TYPING OF SALMONELLA STRAINS ISOLATED FROM WATER AND DIARRHOEIC STOOLS OF CHILDREN UNDER FIVE YEARS IN PARTS OF NORTHWESTERN NIGERIA

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A THESIS SUBMITTED TO THE SCHOOL OF POSTGRADUATE STUDIES, AHMADU BELLO UNIVERSITY, ZARIA IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF DOCTOR OF PHILOSOPHY IN MICROBIOLOGY

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JUNE, 2016

DECLARATION

I declare that the work in this thesis entitled "**TYPING OF SALMONELLA STRAINS ISOLATED FROM WATER AND DIARRHOEIC STOOLS OF CHILDREN UNDER FIVE YEARS IN PARTS OF NORTHWESTERN NIGERIA**" has been carried out by me in the Department of Microbiology, Ahmadu Bello University, Zaria, Nigeria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this project thesis was previously presented for another degree or diploma at this or any other Institution.

Ignatius MZUNGU

______________________	______________________	______________________
Name of Student	Signature	Date

CERTIFICATION

This thesis entitled **"TYPING OF SALMONELLA STRAINS ISOLATED FROM WATER AND DIARRHOEIC STOOLS OF CHILDREN UNDER FIVE YEARS IN PARTS OF NORTHWESTERN NIGERIA"** by **IGNATIUS MZUNGU** meets the regulations governing the award of the degree of Doctor of Philosophy in Microbiology of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

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Dean, School of Postgraduate Studies Signature Date

DEDICATION

To my beloved mother Mrs. Agnes Mzungu, my Late father Mr. Mzungu Sue and my Late sister Eunice Mzungu

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ABSTRACT

Salmonella species are important cause of gastroenteritis and are responsible for a huge global burden of morbidity and mortality. To evaluate the strains of *Salmonella* involved in diarrhoea among children in Northwestern Nigeria, a total of 634 diarrhoea stool samples of children aged five years and below were collected from three randomly selected states within the region and investigated for *Salmonella* infection. Isolates were screened for antibiotic susceptibility by disk diffusion, resistance genes by PCR and sequence analysis of 16S ribosomal RNA for strain determination. The overall prevalence of *Salmonella* species within the study area was 4.1%. The highest frequency of infection occurred among children aged 25-36 months (10.4%). A higher prevalence (6.5%) was observed among children from households that used mainly well water. Bacteriological quality of drinking water in the study area revealed that wells had the highest number of total coliform and faecal coliform counts, and boreholes had the lowest. Two (2.22%) well water samples were positive for *Salmonella* species. Varying degrees of contamination with total coliform and faecal coliform in excess of the maximum allowable limit was observed in all categories of water samples. The *Salmonella* isolates showed 100% resistance to Ampicilin and Amoxycillin, while 87.5% were susceptible to Ciprofloxacin. Multiple antibiotic resistance observed among isolates ranged from 3 to 9 antibiotics. Of the 23 MDR phenotypes, screened for resistance genes, 10(43.5%) were found to harbour *bla*TEM1 gene, 7(30.4%) were carrying *cat*1b gene and 2(8.7%) had *aad*A2 gene. Sequence analysis of the 16S rRNA obtained from the isolates showed that of the 18 isolates sequenced, 11 were affiliated to *Salmonella enterica* subspecies enterica serovar Typhi strain CT18, while 7 were affiliated to *Salmonella enterica* subspecies enterica serovar Typhimurium strain LT2. The percentage identity for all the 16S rRNA sequences ranged between 86 - 99%,

with E-values of zero in all the isolates except one. Phylogenetic analysis demonstrated a common ancestry of the isolates and close genetic relatedness. *Salmonella* still remains one of the major and most important bacterial pathogen of diarrhoea among children in the study area. Provision of adequate potable water to the community, improving sanitary awareness through basic health education, careful surveillance and monitoring incidence and spread of diarrhoeal diseases, may help reduce the disease burden in children.

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LIST OF ABBREVIATIONS

AFLP	Amplified Fragment Length Polymorphism
AP-PCR	Arbitrary Primed Polymerase Chain reaction
ATCC	American Type Culture Collection
BLAST	Basic Local Alignment Search Tool
BSOP	British Standard Operational Procedures
CDC	Center for Disease Control and Prevention
CLSI	Clinical Laboratory Standard Institute
DHFR	Dihydrofolate Reductase
DNA	Deoxyribonucleic Acid
DHPS	Dihydropteroate Synthase
ESBL	Extended-Spectrum Beta-Lactamase
ESC	Expanded Spectrum Cephalosporins
HIV	Human Immunodeficiency Virus
IFN	Interferon
IVAC	International Vaccine Access Center
Kb	Kilobases
LPS	Lipopolysaccharide
M cells	Micro fold cells
MDR	Multi- drug resistant
MLEE	Multi Locus Enzyme Electrophoresis
MLST	Multi Locus Sequence Typing

NADPH	Nicotinamide adenine dinucleotide phosphate
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
rRNA	Ribosomal Ribonucleic acid
RNA	Ribonucleic acid
RNS	Reactive Nitrogen Species
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulfate
SGI	*Salmonella* Genomic Islands
SNP	Single Nucleotide Polymorphism
SPI	*Salmonella* Pathogenicity Island
ST	Sequence Type
THFR	Tetrahydrofolic acids
VNTR	Variable Number of Tandem Repeat Variation
WHO	World Health Organization

CHAPTER ONE

INTRODUCTION

1.1 Background of the Study

Diarrhoeal diseases caused by enteric infections remain a leading global health problem. It is a common cause of infant deaths in developing countries, especially where safe drinking water and adequate sanitation and hygiene is unavailable. Salmonellae are important food-borne pathogens that are responsible for serious cases of food-borne illness leading to millions of cases of diarrhoeal disease, thousands of hospitalizations and deaths annually worldwide. In Nigeria, morbidity associated with illnesses due to *Salmonella* continues to be on the increase and, in some cases, resulting in death.

The global burden of disease for enteric fever have been estimated to be approximately 27 million cases, with 216 000 deaths annually (Crump *et al.*, 2004). In contrast, studies on the global burden of NTS causing gastroenteritis have estimated that, 93.8 million cases of gastroenteritis occur globally every year, resulting in 155,000 deaths (Majowicz *et al.,*2010). The data on which these estimates are based is limited, and come from isolated studies from countries with healthcare structures capable of assessing the burden of *Salmonella* infection. Accurate figures are however, compounded by limitations of diagnostic facilities (Baker *et al.*, 2010; Crump and Mintz, 2010; Franklin *et al.,* 2011).

In addition to the burden of the disease, the emergence of *Salmonella* species resistant to nearly all commonly available drugs exacerbates the morbidity and mortality (Bhutta, 2008;Gonzalez-Escobedo *et al.,* 2011; Tajbakhsh *et al.,* 2012). Factors contributing to the emergence of drug resistance include overuse, misuse and inappropriate antibiotic prescription practices (Singh, 1991; Zhang *et al.,* 2011). The world at large is now

experiencing widespread resistance to multiple first-line antimicrobial drugs such as ampicillin, chloramphenicol, streptomycin, sulfadiazine, tetracycline and trimethoprim (Demczuk *et al.*, 2010). This increase in antimicrobial resistance has reduced the effective treatment options and subsequently increased the treatment costs and the risk of complications and death (Kariuki, 2008). Therefore, antibiotic susceptibility profiles and molecular characterization of resistance determinants are useful epidemiological data which can be used to determine the occurrence, prevalence and spread of resistance genes (van Leeuwen, 2009; Tajbakhsh *et al.,* 2011).

Diarrhoea is defined as the passage of three or more loose or liquid stools per day or more frequent passage than is normal for the individual (UNICEF/WHO, 2009). It is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms. Infection is spread through contaminated food or drinking-water, or from person-to-person as a result of poor hygiene.

Diarrhoeal diseases caused by enteric infections remain a leading global health problem. It is a common cause of infant deaths in developing countries, especially where safe drinking water and adequate sanitation and hygiene is unavailable (Clasen *et al.,* 2007; Galadima and Okolo, 2014). It remains the second leading cause of death among children under five globally (WHO, 2013). Diarrhoeal disease kills 2,195 children every day (about 801,175 each year), accounting for 1 in 9 child deaths worldwide (CDC, 2015). An episode of diarrhoea can last several days, leaving the body without the water and salts that are necessary for survival. Diarrhoea can also have a detrimental impact on childhood growth and cognitive development (Bowen *et al.,* 2012).

The cause of death of persons with diarrhoea is usually associated with severe dehydration and fluid loss (CDC, 2015). Children who are malnourished or have impaired immunity are most at risk of life-threatening diarrhoea (UNICEF/WHO, 2009). Although diarrhoea is a preventable disease, it still remains a major cause of morbidity and mortality among children in Nigeria. As at the end of 2015, Nigeria still ranked second among the top 15 countries with high child mortality due to diarrhoea and pneumonia (IVAC, 2015).

The major causes of diarrhoeal illness include, among others, limited access to or poor quality of water, poor food hygiene, and sanitation. Reports from different parts of the world have implicated various pathogens such as parasites like *Giardia lamblia*, *Entamoeba histolytica.* Others are bacteria such as *Escherichia coli*, *Salmonella* sp., *Shigella* sp., *Campylobacter jejuni*, *Klebsiella* sp., and *Enterobacter* sp. and viruses for example the Rotavirus with the outbreak of infantile diarrhoeal disease (Chatterjee *et al*., 1989; Hald *et al.,* 2003; Diniz-Santos *et al*., 2005; Olesen *et al*., 2005; Parashar *et al*., 2006; Vernacchio *et al.*, 2006). All of these are known to be endemic in essentially all developing countries. However in the developing countries like Nigeria, infantile diarrhoea disease is grossly under-reported and the incidence under-estimated, this is attributed to poverty and ignorance among the affected group who constitute up to 80% of the population of the area (Synder and Merson, 1982), the lack of coordinated epidemiological surveillance system and inadequacy of laboratory facilities for culture. Unsafe water from all sources contribute significantly to the global burden of disease (Pruss-unstun *et al*., 2008). This is principally through the waterborne transmission of gastrointestinal infections such as cholera, typhoid, hepatitis and a wide range of agents that cause diarrhoea (Ahmed *et al.,* 2009).

The report from the WHO and UNICEF Joint Monitoring programme shows that the world is on track to meet goal 7, of the Millennium Development Goals (MDGs) with respect to access to improved water supplies (WHO/UNICEF, 2010). Nevertheless, despite the progress that has been made during the last decade, over 850 million people do not have access to improved source of drinking water, with almost all of them living in developing regions (Ahmed *et al.,* 2009).

Salmonella, a Gram-negative rod-shaped bacterium of the family *Enterobacteriaceae,* causes a wide range of human diseases, such as enteric fever, gastroenteritis, endocarditis, and bacteraemia and in the case of some typhoidal strains, an asymptomatic carrier state (Agbaje *et al.*, 2011). Salmonellae are important food-borne pathogens that are responsible for serious cases of food-borne illness leading to millions of cases of diarrhoeal disease, thousands of hospitalizations and deaths annually worldwide (Ammari *et al.,* 2009; Feasey *et al.,* 2012; Camarda *et al.,* 2013). Although infections with non-typhoidal *Salmonellae* usually cause self-limiting diarrhoeal illness, serious sequelae, including meningitis, sepsis, and death, may occur, especially among infants and elderly persons (Akinyemi *et al.*, 2007).

In recent years, problems related to *Salmonella* have increased significantly, both in terms of incidence and severity of cases of human salmonellosis. While some developed countries have managed to reverse the increasing incidence of human salmonellosis, the situation is different in developing countries of Africa and southern Asia where salmonellosis continues to pose a threat to public health with an estimated incidence of 33 million cases each year (Ivanoff, 1995; Sood *et al*, 1999). Socio-demographic factors (age, education, income), environmental and sanitation factors (poor access to a good water source and poor

sanitation) and climatic factors (rainfall, temperature and humidity) are thought to be related to incidence and spatial distribution of diarrhoea (WHO, 2007). The frequency and gravity of these infections are affected by hygienic conditions, malnutrition, and the excessive use of antibiotics that select for multi-resistant strains (CDC, 2008).

In Nigeria, morbidity associated with illnesses due to *Salmonella* continues to be on the increase and, in some cases, resulting in death. New concerns have been identified as 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, such as chloramphenicol and co-trimoxazole, and the third-generation cephalosporins, have emerged and are threatening to become a serious public health problem (Akinyemi *et al.*, 2005). Although fluoroquinolones have been found to be efficacious both *in vitro* and *in vivo* in the treatment of severe *Salmonella*-associated illnesses, strains with reduced susceptibility to ciprofloxacin among travelers have been reported in some parts of the globe (Hakanen *et al.*, 2001). This resistance results from the use of antimicrobials both in humans and animal husbandry. Multi-drug resistance to "critically important antimicrobials" such as ciprofloxacin and cephalosporins, is compounding the problems.

Reflecting a complex set of interactions with its host, *Salmonella* spp. require multiple genes for full virulence. Many of these genes are found in 'pathogenicity islands' in the chromosome. *Salmonella* Typhimurium possesses at least five such pathogenicity islands (SPI), which confer specific virulence traits and may have been acquired by horizontal transfer from other organisms. Collectively, these packages of virulence cassettes together with a highly conserved plasmid are essential for *Salmonella* pathogenesis (Marcus *et al.*, 2000). A pathogenicity island is considered to be a stable insertion of a large region of

DNA, containing virulence genes, into a site in the bacterial chromosome. This region usually contains complex virulence determinants, which appear to be acquired in a single step.

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 outbreaks 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. Currently, 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 Nigeria have indicated that salmonellosis is a common problem and also have shown the presence of a number of serogroups/ serotypes in humans, animals, animal food products and other foods (Abdullahi *et al.,* 2010; Smith *et al*., 2012).

In order to establish systems for controlling *Salmonella* infection and to develop national and local guidelines for antibiotic treatment, aside continued epidemiological surveillance and timely monitoring for the emergence and re-emergence of multi-drug resistance (MDR), there is need to identify the different strains of *Salmonella* circulating within the

country and to compare with strains in other countries. The need to also investigate the genes involved in drug resistance is equally important.

1.2 Statement of the Research Problem

Diarrhoea alone or in association with malnutrition constitutes major cause of morbidity and mortality among children throughout the tropical world. It is both a cause and effect of malnutrition according to data from the Gambia and Thailand (Guerrant *et al*., 1992; Rauyajin *et al*.,1994).

The magnitude and severity of diarrhoeal disease problem however varies considerably from one continent to another. In the developed world, diarrhoeal diseases do not represent a serious problem either in terms of morbidity or mortality (WHO, 1998; de Quadros and Santos, 2004). However, they have assumed a priority public health significance in the developing world. Diarrhoeal diseases account for 1 in 9 child deaths worldwide, making diarrhoea the second leading cause of death among children under the age of five (CDC, 2015).

The impact of diarrhoeal illness is more felt in sub-Saharan Africa where out of the 25 million children born each year 4.3 million are expected to die by the age of 5years and about 20% of these deaths will be from diarrhoea (Cunliffe *et al.,* 1998). As a sub-Saharan African country, Nigeria not surprising has an appalling under-five mortality rate. As at 2015, Nigeria still ranks second among the fifteen countries having the highest under-5-year mortality, second only to India (IVAC, 2015).

Salmonella food poisoning is one of the most common and widely distributed diseases in the world (WHO, 2005). It is estimated to cause 1.3 billion cases of gastro-enteritis and 3 million deaths worldwide (Bhunia, 2009). Salmonellosis can affect all ages, but the incidence and severity of the disease is higher in young children, the elderly, and people who are immunocompromised or have debilitating diseases.

Salmonellosis in humans caused by non-typhoidal *Salmonella* strains usually results in a self-limiting diarrhoea that does not warrant antibacterial therapy. However, there are occasions when these infections can lead to life-threatening systemic infections that require effective chemotherapy. But of increasing concern is the worldwide emergence of multidrug-resistant phenotypes among *Salmonella* serotypes (White *et al.*, 2001).

1.3 Justification

According to a UNICEF/WHO report in 2009, nearly three quarters of children's deaths due to diarrhoea occur in 15 countries among which Nigeria has the second highest rate of about 151,000 child deaths every year.

A number of different socio-political, and economic factors are present in sub-Saharan Africa which contribute to the constant morbidity from acute and persistent diarrhoea. Previous researches in other parts of Nigeria suggest that routine surveillance, especially for diarrhoeal agents, would be useful in identifying outbreaks and help identify the potential reservoirs and transmission routes of these infections (Nweze, 2010).

Diarrhoeal morbidity has also been shown to slow growth in children well beyond the weaning age (Torres *et al.*, 2001). This suggests that diarrhoea is of great public health

significance and therefore increased attention should be given to its study in children aged less than 5 years.

The importance of salmonellosis in public health sector is a growing concern day by day throughout the world and over the last several decades there have been significant shift in the predominant *Salmonella* serovars associated with human infections (Steven *et al.*, 2011). There is dearth of sufficient studies emphasizing isolation and molecular characterization of *Salmonella* serovars from human sources. Moreover, studies on the manifestation of pathogenicity as well as genetic relatedness among isolates from different locations have not been done in the northwestern part of Nigeria. This research on childhood diarrhoea in northwestern Nigeria is intended to provide an overview of the current state of this problem and to highlight key areas for future research. This research will also establish a *S. enterica* database that would serve as reference for routine monitoring and further studies.

1.4 Aim of the Study

The study was aimed at typing of *Salmonella* strains isolated from water and diarrhoeic stools of children under five years in parts of northwestern Nigeria.

1.5 Objectives

The objectives of the study were to;

1. Isolate and characterize salmonellae from water and diarrhoeic stool samples.
2. Determine the sociodemographic and risk factors associated with diarrhoea of children in the study area.
3. Enumerate faecal coliform in drinking water samples.
4. Determine antibacterial susceptibilities of the isolates.
5. Determine the antibiotic resistance genes in the isolates.
6. Determine the genetic relatedness of *Salmonella* isolates.

1.6 Research Questions

1. What *Salmonella enterica* serovars are prevalent among children in Northwest Nigeria?
2. What are the antibacterial resistance patterns of these *Salmonella enterica* serovars?
3. What is the genetic relatedness of *Salmonella* isolates?

1.7 Research Hypothesis

Null hypothesis: *Salmonella* is not prevalent among children with diarrhoea in Northwestern Nigeria.

Alternative hypothesis: *Salmonella* is prevalent among children having diarrhoea in Northwestern Nigeria.

CHAPTER TWO

LITERATURE REVIEW

2.1 Diarrhoea

Diarrhoea is defined as having loose or watery stools at least three times per day, or more frequently than normal for an individual (UNICEF/WHO, 2009). Although most episodes of childhood diarrhoea are mild, acute cases can lead to significant fluid loss and dehydration, which may result in death or other severe consequences if fluids are not replaced at the first sign of diarrhoea. It is a common cause of infant deaths in developing countries, especially where safe drinking water and adequate sanitation and hygiene are unavailable (Clasen *et al.,* 2007; Galadima and Okolo, 2014). It remains the second leading cause of death among children under five globally (WHO, 2013). Nearly one in five child deaths (about 1.5 million each year) is due to diarrhoea. It kills more young children than AIDS, malaria and measles combined (UNICEF/WHO, 2009).

There are three main forms of acute childhood diarrhoea, all of which are potentially life-threatening and require different treatment courses: Acute watery diarrhoea includes cholera and is associated with significant fluid loss and rapid dehydration in an infected individual. It usually lasts for several hours or days. The pathogens that generally cause acute watery diarrhoea include *Vibrio cholerae* or *Escherichia coli* bacteria, as well as rotavirus. Bloody diarrhoea, often referred to as dysentery, is marked by visible blood in the stools. It is associated with intestinal damage and nutrient losses in an infected individual. The most common cause of bloody diarrhoea is *Shigella,* a bacterial agent that is also the most common cause of severe cases. Persistent diarrhoea is an episode of diarrhoea, with or without blood, that lasts at least 14 days. Undernourished children and

those with other illnesses, such as AIDS, are more likely to develop persistent diarrhoea. Diarrhoea, in turn, tends to worsen their condition (WHO, 2013).

2.1.1 Causes of diarrhoea

Diarrhoea is a common symptom of gastrointestinal infections caused by a wide range of pathogens, including bacteria, viruses and protozoa. It is a common problem that usually lasts 1 or 2 days and resolves on its own without special treatment (Chen *et al.,* 2010). However, a number of organisms are responsible for most acute cases of childhood diarrhoea (WHO, 1999). Reports from different parts of the world have implicated various pathogens such as parasites like; *Giardia lamblia*, *Entamoeba histolytica*, bacteria such as; *Escherichia coli*, *Salmonella* species, *Klebsiella* species, *Enterobacter* species among others, and viruses like the Rotavirus with the outbreak of infantile diarrhoeal disease (Chatterjee *et al*., 1998; Ali *et al*., 2005; Olesen *et al*., 2005; Diniz-Santos *et al*., 2005; Parashar *et al*., 2006; Vernacchio *et al*; 2006).

