceptible to most tested antibiotics respectively. According to Mache's study (2002), a

total of 31 distinct antibiograms (resistance patterns) were found in the *Salmonella* strains tested. Multiple resistances were frequently encountered to ampicillin, tetracycline, cephalothin, trimethoprim-sulfamethoxazole and kanamycin. One strain in group B and one in group C showed simultaneous resistance to seven antibiotics and one strain in group C showed resistance to eight antibiotics. The resistance antibiograms observed by Gedebou and Tassew in 1981, mostly consisted of sulphadiazine and streptomycin resistance combinations, while in Maches's (2002) study, resistance to ampicillin and tetracycline combinations were dominant, at the same time polymixin B, gentamicin and carbenicillin were found to be the drugs of choice according to in vitro susceptibility.

Later Wolday conducted a study on the increasing incidence of multidrug resistant *Salmonella* in Ethiopia. He studied 110 *Salmonella* isolates (48 *S*. Typhi and 62 non-Typhi salmonellae) collected between 1993 and 1996 from hospitalized patients in Tikur Anbessa Hospital, Addis Ababa. He tested the isolate against commonly used drugs: ampicillin, carbenicillin cephalothin, chloramphenicol, gentamicin, kanamycin, tetracycline, amikacin, cefuroxime and trimethprim/sulphamethoxazole (Wolday, 1998). The study showed that thirty-five (31.8%) isolates were susceptible to all of the antibiotics tested, while 29 (26.4%) were resistant to only one drug (most commonly trimethoprim/ sulphamethoxazole) and five (4.5%) were resistant to two drugs (most commonly tetracycline and trimethoprim/ sulphamethoxazole). The remaining 41(37.3%) were resistant to three or more drugs. Cefuroxime was the only drug to which all strains were susceptible. The majority of non-Typhi salmonellae were resistant to chloramphenicol (61.7%), ampicillin (59.7%), carbenicillin (53.2%) and trimethprim-sulphamethoxazole (51.6%), while approximately 30% were resistant to each of gentamicin, kanamycin, cepahalothin and tetracycline. Only 4.8% of the strains were resistant to amikacin and there was no resistance to cefuroxime. This study demonstrated a marked increase in the incidence of multiple drug resistance among *Salmonella* species isolated in Ethiopia, i.e. 37.3 % compared with 13.9% and 18.1% reported by earlier groups of investigators (Gedebou and Tassew 1981; Gebre-Yohanes, 1985). Another study in Ethiopia showed that that 85% of the *Salmonella* species isolated from blood culture were sensitive to chloramphenicol and trimethoprim-sulphamethoxazole (Asrat and Amanuel, 2001).

There are an increased number of reports on multiple drug resistant salmonellosis both in developed and in developing countries caused by both *S.* Typhi and non-typhoidal species, which has been also associated with the advent of the HIV infection. Wolday and Erge (1998) studied *Salmonella* isolates from both HIV-infected and HIV non-infected patient. Their study findings showed that NTS isolated from HIV-positive patients exhibited a higher incidence of resistance rate (>55%) for most of the antibiotics tested. They also noted that the frequency of multiple drug resistance was significantly higher among the non-typhoidal *Salmonella* recovered from patients who were positive for HIV.

On the other hand similar study was done aimed at isolation of potential enteric bacterial pathogens from the stool of HIV-infected and HIV-non infected patients and their antimicrobial susceptibility patterns in Jimma, Hospital, South west Ethiopia (Awole *et al.*, 2002). This study showed that, 90% of *Salmonella* isolates from both patient groups were resistant to doxycycline, amoxicillin, carbenicillin, and streptomycin. The most extensive drug resistance pattern encountered was to amoxyciline, cephalothin, chloramphenicol, trimethoprim-sulfamethoxazole and doxycycline. This is in agreement with the findings reported from Jimma among the general population (Beyene and Abdisa, 2000).

Salmonellosis caused by drug resistant *Salmonella* strains is a major health problem, especially among children in developing countries like Ethiopia. A study report by Mache (2002), to determine the prevalent *Salmonella* serogroups and resistance pattern of the isolates to commonly used antibiotics among children in Jimma, South West Ethiopia, showed that, all the isolates were 100% susceptible for polymyxin B and gentamicin except one strain in serogroup D and B respectively, while 91% of the isolates were susceptible to nalidixic acid. The susceptibility of all strains in each serogroup to ampicillin and tetracycline was below 48%. Between 52.5%, to 74.6% of the isolates were sensitive to the other antibiotics. Gentamicin, polymyxin B and nalidixic acid were found to be the most effective antimicrobials, at least in vitro, where as tetracycline, ampicilln and cephalothin were the least effective for all serogroups. Ninety three percent of the isolates were found to be resistant to one or more drugs.

Mache (2002) also showed that 31 distinct antibiograms were present in *Salmonella* strains which varied from resistance to a single antimicrobial agent and up to six. This base

line study on susceptibility pattern of serogroup of *Salmonella* in Jimma indicated that, the most common resistance antibiograms among *Salmonella* isolates were those combinations containing ampicillin, tetracycline, cephalothin and chloramphenicol. More than 52% of *Salmonella* isolates in each serogroup were resistant to tetracycline and ampicillin and also 25 to 45% of the isolates were resistant to trimethoprim-sulphamethoxazole, cephalothin, chloramphenicol, and carbenicillin. This is much higher than previous studies reported from Addis Ababa and North West Ethiopia in late 1990's (Mache *et al.*, 1997; Assefa *et al.*, 1997). The resistance to all other antibiotics in the above study is also much greater than that reported by authors in the 1980's.

A recent study on determination of antimicrobial susceptibility and resistance of *Yersinia enterocolitica*, *Shigella*, *E. coli* and *Salmonella* strains from diarrhoeal out-patients in Addis Ababa showed that, all *Salmonella* isolates were sensitive to norfloxacin, polymyxin B and nalidixic acid (Andualem and Geyid, 2005). This susceptibility pattern was comparable with the study result of research in Jimma (Mache, 2002). While 21(95.5%) of the strains were sensitive to gentamycin and Kanamyci resistance level of 86.4% to cephalothin, 36.4% to ampicillin and 27.0% to sulphadiazene were recorded and 22.7% of these isolates showed resistance to both streptomycin and trimethoprim-sulphamethoxazole. It was also found that eleven different combinations of resistance patterns were observed for all the *Salmonella* species.

In Ethiopia *Salmonella* serotypes isolated from food animals and foods of animal origin also showed high level of resistance against different antimicrobial agents, for example, 31.8% of *Salmonella* isolates from sheep and goat (Molla *et al*., 2006b), 44.4 % of isolates from camels, (Molla *et al*., 2004), 52% isolates from cattle and 87.2% isolates from minced beef and chicken (Molla *et al*., 1999) were resistant to one or more antimicrobials commonly used to treat both human and animal infections.

Therefore, the isolation of drug resistant *Salmonella* strains from a wide range of sources, from humans, food animals and animal food products underlines the need for judicious use of antimicrobial agents in both clinical practice and animal husbandry, as well as the need for joint and coordinated routine surveillance and monitoring programs of antimicrobial resistance patterns of important food borne pathogens.

1.3. SIGNIFICANCE OF THE STUDY

Salmonellosis is a major health problem of mainly developing countries where a conditions for the existence of endemic disease are still favorable (WHO, 1998). Generally, it is difficult to estimate the real impact of salmonellosis in many developing countries, because the clinical picture is confusing with other febrile and diarrhoeal illnesses (WHO, 2003). Additionally the disease is under estimated, because there are no sufficient bacteriological laboratories for culturing in most areas of developing countries. These factors are believed to result in many cases going undiagnosed (WHO, 2003). In recent years increasing resistance of *Salmonella* species to commonly used antimicrobial drugs has become a matter of concern. The high rate of resistance is hampering the use of conventional antibiotics, and growing resistance to the newer antimicrobial agents is aggravating the situation. Genetic analysis has indicated that the source of resistance is frequently a transferable plasmid (Couturier *et al*., 1988; Su *et al*., 2004).

So far all base-line and follow up studies conducted by different investigators on salmonellosis in Ethiopia have shown the widespread distribution of multi-drug resistant *Salmonella* isolates in the community (Aseffa *et al*., 1997; Ashenafi and Gedebou, 1985; Asrat and Amanuel, 2001; Gebre-Yohannes, 1985; Gedebou and Tassew, 1979; Mache, 2002; Mache *et al*., 1997). The serovars associated with multiple resistances to clinically relevant drugs are found in different food animals and food products, suggesting the potential source of human *Salmonella* infections (Molla *et al*., 1999; Molla *et al*., 2003; Muleta and Ashenafi, 2001; Nyeleti *et al*., 2000; Tibaijuka *et al*., 2003). Some *Salmonella* isolates present in Ethiopia contain multiple plasmids including plasmids encoding antibiotic resistance (Gebre-Yohannes *et al*., 1987; Mache, 2002; Mache *et al*., 1997). The single serotype study shows that multi-drug resistant *S*. Concord was very common in Ethiopia (Gebre-Yohannes, 1985). This serotype has been rarely described from other countries (Barbour and Nabbut, 1982; Barbour *et al*., 1983). In addition to this, the high rate of resistance to commonly used antibiotics among isolates of *Salmonella* species in Ethiopia against the background of HIV/AIDS epidemic, threaten to undermine the successful treatment of patients infected with these pathogens (Awole *et al*., 2002).

Since the type of serotypes and resistance rate of the isolates may vary greatly in different geographical areas and with time, continuous surveillance must be undertaken both nationally and locally in order to develop national and local guidelines for antibiotic treatment. Identification studies should go to the level of serotypes, so that comparison with serotypes isolated from animals/ food products locally or else where in other countries will be possible and these data can be used for tracing of possible source of human infection. A continuous surveillance of the *Salmonella* serogroups and serotypes should be performed in order to: - observe the periodic change of serogroup and serotype and prevalence with time, detect introduction of new serotypes for each serogroup from other countries and enhance the capability of studying *Salmonella* epidemiology.

All the available information except one study on plasmid profile analysis at Addis Ababa, about *Salmonella* species in Ethiopia is based on phenotypic studies, which has its own limitations. To observe the relatedness of the different strain circulating within the country and to compare and contrast with strains elsewhere, molecular study is very important. Therefore, this study was undertaken to isolate and determine the phenotypic and molecular characterizations of *Salmonella* species from children with acute febrile illness and/or diarrhoea in Tikur Anbessa and Jimma specialized University Hospitals.

1.4. HYPOTHESIS

Salmonellosis in children is a major health problem in Ethiopia and is caused by similar serovars to those reported from Africa and elsewhere.

1.5. OBJECTIVES OF THE STUDY

I. General objectives

- To determine the magnitude of salmonellosis in children with suspected case of typhoid fever and diarrhoeal illness visiting Tikur Anbessa and Jimma University Hospitals.
- To perform phenotypic and molecular characterization on the isolated *Salmonella* species in order to know the circulating serotype, level of antibiotic resistance, molecular basis of resistance and genetic diversity.

II. Specific objectives

Phenotypic Characterization

- To serogroup and serotype the isolated *Salmonella* species using polyvalent and monovalent antisera. The occurrence of different serovars in one country can be of global importance because of travel and trade of breeding animals and food products worldwide.
- To determine the antibiotic susceptibility pattern of the isolated *Salmonella* species to selected antimicrobial agents using disk diffusion method in order to provide basic information for clinicians to treat the infected children with appropriate antibiotics.
- To investigate the presence of extended spectrum β-lactamase (ESBL) in *Salmonella* species using National Committee for Clinical Laboratory Standards (NCCLS) ESBL phenotypic confirmatory tests. *Salmonella* isolates resistant to cephalosporins due to the production of ESBL have emerged. This has also caused concern because cephalosporins are drugs of choice for the treatment of salmonellosis in children, to which fluoroquinolones must not be administered.

Molecular Characterization

- To characterize the isolated *Salmonella* strains using molecular methods such as plasmid analysis and incompatibility grouping, PFGE, MLST and *fliC* gene sequencing. Knowledge regarding the molecular epidemiology of different serovars of *Salmonella* in different countries and geographic regions may assist in the

recognition and tracing of new emerging pathogens and clonal relatedness among the isolates. In addition it aids in the identification of sources of infection during outbreak investigations, detection of cross transmission, recognition of particular virulent strains, and monitoring intervention strategies.

CHAPTER II

MATERIALS AND METHODS

CHAPTER II. MATERIALS AND METHODS

2.1. Study design and period

A prospective cross-sectional study was conducted to determine the magnitude of *Salmonella* infection in children (aged 6 month to 15 years) with febrile illness and diarrhoea from January 2006 to June 2008.

2.2. Study area

Ethiopia is situated in East Africa, bordered by Eritrea, Sudan, Kenya, Somalia and Djibouti (Figure 2.1). The land area of Ethiopia is estimated at about 1.1 million square kilometers and the current population is approximately 74 million, of which 84% live in rural areas (CSA, 2007). An estimated 60 to 80 percent of health problems are due to infectious/communicable diseases and nutritional problems (MOH, 2002/2003). Its capital city, Addis Ababa, lies in the middle of the country in the central highlands at an altitude of over 2300 meters and covers an area of 540 square kilometers. The total projected population of Addis Ababa is estimated to be 2,738,248 of which 1,304,518 (48%) are males and 1,433,730 (52%) are females (CSA, 2007).

The climate of Addis Ababa varies from seasons of summer, 9 months, to cool months of rainfall, lasting for about three months, with an overall average maximum and minimum temperature of 22.9^0C and 10.8^0C, respectively, and total mean rainfall of 1,195.5 mm/year.

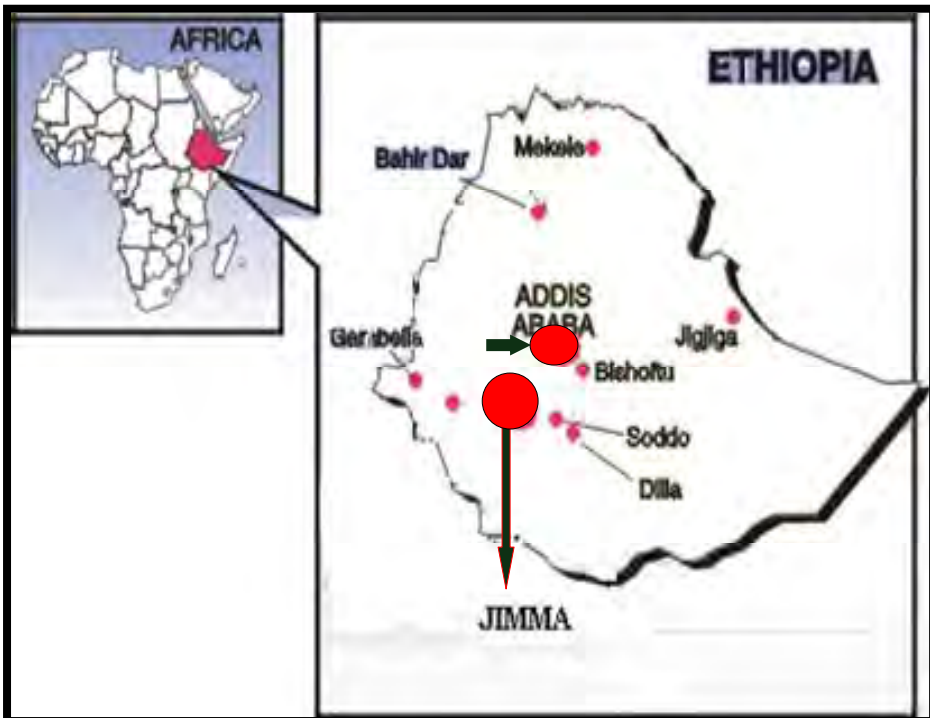

Figure 2.1: (Anonymous, 2009)

Jimma town is located 335km South West of Ethiopia. The town has an average altitude of 1760 meters above sea level with minimum and maximum temperature of 11^0C and 27^0C respectively. According to the 2007 national census, the total population of Jimma town was estimated to be 120,600. Jimma University Specialized Hospital is found in Jimma town. The hospital has 300 beds and provides curative and preventive service for 300-400 patients per day at its Out Patient Department. The Pediatric and Child Health Department with its 65 bed capacity gives inpatient services to patients younger than 15 years of age and manages 80-100 children daily in the Out Patient Department.

Tikur Anbessa Specialized Hospital is found in Addis Ababa and one of the 32 hospitals found in the capital city, and it is a central tertiary as well as a teaching hospital. The hospital was established in 1965 E. C. and has 1,262 rooms and run by Addis Ababa University. As a referral hospital, it provides various health services, including maternal and child health services. The four main departments which provide major services are: Internal medicine, Surgery, Pediatrics and Gynecology and Obstetrics departments using 558 beds.

2.3. Study subjects

A total of 1225 consecutive children (6 month to 15 years of age) with diarrhoeal illness and/or febrile illness from Paediatrics Department of Tikur Anbessa (n=825) and Jimma University (n=400) Hospitals and some selected health centres in Addis Ababa and Jimma were investigated for *Salmonella* infection.

Children below six months and above 15 years of age, whose parents did not agree to participate and took antibiotics before two weeks time for the same complaint, were excluded from this study.

Diarrhoea has been defined as the presence of at least three loose stools or one watery stool per day (Huilan S *et al.*, 1991). Fever has been defined as child whose axillary temperature is $\geq 37.5^0$C.

Sample size determination: - The sample size is determined based on the prevalence rate of the study done by Mache on children at Jimma (Mache, 2002) and calculated with the formula recommended by (Daniel, 1983). The estimate is desired to be with 5% margin of error and 95% confidence.

$$N = \frac{Z^2 \alpha P(1-P)}{d^2}$$

Where N = patient who should be sampled

$Z\alpha$ = the standard normal deviation corresponding, 95% of

Confidence level = 1.96

P = prevalence (15%)

d = the degree of accuracy desired (2%= 0.02)

$$N = \frac{(1.96)^2 \times 0.15 \times (1-0.85)}{(0.02)^2}$$

N = 1,225

Histories were taken from each child from informed and consented parents/guardians before sample collection by the attending qualified health worker. All the relevant data

(demographic, clinical, and laboratory data) were recorded and transferred to the questionnaire prepared for this study (see Appendix I).

In addition to 65 *Salmonella* strains isolated from 1225 children, 48 *Salmonella* strains from stock cultures were also analyzed. These strains were collected from January, 2004 to December, 2005 (before the actual date of data collection) and were isolated from children with similar age groups in Tikur Anbessa Hospital.

2.4. Collection, handling and transport of *specimens*

2.4.1. Stool

Freshly passed stool or rectal swabs specimens were collected, placed immediately in Cary Blair transport medium (Oxoid Ltd, Basingstoke, and Hampshire, England) and transported to the laboratory within 6 hours of collection.

2.4.2. Blood

About 2 ml of venous blood was drawn aseptically by cleaning the skin using tincture of iodine from each patient and placed into Brain Heart Infusion (BHI) broth (Oxoid, Ltd) containing 0.05% sodium polyanetholesulfonate (SPSS) (Oxoid, Ltd). A minimum blood-to-broth ratio of 1 in 10 was maintained (Collee *et al*., 1989). All stool and blood specimens were collected by the laboratory technician.

2.5. Culture and identification of *Salmonella* species

Blood culture broths were incubated at 37^0C and checked for sign of bacterial growth daily up to 7 days, and bottles which showed turbidity (visible signs of growth) were sub-cultured onto Deoxycholate agar (DCA) (Oxoid, Ltd) and Xylose lysin deoxycholate agar (XLD) (Oxoid, Ltd). These media were recommended for isolation of *Salmonella* from clinical specimens (Cheesbrough, 2000).

Blood culture broth with no bacterial growth after 7 days were sub-cultured before being reported as a negative result.

Stool/rectal swab specimens were inoculated on DCA and XLD and for enrichment in Selenite F enrichment broth (Oxoid, Ltd) and incubated at 37^0C for 24 hrs, then subcultured on DCA and XLD agar at 37^0C for 18-24 hrs.

The growth of *Salmonella* species was detected by their characteristic appearance on DCA (pale colonies) and XLD agar (red, black-centered colonies). Confirmatory identification was done by the pattern of biochemical reactions using API 20E identification kits and oxidase test (API systems S.A., Montalieu-Vercieu, France). *Shigella flexneri* (NBLSC 530) and *Salmonella* Typhimurium (NBIS-11) were used for quality control through out the study.

Isolates were kept frozen at -70^{0}C in 15% glycerol broth until phenotying characterizations (serotyping/sergrouping/ antimicrobial susceptibility testing) were done at the core laboratory of Tikur Anbessa and Microbiology Laboratories of Jimma University Hospitals.