Yersinia enterocolitica is an emerging enteric pathogen responsible for a wide spectrum of clinical manifestations including acute gastroenteritis world wide (Okwori *et al.,* 2007), *Cryptosporidium* has been the most frequently isolated protozoan pathogen among children seen at health facilities and is frequently found among HIV-positive patients.

Though cholera is often thought of as a major cause of child deaths due to diarrhoea, most cases occur among adults and older children. For the 1.1 billion people who lack access to improved water supplies, and many more with contaminated water, diarrhoeal disease is highly endemic (Clasen *et al.,* 2007). Nevertheless, the effectiveness of interventions

aimed at improving the quality of drinking water has been questioned (Cairncross, 1989). People can become infected with organisms that cause diarrhoea through multiple pathways, this is because water quality alone may not interrupt transmission. Even in developed countries with improved water supplies, diarrhoea is often endemic (Roy *et al.,* 2006; Colford *et al.,* 2006).

2.1.2 Transmission of diarrhoea

Most pathogens that cause diarrhoea share a similar mode of transmission – through the faecal-oral route. There may be differences, however, in the number of organisms needed to cause clinical illness, or in the route the pathogen takes while travelling between individuals (for example, from the stool to food or water, which is then ingested).

2.1.3 Disease burden

Diarrhoeal disease is a leading cause of child mortality and morbidity in the world, and mostly results from contaminated food and water sources. About 88% of diarrhoea-associated deaths are attributable to unsafe water, inadequate sanitation, and insufficient hygiene (CDC, 2015). Worldwide, 780 million individuals lack access to improved drinking-water and 2.5 billion lack improved sanitation. Diarrhoea due to infection is widespread throughout developing countries (WHO, 2013).

Mortality from diarrhoea has declined over the past two decades from an estimated 5 million deaths among children under five to 1.5 million deaths in 2004, which parallels downward trends in overall under-five mortality during this period (UNICEF/WHO, 2009). Despite these declines, diarrhoea remains the second most common cause of death

among children under five years and is responsible for killing around 760 000 children every year globally (WHO, 2013), following closely behind pneumonia, the leading killer of young children. Together, pneumonia and diarrhoea account for an estimated 40 per cent of all child deaths around the world each year. Nearly one in five child deaths is due to diarrhoea, a loss of about 1.5 million lives each year. The toll is greater than that caused by AIDS, malaria and measles combined. Africa and South Asia are home to more than 80 percent of child deaths due to diarrhoea (UNICEF/WHO, 2009; IVAC, 2015). About 15 countries account for almost three quarters of all deaths from diarrhoea among children under five years of age annually, among these, Nigeria ranked second (UNICEF/WHO, 2009; IVAC, 2015).

Interventions to prevent diarrhoea, including safe drinking-water, use of improved sanitation and hand washing with soap can reduce disease risk. Diarrhoea can be treated with a solution of clean water, sugar and salt, and with zinc tablets (WHO, 2013).

2.2 The Genus *Salmonella*

The genus *Salmonella* belong to the family *Enterobacteriaceae,* salmonellae are ubiquitous pathogens found in humans and livestock, wild animals, reptiles, birds. They are gram-negative, non-spore forming, facultative anaerobic bacilli, they range in size from 0.7 to 1.5 µm in diameter and from 2 to 5 µm in length. Like other members of the family *Enterobacteriaceae*, they produce acid on glucose fermentation; reduce nitrates to nitrite, and do not produce cytochrome oxidase (Farmer, 1995). Most strains of the organism except *S.* Gallinarum and *S.* Pullorum are motile by peritrichous flagellation. Based on their biochemical characteristics; members of the genus *Salmonella* can be differentiated from

other genera within the family *Enterobacteriaceae* (McClelland *et al.*, 2001). The differential metabolism of sugars can be used to distinguish some *Salmonella* serotypes, e.g., most strains of *Salmonella* do not ferment lactose. *S.* Typhi is the only strains of *Salmonella* that does not produce gas in sugar fermentation. Salmonellae are non-capsulated except *S.* Typhi, *S.* Paratyphi C and some strain of *S.* Dublin (WHO, 2003).

2.2.1 Antigenic structures

The typical *Salmonella* species are defined mainly by two sets of antigens: somatic (O) and flagellar (H) antigens that are readily demonstrable by serological reactions in the laboratory (Figure 2.1). 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).

2.2.1.1 O (Somatic) antigen

Somatic antigens represent the side-chains of repeating sugar units projecting outwards from the lipopolysaccharide layer on the surface of the bacterial cell wall. It is a polymer of O subunits and is typically composed of four to six sugars. Differences between O antigens can result from: the sugar components of the O subunit, the nature of the covalent bond between the sugars within the O subunit, or the nature of the linkage between the O subunits that form the O antigen polymer. Specific epitopes within O antigens are divided into two categories: O group antigens and ancillary O antigens. O group antigens are associated with the core sugar configuration of the O antigen structure; and are designated by the primary O epitopes that are associated with the group.

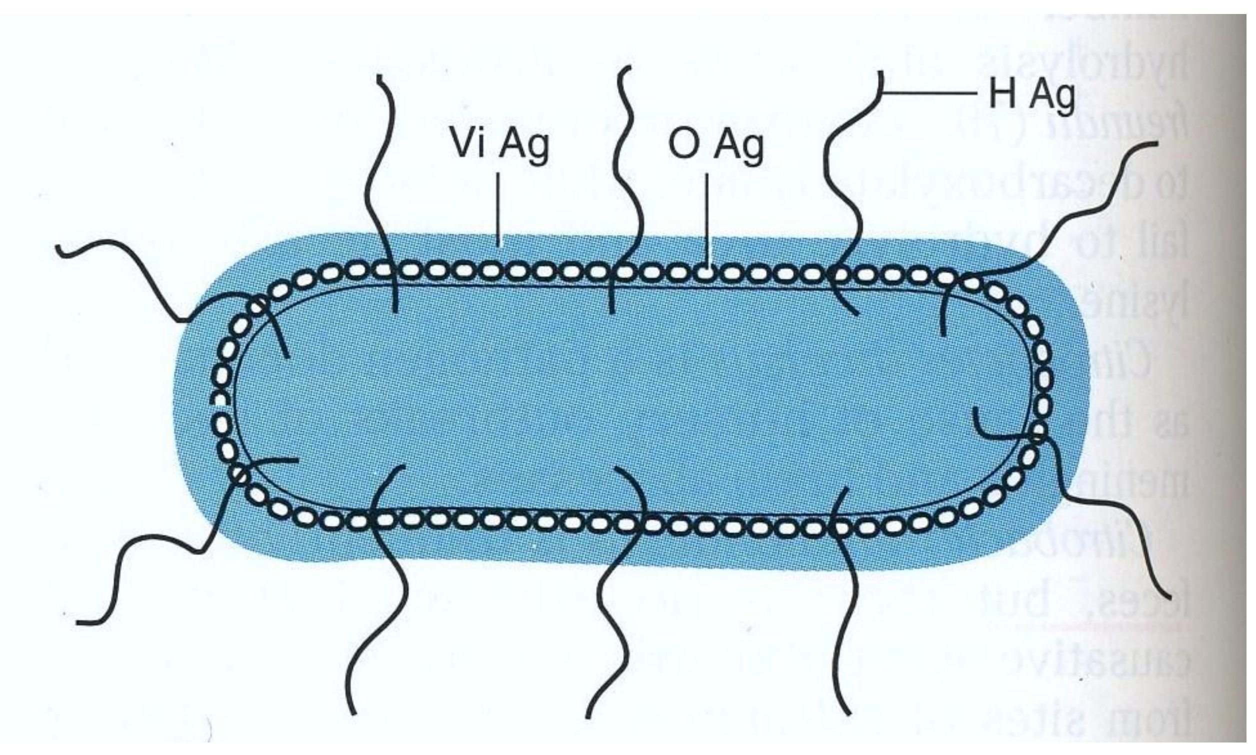

Figure 2.1: Antigenic structure of *Salmonella* species: *These are the antigens used in serologic typing. Figure from Textbook of Diagnostic Microbiology (Mahon and Manuselis, 1995).*

Key:

Vi Ag - Capsular antigen

O Ag - Somatic antigen

H Ag - Flagellar antigen

Ancillary O antigens are additional carbohydrates that are added to the core O antigen structure. They are associated with specific O serogroups and are often variably present or variably expressed (CDC, 2011). Over 60 different O antigens have been recognized and they are designated by Arabic numerals. The O antigens are heat stable, being unaffected by heating for 2.5 hours at 100°C, and alcohol stable withstanding treatment in 96% ethanol at 37°C (Lewis, 1998; Old, 1996).

2.2.1.2 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°C and above detaches the flagella from the bacteria. *Salmonella* is unique among all *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 flagella protein.

There are 114 H antigens, 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:z4 are considered the Z4 complex (Smith and Selander, 1991). The flagellar antigens of phase I

are labeled with lower case letters (a to z and z1 z2 z3 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 in 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)

2.2.1.3 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 of *S*. Dublin (WHO, 2003).

2.3 *Salmonella* Taxonomy

The taxonomy of *Salmonella* is complex because the organisms are a continuum rather than a defined species. The genus *Salmonella* is divided into two species, *enterica* and *bongori*. The species *Salmonella enterica* is further subdivided into six subspecies (Brenner and McWhorter-Murlin, 1998; Popoff and Le Minor, 2001) as follows:

I *Salmonella enterica* subsp. *enterica*

II *Salmonella enterica* subsp. *salamae*

IIIa *Salmonella enterica* subsp. *arizonae*

IIIb *Salmonella enterica* subsp. *diarizonae*

IV *Salmonella enterica* subsp. *houtenae*

VI *Salmonella enterica* subsp. *indica*

As of 2007, 2,579 *Salmonella* serotypes had been described; about 60% belong to subspecies I. In the United States, 99% of reported human *Salmonella* isolates belong to subspecies I (Grimont and Weill, 2007).

Subspecies IIIa and IIIb were originally considered a separate genus, *Arizonae*, and are still sometimes referred to by this name, although it is obsolete. Despite this common history, subspecies IIIb is more closely related to the other *Salmonella enterica* subspecies than to subspecies IIIa (Grimont and Weill, 2007).

Salmonella bongori was originally designated *S. enterica* subspecies V; it has since been determined to be a separate species of *Salmonella*. However, for simplicity and convenience, these strains are still sometimes referred to as "subspecies V". This classification for the genus *Salmonella* has evolved from the initial one serotype-one species concept proposed by Kauffmann on the basis of the serologic identification of O (somatic) and H (flagellar) antigens (Brenner *et al*., 2000). These surface features tend to be variable because of the strong selection pressure from host, and comparative genomics of *Salmonella* serovars have revealed that there could be significant intra-serovar variations among different isolates (Edwards *et al*., 2002, Porwollik *et al*., 2005).

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 accurate 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 have become: O2 (A), O4 (B), O7 (C1), O8 (C2), O9, 12(D1) among others. (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.

2.3.1 *Salmonella* nomenclature

Salmonella nomenclature is complex and still evolving. Currently, the nomenclature system used at the Centers for Disease Control and Prevention (CDC) for the genus *Salmonella* is based on recommendations from the WHO Collaborating Centre. According to the CDC system, the genus *Salmonella* contains two species, *S.enterica*, the type species, and *S. bongori*. In 2005, a new species, "*Salmonella subterranean*" was validly approved by the Judicial Commission (Shelobolina *et al*., 2004). *S. enterica* consists of six subspecies (Brenner *et al*., 1998; Popoff and Le Minor, 2001): I, *S. enterica* subsp. *enterica*; II, *S. enterica* subsp. *salamae*; IIIa, *S. enterica* subsp. *arizonae*; IIIb, *S. enterica* subsp. *diarizonae*; IV, *S. enterica* subsp. *houtenae*; and VI, *S.enterica* subsp. *indica*.

In subspecies I, serotypes (or serovars) are designated by a name usually indicative of the associated diseases, their geographic origins, or their usual habitats. In the remaining subspecies as well as those of *S.bongore*, antigenic formulae determined according to the Kauffmann-White scheme (Popoff and Le Minor, 2001) are used for those unnamed serotypes. Some members of these subspecies may have been named before 1966 and thus their names are retained and cited as those in the subspecies I.

To avoid confusion between serotypes and species, the serotype name is not italicized and starts with a capital letter. When cited at the first time in a report, the genus name is given followed by the word “serotype” (or the abbreviation “ser.”) and then the serotype name, e.g., *Salmonella* serotype or ser. Choleraesuis, and *Salmonella* serotype or ser. Typhi. Afterward the name may be shortened with the genus name followed directly by the serotype name, e.g., *Salmonella* Choleraesuis or *S.* Choleraesuis, and *Salmonella* Typhi or *S.* Typhi (Popoff *et al.,* 2004). For those designated by their antigenic formulae, the subspecies name is written in Roman letters (not italicized) followed by their antigenic formulae, including O (somatic) antigens, H (flagellar) antigens (phase 1), and H antigens (phase 2, if present). A colon is used in between each antigen, e.g., *Salmonella* serotype II 39:z10: z6. For serotypes in *S. bongori* (previously belongs to subgenous V), V is still used for consistency, e.g.,*S.* V 13,22:z35:- (Popoff *et al.,* 2004).

From clinical perspective, the serovars of *Salmonella* which are pathogenic for humans are traditionally divided into two groups. The typhoidal group include 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 2.2). 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.

2.3.2 *Salmonella* serotypes

Serotyping is a subtyping method used to differentiate isolates of *Salmonella* beyond the subspecies level. *Salmonella* serotypes are designated based on the immunoreactivity of two cell surface structures, the O and H antigens. A substantial amount of diversity exists in these two antigens, resulting in the designation of more than 2,500 serotypes and the regular recognition of new serotypes (Bopp *et al.,* 1999).

Historically, serotypes were considered different species (e.g., *Salmonella enterica* serotype Typhimurium was originally designated *Salmonella typhimurium*). It is now known that different serotypes of *Salmonella* can be closely related both phenotypically and genetically; serotypes are not intended as taxonomic designations (CDC, 2011).

Each O epitope is designated by a number; however, many of the common O groups were originally designated by letter and are still commonly referred to this way (e.g., serotype Typhimurium belongs to Group O:4 or Group B, serotype Enteritidis belongs to group O:9 or Group D1; serotype Paratyphi A belongs to Group O:2 or Group A). When multiple O epitopes are present, they are listed sequentially and separated by commas (CDC, 2011).

2.3.2.1 Serotype Identification

Salmonella serotypes are typically identified through a series of tests. Isolates are first identified to the genus and species level. The subspecies is then determined, typically by biochemical testing. O and H antigens are detected in independent agglutination assays using antisera that react with groups of related antigens or a single antigen.

2.3.2.2 Serotype Designation

Salmonella serotypes are designated according to the conventions of the Kauffmann-White Scheme. The Kauffmann-White Scheme is maintained by the WHO Collaborating Centre for Reference and Research on *Salmonella* at the Institute Pasteur and is used by most public health laboratories worldwide (Grimont and Weill**,** 2007). All *Salmonella* serotypes can be designated by a formula; subspecies I serotypes are also given a name (e.g., Typhimurium, Enteritidis, Typhi). The typical format for a serotype formula is: Subspecies [space] O antigens [colon] Phase 1 H antigen [colon] Phase 2 H antigen. Examples:

I 4,5,12:i:1,2 (*S. enterica* serotype Typhimurium)

I 4,12:i:1,2 (*S. enterica* serotype Typhimurium var. O:5-)

I 9,12:g,m:- (*S. enterica* serotype Enteritidis)

II 47:b:1,5 (*S. enterica* serotype II 47:b:1,5)

IV 48:g,z51:- (*S. enterica* serotype IV 48:g,z51:-)

IIIb 65:(k):z *(S. enterica serotype* IIIb 65:(k):z)

2.3.2.3 Other conventions

Some O and H epitopes are variably present. When the variable epitope is known to be encoded by a bacteriophage it is underlined (e.g., O20 is designated O:8,<u>20</u> in some

serogroup C2 serotypes). Bacteriophage-encoded antigens have only been described for O antigens (Grimont and Weill, 2007). When the basis for variability is not known, the antigenic factor is placed in square brackets (e.g., O5 is designated O:4,[5],12 in some serogroup B serotypes). For an individual isolate, if the variable factor is detected it is included in the formula without additional notation (i.e., without underlining or square brackets). If the variable factor is not detected, it is not listed in the formula. Some O and H factors are variably expressed. Weakly recognized antigens are indicated by parentheses (e.g., O antigen (6),14 or H antigen (k)). The term “Serotype” and “serovar” are used interchangeably.

In monophasic isolates, the absence of an H antigen is indicated by a minus sign (“-“) for the particular phase. Variants of serotypes that do not express all the recognized antigens characteristic of that particular serotype are not uncommon. This is a particular issue for subspecies I serotypes, because a serotype name cannot be assigned unless all the antigens specified in the Kauffmann-White scheme for that serotype are identified. Isolates missing one or more antigens are designated by a formula (Grimont and Weill, 2007). For example:

a) Monophasic variants are variants of serotypes that are typically diphasic; they lack the expression of either the flagellar Phase 1 or Phase 2 antigen. These are indicated by a minus sign (“-“) in place of the missing phase (e.g., monophasic variants of serotype Typhimurium lacking the second phase H antigen, 1,2, are designated as *Salmonella* serotype I 4,5,12:i:- or I 4,12:i:-; monophasic variants of serotype Typhimurium that lack the first phase H antigen, i, are designated as serotype I 4,5,12:-:1,2 or I 4,12:-:1,2).

b) Nonmotile variants express no H antigens and are indicated by minus signs in both phases or by “nonmotile” in place of the H antigens (e.g., serotype I 4,5,12:nonmotile or I 4,5,12:-:-)

c) Rough variants are isolates that do not express O antigen. This is indicated by “Rough” in place of the O antigen in the antigenic formula (e.g., serotype I Rough:i:1,2).

d) Mucoid variants express a capsule that prevents immunologic detection of the O antigen. They are indicated by “Mucoid” in place of the O antigen in the antigenic formula (e.g., serotype I Mucoid:i:1,2).

Rarely, isolates express a third H antigen that is noted by a colon followed by the antigen after the Phase 2 H antigen (e.g., serotype II 9,12:g,m,[s],t:1,5,7:z42, in which antigen z42 is the third H antigen).

Figure 2.2 is schematic classifiction of the genus *Salmonella*, as adapted from Langridge, (2008)

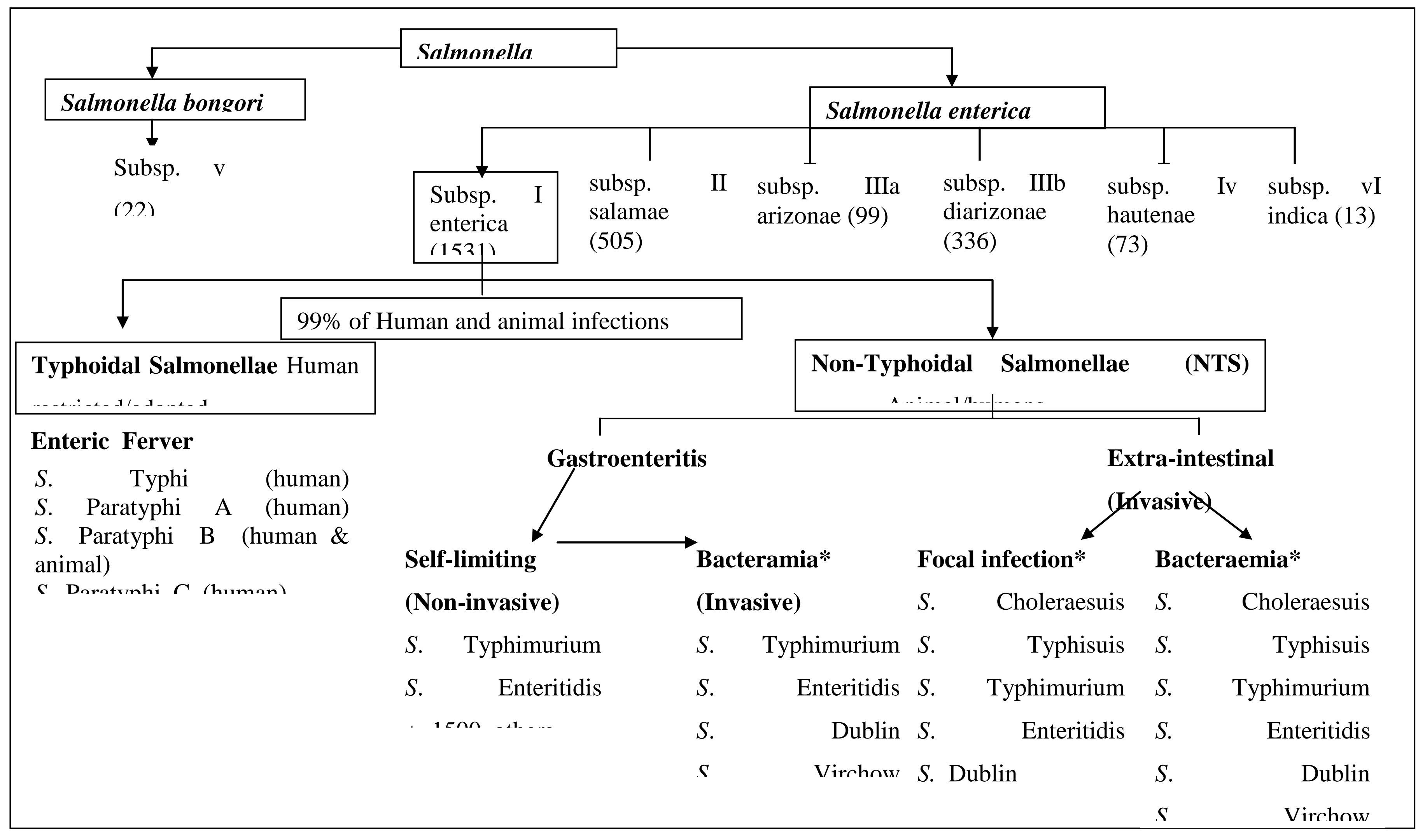

Figure 2.2: Classification of the genus *Salmonella*

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. (Adopted from Langridge *et al.*, 2008).