Strains were kept on nutrient agar (Oxoid, Ltd) slant and butt and transported to The Wellcome Trust Sanger Institute, United Kingdom for molecular characterization (plasmid analysis, incompatibility grouping, PFGE, MLST and *fliC* gene sequencing).

2.6. Microscopic examination of stool specimens

Microscopic examination of stool specimens for ova and parasites was performed using saline and iodine solutions.

2.7. Phenotyping characterization

2.7.1. Serogrouping

Salmonella strains were serogrouped by slide agglutination tests using poly O and single O-groups A, B, C, D, E antisera (Remel, Europe Ltd, UK). These strains were further tested against poly H antisera. Those strains identified biochemically as *Salmonella* Typhi were also tested against Vi antisera.

2.7.2. Serotyping

Serotyping of *Salmonella* species isolates was performed after serogrouping on the basis of phase 1 and phase 2 flagellar antigens by tube agglutination tests with known antisera according to the Kaufmann–White scheme (Kauffmann, 1950).

a. 'O' antigen detection

All *Salmonella* isolates were tested for the presence of O antigen using polyvalent (A-G) and individual (A, B, C1, C2, D, E) O-group anitisera by slide agglutination method. In brief, isolates suspended in 10 µl normal saline on a glass slide and mixed with equal volume of "O" antisera (Remel, Europe Ltd, UK). The slide was rocked gently for one minute, and then examined for the presence of agglutination reaction. The bacterial suspension was mixed with normal saline as a negative control.

b. 'H' antigen detection

Isolates which reacted positively with any single O serum were tested for polyvalent H phase 1 and 2 and polyvalent phase 2 antigens by tube agglutination method. Phase one H antigen was detected using Rapid *Salmonella* Diagnostic 'H' antisera (RSD) plus H-I (Remel, Europe Ltd, UK). Phase 1 and phase 2 H antigens were detected using tube agglutination methods. In brief, the test organism (colony) was incubated overnight in Luria broth (LB) (Oxoid, Ltd) at 37^0C. One ml of this culture was diluted in 9ml LB broth and incubated with shaking at 37^0C for 3-4 hours. The culture was diluted with 10ml 1% Formol saline (until it matches to a MacFarland 0.5 standard). For each agglutination reaction, 1ml of diluted sample was mixed with 5 µl H antisera in Dreyer's tube (VWR International, Lutterworth, UK), and incubated in a 50^0C water bath for two hours. Tubes with presence of agglutination were taken as positive reaction for the respective H antigens.

c. Phase change

Phase conversion was made for those *Salmonella* isolates with only one phase. Ditch plate method was used to convert phase 1 to phase 2 and vice versa. Ditch with the size of 50x20mm was made on a well dried nutrient agar. A sterile filter paper strip was placed across the ditch at right angles. A drop of specific antisera which gave a positive result in the tube agglutination test was placed in the center of the bridge. The organism was inoculated at the very tip of the filter paper. The media was incubated at 37^0C for two days. Organisms that crossed the bridge appeared on the other half of the plate and were then

tested by tube agglutination test (H1; H2), to confirm change in expression of flagellar antigens.

2.7.3. Antimicrobial Susceptibility Testing

a) Disk diffusion testing

Antimicrobial susceptibility testing was performed for all *Salmonella* isolates using the disk diffusion method and results were interpreted using the criteria of the National Committee for Clinical Laboratory Standards (NCCLS) (NCCLS, 2000). Briefly, each frozen isolate was subcultured on MacConkey agar (Oxoid, Ltd). When a pure culture was obtained, a loopful of bacteria was taken and transferred to a tube containing 2 ml tryptic soy broth (TSB) (Oxoid) (pH 7.2) and mixed gently until it formed a homogenous suspension. The turbidity of the suspension was then adjusted to the optical density of a McFarland unit of 0.5 to standardize the inoculum size.

The drugs for disk diffusion testing were obtained from bioMerieux^{R} sa, France in the following concentrations: amoxicillin (AMX) (25μg), ampicillin (AM) (10μg), ceftriaxone (CRO) (30μg), chloramphenicol (C) (30μg), ciprofloxacin (CIP) (5μg), gentamycin (GM) (10μg), nalidixic acid (NA) (30μg), ofloxacin (OFX) (5μg), tetracycline (TE) (30μg) and trimethoprim-sulfamethoxazole (SXT) (1.25+23.75μg). In brief, sterile cotton swab was then dipped into tryptic soy broth and the excess was removed by gentle rotation of the swab against the inside wall of the test tube. The swab was then used to inoculate the bacteria evenly over the entire surface of Mueller Hinton agar (Oxoid, Ltd). The inoculated plates were left at room temperature to dry for 10-15 minutes. With the aid of disk dispenser, a set of antibiotic disks were then placed gently on the surface of the Mueller Hinton agar (ten antibiotic discs per plate). The discs were gently pressed onto the medium surface with sterile forceps to ensure firm contact and incubated at 37^{o}C for 24 hours. Diameters of the zone of inhibition around the antibiotic disc were measured to the nearest millimeter using a metal caliper, and the isolates were classified as sensitive,

intermediate and resistant according to the standardized table supplied by the NCCLS (2000).

Multidrug resistance (MDR) in *S. enterica* serovar Typhi was defined as resistance to the three drugs, namely chloramphenicol, ampicillin and trimethoprim-sulphamethoxazole). MDR for non-typhoid salmonellae species was defined as resistance to any of four or more drugs tested (Cooke and Wain, 2006).

b) E- test

E-test was performed for *Salmonella* Concord against ciprofloxacin using E-test strips (AB, Biodisk, Solna, Sweden). Each frozen isolate was subcultured and processed under the same conditions as described under disk diffusion method. With the aid of a sterile forceps, ciprofloxacin antibiotic strip with the MIC graded scale (0.016-256 μg/ml) was placed gently on the surface of Muller Hinton agar (Oxoid, Ltd) swabbed with the test organism and incubated at 37^{o}C for 24 hours. After the required period of incubation when bacterial growth becomes distinctly visible, the MIC value was read at the point of intersection between the inhibition ellipse edge and the E test strip (E-test technical guide, AB Biodisk, Solna, Sweden). The isolates were classified as sensitive reduced susceptible, intermediately sensitive and resistant according to the E-test application sheet (EAS-013) supplied by the manufacturer (EAS, AB Biodisk, 2000).

A MIC value is defined as the minimum inhibitory concentration of a given antibiotic, which under defined conditions inhibit the growth of a particular organism.

A standard reference strain of *E. coli* (ATCC 25922), sensitive to all antimicrobial drugs being tested was used as a quality control for disk diffusion and E-test.

c) Detection of Extended Spectrum β-lactamase

Salmonella Concord isolates showing zone of inhibition by disc diffusion method for ampicillin and ceftriaxone ≤11 and ≤13 mm respectively were tested for ESBL production and confirmed by E-test. Each suspicious isolate was subcultured and processed under the same conditions as described under E-test. The isolates were tested for susceptibility to

ceftazidime (TZ) MIC graded scale (0.5-32μg/ml) and cefotaxime MIC(CT) graded scale (0.25-16 μg/ml), and in combination with clavulanic acid (L) (4μg/ml); TZL (4-0.064μg/ml) and CTL (1- 0.016μg/ml) (AB BIODISK Solna, Sweden).

ESBL negative control strains, *E. coli* ATCC 35219 and ESBL positive control strain *K. pneumoniae* ATCC 700603 demonstrated the expected zone patterns (NCCLS, 2003).

The result was interpreted as ESBL-positive if the MIC ratio for TZ/TZL was ≥ 8 or CT/CTL was ≥8. The result would be non-determinable if the TZ MIC was > 32μg/ml and TZL > 4 μg/ml, as well as CT was >16 μg/ml and CTL >1μg/ml (Cormican *et al*., 1996).

2.8. Molecular Characterizations

Based on resistance pattern, place and specimen of isolation certain isolates were selected for molecular characterization.

2.8.1. Plasmid DNA extraction and analysis

Plasmid DNA extraction was performed using a Plasmid Mini Prep Kit (Nucleo Bond PC/BAC, MACHEREY-NAGEL, GERMANY) according to the manufacturer's instructions. Briefly, bacterial cells were harvested from overnight grown Luria broth (LB) (Oxoid) by centrifugation at 4,500 x g for 15 min at 4^0C. The pellet was re-suspended in 0.8 ml of Buffer S1 (50mM Tris-HCl, 10 mM EDTA, 100μg/ml RNase A, pH 8.0). Then 0.8ml of Buffer S2 (200mM NaOH, 1% SDS) was added into the cell suspension and incubated for 3 minutes at room temperature. Pre-cooled 0.8ml of Buffer S3 (2.8 M KAc, pH 5.1) was added to the suspension and after mixing , it was incubated on ice for 5 minute and was centrifuged at 13,000 x g for 15 minute at 4^oC. The clear lysate was loaded in to the NucleoBond AX 20 column which was equilibrated with 1 ml of Buffer N2 (100 mM Tris, 15% ethanol, 900 mM KCl). The column was washed twice with 2ml of Buffer N3 (100mM Tris, 15% ethanol, 1.15 M KCl, pH 6.3). The plasmid DNA was eluted with 1 ml of Buffer N5 (100 mM Tris, 15% ethanol, 1 M KCl, pH 8.5). The suspension was centrifuged at 15,000 x g for 30min at 4^0C after adding 0.75 ml of isopropanol. Finally 500 μl of 70% ethanol was added to the pellet and centrifuged at 15, 000 x g for 10min at room temperature. The pellet was dried at room temperature; it was re-constituted with 40μl of TE (pH 8.0) buffer and stored at 4^oC until needed.

Plasmids were separated by electrophoresis on horizontal 0.6% agarose gel made in 1xTBE (45 mM Tris, 45 mM boric acid, 10 mM EDTA pH8) at 80V for 1-2 hrs. DNA bands were visualized with an ultraviolet trans-illuminator, after staining with 0.05% ethidium bromide and were photographed under UV illumination. Plasmid sizes were determined to molecular weights ranging from 147 to 6.9kb carried in *Escherichia coli* K12 strain 39R861.

2.8.2. Plasmid incompatibility grouping

Salmonella Concord isolates were examined for the presence of 18 plasmid replicons using three multiplex panels (Table 2.1). Template plasmid DNA was extracted using a NucleoBond plasmid purification kit (Section 2.8.1). The PCR replicon typing of plasmid was done following the protocol described by Carattoli *et al.* (2005), and Johnson *et al.* (2007). In brief, PCRs were performed in a TETRA DNA Engine Peltier Thermal Cycler (MJ Research) with a reaction consisting of 2.5µl of Mango PCR buffer 10x, 1.5 mM of $MgCl_2$, 25µM of each dNTP, 1.25U of Mango Taq (Bioline), 0.5µM of each primer (the primer list for each panel shown in Table 2.1), 1.0µl of plasmid DNA template (10-100 ng) and nuclease free water to the total reaction volume of 25µl. Conditions used for PCR were as follows: 5 min at 94°C; 30 cycles of 30s at 94°C, 30s at 60°C, and 90s at 72°C; and a final extension of 5 min at 72°C. Amplicons were visualized on 1.5% Tris-acetate-EDTA agarose gels alongside a 1-kb ladder (Bioline), and if an amplicon of the expected size was observed, then an isolate was considered positive for the corresponding replicon (Table 2.1). Genomic DNA of a *Salmonella* isolate without plasmid was used as a negative control.

Table 2.1: Primers used in plasmid replicon typing studies (Johnson *et al.* 2007**)**

Replicon	Direction	Primer sequence (5' to 3')	Annealing Temperature (^{0}C)	Amplicon size(bp)
Panel 1				
B/O	F	GCGGTCCGGAAAGCCAGAAAAC	60	159
	R	TCTGCGTTCCGCCAAGTTCGA		
FIC	F	GTGAACTGGCAGATGAGGAAGG	60	262
	R	TTCTCCTCGTCGCCAAACTAGAT		
A/C	F	GAGAACCAAAGACAAAGACCTGGA	60	465
	R	ACGACAAACCTGAATTGCCTCCTT		
P	F	CTATGGCCCTGCAAACGCGCCAGAAA	60	534
	R	TCACGCGCCAGGGCGCAGCC		
T	F	TTGGCCTGTTTGTGCCTAAACCAT	60	750
	R	CGTTGATTACACTTAGCTTTGGAC		
Panel 2				
K/B	F	GCGGTCCGGAAAGCCAGAAAAC	60	160
	R	TCTTTCACGAGCCCGCCAAA		
W	F	CCTAAGAACAACAAAGCCCCCG	60	242
	R	GGTGCGCGGCATAGAACCGT		
FI1A	F	CTGTCGTAAGCTGATGGC	60	270
	R	CTCTGCCACAAACTTCAGC		
FIA	F	CCATGCTGGTTCTAGAGAAGGTG	60	462
	R	GTATATCCTTACTGGCTTCCGCAG		
FIB	F	GGAGTTCTGACACACGATTTTCTG	60	702
	R	CTCCCGTCGCTTCAGGGCATT		
Y	F	AATTCAAACAACACTGTGCAGCCTG	60	765
	R	GCGAGAATGGACGATTACAAAACTTT		
Panel 3				
I1	F	CGAAAGCCGGACGGCAGAA	60	139
	R	TCGTCGTTCCGCCAAGTTCGT		
FREP	F	GAAGATCAGTCACACCATCC	60	270
	R	TGATCGTTTAAGGAATTTTG		
X	F	AACCTTAGAGGCTATTTAAGTTGCTG	60	376
	R	AT TGAGAGTCAATTTTTATCTCATGTTTT AGC		
HI1	F	GGAGCGATGGATTACTTCAGTAC	60	471
	R	TGCCGTTTCACCTCGTGAGTA		
N	F	GTCTAACGAGCTTACCGAAG	60	559
	R	GTTTCAACTCTGCCAAGTTC		
HI2	F	TTTCTCCTGAGTCACCTGTTAACAC	60	644
	R	GGCTCACTACCGTTGTCATCCT		
L/M	F	GGATGAAAACTATCAGCATCTGAAG	60	78
	R	CTGCAGGGGCGATTCTTTAGG		

2.8.3. Pulsed-Field Gel Electrophoresis (PFGE)

PFGE was performed for *Salmonella* Concord using *Xba*I according to the following protocols: -

I. Cell Preparation

Isolated *Salmonella* strains were subcultured on CLED agar (Oxoid, Ltd) overnight and then a colony was inoculated in 2 ml LB broth (Oxoid, Ltd) and incubated overnight at 37^0C. One ml of the overnight bacterial culture was transferred in to a cuvette and the concentration was adjusted to absorbance (Optical Density) of 1.35 with Spectrophotometer at 610 nm wavelength.

The suspended cells were centrifuged at 13,000 rpm for 5 minutes. The supernatant was removed and the pellet was re-suspended in 1 ml of cell suspension buffer (100 mM Tris: 100 mM EDTA, pH 8.0).

II. Preparation of Agarose plugs

Plugs were prepared by mixing 600 µl of 1.5% low melting agarose (Sigma, USA) made in 1xTE (10 mM Tris:1 mM EDTA, pH 8.0) with 400µl bacterial cell suspension. The mixture was then dispensed into plug moulds and allowed to solidify at 4^0C for 20 minutes. The agarose plugs containing the bacterial cells, were then transferred to bijoux bottle containing 2 ml of lysis solution (50 mM Tris: 50 mM EDTA, pH 8.0 + 1% Sarcosyl) and incubated overnight at 37^0C. The lysis solution was then removed and replaced with 2 ml alkaline lysis solution (1 % (w/v) N-Lauroyl sarcosine, 0.5 M EDTA, 1mg/ml proteinase K, pH 9.5) and incubated at 55^0C in a water bath overnight. The plugs were then washed four times (each time for one hour) at room temperature with 3 ml of 1xTE buffer using gentle shaking or rocking. Finally the plugs were stored in 2ml TE at 4^oC until further use.

III. Restriction digestion

Each plug was cut to a 2.0 to 2.5 mm wide size. Then the plug was equilibrated in 100µl of restriction endonuclease buffer for 30 min on ice as recommended by the manufacturer, the buffer was then aspirated, the tube filled with fresh buffer and digestion of

the DNA was carried out with 20U of *XbaI* (Promega, Madison, Wisconsin, USA). Incubation in ice was carried out for 15 min to allow the enzyme to penetrate into the plug. The reaction was incubated overnight at 37^0C. After digestion, the plugs could be loaded into a gel or stored in 1xTE buffer overnight at 4^0C.

IV. Gel electrophoresis

The plugs containing the digested genomic DNA were loaded into slots of a gel consisting of 1% SeaKem Gold (SKG) Agarose in 0.5x TBE. Electrophoresis of digested DNA fragments was carried out by PFGE using a Contour-clamped homogenous electric field apparatus with a hexagonal electrode array (CHEF-DRII system; Biorad Laboratories, Richmond, California).

PFGE was performed at 10^0C for 24 h at 6 V/ cm with a pulse time ranging 2.2s to 68s and pulse angles were set at 120^0. The run condition was chosen such that the distribution of the fragments was even, facilitating the comparison of the restriction profiles. Digested DNA from *S. enterica* serovar Braenderup H9812 was loaded every five lanes as the molecular marker, as recommended by Pulse net (Swaminathan *et al*., 2001). After a PFGE run, the gel was stained in de-ionized water containing 1 μg/ml ethidium bromide for 10-15 min. The DNA restriction patterns were photographed and were assessed visually.

2.8.4. Extraction of genomic DNA for MLST and fliC gene sequencing

DNA for *fliC* gene sequencing and multilocus sequence typing (MLST) was isolated using the Wizard Genomic DNA Purification Kit (Promega, USA). Briefly:- 1 ml of overnight bacterial culture suspension was centrifuged at 13,000 rpm for 5 minutes and 600μl of Nuclei lysis Solution was added to the pellet, followed by incubation at 80^0C for 10minutes. Three μl of RNase solution was added to the lysate which was then incubated at 37^0C for about 15minutes. Two hundred μl of Protein Precipitation Solution was added to the RNase-treated cell lysate and vortexed. After incubation on ice for 10 minutes it was centrifuged for another 15 minutes. Supernatant was transferred to a new 1.5 ml microcentrifuge tube and 600μl isopropanol was added. It was mixed by inversion until the thread-like strands of DNA form a visible mass. Then it was centrifuged at 13,000 rpm for

15 minutes. After pouring off the supernatant 1 ml of 70% ethanol was added and centrifuged for 2 minutes at 13,000 rpm. The supernatant was discarded and the pellet was air-dried. Finally the DNA pellet was re-suspended with 50 µl of DNA rehydration solution and stored at 4^0C.

2.8.5. Multilocus Sequence Typing (MLST)

Seven house keeping genes (section 1.2.11.3.a, Page 52) were chosen for MLST to allow comparison to an existing database [http://wev. mpiib-berlin. mpg.de/mlst] (Kidgell *et al*, 2002). These seven genes were PCR amplified from chromosomal DNA using the primer combinations as shown in Table 2.2. Amplification and sequencing protocols used were as previously described (Kidgell *et al* 2002). The same primers were used for PCR amplification and sequencing.

MLST data analysis: Sequence were edited and complementary sense and antisense fragments were aligned using GAP4 (Sanger). The sequences were submitted to the MLST database website (http://web.mpiib-berlin.mpg.de/mlst), and assigned an existing or novel allele type number and sequence type (ST) number defined by the database. This multi-microorganism database defines a novel allele type as a sequence containing one or more nucleotide changes from existing allele sequences. Composite STs are assigned based on the set of allele types derived from each of the seven loci.

Table 2.2: Oligonucleotide primer sequences used for amplification and sequencing of MLST (Kidgell *et al* 2002**)**

Primer	**Sequence 5' to 3'**
aroCfor	CCTGGCACCTCGCGCTATAC
aroCrev	CCACACACGGATCGTGGCG
dnaNfor	ATGAAATTTACCGTTGAACGTGA
dnaNrev	AATTTCTCATTCGAGAGGATTGC
hemD	GAAGCGTTAGTGAGCCGTCTGCG
hemD	ATCAGCGACCTTAATATCTTGCCA
hisDfor	GAAACGTTCCATTCCGCGCAGAC
hisDrev	CTGAACGGTCATCCGTTTCTG
purEfor	ATGTCTTCCCGCAATAATCC
purErev	TCATAGCGTCCCCCGCGGATC
sucAfor	AGCACCGAAGAGAAACGCTG
sucArev	GGTTGTTGATAACGATACGTAC
thrAfor	GTCACGGTGATCGATCCGGT
thrArev	CACGATATTGATATTAGCCCG

Primer names consisting of the gene name plus for (forward) or rev (reverse) were used for amplification.