2.4 Genomics of *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,* because of the presence of a large number of serovars in *Salmonella* (Mastroeni, 2006). Genome information can be used to gain insights into the evolution of the genus *Salmonella*, 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 CT18 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. Pairwise 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 2.1) (Edwards *et al*., 2002).

Despite their overall similarity, pairwise 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).

Table 2.1: Average percentage (%) identity of the regions conserved between the five sequenced *Salmonella* serotypes

	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					-

(Adopted from Edwards *et al*., 2002)

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), indicating a common evolutionary origin. The genome size of *S*. Typhimurium LT2 is 4,857 Kbp (McClelland *et al*., 2001) and sequence comparison between these two organisms revealed that 89% of *S*.Typhimurium LT2 CDSs were homologous to *S*.Typhi CT18 at the nucleotide level (McClelland *et al*., 2001; de Jong *et al.,* 2012). 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).

Other molecular studies have revealed that if the DNA sequences of genes in the core genome of different enteric bacteria are compared, *Escherichia coli* and *S.enterica* are found to differ by about 10%, and *Salmonella* serovars within *S.enterica* differ by about 1% (de Jong *et al.,* 2012). This 10% divergence between the core sequences of *E.coli* and *S. enterica* most likely represents evolutionary drift over the 100 million years since the 2 species are separated from a common ancestor (Baker and Dougan, 2007; de Jong *et al.,* 2012). The homology of the 200 pseudogenes in *S.enterica* which are functionally disrupted or inactive, most are functional in *S.*Typhimurium. This, in part, contributes to the host restriction in *S.* Typhi and in NTS serovars may be explained by differences in genome expression leading to differences in host-pathogen recognition (de Jong *et al.,* 2012).

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. Antimicrobial resistance genes can be also found in the 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).

2.5 Salmonellosis

Salmonellosis is an infection with *Salmonella* species, often restricted to the gastro-intestinal tract and is often a self-limiting disease. Salmonellosis in the human host is generally associated with *Salmonella enterica* subspecies *enterica* (also termed subspecies I). Most infected individuals experience mild gastrointestinal illness involving diarrhoea, chills, abdominal cramps, fever, head and body aches, nausea, and vomiting (Honish, 2000). 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 *Salmonella enterica* from human infections can be subdivided into 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 well as *S.* Paratyphi A and Sendai together, whereas *S.* Paratyphi B and C are heterogeneous (Selander *et al.*, 1990).

2.5.1 Typhoidal *Salmonella*

Enteric fever is a severe systemic form of salmonellosis. The symptoms begin after an incubation period of 10 to 14 days after ingestion of contaminated food or water (Giannella, 1996). Enteric fever may be preceded by gastroenteritis, which usually resolves

before the onset of systemic disease. The symptoms of enteric fever are nonspecific and include fever, anorexia, headache, myalgia, and constipation (Bhunia *et al.,* 2009; Bayram *et al.,* 2011).

Initially, there is low fever in which the body temperature rises progressively, and by the second week it is often high (39-40°C) and sustained (Kaur and Jain, 2012).The fever occurs in more than 80% of patients (Khan *et al.,* 1998).The classical disease description includes bacteraemia and fever during the first week, as well as nonspecific symptoms such as chills, headache, anorexia, sore throat, unproductive cough, constipation following diarrhoea, myalgia, psychosis and mental confusion in 5–10% of the cases (Demczuk *et al.,* 2010; Kaur and Jain, 2012). A coated tongue, tender abdomen, hepatomegaly, and splenomegaly are also common.

In the second week, a few rose spots, blanching erythematous maculopapular lesions, approximately 2–4 mm in diameter, appear in 5–30 percent of cases. These usually occur on the abdomen and chest and more rarely on the back, arms,and legs. A relative bradycardia in relation to fever, intestinal constipation or diarrhoea in smaller number of patients (mainly in young children and adults with HIV infection) may occur (Bayram *et al.,* 2011; Kaur and Jain, 2012). Without correct treatment or diagnosis, the typhoid fever may prolong to the third week and the inflammatory lesions become intense in Peyer's patches and intestinal lamina propria (Kaur and Jain, 2012). Ileocaecal ulceration and necrosis (cellular death), with subsequent gastrointestinal bleeding or intestinal perforation may occur. Further complications in 10–15% of patients may result in death, after the third

week of disease. The fever declines in the fourth week of disease in 90% of the survivors, without antibiotic therapy. However, weakness and weight loss may persist for many months (Kaur and Jain, 2012). About 3–5% of those infected progress to a chronic carrier state (Demczuk *et al.,* 2010). *Salmonella* carriage is defined as asymptomatic excretion following acute infection and can be divided into convalescent carriage and chronic carriage (Haeusler and Curtis, 2013).

2.5.2 Non typhoidal *Salmonella*

Non typhoidal *Salmonella* usually cause an acute self-limiting gastroenteritis although in immuno-compromised individuals, serious complications can occur (Boyle *et al.,* 2007). Clinical manifestations of NTS can be broadly divided into four groups: acute gastroenteritis, extra-intestinal infection, non-infectious sequelae and *Salmonella* carriage (Haeusler and Curtis, 2013).

Gastroenteritis usually results in profuse and usually non-bloody diarrhoea which is usually self-limiting (Kariuki *et al.,* 2006; Sanchez-Vargas *et al.,* 2011). However, there are some other associated symptoms which may occur and these are myalgia, headache, fever, abdominal cramping, nausea and vomiting (Giannella, 1996; Pegues and Miller, 2010; Haeusler and Curtis, 2013). Fluid and electrolyte disturbances are the most frequent complication of NTS gastroenteritis. The duration of fever and diarrhoea vary, but is usually 2 to 7 days. Asymptomatic gastrointestinal infection can also occur. However, given the rate of convalescent NTS excretion following acute infection, the true incidence is unknown.

Extra-intestinal infections have been reported in NTS and are common in immunoincompetent individuals with HIV, diabetes mellitus and those on steroid therapy (Feasey *et al.,* 2012; Haeusler and Curtis, 2013). NTS bacteraemia is reported in up to 9% of patients with acute gastroenteritis (Haeusler and Curtis, 2013). Bacteraemia may result in focal NTS infection at any site, including the central nervous system (Haeusler and Curtis, 2013).

2.5.3 Salmonellosis in Nigeria

In Nigeria, 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 Nigeria. However, enteric fevers caused by *S*. Typhi and *S*. Paratyphi are not only endemic but constitute a great socio-medical problem, being responsible for high morbidity and mortality (Umeh and Agbulu, 2009).

A study carried out in Benue State, central Nigeria, to determine the pattern of distribution of *Salmonella* typhoidal serotypes showed that out of 1479 blood samples from patients (569 males and 910 females; age range 0 – 80 years) examined for typhoidal antibodies using slide agglutination technique, 875 (57.6%) were positive for *S.* Typhi antigens, and 389 (26.3%) *S.* Paratyphi antigens. Two hundred and thirty (230, 15.6%) showed a mixture of both typhoid and paratyphoid serotypes (Umeh and Agbulu, 2009).

Abdullahi *et al.* (2010), carried out a study to investigate the incidence of *Salmonella* species among 300 children using stool samples from six hospitals in the metropolitan Kano, Nigeria. The organisms were investigated using cultural, serological biochemical characterization and sensitivity to some antimicrobial agents. A total of 41 *Salmonella* species were isolated from the 300 samples studied, representing a prevalence of 13.67%. These comprised of *Salmonella* Typhimurium (predominant) with 7.67%, followed by *Salmonella* Typhi and *Salmonella* Paratyphi with 4.00%, and 2.00% respectively. Out of the 41(13.67%) *Salmonella* species isolated, 24 (8.00%) were from male and 17 (5.67%) from female patients. The incidence of the bacteria was found to be highest among children of 20-24 months age group and least in 5-9 months age group.

In general studies which center specifically on *Salmonella* as a pathogen of gastroenteritis are rare, however some researches on bacterial aetiology of diarrhoea have implicated *Salmonella* species in many instances. For example, a bacteriological investigation of diarrhoeal diseases was carried out among 60 children between the ages of 0 – 60 months using stool samples from three different hospitals in Ayingba, Dekina Local Government Area of Kogi State. Out of the 60 children, 32 had diarrhoea associated with bacteria. The bacteria isolated were *Escherichia coli* (46.9%), *Salmonella* species (28.1%), *Shigella* species (21.9%), and *Staphylococcus aureus* (3.1%). Statistical association was observed between age and the presence of the bacteria isolates with the age group 13- 24 months having the highest occurrence of the bacterial isolates (21.7%) followed 37 – 48 months (11.7%) and the least occurrence in the age group 0 – 12 months (5.0%) (Okolo *et al.,* 2013)

Another survey to determine the significance of bacterial species as possible pathogenic microorganism that cause diarrhoea was carried out in the Federal Capital Territory Abuja, Nigeria. Stool specimen from 404 children younger than five years of age were collected randomly from five hospitals in Abuja and assessed for microbiological profile of enteric pathogens. The prevalence of bacteria associated diarrhoea ranged from 18.8 to 22.4% among hospitals located in the Municipality, whereas hospitals located in the satellite settlements it ranged from 18.1to 20.9%. Bacteriological analysis showed that at least one bacterial isolate was recovered from 277 out of the 404 stool specimens; 174(62.8%) were strains of *Escherichia coli* others were *Salmonella* Typhi 9 (3.2%), *Staphylococcus aureus* 16(5.8%) and other Enterobacteriacea. (Ifeanyi *et al*, 2010).

Similarly, Ogbu *et al.* (2007) carried out an assessment of microbiological profile of enteric pathogens in pediatric stool specimens in Abakaliki, Nigeria. Of the 150 children diagnosed with diarrhoea, at least one enteropathogen was detected in 122(81.3%). Bacteria detected among cases included *Escherichia coli* (15.3%), *Salmonella* species (11.3%) and *Klebsiella* species (7.3%).

In a related study by Galadima and Kolo (2014), stool samples from children with diarrhoea attending the General Hospital Minna, Nigeria were analysed for the presence of different types of bacteria using standard bacteriological methods. From 253 isolates examined, 139 (54.94%) were identified as *Escherichia coli,* 49(19.37%) *Shigella* species, 42 (16.60%) *Salmonella* species, and 8 (3.16%) *Citrobacter* species. Others were

Enterobacter species 11 (4.34%), and *Vibro cholerae* 4 (1.58%). Thirty eight (15%) samples did not yield any growth. Of the *Salmonella* species isolated, 75% belonged to *Salmonella* Paratyphi A; 19% were positive for *Salmonella* Paratyphi B and 8% for *Salmonella* Typhi.

A prevalence study of enteric pathogens among patients with gastrointestinal presentations in Lagos was done in a Lagos University Teaching Hospital (LUTH), Idi-Araba, Lagos Nigeria. A total of 150 stool samples were collected from patients with gastrointestinal presentations attending LUTH. It was observed that 37.3% of the 150 patients were infected with bacteria. The isolates in order of prevalence included *Proteus* species 53.6%, *Pseudomonas* species 17.9%, *Aeromonas* species 14.3%, *Salmonella* species 10.7% and *Shigella* 3.6% (Ike and Damola, 2014).

2.5.4 Pathogenesis

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* has 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 2.3).

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, severe headache, anorexia, fever and a tendency to constipation rather than diarrhoea (Parry *et al*., 2002). If enteric fever is not treated on time, serious complications like haemorrhage from ulcers can occur during the third week of illness or perforation of the Peyer's patches (PP) can cause generalized peritonitis and septicemia; these are the commonest causes of death in typhoid fever. However, with the introduction of early and appropriate antibiotic therapy, the average case fatality rates for typhoid fever are less than 1% (Everest *et al*., 2001).

2.5.5 Virulence factors

The outcome of a *Salmonella* infection is determined by the immune status of the host, dose of infection and virulence status of the bacterium (Figure 2.3). The virulence status of the bacterium is determined by virulence factors (van Asten and van Dijk, 2005).

2.5.5.1 Salmonella Pathogenicity 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. More than 10 SPIs have been identified on the *Salmonella* chromosome, but SPI-1 and SPI-2 are 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). In addition, *Salmonella* has acquired, by horizontal gene transfer, a type III secretory system encoded by *Salmonella* pathogenicity island 2 that interferes with the trafficking of vesicles containing functional NADPH phagocyte oxidase to the phagosome,

thereby enhancing the survival of *Salmonella* within macrophages. It has been reported that SPI-2 prevents colocalization of the phagocyte oxidase (Vazquez-Torres *et al*., 2000) and the inducible nitric oxide synthase to the *Salmonella*-containing vacuoles (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.

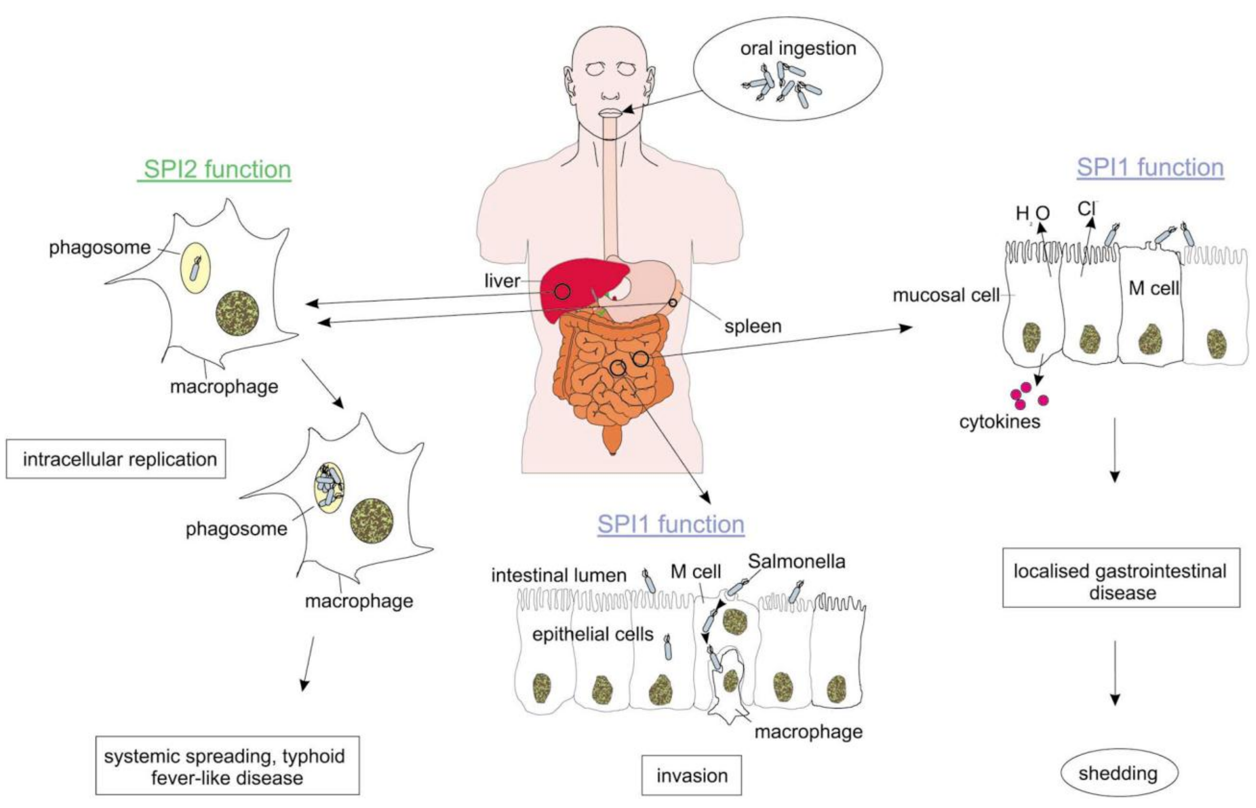

Figure 2.3: Schematic representation of host–pathogen interactions during pathogenesis of *Salmonella* infections.

Salmonella Pathogenicity Island (SPI1) mediate the initial stages of salmonellosis, i.e. the entry of Salmonella into nonphagocytic cells by triggering invasion and the penetration of the gastrointestinal epithelium. Furthermore, its 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 is essential for this phase of pathogenesis (Adopted from Hansen-Wester and Hensel, 2001).

2.5.5.2 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 an indirect translocation of bacterial virulence proteins into the host cell cytoplasm (Figure 2.4). Type III secretion systems are composed of several proteins that form a remarkable needle-like organelle in the bacterial envelope (Galan, 1999). 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).

2.5.5.3 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 acidic 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).

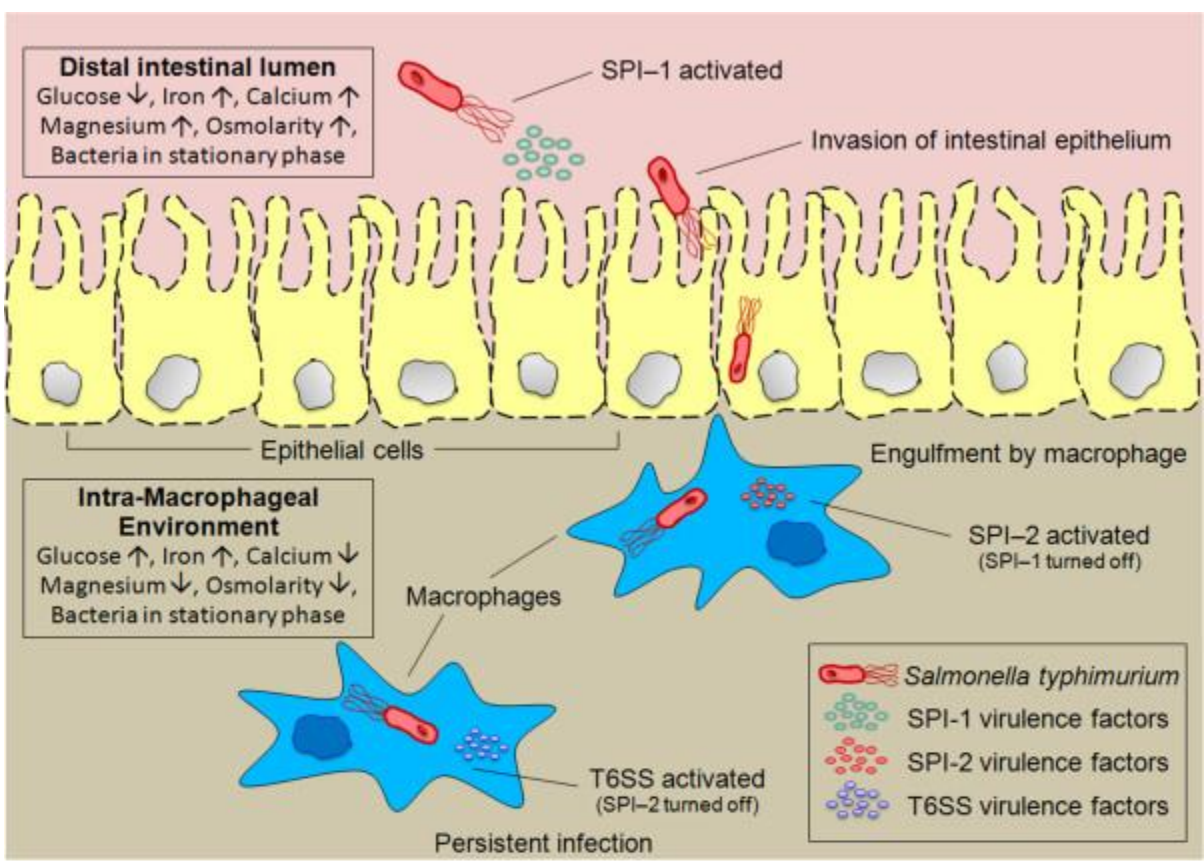

Figure 2.4: ***Salmonella*** **infection**: **Sequential activation of different secretion systems of Salmonella during infection.**

The levels of key environmental factors sensed by the pathogen first in the distal gut lumen, and subsequently in the intra-macrophageal environment, are mentioned ***(Das et al., 2013).***

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).