2.8.6. PCR amplification and sequencing of fliC gene

FliC encodes the phase 1 flagellar (H1) antigen and has been suggested for the use in molecular serology (McQuiston *et al*., 2004; Mortimer *et al*., 2004). Primers *fliC*-1 (5'TGACCATCCAGGTTGGTGCC3') and *fliC*-2(5' AAACGGTTCTGAACCGCACC 3') were designed from the 1506 bp *fliC* coding region (CDS) of the sequenced *S*. Choleraesuis SB-C67 (Gen Bank acc no: NC_006905) to produce an 866 bp amplicon. The 866bp (bp position 431-1297 of the CDS) covers the central variable region of the *fliC* gene (McQuiston *et al* 2004; Mortimer *et al* 2004).

PCRs were performed on a TETRA DNA Engine Peltier Thermal Cycler (MJ Research) with a reaction mix consisting of 47μl PCR Supermix (Invitrogen), one μl of each primer at a concentration of 200nM and one μl of 100ng DNA template. PCR conditions were 95^0C for 50s, followed by 30 cycles of 95^0C for 10s, 58^0C for 1 min, 72^0C for 1 min and a final extension of 72^0C for 1 min.

The PCR products were purified with S-400 columns (GE Healthcare). Direct DNA sequencing of the 866bp products obtained with the *fliC*-1 and *fliC*-2 primers were

performed with an ABI Prism dye terminator cycle sequence kit (Perkin Elmer, Foster City, CA, USA) on an ABI 3730 automated sequencer.

2.9. Statistical analysis

All demographic, clinical and laboratory data obtained from this study were entered, analyzed and interpreted using the Statistical Package for Social Sciences (SPSS) 11.0 (SPSS, 2001). Chi-square was used to test difference between proportion and P-values <0.05 was considered statistically significant.

2.10. Ethical Consideration

The PhD research project was approved by the Department and the Faculty of Research Publications Committee-II (FRPC_II) and endorsed by the Faculty Academic commission, Medical Faculty, Addis Ababa University and ethically cleared. Ethical clearance was also obtained from Armauer Hansen Research Institute (AHRI), Jimma University, Addis Ababa Health Bureau and National Ethical Review Committee of Ethiopian Science and Technology Commission. Written informed consent was obtained from parents/guardians for their children participating in the study (see Appendix II).

CHAPTER III

RESULTS

CHAPTER III RESULTS

3.1. Study Subjects

A total of 1225 children visiting the outpatient pediatric departments with fever alone (222 children, 18.1%), fever and diarrhoea (244 children, 19.9%) and diarrhoea alone (759 children, 62%) were investigated for *Salmonella* and other enteropathogens between January 2006 and June 2008 at Tikur Anbessa and Jimma Specialized University Hospitals. The age and sex distribution of the 1225 patients are shown in Figure 3.1. The age ranges from 6 month to 15 years with mean age of 4.8 (SD±3.93) years. Majority of the patients (66.7%) were between the age of 6 month and 5 years. Six hundred fifty four (53.4%) of the study subjects were males and 571 (46.6%) were females, resulting in an overall female to male ratio of 1:1.5. Of these 1225 patients, 400 (32.7%) were from Jimma and 825 (67.3%) were from Addis Ababa.

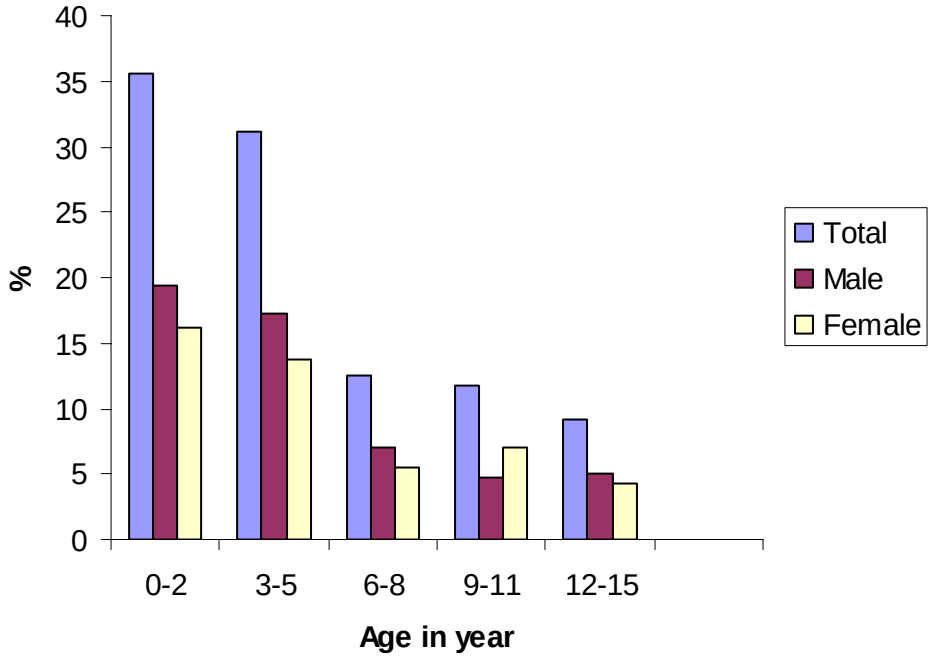

Figure 3.1: Age and sex distribution of 1225 children investigated for *Salmonella* and other enteropathogens in Addis Ababa and Jimma Hospital.

3.2. Clinical Features

Illness symptoms included were diarrhoea (81.3%), fever (38.4%), tenesmus (45.9%), abdominal pain (68.1%), headache (32.3%) and nausea (38.4%) (Table 3.1). Diarrhoea with mucoid consistency and five day duration before visiting health institution were observed in the majority of patients. The average duration of fever was 4 days.

Table 3.1: Clinical findings and their association with culture positive and negative for *Salmonella* species

Clinical findings	Positive (%) (*Salmonella* spp.) n=65	Negative (%) (*Salmonella* spp.) n=1160	Total (%) n=1225	p-values
Fever				
	Yes 32 (49.2)	439(37.8)	471 (38.4)	0.066
	No 33(50.8)	721(62.2)	754(61.6)	
Diarrhoea				
	Yes 48(75.4)	955(82.3)	1003(81.3)	0.084
	No 17(24.6)	205(17.7)	222(18.7)	
Abdominal pain				
	Yes 43(66.2)	791(68.2)	834(68.1)	0.732
	No 22(33.8)	369(31.8)	391(31.9)	
Tenesmus				
	Yes 14(21.5)	548(47.2)	562(45.9)	0.000
	No 51(78.5)	612(52.8)	663(54.1)	
Headache				
	Yes 26(40)	370(31.9)	396(32.3)	0.822
	No 39(60)	790(68.1)	829(67.7)	
Nausea				
	Yes 16(24.6)	454(39.1)	470(38.4)	0.019
	No 49(75.4)	706(60.9)	755(61.6)	
Consistency				
Watery	16(33.3)	186(19.5)	202(20.1)	
Mucoid	9(18.8)	351(36.8)	360(35.9)	
Loose	13(27.1)	312(32.6)	325(32.4)	0.000
Mixed(blood and	10(20.8)	60(6.3)	70(7)	
mucous)	-	46(4.8)	46(4.6)	
Bloody				
Duration of diarrhoea				
0-5 days	38(79.2)	748(78.3)	786(78.4)	
6-11days	8(16.7)	173(18.1)	181(18.)	0.949
≥ 12 days	2(4.2)	34(3.6)	36(3.6)	

3.3. Detection of *Salmonella* and other Enteropathogens from Different Clinical Samples

The number and the percentage of detection of enteropathogens in different clinical samples obtained from pediatrics patients by culture and microscopic examination are shown in Table 3.2. A total of 463 enteropathogens were isolated. Among the 463 total identified enteropathogens, 14 *Salmonella* species and 310 enteropathogens were identified from patients who submitted either only blood or stool respectively. One hundred thirty nine enteropathogens were isolated from patients who submitted both blood and stool sample (Table 3.2). There was no *Salmonella* isolates detected from blood and stool of the same patient.

Table 3.2: Frequency of isolation of enteropathogens from different clinical samples obtained from children, January 2006-June 2008.

Specimen type	Number of samples	Frequency of isolation No (%)
Blood	222	14(3)
Stool	759	310(86.4)
Blood(n=244) and stool (n=244)	488	139(10.6)
Total	1469	463 (100%)

3.4. Enteropathogens

3.4.1. Salmonella species

A total of 65 *Salmonella* species were isolated from febrile and/or diarrheic children. The serogroup distribution of the 65 *Salmonella* isolates is presented in Table 3.3. Serogroup C was the most commonly isolated species (80%), followed by group B (12.3%) and group D (7.7%). Out of 65 *Salmonella* isolates 45 were isolated from stool and 20 from blood. *Salmonella* infections were more common in children at Addis Ababa than it was in Jimma (P = 0.002) (Table 3.3).

3.4.2. Shigella species

A total of 61 *Shigella* species were isolated from the stool. The serogroup distribution of the 61 *Shigella* isolates is presented in Table 3.3. Serogrup B (*S. flexneri)* was the most frequently isolated species (68.9%) followed by group D (*S. sonnei*) (21.3%), group C (*S. boydii*) (9.8%) and there was no *S. dysenteriae* isolate. *Shigella* species were more prevalent among those children under 5years of age (Fig.3.2). *Shigella* infections were more common among children in Jimma than Addis Ababa (P= 0.000) (Table 3.3).

3.4.3. Parasites

Total parasites isolated from the children are presented in Table 3.3. Among the 337 parasites isolated from the stool of children who had complained of either diarrhoea only or diarrhoea and fever, there were 179 protozoan and 158 helminthic parasites.

The parasites isolated were *Giardia lamblia* (108; 8.8%), *Entamoeaba histolytica* (71, 5.8%), *Ascaris lumbricoides* (41, 3.5%), *Trichuris trichuria* (38, 3.1%), *Schistosoma mansoni* (4, 0.3%), Hookworm and *Strongyloides stercoralis* (1; 0.1% each) (Table 3.2). Among the total 337 identified parasites, *G. lamblia* was the most frequently identified parasite in both study sites with the detection rate of 32 %.

The same isolation pattern was also observed in *E. histolytica* and *G. lamblia* infections (common in under five children). Helminthic (except *H. nana*) infections were more prevalent in older children (Figure 3.2).

3.4.4. Concomitant infection

Co-infections were found in 40 (3.3%) of the patients, of these 12 were bacteria/ parasite and 28 were parasite/parasite co-infection. There was no bacteria/bacteria co-infection. *G. lamblia, E. histolytica, A. lumbricoides, H. nana and T. trichuria* were found to be involved in co-infection including with *Salmonella* and *Shigella* isolates.

Table 3.3: Frequency of isolation of enteropathogens from children with diarrhoea and febrile illnesses in Addis Ababa and Jimma (January 2006-June 2008)

Enteropathogens	**Addis Ababa No. (%) n=825**	**Jimma No. (%) n=400**	**Total No. (%) (n=1225)**	**P-value**
***Salmonella* species**				
Serogroup B	7(0.8)	1(0.3)	8 (0.7)	
Serogroup C	43(5.2)	9(2.3)	52(4.2)	0.002
Serogroup D	5(0.6)	-	5(0.4)	
Total	**55(6.7)**	**10(2.5)**	**65(5.3)**	
***Shigella* species**				
Sergroup B *(S.flexineri)*	20(2.4)	22(5.5)	42(3.4)	
Serogroup C *(S.boydii)*	4(0.5)	2(0.5)	6(0.5)	0.000
Serogroup D *(S.sonnei)*	2(0.2)	11(2.8)	13(1.1)	
Total	**26(3.2)**	**35(8.8)**	**61(5)**	
Parasite				
Entamoeba histolytica	63(7.6)	8(2)	71(5.8)	
Giardia lamblia	59(7.2)	49(12.3)	108(8.8)	
Ascaris lumbricoides	16(1.9)	25(6.3)	41(3.5)	0.135
Hymenolepis species	29(3.5)	8(2)	37(3.0)	
Trichuris trichuria	12(1.5)	26(6.5)	38(3.1)	
Schistosoma mansoni	-	4(1)	4(0.3)	
Hookworm	-	1(0.3)	1(0.1)	
Strolongyloides stercoralis	1(0.1)	-	1(0.1)	
Total	**216(26.2)**	**121(30.3)**	**337(27.5)**	

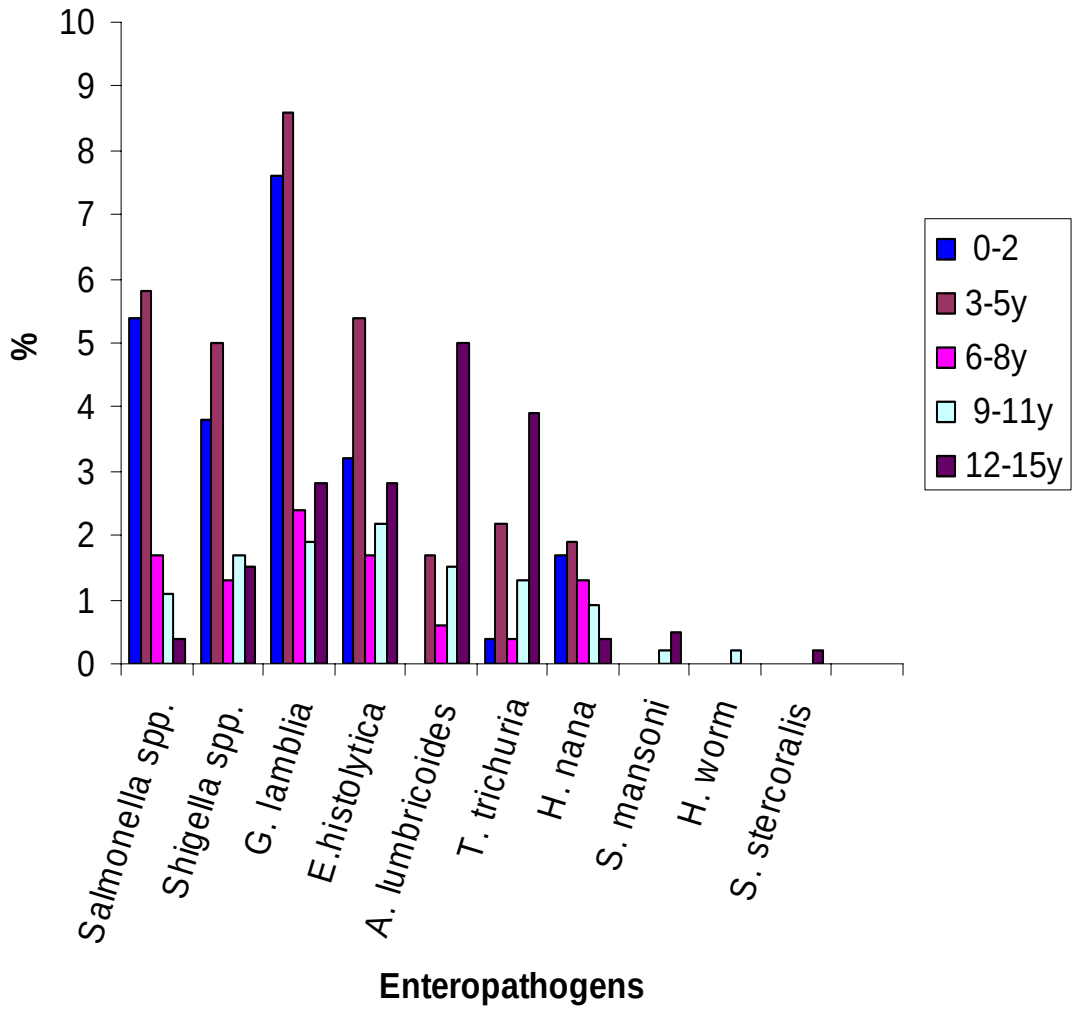

Figure 3.2: Distribution of enteropathogens among different age group of children investigated.

3.5. Salmonellosis

3.5.1. Age and sex distribution

The age and sex distribution among 65 children positive for *Salmonella* infections are shown in Figure 3.3. It was observed that isolation of *Salmonella* was relatively higher in age group 3-5 (41.5%; n= 27) than 0-2() year. Low isolation rate was observed in children > 12 years old (Figure 3.3). Among the 65 children who were positive for *Salmonella* species 37(56.9%) were males and 28 (43.1%) females.

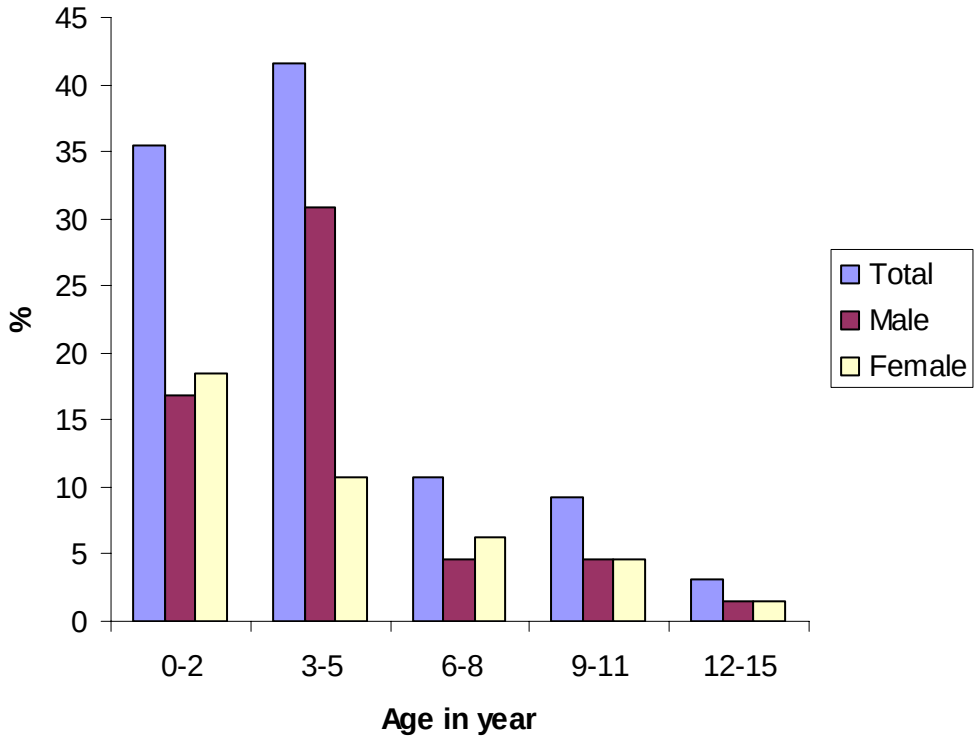

Figure 3.3: Age and sex distribution of children who were positive for *Salmonella* species in Jimma and Addis Ababa (January 2006 – June 2008)

3.5.2. Clinical features

Among 65 children who were positive for *Salmonella*, diarrhoea was the commonest clinical findings 48 (75.4%), followed by abdominal pain 43 (66.2%), fever 32 (49.2%), head ache 26 (40.0%) nausea 16 (24.6%) and tenesmus 14 (21.5%), (Table 3.1)

Even though most children who were positive for *Salmonella* species had a complaint of diarrhoea and abdominal pain, there was no statistically significant difference from culture negative children ($p>0.05$) (Table 3.1).

As it is indicated in Table 3.1, strong association was observed between children presenting with tenesmus and nausea with less likely to isolate *Salmonella* species ($p<0.05$). Though it was not statistically significant, most children with diarrhoea reported within 1 to 5 days duration of the onset. Watery diarrhoea was more common among *Salmonella* positive cases as compared to negatives which was found to be statistically significant ($P<0.0001$). Of the 65 *Salmonella* positive children, 15 (23.1%) had only fever, 34 (52.3%) children had fever and diarrhoea and 16 (24.6%) only with diarrhoea.

3.6. Phenotyping Characterization

3.6.1. Serogroups and serotypes

In addition to 65 *Salmonella* strains, 48 *Salmonella* strains from stock cultures collected from January, 2004 to December, 2005 were serogrouped and serotyped. These strains were isolated from children with diarrhoeal and/ or febrile illness in Tikur Anbessa Hospital, Addis Ababa Ethiopia.

I. Serogroups

Among the 113 *Salmonella* isolates, serogroup C, B, D and E were isolated at a frequency of 89 (78.8%), 13 (11.5%), 9 (8%) and 2 (1.8%) respectively as shown in Table 3.4. Most of the *Salmonella* isolates were from stool 77 (68%) and the rest were from blood 36 (32%).

II. Serotypes

A total of 12 different serotypes were identified with antisera and multilocus sequence typing (Table 3.4). The identified serovars in group C were: *S.* Concord 82 (72.6%), *S.* Colindale 1(0.9%), *S.* Gatow 3 (2.7%), *S.* Laronchelle 1(0.9%), *S.* Garoli 1(0.9%) and *S.* Colorado 1(0.9%). Serogroup B consisted of *S.* Typhimurium 7(6.2%), *S.* Haifa 1(0.9%) and *S.* Paratyphi B 2(1.8%), from serogroup D, *S.* Typhi 2(1.8%) and *S.* Enteritidis 4 (3.5%) and from group E *S.* Butantan 2(1.8%), were the identified serotypes (Table 3.4).

In general 3 isolates each from group B and D were untypable with the available antisera. From the identified serotypes *S.* Concord, *S.* Typhimurium and *S.* Enteritidis were the predominant isolate from group C, B and D respectively (Table 3.4).

Table 3.4: *Salmonella* serogroup and serotypes isolated from children in Addis Ababa and Jimma.

Serogroup	Serotypes	No	Stool	Blood
B	*S.* Typhimurium	7	6	1
	S. Paratyphi B	2	2	-
	S. Haifa	1	1	-
	Not typable	3	3	-
	Total	**13**	**12**	**1**
C1	*S.* Concord	82	56	26
	S. Laronchelle	1	1	-
	S. Colorado	1	1	-
	S. Garoli	1	1	-
	S. Gatow	3	1	2
	S. Colindale	1	1	-
	Total	**89**	**61**	**28**
D	*S.* Typhi	2	-	2
	S. Enteritidis	4	1	3
	Not typable	3	1	2
	Total	**9**	**2**	**7**
E	*S.* Butantan	**2**	**2**	**-**
Totals		**113**	**77**	**36**

3.6.2. Antimicrobial susceptibility

a. Susceptibility to all antibiotics tested

The antimicrobial susceptibility testing was done on all *Salmonella* isolates using disk diffusion method and the results are presented in Figure 3.4 and Table 3.5. Among the 113 *Salmonella* isolates, the overall rates of resistance were high for amoxicillin 94 (83.2%), ampicillin 93 (82.3%), chloramphenicol 92 (81.4%), trimethoprim-sulphamethoxazole 91 (80.5%), ceftriaxone 89 (78.8%), gentamycin 84 (74.3%) and tetracycline 45 (39.8%). Low level of resistance observed against nalidixic acid 9 (8.0%), ofloxacin 1 (0.9%) and ciprofloxacin 1 (0.9%).

Serogroup C showed high level of resistance to most antibiotics tested as shown in Figure 3.4., and the majority of serotypes in group C were resistant to ampicillin 87 (97.8%), amoxicillin 86 (96.6%), chloramphenicol 86 (96.6%) ceftriaxone 85 (95.5%) trimethprim-

sulphamethoxazole 85 (95.5%), gentamycin 83 (93.3%), while 39 (43.8%) were resistant to tetracycline. Only 8 (9%) and 1(1.1%) were resistant to nalidixic acid and ofloxacin respectively (Table 3.5).

Among serogroup C, *S.* Concord showed 100% resistance against ampicillin and trimethoprim-sulphamethoxazole, 98.8% against amoxicillin and ceftriaxone, 97.6% to chloramphenicol, 95.1% against gentamycin, and 42.7% against tetracycline. Low resistance rate was observed for nalidixic acid (9.8%) and ofloxacin (1.2%) and no resistance was observed for ciprofloxacin. In general *S.* Concord showed high level of resistance to the commonly used drugs including third generation cephalosporin (ceftriaxone), while susceptible to second generation flouroquinolones (ciprofloxacin and ofloxacin). *S.* Colorado and *S.* Gatow were resistant to most tested drugs (Table 3.5)

Low percentage of resistance was seen among isolates in serogroup B and D. The two *S.* Butantan isolate in serogroup E were fully susceptible to all tested drugs.

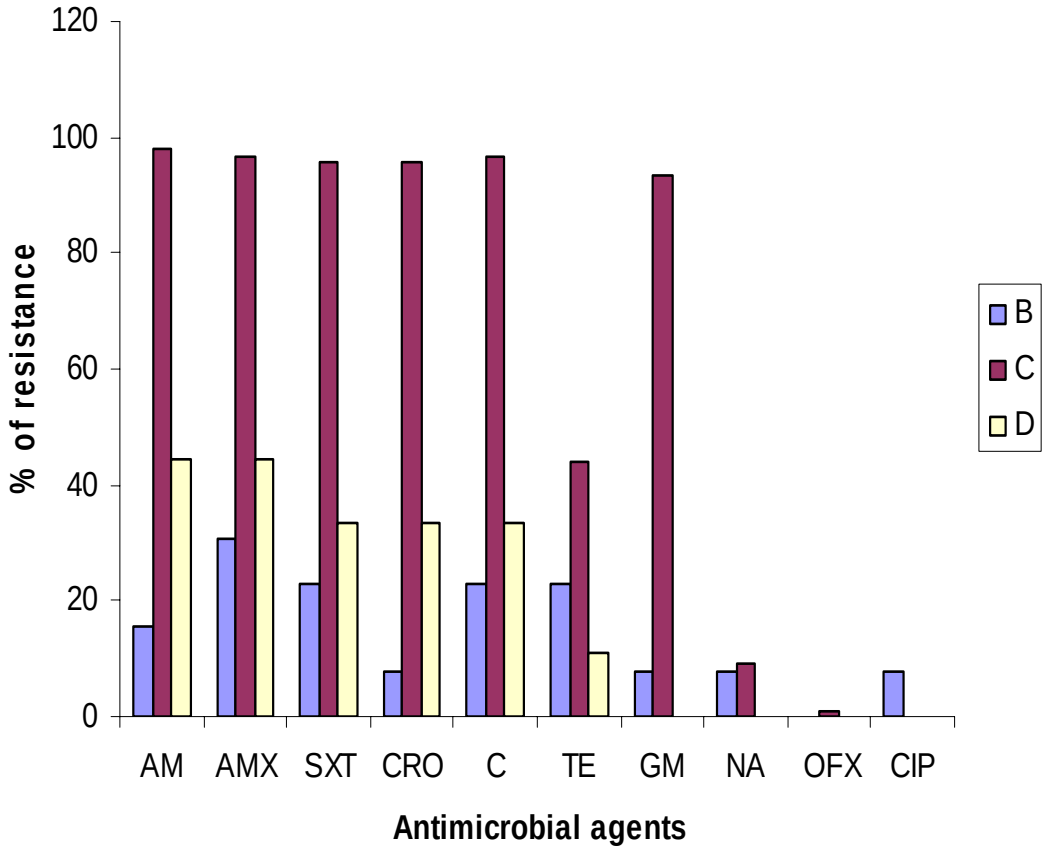

Figure 3.4: Resistance pattern of *Salmonella* serogroups against 10 antimicrobial agents.

AM: Ampicillin; AMX: Amoxicillin; SXT: Trimethoprim-sulphamethaxole; CRO: Ceftriaxone; C: Chloramphenicol; TE: Tetracycline; GM: Gentamycin; NA: Nalidixic acid; OFX: Ofloxacin; CIP: Ciprofloxacin.

Table 3.5: Resistance pattern of *Salmonella* serovars against 10 antimicrobial agents

SG	Serotypes	No.	Number of strain (%) resistance to									
			AM	**AMX**	**SXT**	**CRO**	**C**	**TE**	**GM**	**NA**	**OFX**	**CIP**
	S. Typhimurium	7	2 (28.6)	3 (42.9)	2 (28.6)	1 (14.3)	3 (42.9)	3 (42.9)	1 (14.3)	1 (14.3)	-	1 (14.3)
B(13)	*S.* Paratyphi B	2	-	-	-	-	-	-	-	-	-	-
	S. Haifa	1	-	-	-	-	-	-	-	-	-	-
	Untypeble	3	-	1 (33.3)	1 (33.3)	-	-	2 (66.6)	-	-	-	-
	S. Concord	82	82 (100)	81 (98.8)	82 (100)	81 (98.8)	80 (97.6)	35 (42.7)	78 (95.1)	8 (9.8)	1 (1.2)	-
	S. Garoli	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	- (100)	1 (100)	-	-	-
C_1(89)	*S.*Laronchelle	1	-	-	-	-	1 (100)	1 (100)	-	-	-	-
	S. Colorado	1	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	1 (100)	-	-	-
	S. Colindale	1	-	-	-	-	-	1 (100)	-	-	-	-
	S. Gatow	3	3 (100)	3 (100)	1 (33.3)	2 (66.6)	3 (100)	1 (33.3)	3 (100)	-	-	-
	S. Typhi	2	-	-	-	-	-	-	-	-	-	-
D(9)	*S.* Enteritidis	4	3 (75)	3 (75)	2 (50)	2 (50)	2 (50)	-	-	-	-	-
	Untypable	3	1 (33.3)	1 (33.3	1 (33.3	1 (33.3	1 (33.3	1 (33.3	-	-	-	-
E(2)	*S.* Butantan	2	-	-	-	-	-	-	-	-	-	-
All		**113**	**93 (82.3)**	**94 (83.2)**	**91 (80.5)**	**89 (78.8)**	**92 (81.4)**	**45 (39.8)**	**84 (74.3)**	**9 (8.0)**	**1 (0.9)**	**1 (0.9)**

SG = Serogroup

AM: Ampicillin; AMX: Amoxicillin; SXT: Trimethoprim-sulphamethaxole; CRO: Ceftriaxone; C: Chloramphenicol; TE: Tetracycline; GM: Gentamycin; NA: Nalidixic acid; OFX: Ofloxacin; CIP: Ciprofloxacin

b. Multidrug resistance of *S.* Concord

In this study multi-drug resistance in the NTS was defined as resistance to four or more drugs (Cooke and Wain, 2006). All isolates in serogroup C were resistant to one or more drugs and 96.6 % (86/89) of them were MDR. A total of ten distinct antibiograms (resistance patterns) were found among all isolates of *S.* Concord (Table 3.6). The resistance pattern of *S.* Concord varied from four to eight drugs. Two distinct patterns of resistance were dominant among the *S.* Concord isolates: (i) ampicillin, amoxicillin, Trimethoprim-sulphamethaxole, ceftriaxone, chloramphenicol, Gentamycin (40 isolates) and (ii) ampicillin, amoxicillin, Trimethoprim-sulphamethaxole, ceftriaxone, tetracycline, chloramphenicol, Gentamycin (28 isolates). Only one (1.2%) *S.* Concord isolate was resistant to four antimicrobial agents, 3 isolates (3.7%) to 5, 42 isolates (51.2%) to 6, 31 isolates (37.8%) to 7 and 5 isolates (6.1%) were resistant to 8 drugs respectively.

All the three *S.* Gatow isolates were MDR (resistant to four or more drugs). *S.* Butantan and *S.* Haifa showed susceptibility to all tested antimicrobial agents. Five and three isolates in serogroup B and D respectively were fully susceptible for all the tested drugs.

Table 3.6: Resistance antibiogram pattern of *S*. Concord

SG	Serotype	NST	R≥1	R≥2	R≥4	Resistance antibiogram	No.
	S. Concord	82	82	82	82	AM, AMX, SXT, TE	1
						AM, SXT, CRO, C, GM	1
						AM, AMX, SXT, CRO, GM	1
						AM, AMX, SXT, CRO, C	1
C_1						AM, AMX, SXT, CRO, C, OFX	1
						AM, AMX, SXT, CRO, C, GM	40
						AM AMX, SXT, CRO, TE, C	1
						AM, AMX, SXT, CRO, C, GM, NA	3
						AM, AMX, SXT, CRO, TE, C, GM	28
						AM, AMX, SXT, CRO, TE, C, GM, NA	5
Total		**82**	**82**	**82**	**82**		**82**

SG= serogroup; NST= Number of strain tested; R≥1= number of strains resistant to one or more drugs; R≥2= number of strains resistant to two or more drug; R≥4= number of strains resistant to one or more drugs. AM: Ampicillin; AMX: Amoxicillin; SXT: Trimethoprim-sulphamethaxole; CRO: Ceftriaxone; C: Chloramphenicol; TE: Tetracycline; GM: Gentamycin; NA: Nalidixic acid; OFX: Ofloxacin

c. Susceptibility to nalidixic acid and ciprofloxacin by disc diffusion method

Out of 82 *S.* Concord isolates, 62 (75.6%) were sensitive, 8 (9.8%) were resistant to nalidixic acid, respectively. The remaining 12 (14.6%) isolates showed intermediate susceptibility to nalidixic acid. All were susceptible to ciprofloxacin (100%).

d. Susceptibility to ciprofloxacin by E-test

Of the 82 *S.* Concord isolates, 58 (70.7%) were sensitive (MIC <0.125μg/ml), 22 (26.7%) showed reduced susceptibility (0.125 to <1), one showed intermediate resistance and one isolate was resistant (MIC ≥ 4μg/ml) to ciprofloxacin (Figure 3.5).

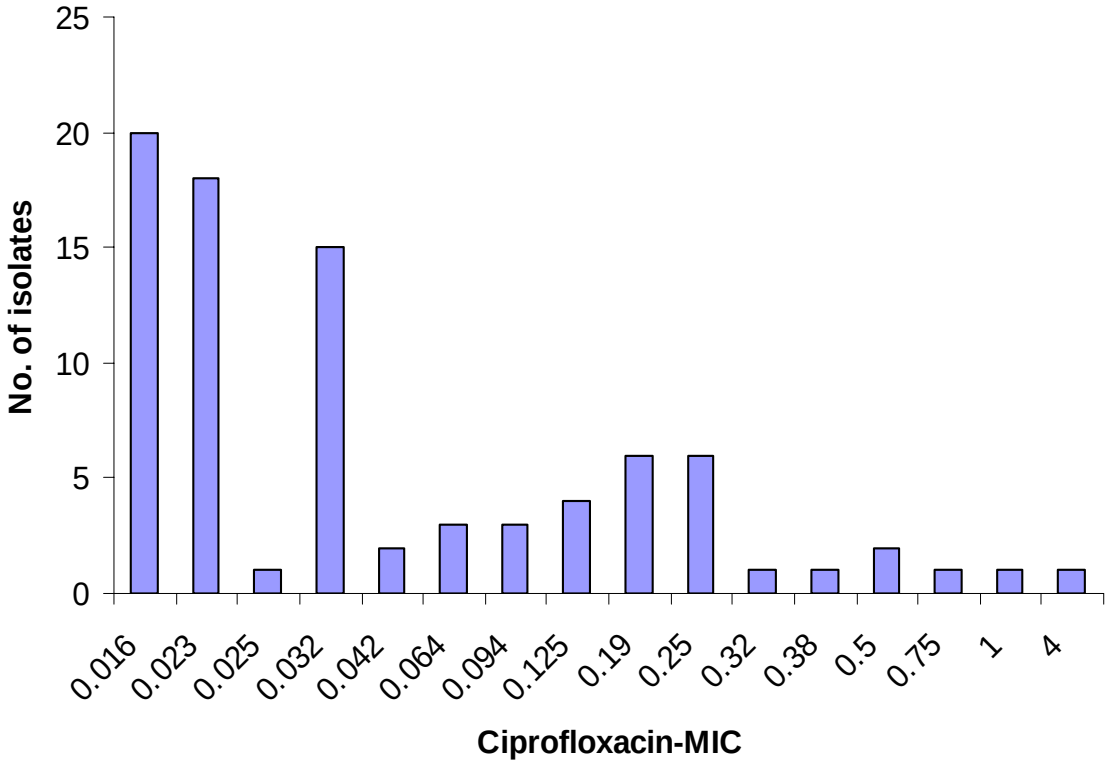

Figure 3.5: MIC values of Ciprofloxacin for *S*. Concord

S=sensitive (MIC<0.125μg/ml), Reduced Susceptibility (MIC 0.125-<1μg/ml), I= intermediate (MIC 1 – 3.9 μg/ml), R= resistant (MIC ≥ 4μg/ml).

e. Nalidixic acid resistance by disc diffusion and ciprofloxacin susceptibility MIC

Of the eight isolates resistance to nalidixic acid, five were with reduced susceptibility, two sensitive and one was resistant to ciprofloxacin. Among the 12 isolates with intermediate result for nalidixic acid, nine showed reduced susceptibility, one intermediate resistance to ciprofloxacin and the other two were sensitive to ciprofloxacin. In general of the 20 *S.* Concord isolates which were either fully resistant or showed intermediate resistance to nalidixic acid, four were susceptible, 14 showed reduced susceptibility, one intermediate and one resistant to ciprofloxacin.

f. Exteneded Spectrum Beta-lactamase producing *Salmonella* Concord

The ESBL screening test showed that, out of the 81 *S.* Concord isolates, 71 (86.5%) were ESBL-positive (MIC ratio for TZ/TZL was ≥ 8 or CT/CTL was ≥8), 10 isolates were non-determinable (ND) (MIC of TZ was > 32μg/ml and TZL > 4 μg/ml, as well as CT was >16 μg/ml and CTL >1μg/ml).

3.7. Molecular Characterization of *S*. Concord

3.7.1. Plasmid profile

Based on resistance pattern, place of isolation and type of specimen of isolation, a total of 13 *S.* Concord isolates were selected for plasmid analysis. All tested *S.* Concord isolates harbored plasmids and it was possible to distinguish ten different profiles with the majority of the isolates carrying multiple large and small plasmids (2-8 plasmids) (Figure 3.6 and Table 3.7). The molecular weight of plasmids varied from less than five to 170 kb with 120, 118 and 95 kb being the most prevalent. The two *S.* Concord isolates from blood carried very large (170 and 150 kb) plasmids and the strains were resistant to seven and eight drugs respectively. Since all *S.* Concord STs (533, 534,599) showed different plasmid profiles, there was no association between plasmid profile, antibiotic resistance pattern and ST types.

3.7.2. Incompatibility testing

Plasmid incompatibility grouping was done by using three multiple panels which can identify 18 plasmid replicons (Figure 3.7). Based on resistance pattern, place of isolation and type of specimen of isolation, seven *S.* Concord were selected for incompatibility testing. Among the *S.* Concord strains tested for all plasmid replicons, all harbored 2 or more plasmid replicon types. Over all plasmid replicon variability was seen, seven replicon

profiles were observed with profile A/C, I1, FI_B being most prevalent. In total seven different types of plasmid replicon genes were observed among the seven Concord isolates with A/C and I1 being most prevalent.

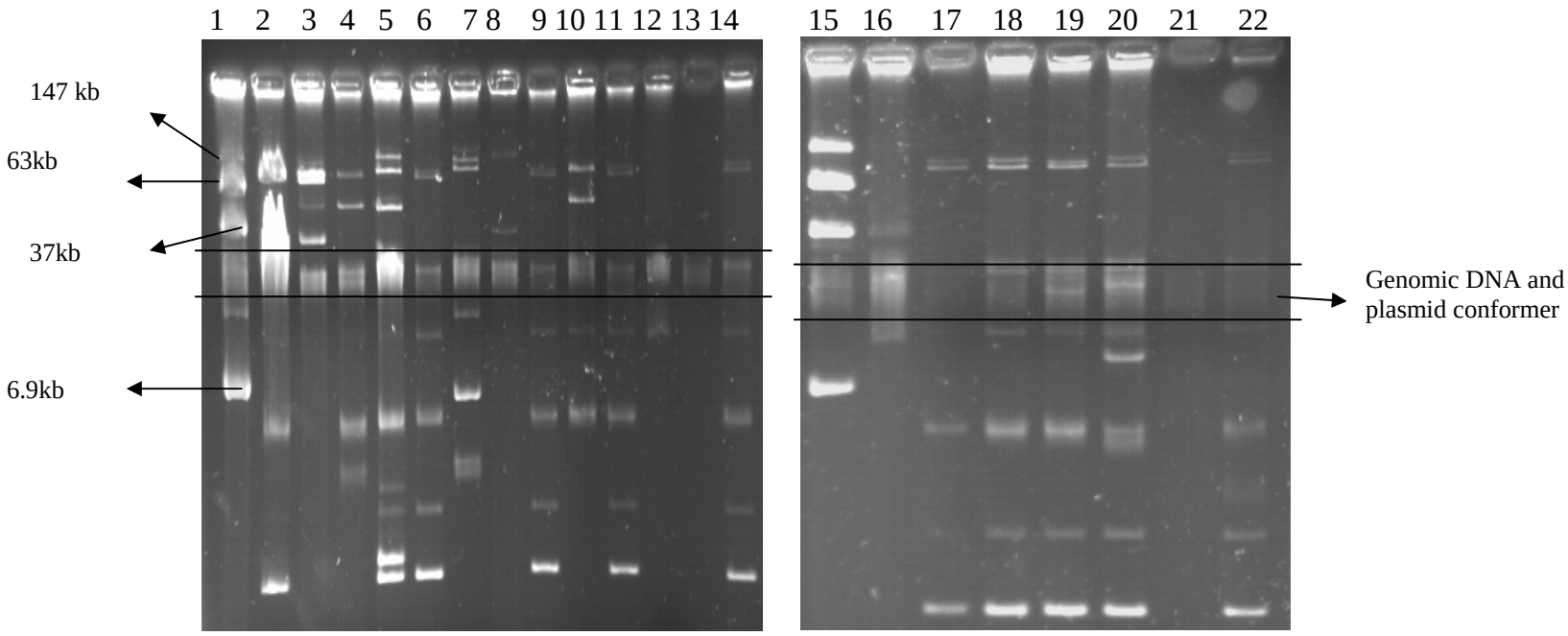

Figure 3.6: Analysis of plasmids from *Salmonella* strains. Lanes: 1 and 15 contains E39R861 size markers; Lanes 12 and 16, Bac_Tracker marker (degraded); Lanes 2, 4-11, 17, 18, 20 and 22, *S*. Concord; Lane 3, *S*. Enteritidis; Lane 13, *S*. Gatow; Lane 14, *S*. Laronchelle; Lane 19 *S*. Colorado and Lane 21, *S*. Butantan. The strain number, number and sizes of plasmids for each isolates are explained on Table3.7.