2.5.6 Epidemiology of *Salmonella* infection

2.5.6.1 Typhoidal Salmonella

Typhoid fever has an insidious onset characterized by fever, headache, constipation, malaise, chills, and myalgia with few clinical features that reliably distinguish it from a variety of other infectious diseases. Diarrhoea is uncommon, and vomiting is not usually severe. Confusion, delirium, intestinal perforation, and death may occur in severe cases.

The aetiologic agent may be recovered from the bloodstream or bone marrow, and occasionally from the stool or urine (CDC, 2013).

Typhoid fever is a global problem and its real impact is difficult to estimate because the clinical picture is similar to those of many other febrile infections, An estimated 21 million cases of typhoid fever and 200,000 deaths occur worldwide (CDC, 2013). *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 (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.

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).

2.5.6.2 Non-typhoidal Salmonella

Unlike *S.* Typhi and *S.* Paratyphi, whose only reservoir is humans; non-typhoidal 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.

The increasing impact and differing epidemiology of NTS, compared with *S.* Typhi, is noted in several African countries (Feasey *et al*., 2010), where NTS infection is expanding but the epidemiology has proved elusive. A systematic review of the literature suggests that NTS are responsible for nearly 60% of bloodstream infections due to *Salmonella*; NTS are now more common than enteric *Salmonella* (Reddy *et al*., 2010).

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 and *Salmonella enterica* serovar 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 non-human isolates, *S.* Typhimurium was the most commonly reported serotype in all 3 years, accounting for 17% of isolates. 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 (Appendix VII) (Galanis *et al*., 2006).

The greatest contributor to disease burden over time is undoubtedly the increase in HIV-NTS co-infection (Green *et al.,* 1993; Kariuki *et al.,* 1996; Rubino *et al.,* 1999 and Hart *et al.,* 2000) associated with a very high mortality (Gordon *et al* 2002; Berkley *et al*., 2005). 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). In a number of sub-Saharan African countries, there is an increasing number of cases of multidrug resistant (MDR) non-typhoidal *Salmonella* (NTS) infection (Feasey *et al*., 2012). Multidrug resistance in NTS has remained a challenge after the turn of this

century, including resistance to extended spectrum beta-lactam agents (Govender *et al*., 2009) and emerging resistance to the quinolones (Kruger *et al.,* 2004).

2.5.7 Immunity to *Salmonella* infection

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 reactive nitrogen species (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 play a pivotal role in activation of macrophage (Mastroeni, 2002). CD8+ T cells are 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).

2.5.8 Laboratory diagnosis of *Salmonella*

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.

2.5.8.1 Culture

A summary of guidance for identification of *Salmonella* species in a diagnostic laboratory is shown in Figure 3.2 (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,

2009). *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 (XLD) agar (Cheesbrough, 2009). *Salmonella* enrichment broths (e.g. selenite broth and tetrathionate broth) may help to recover low numbers of organisms.

2.5.8.2 Biochemical Characterization

Salmonella species are motile (with a few exceptions), facultatively 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 do not ferment lactose. However, approximately 1% of the organism is able to ferment this sugar and thus may not be detected by clinical laboratories that use MacConkey agar. 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 sulphide (Cheesbrough, 2009).

2.5.8.3 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). A study carried out to evaluate the significance of Widal test showed that out of 242 typhoid suspected patients, the Widal qualitative test was positive in 92 (38%) typhoid suspected patients, 10 (43.5%) febrile non-typhoidal patients, and 55 (31.3%) healthy blood donors (Awole, 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. 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**.**

2.5.8.4 Serogrouping/Serotyping

Salmonellae can be characterized by their somatic (O) and flagellar (H) antigens, the latter existing in some serotypes as 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 the purpose of diagnosis.

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 labour intensive. A wide range of antisera are 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 cause enteric fever, gastroenteritis or other infections have the following antigenic compositions (Table 2.2).

Table 2.2: O and H antigens commonly used for serogrouping and serotyping of typhoidal and non-typhoidal *Salmonella* species.

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	1,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

(Adapted from WHO, 2003)

2.5.8.5 Molecular techniques

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 hours if a whole cell PCR is performed or 7 hours 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 Restriction Fragment Length Polymorphism (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, this is a rapid method to distinguish between *Salmonella* Typhimurium and *Salmonella* Typhi, taking 4 hours to make that distinction (Nathisuwan *et al*., 2001; Prakash *et al*., 2005). A comparative study of polymerase chain reaction-based technique with blood culture and Widal test during the first week of illness of 82 suspected cases of typhoid showed a positivity of 71.95%, 34.1%, and 36.5% by PCR, blood culture and Widal test respectively (Haque *et al.,* 1999). A similar 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, and 172 (81%) samples by conventional microbiological methods (Malkawi and Gharaibeh, 2003). Using PCR, *Salmonella* spp. detection could be achieved within 24–36 hours compared to 3–8 days for the conventional microbiological methods(Malkawi and Gharaibeh, 2003).

2.5.9 Treatment

2.5.9.1 Typhoidal Salmonella infection

In *Salmonella* endemic areas, more than 60 to 90 percent of cases of typhoid fever are managed at home with antibiotics and bed rest. For the hospitalized patients, 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 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 with 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).

2.5.9.2 Non-typhoidal Salmonella infections

Infections are usually self-limiting, and antimicrobial treatment is not recommended for uncomplicated illnesses (Aserkoff and Bennet 1969, Gill and Hammer 2001). However, extraintestinal infection can occur, particularly in very young, elderly, and immunocompromised patients (Angulo and swerdlow 1995, Thuluvath and McKendrick 1988). In these cases, effective antimicrobial treatment is essential (Cruchaga *et.al.,* 2001; Parry *et al*., 2002). For susceptible organisms, treatment with an oral quinolone, trimethoprim-sulfamethoxazole, or amoxicillin is adequate. However, 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).

2.6 Antibiotic Resistance

Efforts aimed at identifying new antibiotics were once a top research and development priority among pharmaceutical companies. The potent broad spectrum antibiotics that emerged from these endeavors provided extraordinary clinical efficacy. Success, however, has been compromised as a long list of microorganisms have found ways to circumvent different structural classes of antibiotics 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 importance. Over the years, various studies have reported the presence of genes and mutations conferring resistance to antimicrobial agents in 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 antimicrobial agents, protection, alteration or replacement of the cellular target sites (Schwarz and Chaslus- Dancla, 2001).

2.6.1 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 multi-drug-resistance 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 of which are located on gene cassettes and have mainly been identified in class 1 multi-drug-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).

2.6.2 Trimethoprim-sulphamethoxazole (Cotrimoxazole)

Cotrimoxazole is a combination of trimethoprim and sulphamethoxazole. It has been successfully used to treat *Salmonella* infections. Since these two antimicrobial agents

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 2.5). 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 agent. 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).

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).

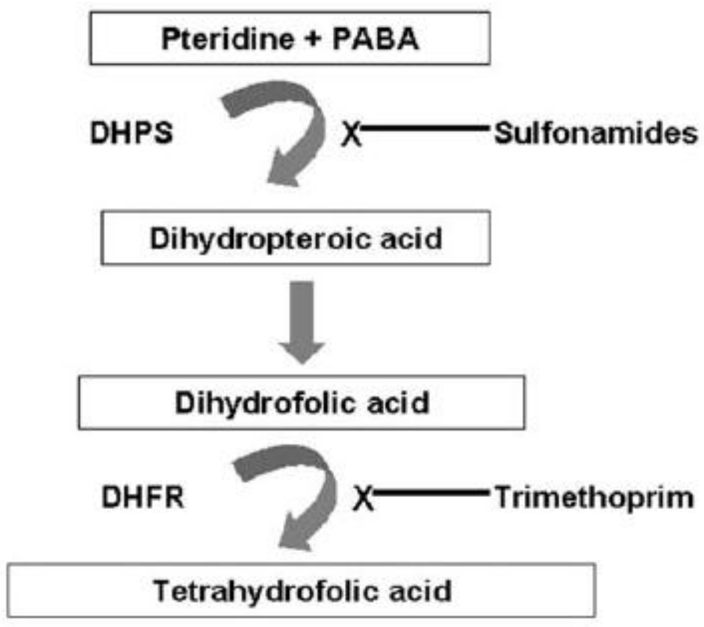

Figure 2.5: Inhibition of folate synthesis by sulfonamides and trimethoprim.

paraaminobenzoic acid(PABA); dihydropteroate synthase (DHPS); dihydrofolate reductase (DHFR). (Adopted from Huang et al., 2004).

2.6.3 Fluoroquinolones

Fluoroquinolones (FQ) represent the second, third and fourth 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 2.6) (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).

A study of over 1000 stored *Salmonella* isolates from Finland, by means of disk diffusion confirmed that resistance to nalidixic acid 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 (Butt *et al.,* 2006).

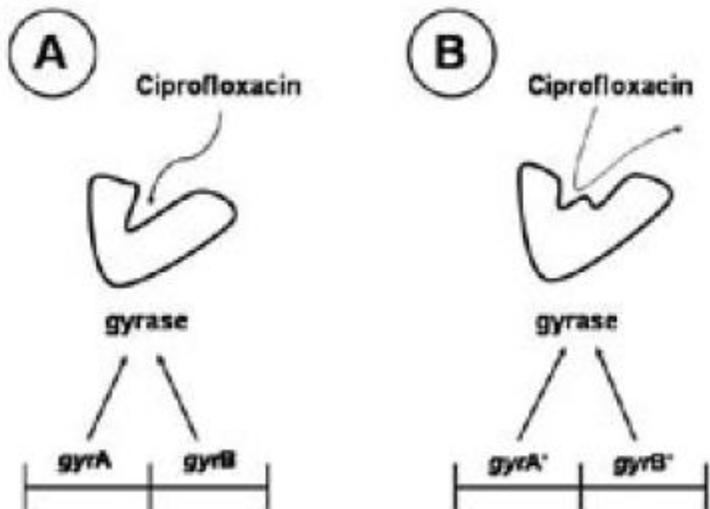

Figure 2.6: Mechanism of ciprofloxacin resistance.

(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 (Adopted from Anderson, 2007).

Mutation in *gyr*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, 1999).

Fluoroquinolones resistance mechanisms also include decreased accumulation due to active efflux and possibly decreased outer membrane permeability. 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.

2.6.4 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 peptidoglycan 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, 1999).

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 (Schwartz *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 β-

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).

2.6.4.1 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).

2.6.4.2 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 into 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-lactam 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 their 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 *Staphylococcus aureus*, α-haemolytic 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).

Extended spectrum β –lactamases 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 antibiotics of choice 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 multiresistant plasmids encoding a variety of extended-spectrum and AmpC-type -lactamases (Miriagou *et al*., 2004).

2.7 Trends in Antibiotic Resistance of *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 areas of the world (Su *et al*., 2004).

2.7.1 *Salmonella enterica* serovar Typhi

The presence of multi-drug resistant (MDR) *S.* Typhi, the causal 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 resistant *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 become 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 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).

2.7.2 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 through the food chain (Angulo *et al*., 2004).

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, gentamicin, 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 serious 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 year (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 resistance 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.

2.7.3 Trends of antibiotic resistance in Nigeria

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). However, the extent of antibiotic resistance in developing countries like Nigeria is difficult to evaluate because *Salmonella* are not routinely cultured and their resistance to the antibiotics, commonly used in both veterinary and human medicine, is seldom assessed. Antimicrobial resistance of *Salmonella* is a common public health problem in developing countries.

A study by Olowe *et al.* (2007) to determine multidrug resistance profile of *Salmonella* Typhimurium to commonly used antibiotics and its infectious nature in Osogbo was carried out on isolates from different samples obtained from Ladoke Akintola University of Technolgy Teaching Hospital, Osogbo. In all, 23 non-duplicate *Salmonella* Typhimurium isolates were recovered from different clinical samples including stool (204), urine (48), blood (52), cerebrospinal fluid (20). High rates of antibiotic resistance were found in most of the isolates studied and resistance rates were 91.3% for amoxicillin and cotrimoxazole, 86.9% for ampicillin, 82.6% for streptomycin and 30.4% for ciprofloxacin, respectively.

The susceptibility patterns reported in 2009 from different locations in Ekiti State showed resistance to streptomycin, chloramphenicol, cefepime, nalidixic acid tetracycline and trimethoprim-sulfamethoxazole. This same trend of resistance was repeated in 2010 and 2011, however, the notable change was a significant increase in the resistance to ampicillin

from 20% to 100% in Ado Ekiti and from 39% to 100% at Ikole Ekiti and also Gentamicin from 18% at Ikere-Ekiti in 2009, to 86% and 84% in 2010 and 2011 respectively. In addition, all the isolates that showed resistance to the antibiotics mentioned earlier contained *qnrB*2, 35(70%) of the isolates contained *blaCMY*-2'; 39 (78%) contained *blaCMY*-23', the mechanisms for extended-spectrum cephalosporin, Aminoglycosides and Quinolone resistance respectively. The genes that code for these resistance have proven to be remarkably mobile and widely distributed within and between species (Ajibade, 2013).

In a related study, Ifeanyi *et al.,* 2010 reported that strains of *Salmonella species* showed substantial resistance to Amoxycillin, Amoxycilin-clavulanic acid, Cephalexin and Cefuroxime.

A study which investigated the incidence rate of *S.* Typhi infection and antimicrobial resistance of clinical isolates of this pathogen to 10 different antibiotics including; pefloxacin (10µg), ciprofloxacin (10µg), augmentin (30µg), gentamicin (10µg), co-trimoxazole (30µg), ampicillin (30µg), streptomycin (30µg), nalidixic acid (30µg), cephalexin (10µg) and ofloxacin (10µg), which are commonly prescribed by physicians in two popular health facilities in Anyigba, Kogi State, Nigeria, by Sule *et al.,* 2012, showed that, of 30 isolates studied, 100% resistance was observed *in vitro* to six antibiotics (pefloxacin, ciprofloxacin, augmentin, gentamicin, co-trimoxazole and ampicillin); each of the isolates showed multi-resistance to, at least, six antibiotics. Eight (26.7%) isolates, however, were resistant to all the antibiotics. The susceptibility pattern of the remaining isolates to those four antibiotics with antibacterial activity against *S.* Typhi was 40.0% (streptomycin), 20.0% (nalidixic acid), 20.0% (cephalexin) and 10.0% (ofloxacin). Though

streptomycin showed the highest antibacterial activity, more than 50% of the isolates were still found resistant to it (Sule *et al*., 2012).

In a recent report by Moses *et al.* (2014) on the prevalence of multiple antibiotic resistance (MAR) and molecular characterization of resistance (*BlaCTX* and *GyrA*) genes in *Salmonella* recovered from stool samples of diarrhoeal patients in Ile-Ife, Osun state, Nigeria. A total of 69 *Salmonella* (*S.*Typhimurium(82.6 %), *S.*Typhi(10.1 %) and *S.*Paratyphi A (7.3 %) were isolated from 187 diarrhoeal stool samples and analysed. Resistance was mostly to nitrofurantoin (100%), ceftriazone (97.2 %), and gentamicin (94.2 %) among others. Sixty seven (97.1 %) of the *Salmonella* isolates were resistant to at least two different classes of antibiotics with 32 antibiotypes. Multiple plasmids of molecular weights (1.46 - 23.13 kbp) and resistance genes (*GyrA*-282 bp, *blaCTX*-480 bp) were detected in the representative MAR isolates. The prevalence of MAR *Salmonella* in diarrhoeal patients' stool samples was high in the study area.

The resistance to antibiotics commonly employed in the treatment of salmonellosis varies. Recently, Galadima and Kolo (2014) reported in their study that all isolates were resistant to chloramphenicol (30ug) and streptomycin (30ug) but were highly sensitive to amoxicillin and ciprofloxacin.

2.8 Prevention and Control of Antibiotic Resistance

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 challenge. 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 nontyphoidal 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).

2.9 Typing of *Salmonella*

Typing is splitting organisms into useful groupings; if the grouping is 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).

2.9.1 Phenotyping

Phenotypic methods are those that characterise the product of gene expression in order to differentiate strains. These typing methods, such as antibiotic susceptibility testing, biotyping, serotyping and phage typing have occasionally been useful in describing the

epidemiology of infectious diseases (Hopkins *et al.,* 2004; Ranjbar *et al.,* 2014). 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, labour intensive, and less typeability properties (Maslow *et al*., 1993; Singh *et al.,* 2006; Maccannell, 2013; Ranjbar *et al.,* 2014).

2.9.1.1 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).

2.9.1.2 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 does not 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).

2.9.1.3 Biotyping

Biotyping refers to analysis of cellular metabolic enzymes. Some diseases often cluster 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).

2.9.1.4 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 analysis.

2.9.2 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).

2.9.2.1 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 to as restriction enzyme analysis of plasmids, 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 genetic elements 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).

2.9.2.2 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, labour-intensive, and incompatible with current highthroughput 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).

2.9.2.3 Pulsed field gel electrophoresis of whole chromosomal DNA

Pulsed field gel electrophoresis (PFGE) is often considered the "gold standard" of molecular typing methods. It 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).

Pulsed field gel electrophoresis 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. The technique 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).

Pulsed field gel electrophoresis 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.

2.9.2.4 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 are mobile DNA elements (Martin, 2001).

2.9.2.5 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 labour 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 labourious 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).

Fadl 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 backs. 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).

2.9.3 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 laboratories 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).

2.9.3.1 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).

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. 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 (Urwin and Maiden, 2003; Sukhnanand *et al*., 2005; Foley *et al*., 2006), *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.

DNA Sequencing is regarded as the "gold" standard for the identification and confirmation of all microorganisms, including *Salmonella*.The most popular PCR target is the 16S-23S rRNA spacer region, also known as the internal transcribed spacer (ITS), a hypervariable region specific for each bacterial species (Pritchett *et al.*, 2000). Once the DNA sequences have been determined, they are compared to a library of known sequences in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Whole genome sequencing has also been used in the identification of *Salmonella* species (Leekitcharoenphon *et al.,* 2012). However, the cost and time involved in performing this technique are major constraints for its routine use (Leekitcharoenphon *et al.,* 2012).

2.9.3.2 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, it 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.

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).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study covered three states in the Northwest geopolitical zone of Nigeria (Figure 3.1). The states includes Kaduna, Katsina and Sokoto. The states were selected on the bases of similarity in climatic conditions, sociocultural and behavioural pattern of the people and their level of infrastructural development.

3.1.1 Kaduna State

Kaduna State is located on the southern end of the high plains of Northwestern region, bound by parallels of latitude $9^{o}03'$ N, and $11^{o}32'$ N, and extends from the upper River Mariga on longitude $6^{o}05'$ E to $8^{o}48'$ E on the foot slopes of the scarp of Jos Plateau. Seasonality in Kaduna is characterised by the cool to hot season being longer than the rainy season. Rainfall varies between 1530mm in the southeast to about 1015mm in the northeast. The 2006 census provisional result put the population of Kaduna State at 6,066,562 with a near 1:1 male/female ratio. Apart from two University Teaching Hospitals, there are three large fully equipped government hospitals and two hundred and sixty nine (269) registered private clinics located within the major towns. There are also comprehensive Primary Health Care Units (PHCU) in some selected rural areas established by the State Government together with the Federal Government and the World Health Organization. These PHCUs cater for childcare, prenatal and antenatal health care (Bello, 2000).

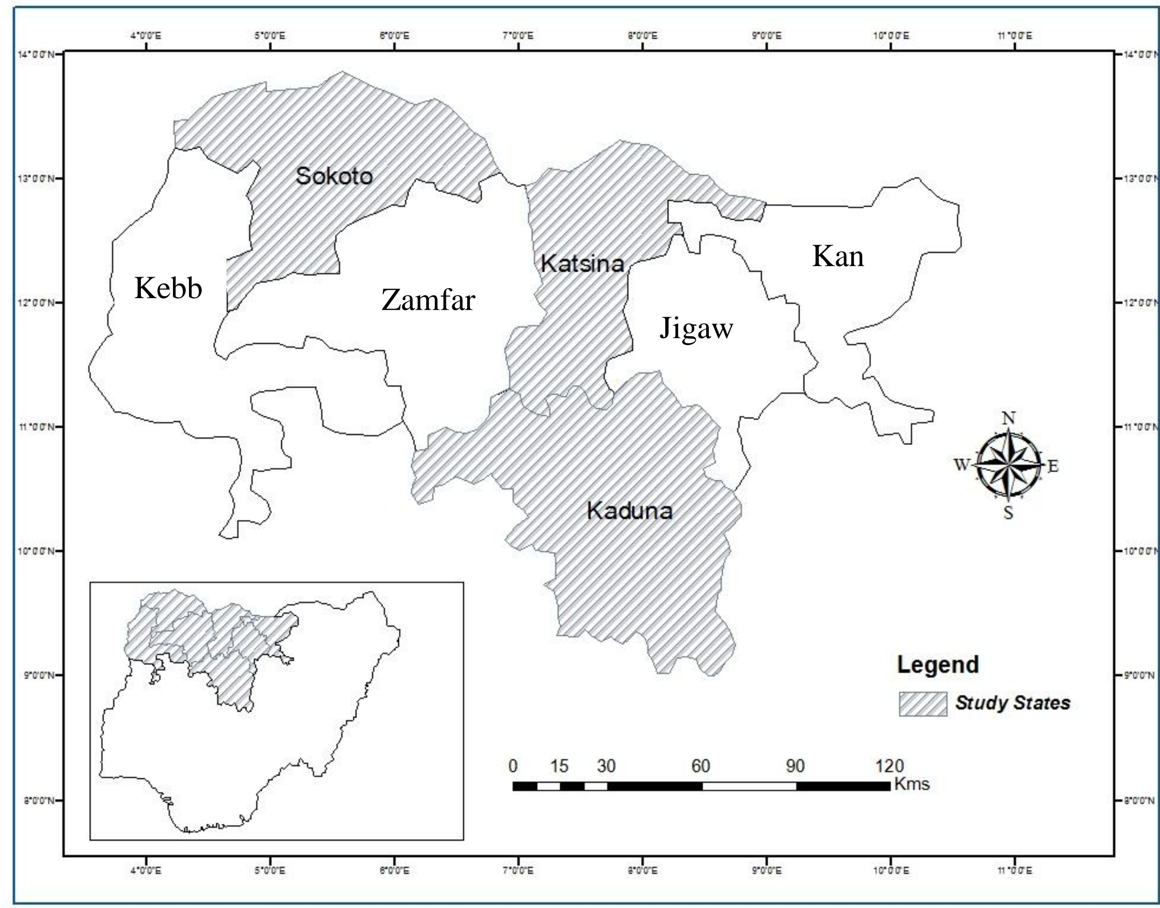

Figure 3.1: Map of Northwestern Nigeria showing the study area as the shaded portions on the map. (Courtesy of GIS Unit Federal University, Dutsin-Ma, Nigeria).

3.1.2 Katsina State

Katsina State is located between latitude 11°08'N and 13°22'N and longitude 6°52'E and 9°20'E and covers a land mass of about 23,938sq km. The state is bounded to the north by Niger Republic, to the east by Jigawa and Kano states, to the west by Zamfara state and to the south by Kaduna state. The state has a climate which varies between a cool dry season (harmattan) from October to February, a hot dry season from March to May and a warm wet season from June to September. Katsina State has 22 General Hospitals, one Specialist Hospital, over 107 Comprehensive Primary Health Care Units and Dispensaries, many Maternity and Childcare Clinics and 87 Private Clinics. Water supply in Katsina State is sourced majorly from damning of rivers and digging of wells and boreholes. Most households own private wells as source of water for drinking and other domestics activities (LGAC, 2002).

3.1.3 Sokoto State

Sokoto State is located to the extreme of the Northwestern region between latitudes 12°N and 13°58'N and longitudes 4°8'E and 6°54'E. The state shares borders with Niger Republic to the north, Kebbi State to the southwest and Zamfara State to the east. It covers a land area of about 32,000 sq. km with a total population of 3,696,999 projected from 2006 census. Rain falls mainly between June and September in the north and from April to October in other parts. As at June 1999, the state has a total of 440 health facilities located in different Local Government Areas of the state, including eight General Hospitals, one each of teaching, psychiatry, army and specialist hospitals (Mamman, 2008).

3.2 Study Design

The study was a hospital and clinic based prospective cross-sectional study conducted to determine the magnitude of *Salmonella* infection in children (aged 5 years and below) with diarrhoea from May, 2013 to April, 2015.

3.3 Study Population

The study population included infants and young children five years and below who reported to the hospitals clinics with diarrhoea symptoms or gastroenteritis.

3.3.1 Control population

These were children of the same age group, who had no history of diarrhoea, at least three weeks preceding sampling. Sixty (60) children in all were included in the study as control population.

3.3.2 Inclusion criteria and exclusion criteria

Children aged below 5 years, presenting with acute diarrhoea and those without diarrhoea attending public and private hospitals within the study area and who gave consent. The exclusion criteria were children above 5years, of both sexes and lack of consent. Diarrhoea in this study was defined as a child passing loose or watery stool or a bloody stool three or more times in 24-hour period as reported by parents.

3.4 Ethical Consideration and Consent

Ethical approval (Appendix I) was obtained from each State Ministry of Health and the Ethics Committee of each hospital under study before the commencement of the study. In the course of this research, individuals' anonymity was maintained, good laboratory practice/quality control were ensured and every finding was treated with utmost confidentiality and for the purpose of the research only.

Histories were taken from each child from informed and consenting parents/caregiver after duely filling the consent form (Appendix II) before sample collection by the attending qualified health worker. Relevant data (demographic, clinical, and laboratory data) were recorded and in the questionnaire prepared for this study (Appendix III).

3.5 Sample Size Determination

The sample size in this study was calculated based on the prevalence rate of 11.3% *Salmonella* in human diarrhoea stools, as per previous studies by Ogbu *et al.* (2007), using the formula; $N = \frac{Z^{2pq}}{L^2}$

Where N is the sample size**;**

Z is the standard normal distribution at 95% confidence interval = 1.96

p is the prevalence rate, which is taken to be 11.3% = 0.113

q is 1-p = 1-0.113 = 0.887

L is allowable error, which is taken as 5% = 0.05

$N = 1.96^2 \times 0.113 \times 0.887/0.05^2$

$N = 154.02$

The calculated sample size was approximately 154, which is the minimum number of samples required for this study. However, to enhance better results, a total of 634 diarrhoea stool samples were collected, that is 207 each from Kaduna and Sokoto and 220 from Katsina. Three well epuiped General Hospitals from each of the states were selected this study (one from each of the three senatorial district of the states). Twenty 20 control (non-diarrhoeic) stool samples were also collected from each of the three states under study (i.e. a total of 60 control samples).

In addition to stool samples, water samples were collected from drinking water sources based on *Salmonella* prevalence of 49.4% (Ateba *et al.,* 2012) calculated using the formula:

$$N = \frac{Z^{2pq}}{L^2}$$

Where N is the sample size

Z is the standard normal distribution at 95% confidence interval = 1.96

p is the prevalence rate, which is taken to be 49.4% = 0.494

q is 1-p = 1-0.494 = 0.506

L is allowable error, which is taken as 5% = 0.05

$N = 1.96^2 \times 0.494 \times 0.506/0.05^2$

$N = 384.10$

However, 246 drinking water samples were collected in duplicate (making a total of 492): 60 samples from running taps, 54 different brands of sachet water, 90 well water samples

and 42 samples from boreholes within the study area, for microbiological quality assessment.

3.6 Collection of Samples

3.6.1 Stool samples

Based on methods specified in Cheesbrough, (2009), about 5-10g of stool was scooped into well labeled sterile wide mouth universal sample bottle using plastic spoon. Where it was not possible to obtain stool, rectal swab was collected using sterile swabs with the aid of a qualified attending nurse, by inserting a sterile cotton wool swab in the rectum of the child for about 10 seconds, this was placed in sterile physiolgical saline in properly labeled sample bottles. Sample collection forms were filled for each sample. This contained information such as: colour of stool, texture (formed, semi-formed, unformed, fluid) and presence of blood, mucus, pus in samples. All samples were transported in ice packs to the Bacteriology laboratory, Department of Microbiology, Ahmadu Bello University, Zaria for analysis.

3.6.2 Collection of water samples

3.6.2.1 Collection of water samples from wells

A weighted bottle was used to collect the sample as follows: a sterile sample bottle was tied on to a weighted length of rope or strong string, using a heavy piece of metal as a weight, with the bottle attached just above the weight.

After aseptically removing the cap from the bottle, it was lowered into the well to a depth of about 1 metre. When no more air bubbles rose to the surface of the water, the bottle was

raised out of the well and the cap carefully replaced. The bottle was labeled appropriately and transported in ice pack to the laboratory for analysis.

3.6.2.2 Collection of water samples from tap

The outside nozzle of the tap was cleaned carefully using a piece of cotton wool, soaked in methylated spirit. The tap was turned on full, and the water allowed to run to waste for 1 minute. This allowed time for the nozzle of the tap to be flushed and any stagnant water in the service pipe to be discharged. The sample bottle was then filled from a gentle flow of water, it was covered and labeled appropriately.

3.6.2.3 Collection of water samples from boreholes

Water from boreholes was similarly collected as in the case of tap water. The outside nozzle of the service pipe was cleaned carefully using a piece of cotton wool, soaked in methylated spirit. The water was pumped out through the nozzle and allowed to run to waste for 1 minute. The sample bottle was then filled from a gentle flow of water, it was covered and labeled appropriately.

3.6.2.4 Collection of Sachet water samples

Sachets of packaged water of different brands were purchased from vendors and transported in cold boxes to the Microbiology laboratory for analysis.

One (1) litre of water was collected twice within an interval of one month from every sampling point for well, taps and boreholes. Two (2) packs of sachet water were collected

from vendors for each brand. All samples were transported in ice pack to the laboratory for analysis within 6 hours of collection.

3.7 Microbiological Analysis of Stool Samples

3.7.1 Media preparation

All media used in this study were prepared, strictly following the manufacturer's instructions.

Selenite F broth was prepared by adding 19g of selenite broth base (Oxoid, UK.) to 1 litre of distilled water, to which 4g of sodium biselenite (Oxoid, UK.) was added. This was warmed to dissolve and dispensed into sterile bujou bottles and sterilized in a boiling water bath for 10minutes.

Salmonella Shigella agar (SSA; Oxoid, UK.) was prepared by suspending 63g of the dehydrated medium in 1litre of distilled water, this was brought to boil on a bunsen burner with frequent agitation and allowed to simmer gently to dissolve the agar. It was then cooled to about 50°C, mixed and dispensed into petri dishes.

Eosin methylene blue (EMB) agar (Oxoid, UK.) was prepared by suspending 37.5g of the dehydrated medium in 1litre of distilled water. This was boiled to completely dissolve and then sterilized by autoclaving at 121°C for 15minutes. It was cooled to about 60°C and shaken in order to oxidize the methylene blue and to suspend the precipitate. It was then asceptically dispensed into petri dishes and allowed to solidify before use.

Nutrient agar (Oxoid, UK.) was also prepared by suspending 28g of the dehydrated medium in 1litre of distilled water. This was boiled to dissolve and dispensed into bujou bottles (in cases of slants preparation) and sterilized at 121°C for 15minutes. It was cooled to about 50°C and dispensed into petri dishes or slanted to solidy.

Xylose lysine desoxycholate (XLD) agar (HKM, China) was prepared by suspending 55.2g of the powder in 1litre of distilled water. This was brought to boil on a bunsen burner with frequent agitation and allowed to simmer gently to dissolve the agar. It was then cooled to about 50°C, mixed and dispensed into petri dishes.

Mueller Hinton Agar (Oxoid, UK.) was prepared by suspending 38g of the powder in 1litre of distilled water and dissolved by boiling and shaking. It was then sterilized by autoclaving at 121°C for 15minutes. This was cooled to 50°C then dispensed into petri dishes and allowed to solidify.

3.7.2 Inoculation

A loopful of faecal sample was inoculated into selenite F broth and incubated for 24 hours at 37°C and then subcultured on *Salmonella Shigella* agar (Oxoid, UK.). The plates were incubated at 37°C for 24 hours and observed for growth. Non fermenters colonies growing, which appeared colourless or pale were purified on Xylose Lysine Desoxycholate agar (Oxoid, UK.), and stored on Nutrient agar (Oxoid, UK.) slants for further studies.

3.8 Biochemical Characterisation of Isolates

A pure culture of all isolates obtained were phenotypically characterised according to methods described in Cheesbrough, (2009) as follows:

3.8.1 Gram staining

A thin smear was made from a pure culture of each isolate by carefully spreading a colony evenly, covering an area of about 15–20mm diameter on a slide. This was allowed to air dry after which it was heat-fixed. The fixed smear was covered with crystal violet stain for 30–60 seconds, then, rapidly wash off with clean water and covered with Lugol's iodine which was washed off after 30–60 seconds with clean water. The smear was decolorized rapidly (few seconds) with acetone–alcohol and washed immediately with clean water. It was counter-stained with neutral red stain for 2 minutes and washed off with clean water, after that it was air-dried and examined with the oil immersion objective of the microscope to determine cell morphology.

3.8.2 Sugar fermentation test

Prepared triple sugar iron agar (Oxoid, UK) slants in test tubes were inoculated by stabbing the butt of the agar and streaking the slope using a sterile straight wire. The tube was loosely capped and incubated at 37 °C overnight. *Salmonella* organisms produce a pink-red slope and yellow butt. Many salmonellae also produce blackening due to hydrogen sulphide production and cracks in the medium due to gas production from glucose fermentation. *Salmonella* Typhi produces only a small amount of blackening and no cracks in the medium.

3.8.3 Urease test

The test organism was heavily inoculated in a bijou bottle containing 3 ml sterile Christensen's modified urea broth (Oxoid, UK) and incubated at 35–37°C for 3–12 hours and observed for a pink colour in the medium.

3.8.4 Oxidase test

This was performed using Microgen™ oxidase reagent strip following the manufacturer's instructions. The strip was moistened with a drop of sterile water, using a piece of plastic stick a colony of the test organism was removed and rub on the strip and observed for development of a red-purple colour within 20 seconds.

3.8.5 Motility test

A semi-solid medium comprising (Per 1litre); Enzyme digest gelatin (10g), Beef extract (3g), Sodium chloride (5g) and Agar (4g) was prepared, dispensed into tubes and sterilized by autoclaving at 121°C. To test for motility, a sterile needle was used to pick a well-isolated colony of the isolates and stab the medium to within 1cm of the bottom of the tube. This was incubated at 37°C for 24 hours. A positive motility test was indicated by a red turbid area extending away from the line of inoculation. Negative test was indicated by growth along the line of inoculation but no further.

3.8.6 Biochemical characterisation with Microgen™ Identification Kit

All isolates were further biochemically characterized using the Microgen™ GnA+B - ID System for Enterobacteriaceae. This is a two panel system (GNA and GNB), comprising 24

biochemical tests. Panel A (GN A) comprise tests for Lysine, Ornithine, H_2S , Glucose, Mannitol, Xylose, ONPG, Indole, Urease, VP., Citrate and TDA. Panel B (GN B) consist of tests for Gelatin, Malonate, Inositol, Sorbitol, Rhamnose, Sucrose, Lactose, Arabinose, Adonitol, Raffinose, Salicin and Arginine. By strictly following the manufacturer's protocol, 18 -24 hour culture of the isolates were emulsified in 3ml sterile 0.85% saline and mixed thoroughly. Using sterile pasteur pipette 3-4 drops (approximately 100μl) of the bacteria suspension was added to each well of the microwell test strip(s). After inoculation, wells 1,2,3, and 9 testing for Lysine, Ornithine, Hydrogen sulphide and Urease on panel A and well 24 (Arginine) on panel B, were overlaid with 3-4 drops of mineral oil. The set up was sealed and incubated at 35-37°C for 18-24 hours. After incubation, 2 drops of Kovac's reagent were added to well 8 (for indole test), 1 drop each of VPI and VPII reagents were added to well 10 (for Voges Proskauer test), and 1 drop of TDA reagent was added to well 12 (for tryptophan deaminase test). The results of the indole, Voges Proskauer and tryptophan deamines were read after 1min, 30min and 1min respectively. Results of the biochemical reactions were read with the aid of a colour chart provided with the kit, and recorded on the result forms. Interpretation of results was carried out with the aid of the Microgen™ Identification System Software (MID version 1.2.5.26). All isolates were stored on Nutrient agar slants for further studies.

3.9 Bacteriological Analysis of Water and Stool Samples

3.9.1 Enumeration of faecal coliform from water samples

The membrane filtration method was used to process all water samples as described by Masters *et al.* (2011). For each water sample, 100ml of the water samples were aseptically

transferred into sterile filtration units fitted with sterile 0.45µm pore size nitrocellulose membrane filters. This unit was connected to a suction machine which enabled efficient and timely filtration process. After filtration, used filters were aseptically transferred onto freshly prepared eosin methylene blue (EMB) ager (Oxoid, UK.) plates and incubated at 35°C for 2 hours to revive cells and then 44.5^{o}C for 22 hours, for enumeration of *E.coli* as well as other faecal coliforms (FC). Counts were made using electronic colony counter, and figures expressed as colony forming unit per millilitre (cfu/ml). Samples were taken in duplicates from each sampling point and mean counts were recorded.

3.9.2 Isolation and identification of *Salmonella spp.* from water samples

For the isolation of *Salmonella spp*., the second membrane filter was aseptically removed using sterile forceps, and placed in 25ml Erlenmeyer flask containing selenite F broth and incubated for 24 hours at 37°C. After the enrichment, a loopful of the broth culture was subcultured on SS agar and incubated at 37°C for 24 hours. Colourless colonies with black centre, presumptive of *Salmonella* were selected and biochemically characterized using Microgen Identification System kit. Confirmed isolates were maintained on nutrient agar slants for further studies.

3.10 Serological Identification of *Salmonella* Isolates

Serological tests were performed on all isolates positive for *Salmonella* by biochemical characterisation, using Microgen™ *Salmonella* latex slide agglutination test kit for the confirmatory identification of the presumptive *Salmonella* colonies from SSA plates. Following the manufacturer's instructions, 1 drop of M40 isotonic saline **(Microgen™)**

was dispensed into a circle of Microgen™ agglutination slide. Using an inoculating wire loop, a colony from the selective agar plate (SSA) was removed and emulsified in the drop of saline to produce a heavy smooth suspension, this was rocked gently for 2 minutes and observed for autoagglutination. If the suspension remained smooth, then a drop of Microgen™ *Salmonella* latex (containing particles coated with polyvalent antisera raised against a wide range of *Salmonella* antigens) was carefully placed next to the bacterial suspension. This was then mixed with the bacterial suspension using a clean mixing stick and gently rocked 2 or 3 times after which it was observed for agglutination within 2 minutes, using indirect lighting over a dark background.

3.11 Determination of Antibiotic Susceptibility of Isolates

Isolates from both water and stool samples, earlier characterized were tested for susceptibility to the following 10 antibiotics, commonly used in humans for the treatment of Gram negative pathogens; Gentamicin (10µg), Ampicillin (10µg), Amoxycillin (30µg), Trimethoprim/Sulphamethoxazole (1:19) (25µg), Ciprofloxacin (5µg), Nalidixic acid (30µg), Tetracycline (30µg), Cefotaxime (30µg), Chloramphenicol (30µg), Amoxicillin/clavulanic acid (2:1) (Oxoid, UK.).

Antibiotic susceptibility testing was performed for all *Salmonella* isolates using the disk diffusion method and results were interpreted using the criteria of Clinical and Laboratory Standards Institute guidelines (CLSI, 2014). Briefly, each stored isolate was subcultured on SS agar (Oxoid, UK.). A loopful of bacteria pure culture was taken and transferred to a tube containing 2 ml 0.85% saline (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. Standard inoculum of 0.1ml of suspension was transferred onto the surface of Mueller Hinton agar (Oxoid, UK.) and evenly spread using a sterile bent glass rod. 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 (3 antibiotic discs per plate). The discs were gently pressed onto the surface of the medium with sterile forceps to ensure firm contact and incubated at 37°C for 24 hours.