Table 3.7: Plasmid profile, plasmid replicon, and sequence type and resistance pattern of selected *S*. Concord/Concord group isolates

Strain No.	Serotype	ST	Plasmid Size(kb)	Lane in Fig. 3.6	Plasmid Replicons	Lane in Fig. 3.7 (panel 1,2,3)	Resistance pattern
002	*S*. Concord	533	120,118,12,<5(3)	2	ND	ND	AMP, AMX, SXT, CRO, C
006	*S*. Concord*	ND	95,47, <5	4	ND	ND	AMP, AMX, SXT, CRO, C, GM
008	*S*. Concord*J	533	170, 95,45, <5(5)	5	A/C, I1, FI_B	1	AMP,AMX, SXT,CRO,C,GM,NA
014	*S*. ConcordJ	ND	95,19, <5(3)	6	A/C, I1, FI_B	3	AMP, SXT, CRO, C, GM
016	*S*. Concord	599	147, 120, 20,7, <5(1)	7	ND	ND	AMP, AMX, SXT, TE
019	*S*. Concord*	599	150,36	8	L/M, HI2, A/C	4	AMP, AMX, SXT, CRO, TE, C, GM, NA
022	*S*. Concord	533	ND		FI_B, I1,A/C, BO	5	AMP, AMX, SXT, CRO, TE, C
025	*S*. Concord	599	ND		L/M, HI2, I1, A/C, FI_B,B/O,FI_C	6	AMP, AMX, SXT, CRO, TE, C, GM
027	*S*. Concord	533	95,19, <5(3)	9	A/C, I1, FI_B	7	AMP, AMX, SXT, CRO, TE, C, GM
029	*S*. Concord*	534	95,44, 18, <5(1)	10	ND	ND	AMP, AMX, SXT, CRO, TE, C, GM, NA
038	*S*. Garoli**	534	ND		L/M, FI_B, HI2, A/C	8	AM, AMX, SXT, CRO, C, GM
047	*S*. Concord	533	ND		A/C, I1, FI_B	9	AM, AMX, SXT, CRO, C, GM
049	*S*. Concord	ND	95,19, <5(3)	11	ND	ND	AMP, AMX, SXT, CRO, GM
051	*S*. Laronchelle**	533	95,19, <5(3)	14	I1, FI_B, A/C, P, T	11	TE, C
052	*S*. Concord	ND	120, 118,20, <5(3)	17	ND	ND	AMP, AMX, SXT, CRO, C, OFX
054	*S*. Concord	533	120,118, 20, <5(3)	18	ND	ND	AMP, AMX, SXT, CRO, C, TE, GM
062	*S*. Concord	ND	120, 118, 20,8, <5(3)	20	ND	ND	AMP, AMX, SXT, CRO, C, TE, GM
073	*S*. Concord*	533	120,118, <5	22	ND	ND	AMP,AMX, SXT, CRO,C,GM,NA

* = Blood isolates

**= Concord Group (have similar STs with *S*. Concord)

J = Isolated from Jimma

( ) = Number of plasmids with less than 5 kb

ND= Not done

ST= Sequence types

AM: Ampicillin; AMX: Amoxicillin; SXT: Trimethoprim-sulphamethaxole; CRO: Ceftriaxone; C: Chloramphenicol; TE: Tetracycline; GM: Gentamycin; NA: Nalidixic acid; OFX: Ofloxacin; CIP: Ciprofloxacin.

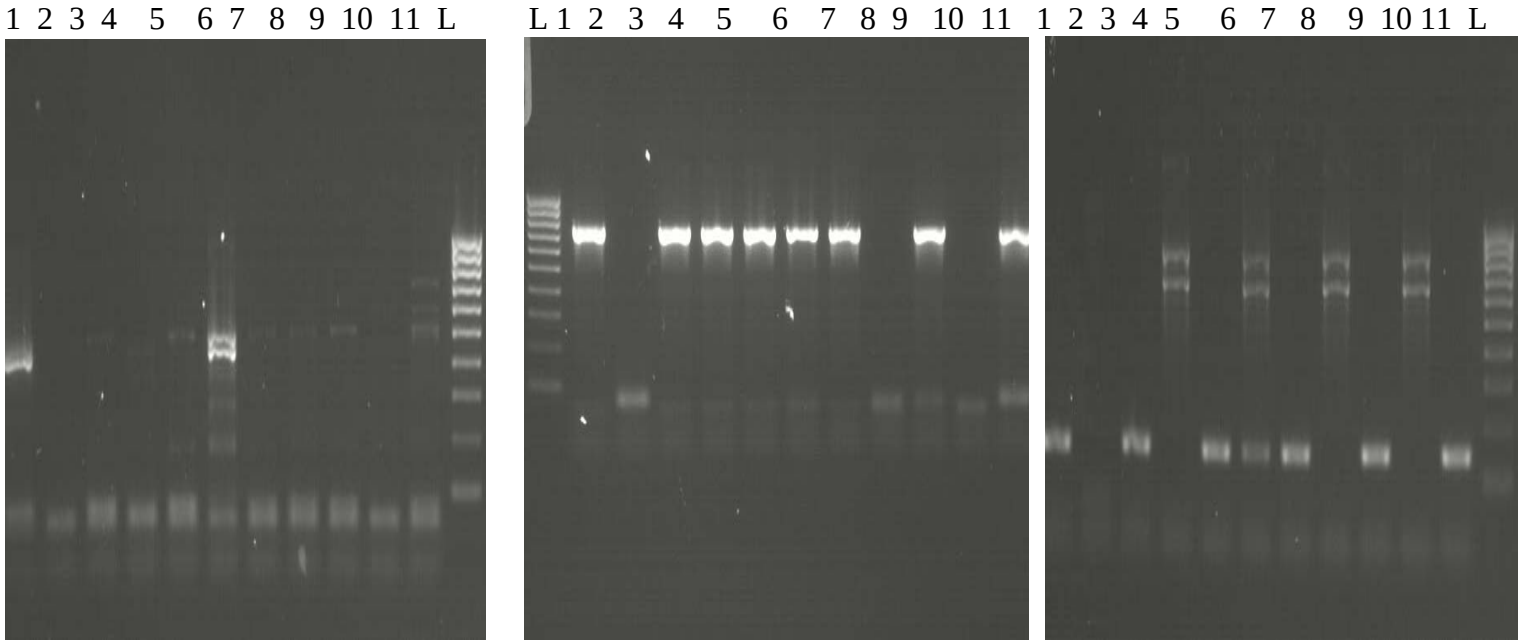

Figure 3.7: Incompatibility grouping of serogroup C *Salmonella* isolates.

Panel 1 (5-plexes): B/O, FI_C, A/C, P and T replicons

Panel 2 (6-plexes), K/B, W, FII_A, FIA, FIB and Y replicons

Panel 3 (7-plexes) I1, F_{rep}, X, HI1, N, HI2 and L/M replicons

Lanes (Panel 1, 2 and 3): L, 1000kb ladder; Lanes 1, 3-7, and 9, *S*. Concord; Lane 8, *S*. Garoli; Lane 11, *S*. Laronchelle; Lane 2, *S*. Haifa (negative control); Lane 10, *S*. Gatow. The types of replicons are shown on Table 3.7.

3.7.3. PFGE analysis

Based on resistance pattern, place and type of specimen of isolation, 20 *S.* Concord isolates were selected for PFGE analysis. PFGE analysis was done to compare the genetic relatedness of *S.* Concord isolated from different geographical locations and body sites (blood and stool). PFGE analysis was done by digesting genomic DNA with *XbaI* enzyme on 20 selected *S.* Concord and other isolates (Figure 3.8). Three isolates (strain no. 005, 010, 070) (Table 3.7) originally identified as *S.* Concord, gave a different band profile from the other *S.* Concord isolates. These isolates were later confirmed as *S.* Enteritidis, *S.* Haifa and *S.* Butantan respectively by the Scottish *Salmonella* Reference Laboratory.

The PFGE analysis of 20 *S.* Concord isolates produced 9-13 fragments which ranged in size from 1135 kb to 50 kb. Over all sixteen different PFGE types/ profiles were seen among all isolates (Table 3.8). Isolates which have the same number and size of DNA band/fragment was considered as clonal and strains with differences in three or more bands were constituted a new PFGE profile (Tenover *et al.*, 1995). Eleven PFGE profiles were seen among the 20 *S.* Concord isolates and 13 profiles among the whole Concord group. Six profiles were observed among the ten blood isolates with profile 3 being relatively common. There was no association between the PFGE profiles of strains that were either isolated from different locations or from different specimens (blood/stool). *S.* Colindale and *S.* Gatow from group C and *S.* Butantan (Group E) showed different profiles either from *S.* Concord or Concord group. Within Concord group, different STs showed different profiles: 1, 4, 5, 10,12,13,14 in 533; 3 and 11 in 534 and profile 6 and 8 in 599 ST).

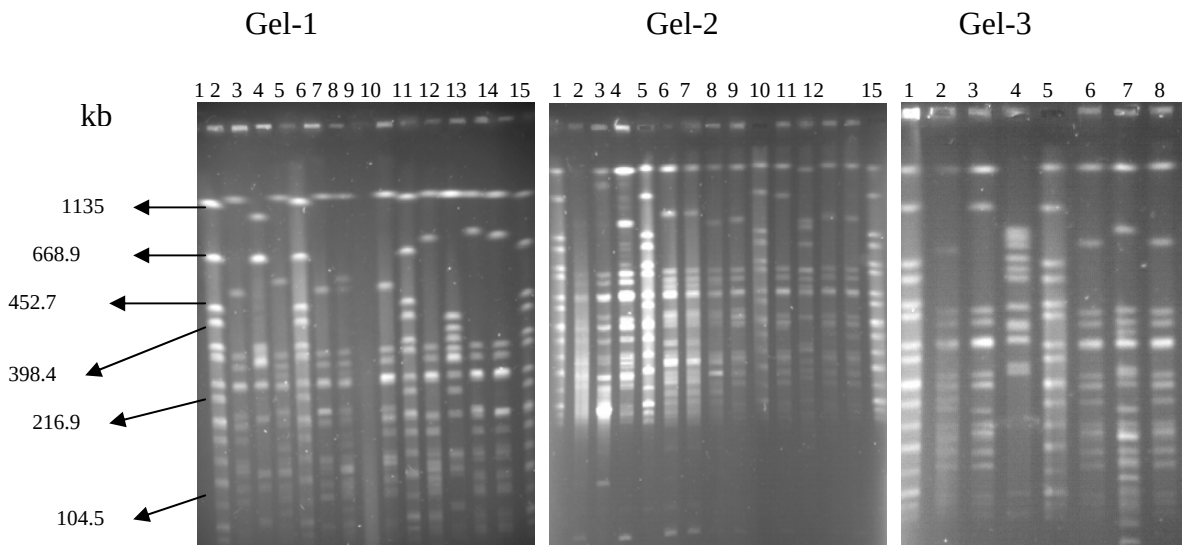

Figure 3.8: PFGE patterns for XbaI-digested genomic DNA of serogroup C *Salmonella* isolates.

Gel-1: Lanes: 1, 5, 10 and 15 *S*. Braenderup global standard markers strain H9812 (CDC). Lanes: 2, 4, 6, 7, 9,11,13,14 are PFGE patterns of *S*. Concord; Lane 3, *S*. Enteritidis; Lane 8, *S*. Haifa; Lane 12, *S*. Colindale.

Gel-2: Lanes: 1, 5, 10 and 15 *S*. Braenderup marker; Lane 2,3,4,6,7,9,11, and 14, *S*. Concord ; Lane 12, *S*. gatow; Lane 8, *S*. Garoli;Lane 13, *S*. Laronchelle

Gel-3: Lanes: 1 and 5 *S*. Braenderup marker; Lanes 3, 6-8, *S*. Concord; Lane 4, *S*. Butantan and Lane 2, *S*. Colorado.

Table 3.8: PFGE patterns and ST types of *Salmonella* species isolated from different location and specimen

Strain	Serotype	Specimen	PFGE Gel	Lane in Figure 3.8	Location of Isolation	PFGE Type	Plasmid profile	ST Type
002	*S*. Concord	Stool	1	2	A. Ababa	1	1	533
005	*S*. Entiritidis	Stool	1	3	A. Ababa	2		-
006	*S*.Concord	Blood	1	4	A. Ababa	3	2	ND
008	*S*. Concord	Blood	1	6	Jimma	4	3	533
009	*S*. Concord	Blood	1	7	Jimma	3		ND
010	*S*. Haifa	Stool	1	8	A. Ababa	Degraded		49
014	*S*. Concord	Stool	1	9	Jimma	5	4	ND
016	*S*.Concord	Stool	1	11	A. Ababa	6	5	599
018	*S*. Colindale	Stool	1	12	A. Ababa	7		535
019	*S*. Concord	Blood	1	13	A. Ababa	8	6	599
020	*S*. Concord	Blood	1	14	A. Ababa	9		ND
022	*S*. Concord	Stool	2	2	A. Ababa	Partially degraded		533
025	*S*. Concord	Stool	2	3	A. Ababa	6		599
027	*S*. Concord	Stool	2	4	A. Ababa	10	4	533
029	*S*. Concord	Blood	2	6	A. Ababa	3	7	534
031	*S*. Concord	Blood	2	7	A. Ababa	3		ND
038	*S*. Garoli*	Stool	2	8	A. Ababa	11		534
047	*S*. Concord	Blood	2	9	A. Ababa	5		533
049	*S*. Concord	Stool	2	11	A. Ababa	9	4	ND
050	*S*. Gatow	Stool	2	12	A. Ababa	16		536
051	*S*. Laronchelle*	Stool	2	13	A. Ababa	13	4	533
052	*S*. Concord	Stool	2	14	A. Ababa	13	8	ND
059	*S*. Colorado*	Stool	3	2	A. Ababa	1	8	533
060	*S*. Concord	Stool	3	3	A. Ababa	14		533
070	*S*. Butantan	Stool	3	4	A. Ababa	15		600
073	*S*. Concord	Blood	3	6	A. Ababa	12	9	533
081	S. Concord	Stool	3	7	A. Ababa	3		534
082	*S*. Concord	Blood	3	8	A. Ababa	12		533

* = Concord Group
ND= Note done

3.7.4. Multilocus sequence typing profile

The seven gene MLST scheme identified seven STs (sequence types) among the 58 *Salmonella* isolates tested (Table 3.9), with ST533 encompassing 65.5% (38/58). The second most common sequence type was ST534 (15.5%) followed by ST599 (8.2%) with each sharing six alleles with ST533, hence belonging in the same MLST group. *S.* Laronchelle and *S.* Colorado had the same ST with that of *S.* Concord ST533, while *S.* Garoli has the same ST with *S.* Concord ST534. Therefore these serovars (Laroncelle, Colorado and Garoli) were considered and characterized as Concord group.

Three types of STs (533, 534, 599) were observed among the 52 *S.* Concord group of isolates with ST533 being the majority (38/52). ST536 (*S.* Gatow) and ST535 (*S.* Colidale), were not closely related to the *S.* Concord group. Eight strains which were untypable by serology (non-motile) isolates exhibited three types of STs (533, 534, and 536). The untypable isolates of the ST533/ 534 group are probably non motile variants of *S.* Concord whereas the untypable ST536 was not considered to be *S.* Concord, but most likely to be a non- motile variants of *S.* Gatow. *S.* Butantan and *S.* Haifa which were from other serogroups showed completely different new sequence types from Concord groups.

Table 3.9: Serovar distribution and STs of selected serogroup C isolates.

Serovar	**ST**	**No. isolates**	**Allelic profile**						
			aroC	***dnaN***	***hemD***	***hisD***	***purE***	***sucA***	***thrA***
S. Concord	533	30	14	7	3	191	6	19	12
S. Colorado	533	1	14	7	3	191	6	19	12
S. Laronchelle	533	1	14	7	3	191	6	19	12
Untypeble(Group C)	533	6	14	7	3	191	6	19	12
S. Concord	534	7	181	7	3	191	6	19	12
S. Garoli	534	1	181	7	3	191	6	19	12
Untypable(Group C)	534	1	181	7	3	191	6	19	12
S. Concord	599	5	14	7	3	12	6	19	12
S. Colindale	535	1	17	2	16	166	160	171	119
*S.*Gatow	536	2	111	109	17	149	161	13	23
Untypable(Group C)	536	1	111	109	17	149	161	13	23
S. Butantan	600	1	192	11	49	42	161	13	3
S. Haifa	49	1	5	14	21	9	6	12	17

3.7.5. fliC gene sequencing

Genes encoding flagellin (*fliC* – phase 1 antigen) are typically conserved at the 5' and 3' ends in *Salmonella*. In this study the partial sequence of the variable central region of *fliC* for 39 strains was analyzed (Table 3.10). Alignments of the *fliC* sequences from the different STs generated are shown in annex III.

Alignments indicate that there are 3 *fliC* profiles. *fliC* profile 1 includes all the different ST types (533, 534, 599, g533 and g534) (Table 3.9). *fliC* profile 2 which has 1bp change (C → G) as compared to *fliC* profile 1 at position 381 of the sequenced fragment (annex II) is also represented by the ST533 types in this study. Both *fliC* profile 1 and 2 are represented by serovars belonging to the *S*. Concord group which agrees with the MLST data in indicating that these isolates are closely related (Table 3.10).

fliC profile 3 (Table 3.9) which belongs to *S*. Haifa (group B) has multiple base pair changes compared to the other 2 profiles (annex III).

Table 3.10. Co-relation between *fliC* profiles and ST types

Strain	Serotype	*fliC* profile	ST Type
032	Concord	1	599
054	Concord	1	533
065	Concord	1	533
075	Concord	1	533
025	Concord	1	599
082	Concord	1	533
035	Concord	1	534
012	Concord	1	534
016	Concord	1	599
058	Concord	1	533
047	Concord	1	533
076	Concord	1	533
078	Non-motile Group C	1	g533
003	Concord	1	533
002	Concord	1	533
081	Non-motile Group C	1	g534
059	Colorado	1	533
051	Laronchelle	1	g533
013	Concord	1	533
019	Concord	1	599
028	Concord	1	534
029	Concord	1	534
038	Garoli	1	534
061	Non-motile Group C	1	g533
040	Concord	1	533
064	Concord	1	533
069	Concord	1	533
071	Concord	1	533
077	Non-motile Group C	1	g533
092	Concord	1	599
027	Concord	2	533
072	Concord	2	533
024	Concord	2	533
022	Concord	2	533
063	Concord	2	533
073	Concord	2	533
086	Concord	2	533
080	Concord	2	533
010	Haifa	3	49

CHAPTER IV

DISCUSSION

CHAPTER IV. DISCUSSION

Diarrhoeal infections can be caused by many etiological agents, but mainly by enterobacteria such as *Escherichia coli, Salmonella* spp., *Shigella* spp., *Campylobacter jejuni* and *Vibrio cholerae*. Parasites such as *Entamoeba histolytica* and *Giardia lamblia*, and some rotaviruses are also important agents (Okeke et al., 2003).