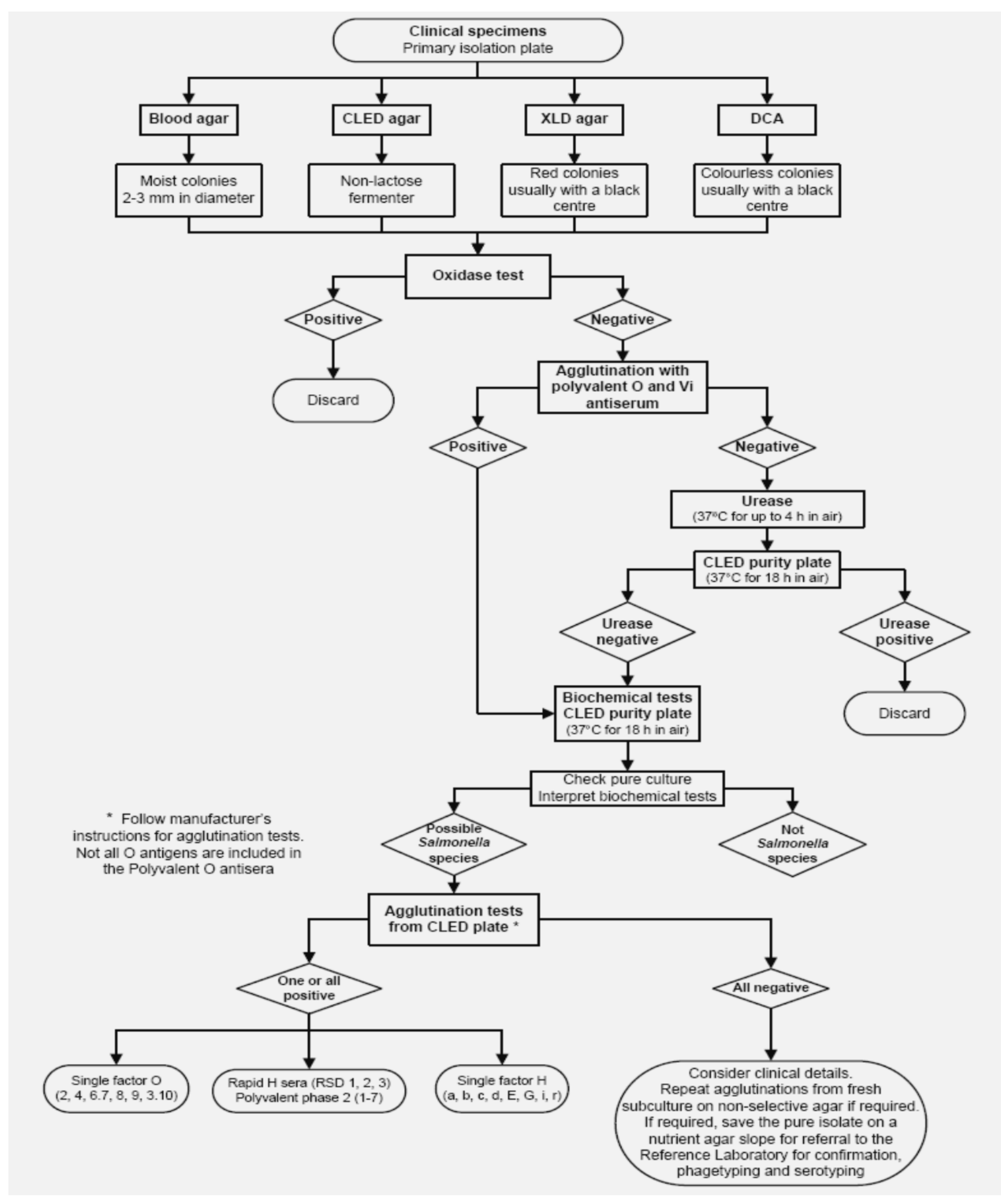

Figure 3.2: Flow chart for identification of *Salmonella* species (Adopted from BSOP, 2007)

Diameters of the zone of inhibition around the antibiotic disc were measured to the nearest millimeter using a meter rule and the isolates were classified as sensitive (S) or resistant (R), based on CLSI break points (Appendix VII). Intermediates were reported as isolates with reduced susceptibility.

3.12 Molecular Typing of *Salmonella* Strains

3.12.1 Extraction of DNA from isolates

The DNA from isolates was extracted using Zymo Research (ZR) Fungal/Bacterial DNA MiniPrep™. Twenty four hour (24hr) cultures of isolates in Luria Bertani broth were resuspended in 200µl isotonic buffer (Phosphate buffered saline). One hundred milligram (wet weight) of the resuspended bacterial cells was added to a ZR BashingBead™ Lysis Tube and 750µl Lysis Solution was added to the tube. This was secured in a bead beater fitted with a 2ml tube assembly and shaken at maximum speed for 5minutes. The ZR BashingBead™ Lysis Tube was centrifuged in a microcentrifuge at 10,000x*g* for 1minute. The supernatant (400µl) was transferred into a Zymo-Spin™ IV Spin Filter in a collection tube and centrifuged at 7,000x*g* for 1minute. To the filtrate in the Collection Tube, 1,200µl of Fungal/Bacterial DNA Binding Buffer was added and 800µl of the mixture was transferred to a Zymo-Spin™ IIC Column in a collection tube and centrifuged at 10,000x*g* for 1minute. The flow through was discarded and the step was repeated, after which 200µl DNA Pre-Wash Buffer was added to the Zymo-Spin™ IIC in a new collection tube and centrifuged at 10,000x*g* for 1minute, then 500µl of Fungal/Bacterial DNA Wash Buffer was added to the Zymo-Spin™ IIC Column and centrifuged at 10,000x*g* for 1minute. The DNA was eluted by transferring the Zymo-Spin™ IIC Column into a clean 1.5ml

microcentrifuge tube and 100µl DNA Elution Buffer was added directly to the column matrix and centrifuged at 10,000x*g* for 30 seconds to elute an ultra pure DNA. This was stored at -20°C for further use.

3.12.2 Screening for *integrons* and antibiotic resistance genes by PCR

The isolates were screened for class 1 integrons, *bla*TEM1, *cat*1b and *aadA*2 by PCR as described by Levesque *et al.* (1995). The primers for *bla*TEM1, *cat*1b, *aadA*2 and Class 1 integron and annealing temperatures are shown in Table 3.1. In each case, the reaction mixture was constituted by adding to 5.5µl nuclease free water in the PCR reaction tube; 12.5µl PCR master mix 2X, which comprised 0.05u/µl Taq DNA polymerase, PCR buffer, 4 mM $MgCl_2$, 0.4 mM of each dNTP (Thermo Fisher Scientific Inc,NYSE: TMO , USA), 2.5µl of each primer and 2µl of template bacterial DNA. This was mixed gently and loaded onto a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA). The system was programmed to run the amplification reactions asfollows; an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 30 seconds, annealing at 55°C, 56°C, 62°C and 50°C for 30 seconds for *bla*TEM1, *cat*1b, *aadA*2 and Class 1 integron respectively. This was followed by extension at 72°C for 2 min 30 seconds, and a final extension at 72°C for 10 minutes. The amplified products were separated by gel electrophoresis on 1.5% agarose and were detected by comparison against a 100bp DNA ladder as a size marker and visualised under UV light on a Biotop SC - 645Gel Documentation system (Biotech Co. Ltd, Shanghai China).

3.13 Sequence Analysis of 16S rRNA

The DNA was subjected to the cocktail mix in Table 3.2 with the following forward and reverse primer sequences for the 16S rRNA. 27F : AGAGTTTGATCMTGGCTCAG, 1525R: AAGGAGGTGWTCCARCCGCA. The 16S Forward is 27F and the 16S Reverse is 1525R.

3.13.1 PCR conditions for Sequencing

The PCR conditions were: Initial denaturation at 94°C for 5min., denaturation at 94°C for 30sec., followed by annealing at 56°C for 30sec. and extension at 72°C for 45sec. This was run for 36 circles with a final extension at 72°C for 7min. for each isolate. The amplicons from the reactions above were loaded on 1.5% agarose gel for electrophoresis using 100bp ladder (Invitrogen).

3.13.2 PCR product purification

The PCR product was purified with the following protocol: 20µl of absolute ethanol was added to the PCR product and incubated at room temperature for 15minutes, after which it was spun at 10000rpm for 15min. The supernatant was decanted, and it was further spun at 10000rpm for 15min, this was followed by the addition of 40µl of 70% ethanol and the supernatant was decanted. The product was air dried after which 10µl of ultrapure water was added and the amplicons were viewed on 1.5% agarose gel. The product from the purification process was loaded on the 3130xl genetic analyzer (Applied Biosystems) to give the nucleotide sequences. All data (nucleotide sequences) were viewed and analysed using the BioEdit sequencing software.

Table 3.1: Primer sequences for PCR

Gene	5′-3′ DNA sequence of Primer	$T_m(^oC)$	Reference
bla(Tem)	F CATTTCCGTGTCGCCCTTAT	55	Walker *et al.,* 2001
	R TCCATAGTTGCCTGACTCCC		
cat1b	F CCTATAACCAGACCGTTCAG	56	Randall *et al.,* 2004
	R TCACAGACGGCATGATGAAC		
aadA2	F TGTTGGTTACTGTGGCCGTA	62	Walker *et al.,* 2001
	R GATCTCGCCTTTCACAAAGC		
Integron A	F GGCATCCAAGCAGCAAGC	50	Sandvang *et al.,* 1997
	R AAGCAGACTTGACCTGAT		

Key: F - Forward

R - Reverse

Table 3.2: The PCR cocktail mix for sequencing.

Reagent	Quantity
10× PCR buffer	1.0
25mM $MgCl_2$	1.0
5pMol forward primer	0.5
5pMol reverse primer	0.5
DMSO	1.0
2.5Mm DNTPs	0.8
Taq 5u/ul	0.1
10ng/µl DNA	2.0
H_2O	3.1
Total	10µL

Key: PCR - Polymerase chain reaction
DMSO - Dimethyl sulfoxide
DNTPs - Deoxy-nucleoside triphosphate
DNA - Deoxyribonucleotite acid

3.13.3 Determination of genetic relatedness

The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown above the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2015).

3.14 Data Analysis

All demographic, clinical and laboratory data obtained from this study were entered, analyzed and interpreted using the Statistical Package for Social Sciences (SPSS) version 21. Chi-square was used to test difference between proportion and P-values <0.05 was considered statistically significant. Means and Mean standard deviation were calculated for coliform counts in drinking water samples. Evolutionary analyses by sequence alignment were conducted in MEGA7 software.

CHAPTER FOUR

RESULTS

4.1 Prevalence of *Salmonella* Species among the Study population

A total of 634 stool samples from children aged five years and below, presenting with diarrhoea, were collected and investigated for *Salmonella* infection. Samples from 60 apparently healthy children within the study area were also collected as control. Of the 634 study subjects, 344(54.3%) were males and 290(45.3%) were females, with 207 (32.6%) each of the subjects from Kaduna and Sokoto States, while 220 (34.8%) were from Katsina State. The overall prevalence of *Salmonella* species within the study area was 4.1% with prevalence of 3.9%, 3.4% and 5.0% in Kaduna, Sokoto and Katsina States respectively.

The distribution of positive culture samples among the states under study is shown in Table 4.1. Of the three states, Katsina had the highest number of positive culture samples (5.0%), with Sokoto having the lowest (3.4%). There was no statistically significant difference in the occurrence of *Salmonella* species among the states ($P>0.05$).

The prevalence of *Salmonella* species in relation to age and sex of study subjects is shown in Table 4.2. The highest frequency of isolation occurred among children aged 25-36 months (10.4%) and no positive case in 49-60 months age group. In terms of gender, males had a higher prevalence (4.9%) of *Salmonella* infection than their female counterparts.

Table 4.1: Occurrence of *Salmonella* species in relation to the states under study

Location	No examined	No positive (%)
Kaduna	207	8 (3.8)
Sokoto	207	7 (3.4)
Katsina	220	11 (5.0)
Total	634	26 (4.1)

$\chi^2 = 0.754$, df = 2, P=0.686

Table 4.2: Prevalence of *Salmonella* species in relation to age and sex of study subjects.

Age group (months)	No. examined	No. (%) positive	No. Male	No. (%) positive	No. Female	No. (%) positive
0-12	222	4(1.8)	131	2(1.5)	91	2(2.2)
13-24	205	7(3.4)	102	5(4.9)	103	2(1.9)
25-36	96	10(10.4)	47	7(14.9)	49	3(6.1)
37-48	74	5(6.8)	45	3(6.7)	29	2(6.9)
49-60	37	0(0.0)	19	0(0.0)	18	0(0.0)
Total	634	26(4.1)	344	17(4.9)	290	9(3.1)

$\chi^2 = 15.876$, df = 4, P = 0.003

Table 4.3 compares the occurrence of *Salmonella* infection among study subjects in relation to the educational status of their caregivers. Higher prevalence was observed among children whose caregivers had primary (5.3%) and low in subjects whose caregivers had no formal education(3.4%). However there were occurrences of positive culture samples among all educational categories and no statistically significant association between educational status of caregivers and occurrence of the infection (P = 0.747).

4.2 Association of Certain Factors with Occurrence of *Salmonella* Infection

Certain factors considered in the study in relation to the occurrence of *Salmonella* infection were; other symptoms observed in the study including vomiting (45.3%) and fever (51.9%). Type of toilet used by household members, source of water for domestic use, and if water treatment of any form was carried out prior to use. A high isolation rate (5.9%) of *Salmonella* species was observed among patients from households that use open field defaecation, however, there was no statistically significant association of the risk factors to the infection. There is, however a statistically significant difference in the source of water where 6.5% prevalence was observed among those who used well water as a major source of water. There was a higher isolation rate among patients who did not treat water prior to use than those who treated their water, but there was no statistically significant association (Table 4.4)

The prevalence of *Salmonella* in relation to texture or consistency of stool is presented in Table 4.5. On the basis of stool consistency, those with watery stools had the highest *Salmonella* species isolation rate (5.9%).

Table 4.3: Occurrence of *Salmonella* infection among children in relation to their caregiver's level of education

Level of education	No of caregivers	No. positive (%)	P-value
None	267	9(3.4)	
Primary	187	10(5.3)	0.747
Secondary	161	6(3.7)	
Tertiary	19	1(5.3)	

$\chi^2 = 1.224$, df = 3, P = 0.747

Table 4.4: Association of certain factors with occurrence of *Salmonella* infection

Factor	No examined	No(%) positive	Odds ratio (C.I)	χ^2	P-value
Vomiting					
Yes	287(45.3%)	10(3.5)	0.747 (0.443-1.293)	0.507	0.476
No	347(54.7%)	16(4.6)			
Fever					
Yes	329(51.9%)	11(3.3)	0.669 (0.650-1.424)	0.998	0.318
No	305(58.1%)	15(4.9)			
Duration of diarroea					
0-5 days	359(56.6%)	13(3.6)			
6-10days	213(33.6%)	10(4.7)		0.487	0.784
>11 days	62(9.8%)	3(4.8)			
Type of toilet					
Pit latrine	446	16(3.6)			
Open field	101	6(5.9)		1.061	0.588
Water closet	87	4(4.6)			
Source of water					
Pipe borne	127	0 (0.0)			
Well	325	21(6.5)		9.363	0.025*
Borehole	116	4(3.4)			
Others	66	1(1.5)			
Treatment of water					
Yes	118	4(3.4)		0.186	0.666
No	516	22(4.3)			

* = P-value ≤ 0.05, χ^2 = Chi square, C.I = Confidence interval

Table 4.5: Prevalence of *Salmonella* in relation to texture/consistency of stool

Texture	No.(%) examined	No.(%) positive	χ^2	P-value
Loose	176(27.8%)	3(1.7)		
Mucoid	162(25.6%)	8(4.9)	6.670	0.083
Watery	255(40.2%)	15(5.9)		
Bloody	41(6.4%)	0(0.0)		

$\chi^2 = 6.670$, P-value > 0.05

The result presented on Table 4.6 shows that 38.6% of patients were treated with antibiotics, 27.1% used Oral rehydration solution (ORS) and 34.2% were not given any treatment. There were more positive culture samples among patients who received ORS treatment (5.2%), followed by those who received no treatment (5.1%). The least positive culture samples were among those who received treatment (2.4%). However there was no statistical association between positive culture samples and treatment type.

4.3 Bacteriological Quality of Drinking Water in the Study Area

The result on Table 4.7 shows the mean total coliform counts of water samples from well water, borehole water, Sachet water and tap water. Wells had the highest total coliform counts, and Borehole water had slightly lower coliform counts than sachet water in all the states. Analysis using ANOVA showed no statistically significant difference in values of total coliform counts among the states for all the sources of water.

The faecal coliform count of the water samples also showed similar trend as the total coliform counts, with wells having the highest faecal coliform counts followed by tap water, sachet water and borehole water in that order. No statistically significant difference was observed among the states for the various water sources ($P = 0.712$), the result is presented in Table 4.8.

Table 4.6: Association of types of management practices with occurrence of *Salmonella* infection.

Management	No examined	No. positive (%)
Antibiotics	245(38.6%)	6(2.4)
ORS	172(27.1%)	9(5.2)
None	217(34.2%)	11(5.1)
Total	634	26(4.1)

χ^2=4.004, P = 0.135,

Key: ORS - Oral Rehydration Solution.

Table 4.7: Mean total coliform count from well, borehole sachet and tap water from each of the states under study

State	Type of water	No. of examined	Maximum count (cfu/100ml)	Minimum count (cfu/100ml)	Mean count (cfu/100ml)	S.D
Kaduna	Well	n=30	130	23	61.90	31.983
	Borehole	n=14	36	2	15.20	11.531
	Sachet	n=18	48	0	16.31	15.358
	Tap	n=20	150	2	43.90	42.976
Sokoto	Well	n=30	91	19	39.00	21.299
	Borehole	n=14	47	1	25.90	16.587
	Sachet	n=18	60	0	19.40	21.448
	Tap	n=20	72	10	30.30	18.136
Katsina	Well	n=30	110	12	44.7	35.883
	Borehole	n=14	24	1	9.75	8.779
	Sachet	n=18	56	0	21.9	15.600
	Tap	n=20	116	10	40.60	34.452

Table 4.8: Mean faecal coliform counts from well , borehole, sachet and tap water from each of the states under study.

State	Type of water	No. of examined	Maximum count (cfu/100ml)	Minimum count (cfu/100ml)	Mean count (cfu/100ml)	S.D
Kaduna	Well	n=30	71	1	31.40	23.197
	Borehole	n=14	10	0	5.00	5.477
	Sachet	n=18	14	0	3.30	5.315
	Tap	n=20	14	0	4.50	4.378
Sokoto	Well	n=30	64	1	21.60	16.405
	Borehole	n=14	3	0	1.60	1.512
	Sachet	n=18	21	0	4.60	8.516
	Tap	n=20	26	0	7.80	9.636
Katsina	Well	n=30	72	2	29.70	21.046
	Borehole	n=14	12	0	2.60	4.033
	Sachet	n=18	17	0	5.00	6.423
	Tap	n=20	31	0	8.50	10.947

4.4 Prevalence of *Salmonella* Species from Drinking Water in the Study Area

Figure 4.1 shows the prevalence of *Salmonella* species in the various water samples. It was observed that out of 90 well water samples analysed, 2 (2.22%) were positive for *Salmonella* species. There were no positive samples among any of borehole, sachet, and tap water. One positive sample each was from Kaduna and Katsina States. None was isolated from Sokoto State in this study. However, 100% of well water in the study had total coliform counts (TCC) and faecal coliform counts (FCC) in excess of the maximum allowable limit of 10.0 cfu/100 ml for TCC and 0.0 cfu/ml for FCC. For borehole water samples, 71.4% and 14.3% had TCC and FCC respectively above standard limits.

4.5 Antibiotic Susceptibility of *Salmonella* Species Obtained in the Study

The result of antibiotic susceptibility of *Salmonella* isolates from stool samples is presented in Table 4.9. The organisms showed 100% resistance to the β-lactams: Ampicilin, Amoxycillin and 93.2% resistance to Amoxicillin-clavulanic acid. The isolates were 100% susceptible to the aminoglycoside - Gentamicin and 87.5% susceptible to Ciprofloxacin. A high sensitivity of 70.8% to Chloramphenicol was also observed.

Table 4.10 shows the antibiotic susceptibility of the two *Salmonella* isolates from well water. All two isolates were resistant to Ampicillin and Amoxicillin, one was susceptible to Amoxicillin clavulanic acid and one showed reduced susceptibility. The two isolates were however, 100% susceptible to Gentamicin, Ciprofloxacin, Nalidixic acid, Chloramphenicol, Tetracycline, Sulphamethoxazole Trimethoprim. and Cefotaxime.

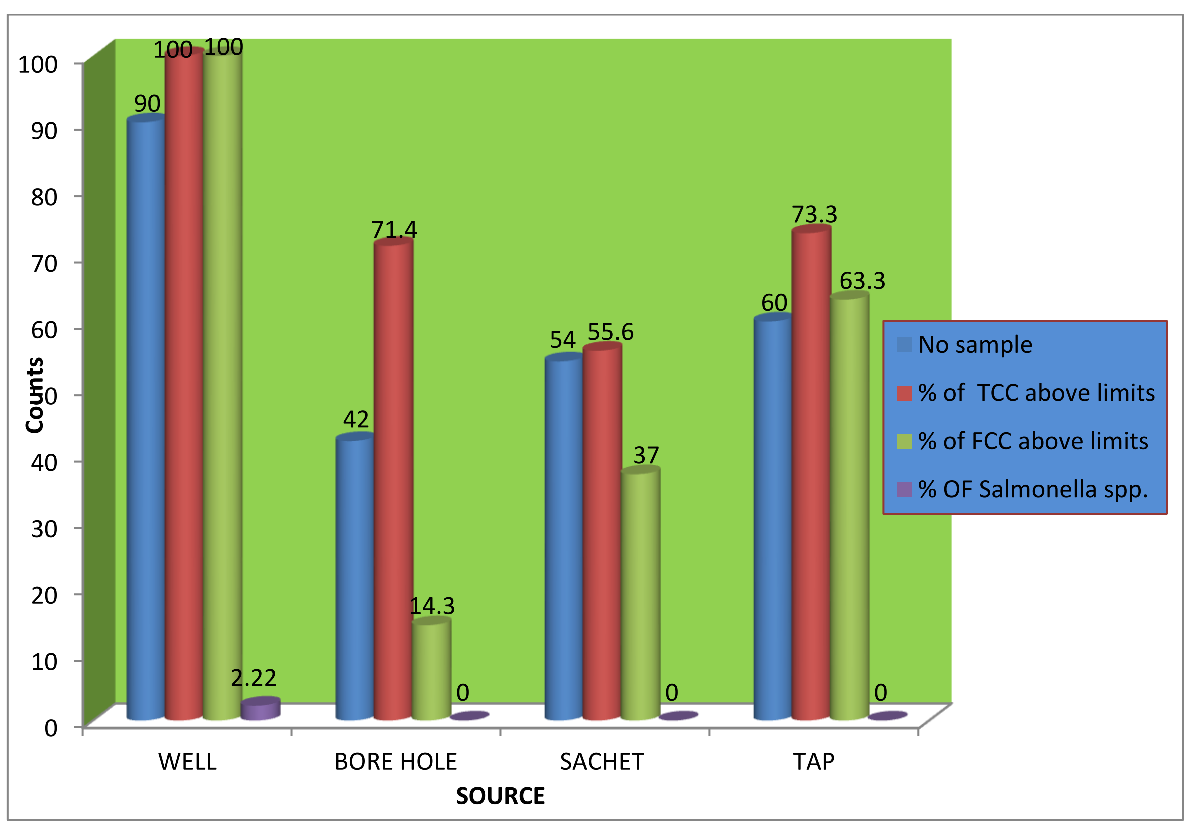

Figure 4.1: Prevalence of *Salmonella* spp. and the proportion of water samples with TCC and FCC higher than maximum allowable limits.

Key: TCC - Total coliform count

FCC - Faecal coliform count

Table 4.9: Antibiotic susceptibility of *Salmonella* species isolated from diarrhoeic stools

Antibiotic (Concentration)	Susceptibility of isolates to antibiotics (n =27)		
	Susceptible(%)	Intermediate(%)	Resistant(%)
Ampicilin (10µg)	0(0.0)	0(0.0)	27(100)
Amoxycillin (10µg)	0(0.0)	0(0.0)	27(100)
Amoxicillin Clavulanic acid(30µg)	0(0.0)	0(0.0)	27(100)
Gentamicin (30µg)	27(100)	0(0.0)	0(0.0)
Sulphamethoxazole Trimethoprim (30µg)	19(72.4)	0(0.0)	8(27.6)
Chloramphenicol(30µg)	20(75.9)	2(6.9)	5(17.2)
Cefotaxime (10µg)	3(17.2)	9(31.0)	15(51.7)
Tetracycline(30µg)	13(51.7)	0(0.0)	14(48.3)
Ciprofloxacin(5µg)	24(89.7)	1(3.4)	2(6.9)
Nalidixic acid(30µg)	19(72.4)	4(13.8)	4(13.8)

Table 4.10: Antibiotic susceptibility of *Salmonella* species isolated from water

Antibiotic (Concentration)	Susceptibility of isolates to antibiotics (n =2)		
	Susceptible(%)	**Intermediate(%)**	**Resistant(%)**
Ampicilin (10µg)	0(0.0)	0(0.0)	2(100)
Amoxycillin (10µg)	0(0.0)	0(0.0)	2(100)
Amoxicillin Clavulanic acid(30µg)	1(50)	1(50)	0(0.0)
Gentamicin (30µg)	2(100)	0(0.0)	0(0.0)
Sulphamethoxazole Trimethoprim (30µg)	2(100)	0(0.0)	0(0.0)
Chloramphenicol(30µg)	2(100)	0(0.0)	0(0.0)
Cefotaxime (10µg)	2(100)	0(0.0)	0(0.0)
Tetracycline(30µg)	2(100)	0(0.0)	0(0.0)
Ciprofloxacin(5µg)	2(100)	0(0.0)	0(0.0)
Nalidixic acid(30µg)	2(100)	0(0.0)	0(0.0)

The antibiotic resistance patterns of *Salmonella* isolates is shown in Table 4.11. Four isolates showed resistance to three antibiotics and one isolate showed resistance to 9 antibiotics. The two isolates from water were resistant to two antibiotics each. Various resistance patterns were exhibited by the isolates.

4.6 Occurrence of Antibiotic Resistance Genes among Isolates

The result of PCR detection of antibiotic resistance genes is presented in Table 4.12. Out of 23 isolates screened, 10(43.5%), were found to harbour *bla*TEM1 gene, 7(30.4%) were carrying *cat*1b gene and 2(8.7%) had *aad*A2 gene.

Table 4.13 shows the occurrence of multiple resistance among *Salmonella* isolates in relation to the presence of antibiotic resistance genes. Three isolates with multiple antibiotic resistance carried *bla*TEM1 gene, 1 isolate carried cat1b gene, 4 isolates harboured both *bla*TEM1 gene and *cat*1b gene, 1 isolate had *cat*1b gene and *aad*A2 genes while another isolate harbour the three genes.

Plate I is a gel picture showing the detection of *bla*TEM1. The gene amplified at 643bp. Ten isolates were positive as shown on the gel picture (lanes 1,2,13,15,16,17,18,19,20 and 24).

Plate II is the gel picture showing amplicons for phenicol resistance gene *cat*1b. Lanes 15,16,17,18,19, and 23 showed positive amplicons 508bp in size.

Two isolates were positive for aminoglycoside resistance gene *aad*A2 (380bp). The result is shown on plate III (lane 15 and 23).

Table 4.11: Antibiotic resistance pattern of *Salmonella* species isolated from diarrhoeic stools and water samples

S/N	Resistance pattern	Number of isolates
1	AMP, AML, CTX	1
2	AMP, AML, AMC	3
3	AMP, AML, AMC, CTX	8
4	AMP, AML, AMC, TET	1
5	AMP, AML, AMC, CHL, TET	1
6	AMP, AML, AMC, CTX, CHL,NAL	1
7	AMP, AML, AMC, TET, SXT	3
8	AMP, AML, AMC, TET, SXT, NAL	1
9	AMP, AML, AMC, TET, SXT, CTX, CHL	2
10	AMP, AML, AMC, TET, NAL, CTX, CHL	2
11	AMP, AML, AMC, TET, SXT, NAL, CHL	1
12	AMP, AMC, AML, CTX, TET	1
13	AMP, AML, AMC, CTX, TET, SXT, CHL, NAL, CIP	1
Isolates from water		
14	AMP, AML	2

Key: AMP- Ampicilin, AML- Amoxycillin, AMC- Amoxicillin Clavulanic acid, SXT- Sulphamethoxazole Trimethoprim, CHL- Chloramphenicol, CTX- Cefotaxime, TET- Tetracycline, CIP- Ciprofloxacin, NAL- Nalidixic acid.

Table 4.12: Occurrence of antibiotic resistance genes among isolates

Resistance gene	No. of isolates screened	No. positive (%)
bla TEM1	23	10 (43.5)
*cat1*b	23	7 (30.4)
*aad*A2	23	2 (8.7)

Table 4.13: Antibiotic resistance patterns in relation to presence of antibiotic resistance genes

Isolate designation (gel lane)	Resistance pattern	*bla* TEM1	*cat*1b	*aad*A2
1	AMP, AMC, CTX, AML	+	-	-
2	AMP, AMC, CTX, AML	+	-	-
8	AMP, AMC, TET, AML	-	+	-
15	AMP, AMC, CTX, AML, NAL, CHL	+	+	+
16	AMP, AMC, TET, AML, NAL, CHL	+	+	-
17	AMP, AMC, CTX, AML, TET, SXT, CHL	+	+	-
18	AMP, AMC, SXT, AML, TET, CHL	+	+	-
19	AMP, AMC, CTX, AML	+	+	-
20	AMP, AMC, CTX, AML	+	-	-
23	AMP, AMC, CTX, AML	-	+	+
Total(%)		8	7	2

Key: AMP- Ampicilin, AML- Amoxycillin, AMC- Amoxicillin Clavulanic acid, SXT- Sulphamethoxazole Trimethoprim, CHL- Chloramphenicol, CTX- Cefotaxime, TET- Tetracycline, NAL- Nalidixic acid.

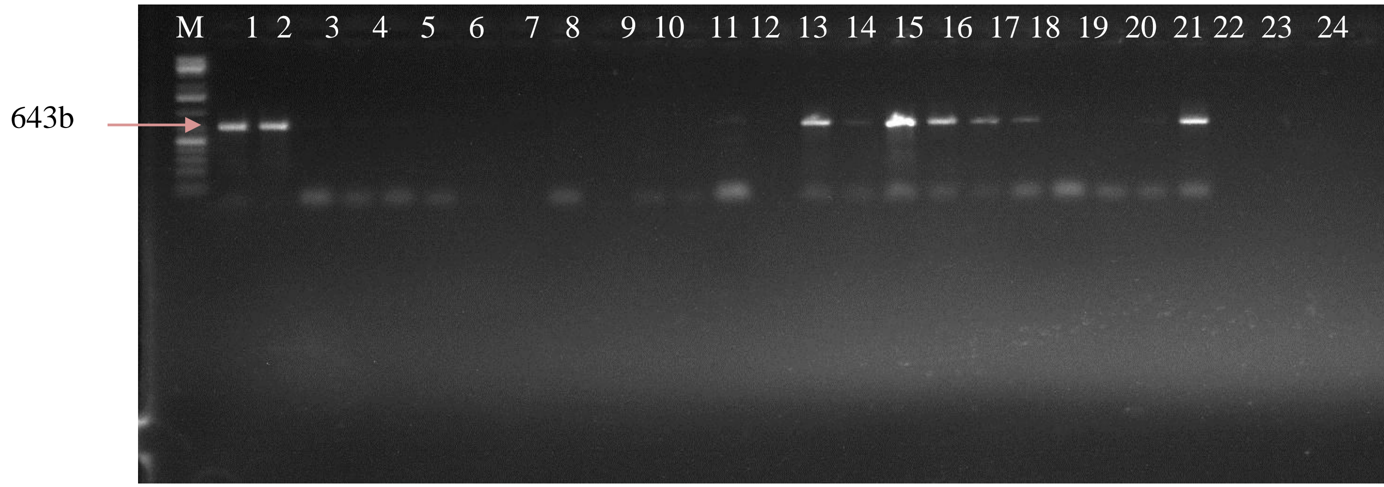

Plate I: *bla*TEM1 gene among the *Salmonella* isolates. Lane M: Molecular marker(1kb), Lanes 1-11 and 13-24, are DNA from isolates; Lane 12 is negative control.

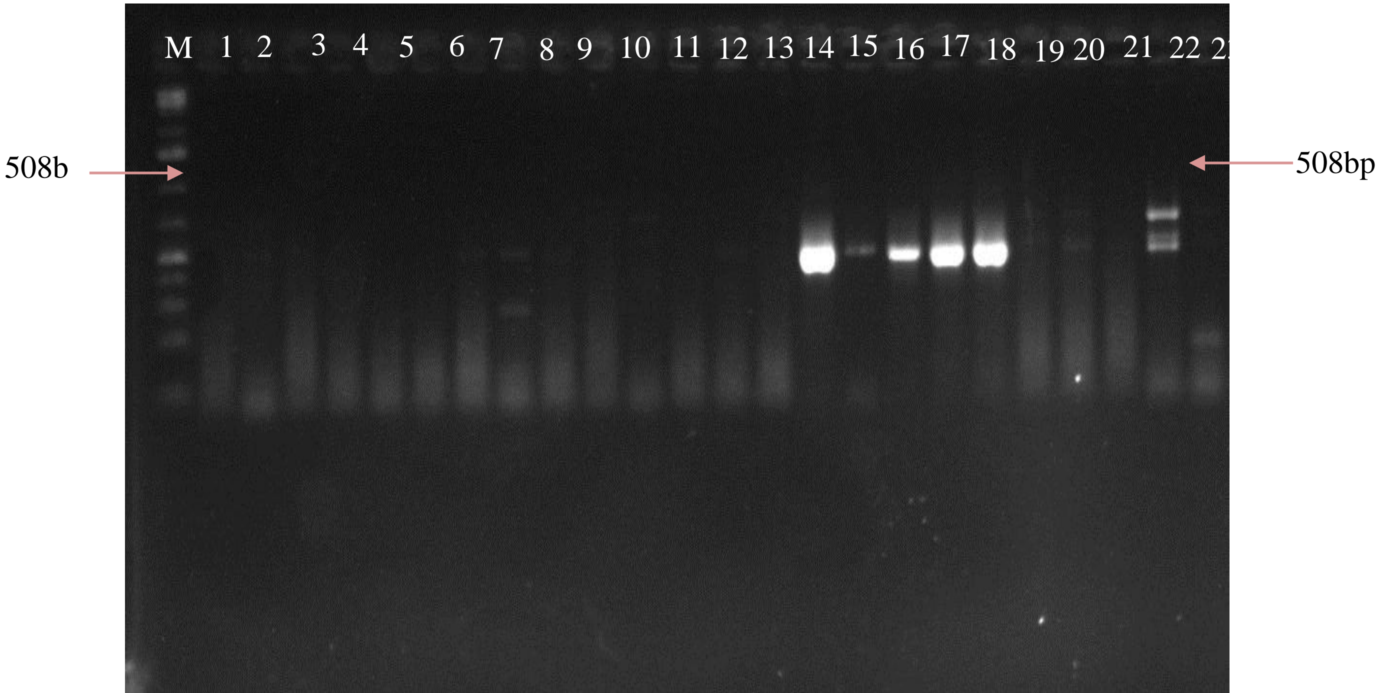

Plate II: *cat*1b gene among the *Salmonella* isolates. Lane M: Molecular marker (1kb), Lanes 1-11 and 13-24, are DNA from isolates; Lane 12: negative control.

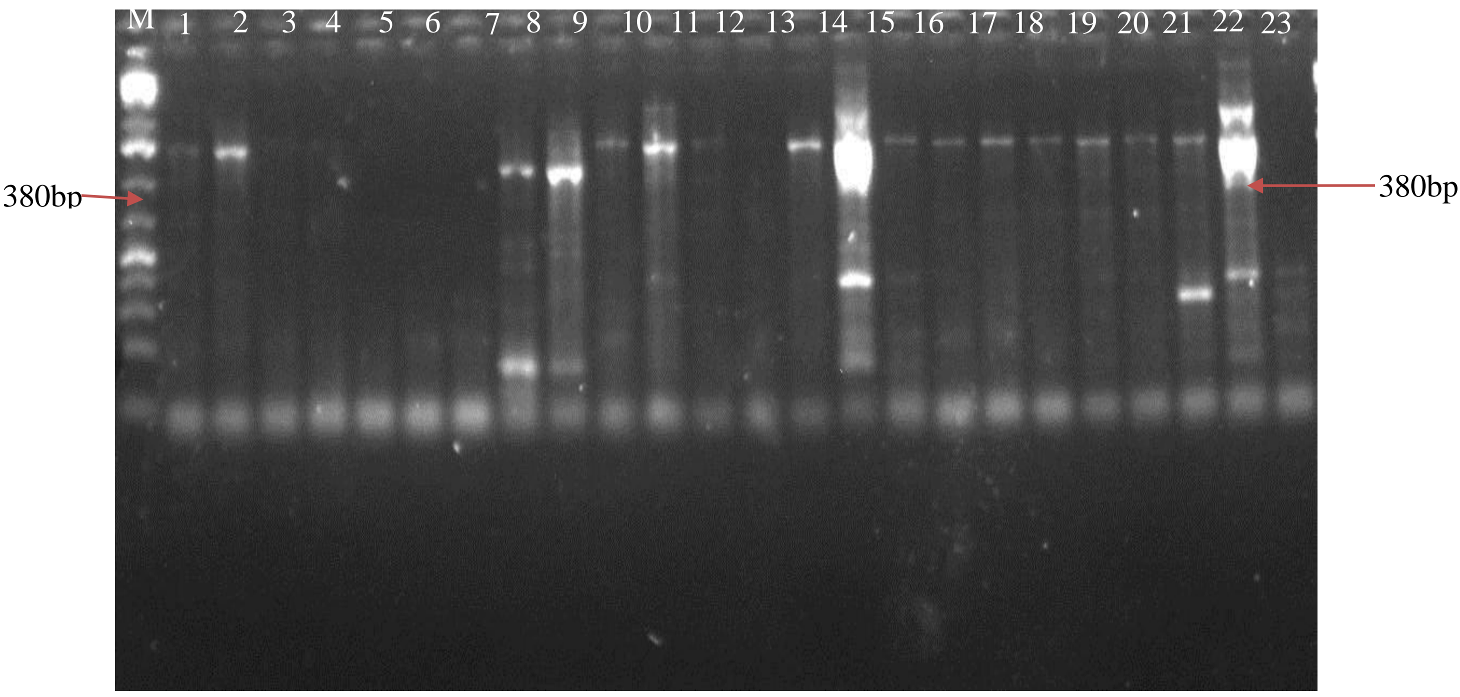

Plate III: *aad*A2 gene among the *Salmonella* isolates. Lane M: Molecular marker (1kb), Lanes 1-11 and 13-24, are DNA from isolates; Lane 12 is negative control.

Analysis for *class1 integron* revealed that 17 isolates (lanes 2,4,6 and 10-23) harboured the *class 1 integron* (750bp), while lanes 8 and 9 showed positive amplicons for the gene cassette 650bp in size (Plate IV). The base size of *class 1 integron* is known to vary between 650bp-750bp, both variants were observed among the *Salmonella* isolates in this study.

4.7 Multiple Antibiotic Resistance Pattern of *Integron* Positive *Salmonella* Isolates

Table 4.14 shows the multiple antibiotic resistance patterns of *Salmonella* isolates that habouring integron 1 gene. A total of 18 (72%; see plate IV) isolates with multiple antibiotic resistance were positive for integron 1. Six (6) of them were resistant to 4 antibiotics, 3 were resistant to 5 antibiotics, and 9 were resistant to at least 6 antibiotics.

4.8 Closest Affiliations of 16S rRNA Sequences Obtained from Isolates of *Salmonella*

The BLAST analysis of the 16S rRNA sequences obtained from the isolates are as shown in Table 4.15. Of the eighteen sequences analysed, eleven were closely related to *Salmonella enterica* subspecies enterica serovar Typhi strain CT18, while seven were affiliated to *Salmonella enterica* subspecies enterica serovar Typhimurium strain LT2. The percentage identity for all the 16S sequences ranged between 92-99%, except for isolate 15(*Salmonella enterica* subspecies enterica serovar Typhimurium strain LT2), which had the percentage identity of 86%. The E-values were also zero in all the isolates except isolate 15(*Salmonella enterica* subspecies enterica serovar Typhimurium strain LT2), which had an E-value of 2e-119.