Among the bacteria causing diarrhoeal diseases, *Salmonella* spp. continues to be a major public health problem. Although most *Salmonella* infections are self-limiting, serious sequelae, including systemic infection and death, can occur. In addition, various *Salmonella* spp. resistant to the commonly available drugs have been reported with increasing frequency through out the world (Parry *et al*., 2003). In Ethiopia, both published and unpublished data from different health institutions have previously reported on the isolation of different enteric pathogens from febrile and diarrheic children and the presence of enteric bacteria particularly *Salmonella* species which are resistant to the commonly used drugs. In the present study a total of 463 entropathogens were isolated from stool and blood samples (Table 3.2).

INTESTINAL PARASITES

Of the nine different intestinal parasites isolated in the present study, *G. lamblia* and *E. histolytica* were the dominant parasites with prevalence rates of 8.8% and 5.8% respectively (Table 3.3). Since all samples were taken from children with diarrhoea, high isolation rates of these protozoa may not be surprising. In fact these two parasites are well known etiologic agent of diarrhoea (Vargas *et al*., 2004). Even though 71 E. histolytic were reported, the actual figure will be very much less, since it needs special training to identify using the conventional method or needs more sensitive and specific molecular methods as it was witnessed with the study from Ethiopia (Kebede, 2005) . The data also indicated that there is an age dependent association with soil transmitted helminthes like *A. lumbricoides*, *T. trichuria* and *S. mansoni* (may be acquired through swimming), which were more frequent in older children (Figure3.2). Older children have more mobility and spend more time outside the house and so have higher exposure to contaminated soil or river water, the

probable sources of these helminthes. In contrast protozoan and bacterial pathogens were isolated more frequently from younger children suggesting transmission through contamination of water and food at home. Comparison with previous study results conducted in different parts of Ethiopia is difficult since the parasite prevalence varies with agro-ecozone, altitude and other environmental factors which are not studied here.

Many studies have previously demonstrated the high prevalence of parasitic infections among Ethiopian children in various parts of the country (Ali et al., 1999; Hileamlak, 2005; Merid et al., 2001; Roma and Worku, 1997; Roma et al., 2000; Tadesse, 2005). The intestinal parasite prevalence rate of 27.5% is similar to that reported by Tadesse (2005) (27.2%) in Eastern Ethiopia from school children but lower than that reported by Haileamlak (2005) (54.7%) in Southwest Ethiopia on children aged 1-5 years. The low prevalence rate in our study could be ascribed to the simple microscopic techniques we used compared with the concentration techniques used by others; furthermore we took samples only from children with diarrhoea. Mixed infections (4%) were less frequent than mono-infections, a finding that has been previously confirmed (Prats *et al*., 1997).

In this study, *A. lumbricoides* (3.5%) and *T. trichuria* (3.1%) were found to be the dominant soil transmitted helminthes (Table 3.3). Studies done by Ali *et al.* (1999) and Roma and Solomon (1997) reported higher rates of *A. lumbricoides* (54.6% vs. 75.2%) and *T. trichuria* (21% vs. 24.4%) respectively. Our study data showed comparable prevalence rate with previous report by Tadesse (2005) where the prevalence rate of *A. lumbricoides* and *T. trichuria* were 3.6% and 3.9%, respectively.

A prevalence rate of 3% for *H. nana* is higher than earlier findings, which were 1.1% (Roma and Worku, 1997), 1.3% (Assefa *et al.*, 1998) and 2.8% (LO *et al*., 1989) and lower than with the findings of Tadesse (2005) (10.1%) and Haileamlak (2005) (4.3%) .

SHIGELLA SPECIES

Shigellosis is primarily a pediatric disease, with more than half of all infections occurring in children between six month to 10 years of age as observed in previous Ethiopian study (Mache, 2001). The isolation of *Shigella* species (5%) in our study is lower than reported by Mache (2001) (20.1%) from similar study subjects and area (Table 3.3).

The low isolation of *Shigella* in this study compared to the previous study in Jimma could be due to increased awareness of the community about personal and environmental hygiene from the continuous interventions made by the health science students from Jimma University during their field practice. Since this is a preliminary study, it should be verified by further study of communities' knowledge, attitude and practice of post intervention or further epidemiological study should be done.

Even though, the study was conducted in a different age groups, our prevalence rate of 5% was closer to 5.8% (Andualem and Geyid, 2003) in Addis Ababa and 8.7% frequency of *Shigella* isolates in Gondar (Andualem *et al*., 2006), but lower than that reported by Ashenafi (1983) (9%) and 11.7% isolation rate reported by Asrat *et al*. (1999) at Tikur Anbessa, Ethio-Swedish children's hospital. The isolation rate of *S. sonne*i (21.3%) in our study seems high. In developed countries, higher frequencies of *S. sonnei* have been reported, but these frequencies are gradually decreasing (Gross *et al*., 1994).

In our study, serogroup B (*S. flexner*i) was the most commonly isolated species at 68.9 % (Table 3.3). The dominance of *S. flexneri* has been reported in previous Ethiopian studies, which is similar with our study result (Mache, 2001; Roma et al., 2000). The predominance of *S. flexneri* in developing countries has also been reported else where (Murray, 1989).

Differences in prevalence and serogroups findings among different studies can be explained by variations in geographical, socio-economic conditions, and cultural practices of the communities under consideration. The category of the study population, the duration and seasons of the year in which the study took place may also contribute to the variation in results.

SALMONELLA SPECIES

Prevalence

Epidemiological investigation of salmonellosis in developing countries like Ethiopia is difficult because of the very limited scope of the studies and lack of coordinated surveillance systems. The over all prevalence of *Salmonella* in this study was 5.3% (Table 3.3). This is comparable with studies conducted in Ethiopia at different times, 4.5% (Ashenaffi and

Gedebou, 1985), 6.4 % (Mache *et al*., 1997), 4.5 % (Ashenafi, 1983) and higher than the findings reported by Asrat *et al*. (1999) (3.8%) and Aseffa *et al.* (1997) (1%) which could be difference in study time, place, age group of the study subject, study objectives and other factors which were not studied/ considered in this study.

But lower than reported by Mache (2002), (15 %) and Awol (2002) (8.1%). The low isolation of *Salmonella* in this study could be due to the use of antibiotics prior to visiting health institutions. It was found that recent antibiotic use reduced blood culture yields by 62 to 73% in patients with sever or fatal disease (Berkley *et al*., 2005). In this study 10% of the patients had evidence of recent antibiotic use, but it is likely that many more patients had been exposed to antibiotics in the few days preceding to presentation at the health institutions. In this study, it was observed that isolation frequency of *Salmonella* was high in children between 2-5 years of age (Figure 3.3).

Clinical features

The clinical features of *Salmonella* infections commonly presented with diarrhoea, fever, head ache and abdominal cramping (Hohmann, 2001), which is similar to our study where abdominal pain and diarrhoea were the dominant symptoms of culture positive cases. The incubation period for *Salmonella* gastroenteritis is typically from 12 to 72 hrs (Hohmann, 2001), which is in agreement with this study where the duration of diarrhoea was between 1 to 5 days in majority of patients (Table 3.1).

Serogroups

Previous studies conducted in Ethiopia on *Salmonella* in humans, animals, animal food products and other foods indicated the presence of a number of serogroups/serotypes (Erku and Ashenafi, 1998; Gedebou and Tassew, 1981; Mache *et al*., 1997; Molla *et al*., 1999; Molla *et al*., 2003). In Ethiopia, all the major serogroups (A, B, C, D and E) including *S.* Typhi (which is usually identified by serotyping in most studies), have been reported by different investigators and among the isolated serogroups, A and E were reported with very low frequency, where as serogroup B and C interchangeably were the dominant isolates (Gebre-Yohannes, 1985; Gedebou and Tassew, 1981; Mache, 2002; Mache *et al*., 1997).

Among the 113 *Salmonella* isolates in this study, serogroup C comprised 78.8%, B 11.4%, D 8% and E, 1.8% (Table 3.4). There was no group A isolate. The high prevalence of *Salmonella* serogroup C (78.8%) isolates in this study is similar to previous findings reported in Addis Ababa (Ashenafi and Gedebou, 1985: Mache *et al*., 1997), but different from studies conducted in Gondar, Jimma and Addis Ababa, where serogroup B was the dominant isolate (Assefa *et al*., 1997; Mache, 2002; Asrat, 2008). The difference in the pattern of serogroup may be due to ecological (animal reservoirs) or geographical differences, study time, or differences in the human host.

Serovars

In developing countries NTS account for a steadily increasing proportion of human infections and are the major agents of food poisoning and acute gastroenteritis; however, they can cause severe diseases such as septicemia and local infections at any site of the body. This study showed that 96.3 % (Table 3.4) of the isolates were NTS which is in agreement with earlier studies done in Addis Ababa (84.4%) (Mache *et al*., 1997) and Jimma (78%) (Mache, 2002). This is in contrast with the findings of hospital based studies conducted by Gedebou and Tassew (1981) where *S.* Typhi constituted 75% of the total isolates and much higher from that of Gebre-Yohannes (1985), where NTS accounted for 43.1 % of the isolates. Compared to the prevalence of *S.* Typhi the isolation rate of non-Typhi isolates is increasing over time in Ethiopia; (24.8%) (Gedebou and Tassew, 1981), 51.4% (Gebre-Yohannes, 1985), (56.4%) (Wolday, 1998), (84.4%) (Mache *et al*., 1997) and (78%) (Mache, 2002). This is also true in other parts of the world where the incidence of *S.* Typhi has decreased substantially to non-significant levels, while the incidence of NTS has increased dramatically and become a major public health concern (Weinberger and Keller, 2005).

It is well known fact, that humans are the only host to *S.* Typhi and transmission via food and water contaminated with human wastes can be interrupted by improving personal and environmental sanitation, whereas in the case of NTS, the presence of multiple reservoir hosts makes prevention more complex. It was found that most human NTS infections in the United States are related to ingestion of contaminated food products rather than person-to-

person transmission or direct fecal-oral transmission and many outbreaks have been traced to ingestion of contaminated animal products and in some cases, traced to specific farms, flocks, or herds of animals (Altekruse *et al*., 1993; Winokur *et al*., 2000). The other reason for increased prevalence of NTS could be suppression of the immune system. NTS are especially problematic in a wide variety of immunocompromised individuals, including patients with malignancy, HIV, or diabetes, extreme ages and those receiving corticosteroid therapy or treatment with other immunotherapy agents (Hohmann, 2001).

An effective T helper 1 cell (Th1) response is necessary for killing of intracellular *Salmonella* by macrophages. Thus young age and conditions that suppress cell-mediated immunity and reduce intestinal mucosal integrity such as malnutrition and HIV infection increase invasive salmonellosis and mortality (Graham, 2000). Approximately 5% of individuals with gastrointestinal illness caused by nontyphoidal *Salmonella* will develop bacteremia, a serious and potentially fatal problem. Bacteremia is more likely to occur in immunologically compromised patients and these hosts are also more likely to develop focal infection (Hohmann, 2001). Several studies in tropical Africa investigating invasive diseases in children have shown that NTS are important causes of life threatening infection particularly in infants and young children below the age of 5 years (Berkley *et al*., 2005). The most common manifestation of invasive NTS is bacteraemia. In contrast, studies from most parts of the developed world found that most NTS infections in children presented as gastroenteritis and the reported incidence of bacteraemia among children with gastroenteritis caused by NTS varies between 3.3% and 41% (Graham, 2002), although majority of studies reported incidences at no more than 5%.

In our study 96.3% of the isolates were NTS and 35% were isolated from bacteremic cases. Recent data from Malawi also showed that NTS were the most common blood culture isolates (40%), and NTS bacteraemia was diagnosed in 299 children during a 2- year period with a case fatality rate of 24% (Graham *et al*., 2000). In our study a higher proportion of children under 5 years of age (as compared with children over 5 years) presenting with NTS infection developed bacteraemia and this may be attributed to lower immune status in the younger children. HIV is another important predisposing factor for invasive NTS infections. Although the reason for this is not clear, published reports have demonstrated an association

between HIV and bacterial infections (Nathoo *et al*., 1996). Another similar study has shown that in USA and Europe *Salmonella* bacteraemia is commonly associated with childhood HIV infection (Rongkavilit *et al*., 2000). Although the HIV status of the study subjects was not investigated in the present study the over all prevalence of HIV infection in all age groups of Ethiopian population is about 3.5% (MOH, 2005).

Dissemination of NTS might also be enhanced by intestinal inflammation resulting from chronic diarrhoeal disease, parasitic infection, or suboptimal nutrition (Homman, 2001). A study conducted in Kenyan children found an association between bacteraemia caused by NTS with severe malnutrition, HIV infection and parasite infection including malaria (Berkley *et al*., 2005).

The increased occurrence of *Salmonella* infections within the last decades has accentuated the need for serotyping as a base for proper diagnosis, identification of sources of infection, control of products and to gain better understanding of the global epidemiology of *Salmonella*. In Ethiopia, there is no systematic surveillance system that helps to know the types of serovars that circulate in the country, to document the occurrence and trends of serovars, to detect local, regional, and even national out breaks, to find and eliminate the source and suggest preventive actions. Few published information is available regarding the different *Salmonella* serotypes that circulate within the country (Gebre-Yohannes, 1985). Findings from this study showed that among the 216 *Salmonella* isolates 26 different serotypes were observed, of these, *S.* Typhi (48.6%) was the dominating serotype followed by, *S.* Concord (12.5%) and *S.* Typhimurium (11.1%) (Gebre-Yohannes, 1985).

In the present investigation, the dominant serotype of *Salmonella* from both blood and stool samples were *S. Concord* (72.6 %) followed by *S.* Typhimurium (6.2%) (Table 3.4). *S.* Concord was reported in Ethiopia for the first time more than two decades ago from a bone-processing factory in Addis Ababa (Pegram *et al*., 1981). Later, Gebre Yohannes (1985) reported that *S.* Concord and *S.* Typhimurium were the commonest isolated among the non-Typhi *Salmonella* isolates and commented that the high isolation of *S.* Concord in Ethiopia during that time is unusual and needs further study to clarify the animal or food

source associated with its epidemiology. There was also a report of a single isolation *S.* Concord from Ethiopian migrant in Ireland (Morris *et al*., 2006).

The high percentage of isolation of *S.* Concord in Ethiopia in the previous and present studies is unique when compared to none or low isolation rate of this serotype in other countries of the world where other serotypes were predominantly isolated. For example in Zaire (Cheesbrough *et al*., 1997) and Rwanda (Lepage *et al*., 1990) *S.* Typhimurium was the predominant isolate. In Kenya, surveillance from 1994 to 1997 showed that *S.* Typhimurium predominated (prevalence of 75%) among cases of NTS bacteraemia and *S.* Enteritidis made up only 4.8%. However, after 1997 the proportion of *S.* Enteritidis rose steadily and by 2003 the two commonly isolated NTS serotypes were in almost equal proportions (Kariuk, *et al*, 2005). In other parts of Africa (Cameroon, Mali, Morocco, Senegal and Tunisia), *S.* Enteritidis and *S.* Typhimurium were reported in equal proportion in all age groups (Galanis *et al*., 2006). The difference in the pattern of serotypes may be due to ecological (animal reservoirs) or geographical differences in the Africa sub-region, or differences in the human host.

A study conducted by Galanis and his colleagues (2006) showed that different geographical regions harbor different serovars, and different serovars predominate at different periods of time. Nevertheless, a World Health Organization survey in all age groups on the global distribution of *Salmonella* between 2000 and 2002 showed that among human isolates, *S.* Enteritidis was the most common serovar, accounting for 65 % of all isolates followed by *S.* Typhimurium at 12 % and *S.* Newport at 4 % (Galanis *et al*., 2006). However there have been reports of low isolation rate of *S.* Concord else where (Erdem et al., 2005); (Hasman et al., 2005); (Barbour and Nabbut, 1982).

Antimicrobial susceptibility testing

Infection with non-typhoidal *Salmonella* in infants and children commonly produces self-limited diarrhoea. Studies have indicated that antimicrobial treatment for uncomplicated gastroenteritis does not shorten the duration and severity of symptoms; in contrast, it may prolong fecal excretion, increase the risk of relapse, and result in the emergence of antibiotic resistance (Chiu *et al*., 1999). Nevertheless, if extra-intestinal complications occur, effective

antimicrobial treatment is essential. Multidrug resistant phenotypes have been increasingly described among *Salmonella* species worldwide, according to the infectious disease report released by the WHO in 2000 (WHO, 2000).

In this study, majority of *Salmonella* isolates were resistant to ampicillin (82.3%), amoxicillin (83.2%), trimethiprim-sulphamethoxazole (80.5%), ceftriaxone (78.8%), chloramphenicol (81.4%), gentamycin (74.3%) and tetracycline (39.8%) (Table 3.5 and Figure 3.4). This observation is in contrast with findings of Gedebou and Tassew (Gedebou and Tassew, 1981) where most of the isolates were sensitive to ampicillin (86.7%), carbenicillin (83.6%), cephalothin (89.7%), chloramphenicol (88.5%), gentamicin (100%), kanamycin (89.7%), polymyxin (98.8%), tetracycline (88.5%) and trimethiprim-sulphamethoxazole (100%). Resistance to one or more drugs was observed in 88.5% of the isolates in this study which is comparable with previous study done in Ethiopia where 93% of the isolates were resistant to one or more drugs (Mache, 2002). Only 9.7% (11/113) of our isolates were sensitive to all drugs. High rate of resistance was seen among the isolates in serogroup C, where all isolates (100%) were resistant for one or more drugs which is higher than the previous studies done in Ethiopia, where 94% (Gedebou and Tassew, 1981) and 92.9% (Mache *et al*., 1997) of the serogroup C isolates were resistant to all antibiotics tested.

In this study all *S.* Concord isolates were resistant to between five and eight drugs (Table 3.6). This pattern of resistance has been observed for at least two decades (Gebre-Yohannes, 1985). The single isolate of *S.* Concord from an Ethiopian migrant in Ireland was also resistant to ampicillin, chloramphenicol, streptomycin, sulphonamide, tetracycline, trimethoprim and gentamicin (Morris *et al*., 2006).

In this study resistance to ampicillin, amoxicillin, trimethiprim-sulphamethoxazole, gentamicin, chloramphenicol and ceftriaxone combinations were dominant, signifying the resistance of *S.* Concord isolates to the most common multiple first line drugs including ceftriaxone, a third generation cephalosporin (Table 3.6). This high rate of resistance of *S.* Concord to commonly used drugs indicates probably the extensive use of these antibiotics for therapeutic and prophylactic purposes both for *Salmonella* and other infections. *S.*

Concord isolates showed 9.8% and 1.2% resistance to nalidixic acid and ofloxacin respectively. All isolates were susceptible to ciprofloxacin at least with disk diffusion test.

In Ethiopia the unregulated over-the-counter sale of these antimicrobials, mainly for self-treatment of suspected infection in humans, and to a lesser extent for use in animals without prescription, would inevitably lead to emergence and rapid dissemination of resistance. In addition availability of cheaper generic drugs of variable quality in the market for treatment of bacterial infections may also contribute to the increased level of resistance. A study on practice of self-medication in Jimma town showed that out of the 152 ill people, 27.6% were self-medicated (Worku and G/Mariam, 2003). The relative lesser cost (35.7%) was the major reason for using self medication.

Fluoroquinolones are broad-spectrum antimicrobial agents that target DNA gyrase (*gyrA* and *gyrB*) and topoisomerase IV genes (*parE* and *parC*) (Ercis *et al.*, 2006*)*. Isolates of *Salmonella* with nalidixic acid resistance commonly showed decreased susceptibility to ciprofloxacin (Oteo *et al*., 2000). Most isolates with nalidixic acid resistance have a single point mutation in the quinolone resistance determining region (QRDR) of *gyrA*; isolates with ciprofloxacin resistance have two QRDR point mutations (Oteo *et al*., 2000). In this study eight strains of *S*. Concord were fully resistant to nalidixic acid by disk diffusion test and of these, five (62.5%), one (12.5%) and two (25%) isolates showed decreased susceptibility, resistance and sensitivity to ciprofloxacin by E-test, respectively (Table 3.5 and Figure 3.5). Out of 12 strains which showed intermediate resistance using disk diffusion test for nalidixic acid 9 isolates showed decreased susceptibility, one intermediate and the other two were sensitive to ciprofloxacin by E-test. In this study of the total 22 isolates with reduced susceptibility to ciprofloxacin, 15 (68.2%) isolates were either resistant or intermediate to nalidixic acid susceptibility and the other seven isolates (31.8%) were susceptible to nalidixic acid. This indicates that all strains which show resistance/reduced susceptibility to nalidixic acid may not be resistant/ reduced susceptible to ciprofloxacin. On the other hand all isolates which show reduced susceptibility to ciprofloxacin may not be resistant to nalidixic acid which could be presence of resistance mechanism of ciprofloxacin other than mutation on DNA gyrase and MICs for NA will always be higher than for ciprofloxacin as resistance develops by step wise mutations (Cebrian *et al.,* 2005*)*. In our

study the isolates which were either resistant or intermediate to nalidixic acid by disk diffusion test showed high MIC for ciprofloxacin. There is also a report which makes the value of nalidixic acid disk diffusion test to screen for reduced susceptibility test for fluoroquinolones questionable (Hakanen *et al*., 2005).

A study from Finland indicated that out of 16 *Salmonella* isolates which had reduced ciprofloxacin susceptibility, eight were fully susceptible and the other eight isolates showed intermediate result to nalidixic acid by both E-test and disk diffusion test (Hakanen *et al*., 2005). In this study 70 % (14/20) of the Concord resistant/intermediate to nalidixic acid were reduced susceptible to ciprofloxacin which emphasizes the usefulness of nalidixic acid in monitoring emergence of ciprofloxacin resistance.

The presence of nalidixic acid sensitive strains with reduced susceptibility to ciprofloxacin in this and previously mentioned studies creates a problem especially in clinical laboratories that use nalidixic acid disk diffusion test to screen for reduced ciprofloxacin susceptibility in *Salmonella* isolates. Our data on reduced susceptibility to ciprofloxacin 26.8% is similar the findings reported in Taiwan (27.4%) but higher than reports from Australia (3.9%) (Parry, 2003) and Turkey (12.3%) (Ercis *et al*., 2006), but lower than the report from America (91%), (Stevenson *et al*., 2007). The development of nalidixic acid resistance strain is a problem of even the developed countries. A seven year (1996-2003) study in America, showed that 1.6% of the isolates were nalidixic acid resistant and 14(7%) of those were ciprofloxacin resistant and 91% of the 203 isolates showed decreased susceptibility (Stevenson *et al*., 2007). In a European surveillance study of 27,000 isolates in 2000, low-level ciprofloxacin resistance was found in 13% of *S.* Typhimurium, 8% of *S.* Enteritidis, 53% of *S.* Virchow and 57% of *S.* Hadar isolates (Threlfall *et al*., 2003).

Several studies have also suggested that the use of fluoroquinolone in veterinary medicine contributes to the emergence and dissemination of nalidixic acid resistance in *Salmonella* among food animals, which may be transmitted to humans (Angulo *et al*., 2004b). In Ethiopia 42.9% of *Salmonella* isolates from slaughtered pigs had developed resistance to both nalidixic acid and ciprofloxacin (Molla *et al*., 2006a). In our study, there were 12 Concord isolates with intermediate susceptibility to nalidixic acid of which 9

isolates showed reduced susceptibility to ciprofloxacin. This indicates that 'intermediate' result should be also considered as indicator of reduced susceptibility for ciprofloxacin.

The reason for the isolation of nalidixic acid sensitivity and ciprofloxacin resistance/reduced susceptibility strain is the involvement of other resistance mechanisms (mutations at other locations of the QRDR remain possible), such as efflux pumps. It has been shown that exposure to low concentrations of fluoroquinolones may lead to activation of the efflux pump system and a reduction in susceptibility, even when there are no mutations in *gyrA* (Cebrian *et al.*, 2005*)*. Moreover, a possible role of plasmid-mediated resistance in these isolates must be kept in mind, considering that plasmid coded resistance was observed in multiple serovars (Jacoby, 2005). We found that most of our nalidixic acid resistant Concord isolates were from blood, suggesting that nalidixic acid resistant *Salmonella* may be associated with more severe infections.

The overall ciprofloxacin resistance rate in Concord (1.2%) seems low however the percentage of reduced susceptibility for ciprofloxacin (26.8%) indicated that the development of resistant to this drug is imminent. Thus, the emerging resistance and reduced susceptibilities to fluoroquinolone in our *Salmonella* isolates is of great concern for Ethiopia. This heralds the need for local as well as national surveillance for resistance to nalidixic acid which is useful in monitoring emerging fluoroquinolone resistance.

Extended spectrum cephalosporins, especially ceftriaxone are commonly used to treat children with invasive infections or severe diarrhoea caused by *Salmonella enterica* (Hohmann, 2001). However resistance to expanded-spectrum cephalosporins (ESCs) like ceftriaxone among NTS has been recognized since the late 1980s and currently, ESC resistant *Salmonella* strains are reported world wide and in some countries their incidence is significant (Miriagou *et al*., 2004). The emergence of resistance to the first generation cephalosporins (cephalothin) in Ethiopia had been reported earlier, the prevalence of resistance was: 88.4% (Gedebou and Tassew, 1981), 78.6% (Mache *et al*., 1997), and 53.8% (Mache, 2002). To our knowledge there is no recent published data on the status of susceptibility of *Salmonella* species to third generation cephalosporins (ceftriaxone, ceftazidime, etc.) in Ethiopia. In this study, 98.8 % of *S.* Concord isolates were resistant to ceftriaxone (Table 3.5). This is much higher compared to earlier studies conducted in

Thailand 30%, (Boonmar *et al*., 1998), Yemen 42% (Banajeh *et al*., 2001), Kenya 15% (Oundo *et al*., 2000) and South Africa 15.6% (Kruger *et al*., 2004). The major concern is that 26.4% of the isolates which were resistant to ceftriaxone showed reduced susceptibility to ciprofloxacin in the present study. This figure is lower than from study result in England where it was found that 62% of ceftriaxone resistant isolates were resistant to ciprofloxacin (Threlfall *et al*., 2000). The high resistance of Concord to ceftriaxone could be, either the bacteria acquired the resistance from other bacteria (since it is plasmid mediated) or resulted from mis-use of this drug to treat wide range of bacterial infections. To date resistance to ESCs has been reported in 53 serotypes. Unlike our study where the *S.* Concord is predominant, the most frequently incriminated resistant serotypes in other countries are *S.* Typhimurium and *S.* Enteritidis (Arlet *et al*., 2006).

Production of ESBLs is predominantly associated with *Enterobacteriaceae* particularly *E. coli* and *K. pneumonia.* ESBLs production in *Salmonella* species was first identified in 1988 and then increased in prevalence world wide (Hammami *et al*., 1991). Isolation of *Salmonella* spp. with ESBL enzymes has occurred in diverse geographic areas of the world. Recent reports have documented several regions that have discovered ESBL enzymes, which include Europe, North America, Africa, Turkey, and the Asia-Pacific in both typhoidal and nontyphoidal species (Biedenbach *et al*., 2006). To our knowledge there is no previous study about ESBL detection in *Salmonella* species in Ethiopia. However there was a previous study that detected ESBL enzymes in clinical isolates of *Klebsiella* species from Harar, Ethiopia (Seid and Asrat, 2005).

The present study showed that 98.8% of *S.* Concord isolates were positive for production of ESBL. Beside this it was found that all strains which were nalidixic acid resistant/intermediate were also ESBL producers. Therefore, the presence of isolates with extended-spectrum beta-lactamase (ESBL) phenotypes and either reduced susceptibility or resistance to fluoroquinolone become an important clinical concern that could further compromise the use of ceftriaxone and fluoroquinolones such as ciprofloxacin for empirical treatment. This is particularly critical when treating at-risk patients including children who are more prone to complications related to infections caused by *Salmonella* species.

S. Concord which was isolated from Ethiopian migrant in Ireland was ESBL producer and resistant to seven drugs: ampicillin, chloramphenicol, streptomcycin-sulfonamides, tetracycline, trimethoprim, entamicin (Morris *et al*., 2006). Published data regarding ESBL producing *Salmonella* species in Africa is scarce. One study from South Africa showed that 5.6% of NTS produced ESBLs, (Kruger *et al*., 2004). These ESBL positive isolates were *S*. Typhimurium, *S*. Isangi and *S*. Muechen.

The production of plasmid-mediated ESBL and other beta-lactamase enzymes in *Salmonella* species is also an epidemiologic problem because resistance can be transferred between the *Enterobacteriaceae* including *Salmonella* species and clonal dissemination could cause large community outbreaks and the spread of resistant pathogens to the hospital setting (Su *et al*., 2003).

Overall, the high frequency of resistant *Salmonella* serovars to the various antimicrobials could be an indication of indiscriminate and continuous uses of sub-therapeutic doses of commonly available antimicrobials both in the veterinary and public health sectors in Ethiopia. It has been reported that the misuse or overuse of antimicrobials for treatment, prophylaxis and growth promotion in food animals has contributed to the emergence and spread of resistance to food-borne pathogens including *Salmonella* (Molla *et al*., 2006a). This is particularly striking in developing countries where there is a widespread misuse of antimicrobials due to the lack of access to appropriate treatment and under use due to inadequate dosing, poor drug quality and incomplete treatment courses. NTS infections in many developing countries including Ethiopia cause high morbidity and mortality, particularly in the young and in immunosuppressed individuals. It appears that more expensive and less readily available antimicrobials may be required in future to treat these infections.

MOLECULAR CHARACTERIZATION

Plasmid profile

The antibiotic resistance data in this study showed that all isolates of *S.* Concord were MDR. Therefore to understand the resistance mechanisms, selected *S.* Concord isolates were investigated for plasmid profile. All tested isolates carried multiple (2-8) large (118,120,150,170kb) and small (less than 5kb) plasmids (Table 3.7 and Figure 3.6). The plasmid analysis data was in agreement with previous study report in Ethiopia by Mache *et al*. (1997) where all tested MDR *Salmonella* isolates contained multiple (5-9) plasmids per strain. In the present study, two *S.* Concord isolates from blood carried large plasmids (170 and 150kb), which could be associated with invasiveness of the strain (Guerra *et al*., 2002). Most of the isolates carried relatively large (120, 118, 95kb) plasmids, which could be a fusion of virulence plasmids and R-plasmids. A 140 kb self transferable plasmids of the IncFI1 coding for chloramphenicol and tetracycline resistance was found to encode *Salmonella* virulence plasmids (*spv ABC* and *rck*) (Guerra *et al*., 2002). On the other hand *S.* Butantan which was susceptible to all antibiotic was found to be free of plasmids in this study.

It has been previously reported that several types of β-lactamase were found on plasmids of *Salmonella* isolates belonging to different incompatibility groups which can be transferred horizontally or vertically to different species (Llanes *et al*., 1999). The *S.* Concord isolates that carried multiple plasmids were also positive for ESBL production that could be transferable to different or the same species extends those observations.

Plasmid profile data indicated no strict association between antibiotic resistance and plasmid type. The heterogenity of the plasmid profile is thought to reflect the sporadic and non-repetitive nature of the isolates. Correlations were not seen between plasmid profile and PFGE. Two *S.* Concord isolates with the same plasmid profile had different PFGE pattern. Usually plasmids don't provide enough discrimination due to instability and low level of diversity of extra-chromosomal DNAs harbored among *Salmonella* isolates (Fernandes *et al*., 2003). *Salmonella* serovars of subspecies I usually carry a large low copy number plasmid that contains virulence genes that required triggering systemic infection. A trans-conjugative experiment showed that large plasmids of *Salmonella* can be involved also in

antibiotic resistance (Rychlik *et al*., 2006). Even though we didn't do virulence or conjugative experiments, the multiple plasmids in our strains may be involved in these activities.

Plasmid incompatibility

Classification of plasmids into incompatibility groups is desirable because specific plasmids types have been associated with virulence and antimicrobial resistance. Our data indicated that replicons A/C and IncFI were the most common replicons (Table 3.7 and Figure 3.7). Different plasmid studies indicated that these two replicons are associated with MDR *Salmonella* isolates. Class 1 integrons have been identified on Inc FI and L/M plasmids which accounts for almost the entire resistance phenotype observed in *Salmonella* isolates (Tosini *et al*., 1998). The IncFI plasmids were very common in different serotypes of *Salmonella* when the resistance type was to-ampicillin-chloramphenicol-kanamycin-sulfonamide-streptomycin-tetracycline. Other similar studies have shown that Resistance (R-) plasmids of the IncFI incompatibility group have been associated with emergence of MDR *S. enterica* serotypes (Anderson *et al*., 1977). For example, IncFI plasmids were found most often in Middle Eastern isolates of *S.* Typhimurium, but similar plasmids were also identified from Africa, Europe, and North America (Anderson *et al*., 1977).

A study done elsewhere proved that epidemiologically unrelated *Enterobacteriaceae* demonstrate the dominancy of incompatibility A/C carrying some emerging resistance determinants to extended-spectrum cephalosporins and carbapenems (Carattoli *et al*., 2006). Similar plasmids replicons were observed in *S.* Concord isolates from Jimma and Addis Ababa, which demonstrate that plasmids encoding resistance to clinically relevant antimicrobial agents can spread among different regions (Table 3.7). Except one isolate which was resistant to two drugs the rest were resistant to four or more drugs. This is in agreement with the study done previously where all three isolates of *S.* Typhimurium with A/C plasmid replicon showed resistance to five or more drugs including ampcillin, ceftriaxone-ceftazidime, cefotaxime and cefoxitin (Carattoli *et al*., 2006). Our data indicate the presence of multiple replicons which could be responsible for the development of MDR *S.* Concord. A systematic search for integrons and transposable elements and relating with the type of replicons could provide a useful genetic basis for MDR in *S.* Concord isolates.

PFGE

Currently PFGE is the standard method for *Salmonella* outbreak investigation and is suitable for examining epidemiologically related *Salmonella* strains (Foley *et al*., 2006). In this study 16 PFGE profiles were seen among the 23 Concord group and other 5 non Concord isolates (Figure 3.8). Every ST had their own PFGE profile (e.g. ST 533: 1, 4, 5, 10, 12, 13, 14; 534, 3 and 11; 599, 6 and 8). This indicates that *S.* Concord in Ethiopia is in an endemic situation, rather than a spread of a clonal type (has many different point sources/reservoirs). The same ST and PFGE types were found in blood and stool suggesting there was no association between genotype and invasiveness. The same ST and PFGE types are found in Addis Ababa and in Jimma suggesting movement of infected people between the two cities (Table 3.8).

In the present investigation, among the 13 Concord group isolates which were tested for both PFGE and plasmid analysis, there were 10 PFGE and nine plasmid profiles. Among the 27 *Salmonella* isolates tested for both PFGE and MLST there were 15 PFGE profiles and seven STs. This showed that PFGE has high discriminatory power in investigating epidemiological relationship of *Salmonella* isolates. In this study, isolates which have the same number and size of DNA band/fragment was considered as clonal. However, Davis and his colleagues probed the individual fragment of *E. coli* 0157 and found that fragments which migrated the same distance were not always genetically homologous (Davis *et al*., 2003). In addition, the limitation of the power of PFGE to resolve fragments of similar sizes augments the problem, as fragments which are similar, but not identical, in size may migrate together. The authors concluded that for *E. coli* 0157, to infer genetic relationship in the absence of epidemiological data, at least six restriction enzymes would be needed to provide a reasonable estimate using PFGE. Despite these limitations, PFGE is used for epidemiological analysis of different *S. enterica* serovars. Murase and his colleagues (1995) used PFGE for the epidemiological analysis of *Salmonella* infections caused by serovar Typhimurium, serovar Thompson and serovar Enteritidis and found that this method is useful.

Although PFGE is the current benchmark for molecular typing of Salmonella, it has considerable practical disadvantages. A significant proportion of fragments comigrates or is too small to visualize, so the Tenover *et al*. (1995) criteria could misclassify unrelated isolates as closely related. There is also potential for the Tenover *et al*. (1995) criteria to misclassify closely related isolates (*S*. Typhi) as unrelated if two cells with a recent common ancestor each undergo a minor genetic event and differ from the parent by three bands but differ from each other by six bands. It should be noted that Tenover *et al*. (1995) cautioned against the use of their criteria for the interpretation of PFGE in the absence of epidemiologic data or for extensive sets of isolates.

MLST

Multilocus sequence typing utilizes variability in the sequences of particular genes, due to mutation or recombination events, to determine the relatedness of bacteria. With MLST, multiple genes with conserved sequences are compared for nucleotide base changes (Foley *et al*., 2006). Housekeeping genes (genes required for basic cellular functions) are most often sequenced because they are present in all isolates and are not subject to strong selective pressures that can lead to relatively rapid sequence changes; the variation detected by MLST therefore represents evolutionary distance. MLST analyzes nucleotides within the targeted genes, and so multiple genes from various regions of the bacterial chromosomes are needed (Kotetishvili *et al*., 2002). In the present study, we used seven housekeeping genes previously used to analyze *S*. Typhi isolates, on the basis that they are scattered around the chromosome, are flanked by genes of known function and neither the gene chosen for sequencing nor the flanking genes are likely to be under diversifying selection (Kidgell *et al*., 2002). The MLST analysis in this study showed the presence of a total of seven STs among 58 isolates (Table 3.9). ST533, ST534, ST599 are single locus variants and because they differ in only one of the seven loci are closely related genetically and make a single Concord group. Probably serotypes like Concord, Laronchelle, Garoli and Colorado share the common ancestors.

There were more antibiotic susceptibility profiles, plasmids and PFGE patterns than sequence types which indicates that MLST scheme have a limited ability to allow for sensitive subtypes discrimination within *Salmonella* serotypes but is comparable to the level

of variation detected by serotyping and provides a natural classification equal to serotyping in uniform serotypes such as *S.* Typhi (Kidgell *et al*., 2002) and superior to serotyping in non-uniform serotypes such as the group C1 isolates described in this study.

Different serotypes like Gatow, Colindale, Haifa, and Butantan had STs that differed by more than one allele from Concord group and so with MLST we were able to describe them as not closely related to *S.* Concord. Furthermore isolates which could not be typed by serology, due to lack of antigen expression, could be typed by MLST. Thus MLST was able to identify and classify *Salmonella* isolates which were difficult to identify by serotyping. Therefore MLST could replace the classical serotyping method and group the isolates into natural (genetically related) clusters rather than artificially as observed in ordinarily serotyping method.

***fliC* gene sequencing**

Sequencing of the *fliC* gene showed that the Concord group and *S*. Haifa which belong to different serogroups had different sequence profiles (Table 3.10 and Annex III). This indicates the possibility for its role in molecular serology and to complement MLST for classification and strain differentiation. For example the *fliC* profile 2 has 1 bp difference as compared to *fliC* profile 1. Most likely a synonymous base pair change (does not result in amino acid substitution) as the same phase 1 antigen (lv) is produced between isolates belonging to *fliC* profile 1 and 2 (based on serology). *S*. Concord has the following antigenic formula 6, 7: lv: 1, 2. Based on the *fliC* sequencing, we suggest that all the isolates from the Concord group have evolved from a common ancestor but is now slowly diverging to form different ST types (533, 534, 599, g533, g534). *S*. Concord has therefore been present in Ethiopia for a considerable time or has been imported into Ethiopia on multiple occasions.

LIMITATIONS OF THE STUDY

The study was hospital based and may not represent a true reflection of prevalence of salmonellosis in the community and all eligible study subjects in the study may not be included in our study. Serotyping was limited by the antisera available in our laboratory, but the sequence typing has been used to overcome this problem. Due to limited resources, molecular analysis was done mainly for *S.* Concord not for other serotypes isolated in this study, which may not reflect the characteristics of all isolates.

CONCLUSIONS

A cross sectional study was conducted to determine the prevalence of enteric pathogens with special emphasis on *Salmonella* species in children who clinically presenting with diarrhoea and/or febrile illness in Tikur Anbessa and Jimma University hospitals. The overall prevalence of salmonellosis was 5.3%. Among the four identified serogroups of *Salmonella* isolates, group C had the highest percentage (78.8%) and *S.* Concord accounted for 89% of group C isolates. Thus the major non-typhoidal *Salmonella enterica* from humans in Addis Ababa and Jimma was *S.* Concord. This is in contrast with other countries where *S.* Enteritidis and *S.* Typhimurium were the most common serovars isolated in most clinical cases.

High antibiotic resistance was observed among group C *Salmonella* and particularly in *S.* Concord isolates. All *S.* Concord isolates were resistant to ampicillin, trimethoprim-sulfamethoxazole, ceftriaxone, amoxicillin, chloramphenicol, gentamicin, and tetracycline. In addition ESBL was detected in 98.8% of Concord isolates and of these ESBL producers, 1% showed resistance, 1% intermediate and 27% reduced susceptibility to ciprofloxacin. Our data also showed that isolates that express ESBL are frequently resistant to other antimicrobial agents, since many of these additional resistance genes are encoded on same plasmids.

Plasmid profile analysis indicated that *S.* Concord carried multiple copies of small and large plasmids. Some plasmids contained replicons that are associated with MDR and ESBL production. It is believed that the large molecular weight plasmid could be cointegration of virulence and R-plasmids. The co-selection of antimicrobial and virulence markers following recombination, as with another group C *Salmonella S.* Choleraesuis, can

lead to the emergence of strains that may be more virulent and difficult to treat (Fluit, 2005). This is probably happening with *S.* Concord in Ethiopia.

The presence of different PFGE types among isolates of the *S.* Concord group indicates a high degree of variability. Based on these findings it is plausible that the *S.* Concord group has evolved from a common ancestor and is slowly diverging. The amount of variation seen showed that *S.* Concord is endemic and suggests it has been in Ethiopia for a very long time. This is not an outbreak or a spread of a single clonal group from a recently imported case but is coming from many sources which are completely unknown.

Furthermore, multi locus sequence typing (MLST) showed that some serotypes (*S.* Laronchelle, *S.* Garoli and *S.* Colorado) had, at most, a single allele difference from *S.* Concord and should be considered as part of the Concord group. Other serotypes had sequence types that differed by several alleles (*S.* Gatow, *S.* Colindale, *S.* Haifa, and *S.* Butantan) and were really different serotypes. Sequencing of the *fliC* gene, which encodes the phase 1 flagella antigen (H1), confirmed the MLST results and suggests that *S.* Concord, *S.* Laronchelle, *S.* Garoli and *S.* Colorado should be considered as a single serotype.

In summary *S.* Concord was the dominant serotype, which is invasive and highly resistant to the commonly as well as for the newer drug used o treat Salmonella infections. Molecular characterizations also indicated that it contains plasmids which associated with both resistance and invasiveness and that could be also transferable to the same or different bacterial species. Therefore concerted and holistic effort should be done to combat this virulent and resistant pathogen (see recommendation section).

RECOMMENDATIONS

1. The major non-typhoidal *Salmonella enterica* from humans in the study area is *S.* Concord which is highly resistant to the widely available drugs including to the newer drugs. The presence of such invasive and highly resistance strain in resource poor countries like Ethiopia will cause high mortality and morbidity especially in young children and immuno-suppressed individuals. The high isolation rate of *S.* Concord seems unique to Ethiopia. Therefore further urgent epidemiological research/ surveillance should be conducted to detect source of infection, mode of transmission,

distribution within the country. Emphasis also should be given towards vaccine development against such invasive and MDR isolates.

2. Further phenotypic and molecular characterization should be done to determine the phylogenetic relation ship between Concord group and *S*. Concord
3. The presence of multidrug resistant strains containing genes for ESBL production and the emergence of reduced susceptibility to ciprofloxacin is a major concern and causes treatment failures. Therefore further strategy should be taken to avoid unnecessary and inappropriate antibiotic use both in human and veterinary sector.
4. To avoid any treatment failures, especially *Salmonella* isolates from invasive infections should be routinely screened for the presence of ESBL production and decreased ciprofloxacin susceptibility.
5. The epidemiology of salmonellosis in Ethiopia has not been well explored. Since the serotypes and resistance pattern of the isolates may vary greatly in different geographical areas, continuous surveillance must be undertaken in order to:-
    - Observe the periodic change of serogroup and serotype and prevalence with time.
    - Detect introduction of new serotypes for each serogroup from other countries
    - Trace the source of infection
    - Develop guidelines for antibiotic treatment
6. Identification studies should go to the level of serotypes, so that comparison with serotypes isolated from animals/ food products could be possible, which in turn is important for detection of source of infection.
7. Concentrating on antimicrobial therapy does not seem to be a solution to the control of salmonellosis as there are strong resistance patterns within the different isolates of salmonellae. Therefore, to decrease the incidence of salmonellosis in Ethiopia, besides giving attention in identification, susceptibility testing and reporting during routine bacteriological analysis, public health measures such as improving personal and food hygiene and intensive health education has to be given.
8. A central bacteriological reference laboratory should be strengthened to be able to identify *Salmonella* to the level of serovar and measure quantitatively antibiotic susceptibility, so that comparison with serovars isolated from humans, animals and food

products could be possible. In the longer term the introduction of molecular typing tools is essential.

9. Establishment of *Salmonella* Surveillance Network particularly with the neighboring countries, where there is migration of people that may contribute for the spreading of the disease, including resistant strain is very important. Such surveillance is crucial in sharing information on serotypes found in their countries that will help in preventing disease outbreak and raise awareness among health authorities, food producers, food regulators, and consumers.

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APPENDIX I. Questionnaire

Questionnaire for investigation of salmonellosis in children at Tikur Anbessa University Specialized and Jimma University Specialized Hospitals

I. PATIENT IDENTIFICATION

Serial number _____ Sex _____ Age____ Card no._____ Date_____

Site/place of collection _____

Region_____ Woreda _____ Kebele _____

II. CLINICAL DATA

	YES	NO
Diarrhoea	☐	☐
If yes, specify duration _____		
Nausea	☐	☐
Tenesmus	☐	☐
Abdominal pain	☐	☐
Headache	☐	☐
Fever	☐	☐
If yes, specify _____ and duration _____		
History of antibiotic treatment within the last month	☐	☐

If yes, name of antibiotics _____, duration _____

Clinical Diagnosis

AFI to rule out typhoid fever_____

Gastroenteritis _____

Any other_____

III. LABORATORY DATA

Type of specimen Blood ☐ Stool ☐ Both ☐

Consistency of stool	**Yes**	**No**
Watery	☐	☐
Mucoid	☐	☐
Bloody	☐	☐
Mixed (blood and mucus)	☐	☐
Loose	☐	☐

Other, specify _____-

Microscopic examination

Stool: _____

Culture and identification

Stool: _____

Blood: _____

Phenotypic characterization

Serogroup:_____

Serotype/serovar:_____ _____

Antibiotics susceptibility result_____

Antimicrobials	***Ampicillin***	***Amoxacillin***	***Cotrimoxazole***	***Nalidixic acid***	***Ofloxacin***	***Ceftriazone***	***Tetracycline***	***Chloramphenicole***	***Gentamicin***	***Ciprofloxacin***
Sensitive (S) **Intermediate (I)** **Resistant (R*)***										

E-test

ESBL _____

CIP_____

Molecular Characterization of *Salmonella* isolates

Strain no.	Serotype	Plasmid Profile	Incompatibility group	PFGE profile	MLST ST	*fliC* Profile	Comment

APPENDIX II

PATIENT INFORMATION SHEET/ CONSENT FORM

Title of the project:

"Phenotypic and molecular characterization of *Salmonell* species in Ethiopia**"**

Name of Principal Investigator:

Getenet Beyene

Organization:

Department of Microbiology, Immunology, and Parasitology (DMIP), Faculty of Medicine, Addis Ababa University

Name of sponsor:

School of Graduate Studies, Addis Ababa University

This information sheet is prepared for febrile and diarrheic children younger than 15 years of age (the study subject) and to their parent/caretaker who will visit Pediatric Out patient Department of Tikur Anbessa and Jimma Specialized Hospitals, during the sample collection time.

Purpose of the study

The main aim of this study is to isolate and to see the prevalence of *Salmonella* species among febrile and diarrheic children and to make phenotypic and genotypic characterization

Benefit of the study

This study will give information on the prevalence of mainly *Salmonella* species and some enteric pathogens among the study age groups. It also provides the current antimicrobial susceptibility pattern of the pathogen. Over all it will give information that will help to develop a rational prevention strategy to combat typhoidal and non-typhoidal salmonellosis.

Procedures

In order to undertake the aforementioned study, blood and stool specimen will be taken from children who have fever and diarrhoea (salmonellosis suspected) for culture and susceptibility testing. The study subjects are expected to give blood, stool samples, and information related to their illness.

Risk and Discomforts

Your child/adopted child will be examined by qualified and the specimens will be collected by the experienced health personnel. Since all procedures will be carried out in aseptic techniques with sterilized instruments, your child/adopted will not face any risk. There will be slight temporary pain during blood collection.

Benefits

If your child/adopted child participate in the study, he/ she will be examined by qualified health worker and if the disease-causing agent is identified, he/she will be treated freely. Your child/adopted child participation is likely to help in developing a rational preventive strategy to decrease problems caused by *Salmonella* infections in children.

Confidentiality

All information that all be collected from the study subjects will be kept confidential. Any information about the participant that will be collected from the study will be stored in a file that will not bear a name on it, but only a number assigned to it instead.

Right to refuse or withdraw

Participation in this study is on voluntary basis and participants have the right to refuse to participate in the study and refusal or withdrawal your consent will not affect your child/adopted child future relationship with the medical professionals.

Whom to contact

If the study participants (parent/caretaker) have any questions they may ask now or any time later. If they wish to ask later, they may contact with the following address:

Ato /Getenet Beyene,

Department of Microbiology, Immunology and Parasitology, Faculty of Medicine, Addis Ababa University, P. O. Box 1176, Telephone number: 52 87 26 or 09-64 40 93.

This study will be conducted only after getting approval from ethical committee of Faculty of Medicine (Addis Ababa University), Jimma University, Armauer Hansen Research Institute and National Ethical Review committee of Ethiopian Science and Technology Commission.

CONSENT FORM

Participant code number .....

Name of participant .....

Name of parent/caretaker .....

_____, who is the parent/caretaker of the child have got enough information on the proposed study and informed that blood and stool specimen will be taken from my child/adoptive child for the research. I understood that all information collected from my child/adoptive child will be kept confidential and have the right to refuse or withdraw from the study at any time without affecting my child/adoptive child medical care. I have read the foregoing information, or it has been read to me in a language I understand. I had the opportunity to ask questions about it and any questions that have asked and have been answered to my satisfaction. I have consented voluntarily that my child/ adoptive child to participate as a subject in this study.

Signature of the parent or caretaker **Signature of the principal investigator**

_____ _____

Date _____

የምርምር ተሳታፊዎች ጥናት ማብራሪያ ቅፅ

የጥናቱ ርዕስ፤ በኢትዮጵያ ውስጥ የሚገኙ የሳልሞኔላ ባክቴሪያ ዝርያ አይነቶች

የዋና ተመራማሪው ስም፡ ጌትነት በየነ

የድርጅቱ ስም ፡ ማይክሮባዮሎጂ፤ኢሚኖሎጂና ፓራሳይቶሎጂ ዲፓርትመንት፤ ህክምና ፋኩልቲ፤ አዲስ አበባ ዩንቨርሲቲ

ምርምሩን በገንዘብ ያገዘው ድርጅት፡ ድህረ ምረቃ ት/ቤት አዲስ አበባ ዩኒቨርሲቲ

በአዲስ አበባ ዩንቨርሲቲ ህክምና ፋኩልቲ፤ ጥቁር አንበሳ አጠቃላይ ስፔሻላይዝድ ሆስፒታል በጅማ ዩኒቨርስቲ ስፔሻላይዝድ ሆስፒታል እና በጂማ አከባቢ በገጠር በሚገኙ ጤና ጣቢያዎች ለህክምና ከሚመጡ እድሜአቸወ ከ15 ዓመት በታች ከሆኑና ተቅማጥ በሽታ ከአለባቸው ልጆች ላይ ሳልሞኔላ በተሰኘ ባክቴሪያ ላይ በሚደረገው ጥናት ውስጥ ተሣታፊ ለሚሆኑ ልጆችና ለወላጆቻቸው የተዘጋጀ ማብራሪያ ቅፅ ::

የጥናቱ ዓላማ

የዚህ ጥናት አላማ ሳልሞኔላ በሚባል የሚታወቀውንና የተለያዩ የጤና ችግሮችን የሚያስከትለውን በተለይም በሕፃናት ላይ የአንጀት ተስቦና ተቅማጥ በሽታ መንሥዔ የሆነውን ባክቴሪያ በከተማና በገጠር በልጆች ላይ ያለውን ሥርጭት ፡ የዝርያ አይነት እንዲሁም በዚህ ባክቴሪያ የሚመጣዉን በሽታ ለማከም የሚረዳንን የመድኃኒት አይነት ለማወቅ የሚያስችል ነው::

ጥናቱ የሚያስገኘው ጥቅም

ይህ ጥናት በልጆች ላይ የአንጀት ተስቦ እና የተቅማጥ በሽታ ሚያመጣውን ባክቴሪያ ስርጭት ፡ ዝርያ እንዲሁም በሽታውን ለማከም የሚያገለግለውን መድኃኒት ለማወቅ አስተዋፅኦ ያደርጋል:: ይህንንም በማጥናት ወደፊት በዚህ ባክቴሪያ የሚመጣውን የጤና ችግር ለመከላከል የሚያስችል ዘዴ ለመቀየስ ይረዳል::

የሥራው / የአካሄድ ቅደም ተከተል

ከላይ የተጠቀሰውን ጥናት ለማሳካት እድሜአቸው ከ15 ዓመት በታች ከሆኑና ትኩሳትና የተቅማጥ በሽታ ካላቻው ልጆች ወይም የሳልሞኒላ በሽታ አለባቸዉ ተብሎ ከሚጠረጠሩ ልጆች ላይ ደምና ሰገራ ናሙና በመውሰድ የተለያዩ የቤተ ሙከራ ምርመራዎች ይካሄዳሉ ሌሎችንም ከበሽታዉ ጋር የተዛመዱ መረጃዎች ይወስዳሉ :: ስለዚህ የጥናቱ ተሣታፊዎች የደምና ሰገራ ናሙና እንዲሁም አንዳንድ መረጃዎችን በመስጠት የጥናቱ ተባባሪ መሆን ይኖርባቸዋል ::

አላስፈላጊ ጉዳት ወይም ምቾት ማጣት

ልጅዎን የሚመረምረዉና ናሙና እንዲሰጥ የሚያዘዉ የህጻናት ሀኪም ነዉ፡፡ ናሙና የሚወስደዉ ልምድ ባለዉ የጠና ባለሙያ ነዉ፡፡ የደም ናሙና የሚወሰደዉ ፍጹም ንጽህናዉን በጠበቀ መሳሪያ ነዉ፡፡ የደም ናሙና በሚወሰድበት ጊዘ መጠነኛ ህመም ከመሰማቱ ዉጭ ከሚወሰደዉ ደም መጠን ጋርም ሆነ በሚወሰደበት መንገድ በልጅዎ ላይ የሚደርስ ጉዳት የለም፡፡

ጥቅማጥቅሞች

ልጅዎ በዚህ ጥናት ቢሳተፍ/ብትሳተፍ በሽታውን የሚያመጣው ባክቴሪያ ተለይቶ ይታወቃል፡፡ መድኃኒቱ ስለሚታወቅና ውጤቱም ለሐኪሙ ሥለሚሰጥ ትክክለኛ ሕክምና ልጅዎ ያገኛል ብለን እናምናለን ፡፡ መድሀኒትም በነጻ ያገኛል/ ታገኛለች እንዲሁም በጥናቱ ላይ ልጅዎ በመሣተፉ / በመሣተፏ ወደፊት ይሄንን በሽታ በቁጥጥር ስር ለማድረግ የሚረዱ የተለያዩ ዘዴዎችን ለማግኘት ይረዳል በዚህም ምክንያት የዜግነት ግዴታዎን ሊወጡ ይችላሉ ፡፡

የሚሥጢር አጠባበቅ

በዚህ ምርምር የሚገኝ ማንኛውም መረጃ በሚስጢር የሚጠበቅ ይሆናል ፡፡ የሚሰበሰበው መረጃ ሁሉ በስም እንዳይሆን እናደርጋለን ፡፡ ይህ የሚደረገው ለእያንዳንዱ የጥናት ተሳታፊ የተለየ ቁጥር በመስጠት ይሆናል፡፡

በጥናቱ ያለመሣተፍ ወይም ከገቡ በኋላ የመውጣት መብት

በዚህ ጥናት መሣተፍ በፍቃደኝነት ላይ የተመሠረተ ነው፡፡ በጥናቱ ላይ ልጅዎ ባለመሣተፉ / ባለመሣተፏ ወደፊትም ሆነ አሁን ከሕክምና ባለሙያዎችም ሆነ ከሌሎች ሠዎች የሚደርስባቸው ችግር የለም ፡፡

ሰስፈላጊ ሆኖ ከአገኙት ወይም የምትጠይቁት ጥያቄ ካልዎት

አቶ [ጌትነት](#) በየነ

ማይክሮባይሎጂ፤ ኢሚኖሎጂና ፓራሳይቶሎጂ ዲፓርትመንት ት/ክፍል

ህክምና ፋኩልቲ ፤ አዲስ አበባ ዩኒቨርሲቲ

ስልክ 52 87 26 ወይም 09 64 40 93 ማነጋገር ይቻላሉ፡፡

ይህ የምርምር ሥራ አዲስ አበባ ዩኒቨርስቲ ጅማ ዩኒቨርስቲ በአህሪ አለርት በኢትዮጵያ ሳይንስና ቴክኖሎጂ ኮሚሽን የስነምግባር ኮሚቴ ታይቶ ጉዳት የሌለው መሆኑ ታውቆ የሚፈቀድ ነው፡፡

የሥምምነት ቅፅ

የተሣታፊው ልዩ መለያ ቁጥር ----------

የተሣታፊው ሥም ----------

የወላጅ ወይም ያአሣዳጊ ሙሉ ሥም ----------

እኔ ---------- ስሙ /ስሚ ከላይ የተጠቀሰው ወላጅ ወይም አሣዳጊ የሆንኩ ሳልሞኔላ በሚባለው ባክቴሪያ ላይ ሊደረግ በታሰበው ምርምር ላይ በቂ መረጃ አግኝቻለሁ ። ለዚህም ጥናት ከልጄ /ከማሳድገው/ጋት ልጅ የደምና ሠገራ ናሙና እንደሚፈለግ ተረድቻለሁ ። ናሙና የመውሰዱ ጥረት በልጄ /በሳማድገው/ጋት ልጅ ላይ ምንም አይነት ጉዳት እንደማያደርስ ተረድቻለሁ ። ከልጄ / ከማሳድገው/ጋት ልጅ የሚወሰደው ማናቸውም መረጃ በሚስጢር እንደሚያዝ ተገልጸልኛል ። እንዲሁም ልጄን/ የማሳድገውን/ጋት ልጅ በተመለከተ የምጠየቀውን መረጃ ያለመስጠትና በጥናቱ ያለመተባበር ከጥናቱ በማናቸውም ወቅት ማግለል እንደምችል የተገለጸልኝ ሲሆን ይህንንም በማድረጌ በልጄ/ በማሳድገው/ጋት ልጅ ላይ ወደፊትም ሆነ አሁን ከሕክምና ባለሙያዎችም ሆነ ከሌሎች ሰዎች የሚደርስ ችግር እንደሌለ ተገንዝቤአለሁ።

ከዚህ በላይ የተገለፀውን መረጃ ከአነበብኩ ወይም ከተነበበልኝ በኋላ አስፈላጊ መስለው የታዩኝን ጥያቄዎች ጠይቄ ተገቢው መልስ ከተሰጠኝ በኋላ ልጄ /የማሳድገው/ጋት ልጅ በዚህ ምርምር እንዲሳተፍ/ እንድትሳተፍ ወስኛለሁ ።

የወላጅ ወይም የአሳዳጊ ፊርማ **የዋናው ተመራማሪ ፊርማ**

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Appendix III*:* Sequence of *fliC* gene.

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  116                                                                 165
010 GTTACTCAATCTCTTGATTTAAAAACTGCTGGAATTACTG---GTGCTAC
027 ACAACGAAAGCTTATGCCAATAATGGTACTACACTGGATGTATCGGGTCT
072 ACAACGAAAGCTTATGCCAATAATGGTACTACACTGGATGTATCGGGTCT
024 ACAACGAAAGCTTATGCCAATAATGGTACTACACTGGATGTATCGGGTCT
022 ACAACGAAAGCTTATGCCAATAATGGTACTACACTGGATGTATCGGGTCT
063 ACAACGAAAGCTTATGCCAATAATGGTACTACACTGGATGTATCGGGTCT
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002  TCAAAATGTCTTATACCGATAAAAATGGTAAGACAATTGAAGGCGGTTAT
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      566                                                        615
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002   GCGCTTAAAGCTGGCGATAAGTATTACGCCGCAGATTACGATGAAGCGAC
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5’ to 3’ partial *fliC* sequence and its alignment showing regions of base pair variations among the different *Salmonella* strains analyzed in the study.
Base pair (bp) that differ from the consensus are indicated by substitutions (in red bold). The sequence represents 500 bp of the central variable region of the *fliC* gene (numbers indicated above the sequences).