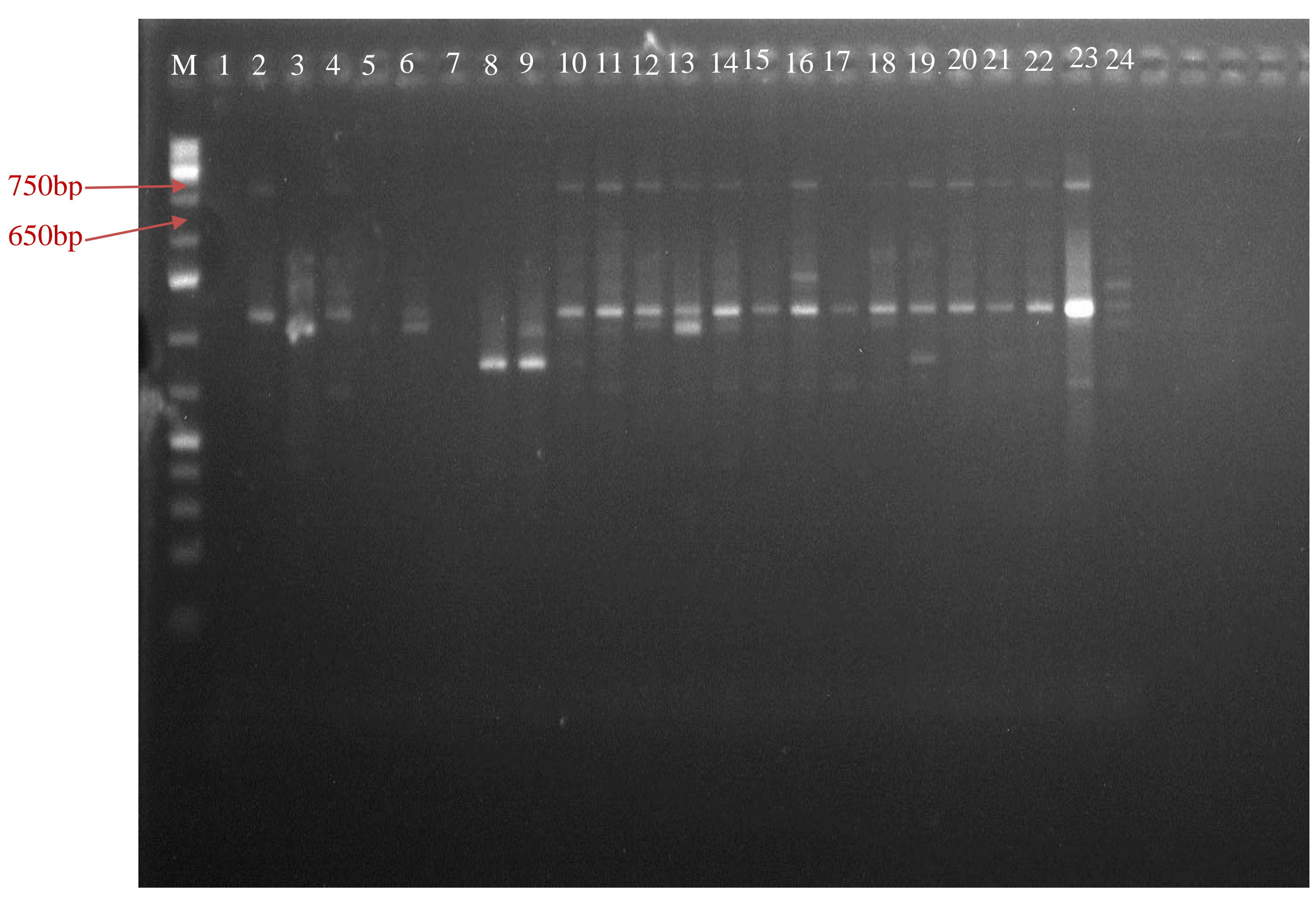

Plate IV: Class 1 *integron* among the *Salmonella* isolates. Lane M: Molecular marker (1kb), Lanes 2-24, are DNA from isolates; Lane 1: negative control.

Table 4.14: Multiple antibiotic resistance pattern of Integron positive *Salmonella* isolates

Isolate designation (gel lane)	Pattern of antibiotic resistance
2	AMP, AMC, CTX, AML
3	AMP, AMC, CTX, AML
4	AMP, AMC, CTX, TET, NAL, CHL, AML
8	AMP, AMC, TET, SXT, AML
9	AMP, AMC, TET, AML
10	AMP, AMC, CTX, TET, SXT, CHL, AML
11	AMP, AMC, TET, SXT, AML
12	AMP, AMC, CTX, NAL, CHL, AML
13	AMP, AMC, CTX, TET, CIP, NAL, SXT, CHL, AML
14	AMP, AMC, CTX, TET, SXT, CHL, AML
15	AMP, AMC, CTX, AML, NAL, CHL
16	AMP, AMC, TET, AML, NAL, CHL
18	AMP, AMC, SXT, AML, TET, CHL
19	AMP, AMC, CTX, AML
20	AMP, AMC, CTX, AML
21	AMP, AMC, CTX, TET, NAL, CHL, AML
22	AMP, AMC, TET, SXT, AML
23	AMP, AMC, CTX, AML

Key: AMP- Ampicilin, AML- Amoxycillin, AMC- Amoxicillin Clavulanic acid, SXT- Sulphamethoxazole Trimethoprim, CHL- Chloramphenicol, CTX- Cefotaxime, TET- Tetracycline, CIP- Ciprofloxacin, NAL- Nalidixic acid

Table 4.15: Closest affiliations of 16S rRNA sequences obtained from *Salmonella* isolates

S/N	Isolate designation	Description	E-Value	% ID	Accession Number.
1	1	*Salmonella ent.* subsp. ent. ser. Typhimurium str. LT.2	0.0	99	NC003197.1
2	2	*Salmonella ent.* subsp. ent. ser. Typhimurium str. LT.2	0.0	98	NC003197.1
3	6	*Salmonella ent.* subsp. ent. ser.Typhimurium str. LT.2	0.0	92	NC003197.1
4	12	*Salmonella ent.* subsp. ent. ser. Typhi str. CT18	0.0	99	NC003198.1
5	13	*Salmonella ent.* subsp. ent. ser.Typhi str. CT18	0.0	99	NC003198.1
6	14	*Salmonella ent.* subsp. ent. ser. Typhi str. CT18	0.0	98	NC003198.1
7	15	*Salmonella ent.* subsp. ent. ser. Typhimurium str. LT.2	2e-119	86	NC003197.1
8	16	*Salmonella ent.* subsp. ent. ser. Typhi str. CT18	0.0	98	NC003198.1
9	19	*Salmonella ent.* subsp. ent. ser. Typhimurium str. LT.2	0.0	96	NC003197.1
10	20	*Salmonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	98	NC003198.1
11	21	*Salmonella* ent. subsp. ent. ser.Typhi str. CT18	0.0	99	NC003198.1
12	23	*Salmonella* ent. subsp. ent. ser. Typhimurium str. LT.2	0.0	99	NC003197.1
13	24	*Salmonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	97	NC003198.1
14	25	*Salmonella* ent. subsp. ent. ser. Typhimurium str. LT.2	0.0	99	NC003197.1
15	26	*Salmonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	98	NC003198.1
16	27	*Salmonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	98	NC003198.1
17	29	*Salmonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	99	NC003198.1
18	30	S*almonella* ent. subsp. ent. ser. Typhi str. CT18	0.0	99	NC003198.1

4.9 Phylogenetic Relationship Between the Isolates of *Salmonella* Obtained from Water and Diarrhoeic Stools

Figure 4.2 is a phylogenetic tree constructed from the sequenced 16S rRNA gene of the *Salmonella* isolates. The evolutionary history was inferred by using the Maximum Likelihood method based on the Tamura-Nei model (Tamura and Nei, 1993). The bootstrap consensus tree inferred from 2000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (2000 replicates) are shown above the branches (Felsenstein, 1985). Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using the Maximum Composite Likelihood (MCL) approach, and then selecting the topology with superior log likelihood value. The analysis involved 18 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 421 positions in the final dataset. Evolutionary analyses were conducted in MEGA7 (Kumar *et al*., 2015).

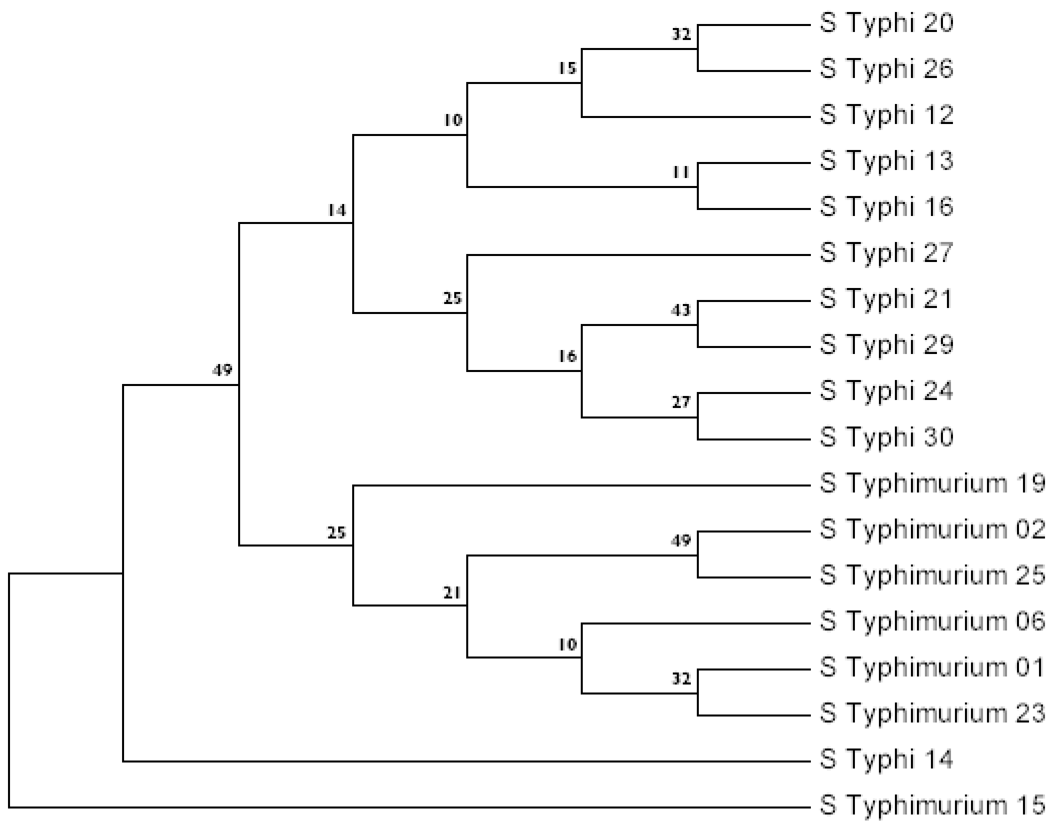

Figure 4.2: Molecular phylogenetic analysis by Maximum Likelihood method of *Salmonella* isolates.

CHAPTER FIVE

DISCUSSION

Acute diarrhoea due to bacterial infections is an important cause of morbidity and mortality in infants and young children in most developing countries including Nigeria (Adegunloye, 2006). Diarrhoea can result from infections with many aetiological agents, but mainly by members of the family *Enterobacteriaceae* such as *Escherichia coli, Salmonella* spp., *Shigella* spp., *Campylobacter jejuni* and *Vibrio cholerae*. Parasites such as *Entamoeba histolytica* and *Giardia lamblia*, and rotaviruses are also important agents. Identification of the enteropathogens involved in diarrhoeal diseases in the country is an essential step towards the implementation of effective primary health care activities against the disease (Olowe *et al.,* 2003).

Among the enterobacteria causing diarrhoeal diseases, *Salmonella* spp. continues to be the major public health problem. Although most *Salmonella* infections are self-limiting, serious sequelae, including systemic infections and deaths, can occur. Additionally, various *Salmonella* spp. resistant to the commonly available drugs have been reported with increasing frequency throughout the world (Parry *et al*., 2003).

In this study, a prevalence of 4.1% was observed for *Salmonella* species among 634 children with diarrhoea. This low prevalence reflect the fact that diarrhoeal disease in children has multiple aetiologies ranging from viruses, parasites and other bacteria agents. These have been reported with varying prevalence around the world including Nigeria. Ogbu *et al.* (2007) reported the occurrence of Rotavirus (23.3%), *E. coli* (15.4%),

Salmonella species (11.3%), *Klebsiella* species (7.3%) and *Enterobacter* species (9.6%) as the most predominant aetiological agents of children diarrhoea in Abakaliki south-east Nigeria. *Shigella, Yersinia enterocolitica*, *Entamoeba histolytica* and *Giadia lamblia* were also recovered in that study. Similarly, Okolo *et al.* (2013) reported the occurrence of *E.coli, Salmonella, Campylobacter* and *Shigella* species among children in Anyigba North Central Nigeria. Ifeanyi *et al*. (2010) observed *E.coli, Salmonella* Typhi, *Klebsiella pneumoniae*, *Staphylococcus aureus* among others; and Akingbade *et al.* (2013) isolated *E.coli, Salmonella* and *Shigella*. However, Korie *et al*. (2012) observed only Enteropathogenic *E.coli* from their report in Enugu.

There was no case observed among the 60 control subjects included in the study. This indicate a low carrier rate of the agent in children of this age (≤5years) probably due to their lower immune status and virulence of *Salmonella* species, children who get infected usually come down with the disease. However, carrier cases have been reported among primary school pupils in a study by Adegunloye (2006) in Akure.

The prevalence of *Salmonella* species in the study subjects agrees with the reports of similar studies in Lagos where Ogunsanya *et al.* (1994) reported a *Salmonella* prevalence of 3.3%, Bangladesh (Albert *et al*., 1999), Korea (Seung-Hak *et al.,* 2006), and Ghana (Reither *et al.,* 2007). However, the prevalence rate observed in this study (4.1%) is higher than that obtained by Ifeanyi *et al.* (2010) who reported a prevalence of 2.2% from Abuja, Nigeria. This could be due to the differences in infrastructural development, socioeconomic status and educational level of people living in the area of study (Abuja). This report however, shows lower prevalence than that reported by Ogbu *et al.* (2007), who reported a prevalence of 11.3% in Abakaliki, South-Eastern Nigeria, Duru *et al.* (2014) reported

10.0% prevalence Port-Harcourt, South-South Nigeria, Ike and Damola (2014) obtained 10.7% in their study in Lagos, South-Western Nigeria. The disparity may be attributable to differences in study design, patients' selection, differing environmental conditions, socio-cultural and behavioral patterns of people in those regions.

A comparison of occurrence of *Salmonella* species in the study subjects among states showed no statistically significant difference. This is probably due to the similarity in the socio-cultural behaviour and infrastructural development of the area. Children are therefore, exposed in most cases to similar risk factors associated with infection across the states under study.

The result from this study showed that the majority of *Salmonella* species were detected among children of between the ages 25-36 months. This could be due to combined effects of declining levels of maternally acquired antibodies, the lack of active immunity in the infant, the introduction of foods that may be contaminated with faecal bacteria and direct contact with human or animals faeces when the infant starts to grow. This age bracket also represent a very active stage in the growth and development of a child. It may also be said to correspond to the period when the child's contact with the environmental pathogens increases dramatically (Duru *et al.,* 2014), typically due to ignorance on the rudiments of aseptic or hygienic practices (Sule *et al.,* 2011). All these, including the high virulence of *Salmonella* and its low infective dose make exposed children more vulnerable to infections. Detection of *Salmonella* diminished in the age bracket: 37-48 months (6.8%) and 49-60 months (0.0%). This may signify both an improvement in the immune status of the children and their eating habits. Most enteric pathogens stimulate at least partial immunity against

repeated infection or illness, which helps to explain the declining incidence of disease in older children and adults (Patwari *et al.,* 1993). Low prevalence in the 0-12 months age group could be due to breast feeding. Antibodies in breast milk protect them from infectious agents. Bacteria like the *Salmonella* species, are associated with dairy products, faecally contaminated food or water, hence proper hygiene and sanitation must be practiced to reduce infection by these pathogens. Similar trend in age related prevalence was observed in the reports of Abdullahi *et al.* (2010), Ifeanyi *et al.* (2010) and Okolo *et al.* (2013).

Male children were more infected than their female counterparts, probably due to their more active nature compared to the females. In addition female children within the study area are more protected than the males, this could also increase the chances of infection in the male children, although the difference is not statistically significant ($\chi^2 = 1.352$ and $P > 0.05$). This finding is similar to the works of Ngozi and Onyenekwe (2003); al-Jurayyan (2004); Adkins and Santiago (2006) and Abdullahi *et al.* (2010).

There was no statistical association of the infection with the educational status of mothers or caregivers in this study. Positive cases were found among children whose caregivers were both literate and semi-literate. This suggests that other factors such as source of water, age of children and sanitary conditions of the environment may have played greater role in the infection with *Salmonella* species.

The physical appearance of the stool samples was used to categorize diarrhoea samples into watery, loose, mucoid, and bloody diarrhoeic stools. This categorization is necessary as different sample appearance is associated with different causative agents. However, the appearance must be differentiated from normal liquid sample from exclusively breast fed infants who may pass several soft, semi liquid stools each day. For such, it is practical to define diarrhoea as an increase in stool frequency or liquidity that is considered abnormal by the mother. Bacterial aetiologies of watery diarrhoea may be *Escherichia coli, Shigella, Campylobacter jejuni, Salmonella, Vibrio cholerae*. *Shigella* is the most important cause of diarrhoeic stools with bloody flecks and mucus. In this study, watery stools were predominant (n = 255; 40.2%), followed by those with loose stool (n = 176; 27.8%) and mucoid appearance (n = 162; 25.6%). There were 41(6.4%) diarrhoeic stools with blood. Watery stool was the most associated with *Salmonella* infection with a prevalence of 5.9%, which correlates with the nature or appearance of stool sample and the aetiologic bacterial agent. However, it was not statistically significant.

There was a statistically significant association between the source of water and occurrence of positive culture samples, where a prevalence of 6.5% was observed among those who used well water as a major source of water. This is particularly significant considering the fact that majority of the study subjects were from households whose main source of drinking water is the well. Wells that are not properly constructed or left open are prone to contamination from run-off and other human activities and therefore, require treatment prior to use.

The significance of water treatment was further shown in this study, with a higher *Salmonella* infection among patients who did not treat water prior to use than those who treated their water, though there was no statistically significant association. Patients from household that used borehole surprisingly showed a high infection rate (3.4%). This could be due to poor maintenance of boreholes which often breakdown forcing people to seek alternative sources of water which are mostly wells or even reservoirs like dams. Also, transmission of the disease is not limited to water contamination, food and animal products are other sources of infection, as well as carriership of the agent among household members. No household in the study area may claim exclusive use of only one source of water.

A high prevalence of *Salmonella* species was also observed among children whose households had no toilet facilities than those that used pit latrines or water closet, however, there was no statistically significant association. Open defaecation may increase the risk of environmental contamination when such faecal material flows into nearby water bodies.

Administration of antibiotics or other herbal remedies could affect the isolation rate of pathogens. The low isolation of *Salmonella* in this study could be due to the use of antibiotics prior to visiting health institutions. It has been found that recent antibiotic use reduces culture yields by 62 to 73% in patients with severe or fatal disease (Berkley *et al*., 2005). In this study 38.6% of the patients had been treated either with antibiotics or herbs, preceding to presentation at the health institutions. Sensitivity specimen used for isolation of *Salmonella* could also affect the isolation rate as bone marrow and blood are known to yield better isolation rate than stool.

It is significant to note that most of the mothers or caregivers in the study had different understanding about the aetiology of diarrhoea. Some thought that diarrhoea was caused by teething, sugary food, bad water, or is part of normal development of the child. This type of knowledge lays a foundation for either wrong intervention or non-intervention by the mothers when their children develop diarrhoea with sometimes disastrous consequences. This understanding gives rise to higher mortality and morbidity rates in any community since the necessary early and accurate intervention for the proper management of childhood diarrhoea is not applied. For instance only 27.1% of the mothers used or had knowledge about the use of ORS (SSS/ORT) for the management of their children who had diarrhoea at home. The reason for this low level of knowledge of the use of ORS as shown by this study is most likely to be a drop in the level of education and awareness of the mothers on diarrhoeal management due to poor public enlightenment on this topic. Over two decades since the introduction of ORT in Nigeria and some other African countries, the level of awareness may be on the decline due to the lack of consistency in maternal education on this issue (Adimora *et al.,* 2011).

The presence of total coliform and faecal coliform in water is a clear indication of groundwater contamination by human or animal faeces. Faecal contamination of groundwater is responsible for most water borne diseases such as cholera, typhoid and diarrhoea (Amadi, 2009; Egharevba *et al.,* 2010). Poor sanitary situation of an area such as close proximity of unlined soakaway/pit-latrine can introduce total coliform and faecal coliform into the shallow aquifer via infiltration.

In this study it was observed that the total coliform and faecal coliform counts had concentrations in most locations above the recommended maximum permissible limits of 10 cfu/100ml for total coliform counts and 0 cfu/100ml for faecal coliform counts as indicated by WHO (2006) and NSDWQ (2007). Well water samples had the highest level of contamination. This contamination may be due to several factors such as the sanitary habit of well owners and users. Uncovered wells also stand the risk of contamination in this way. In most cases, practices such as washing of clothes, household utensils, various materials and objects are done close to the wells. When the washed dirt is poured on the ground, close to the well it becomes unsanitary. Shallow wells are susceptible to contamination by surface and soil microorganisms (Ijostem *et al.,* 1997).

Pollution of water from wells may also be attributed to the deliberate discharge of sewage effluents around the well, while some wells may have been contaminated from its inception by the uncleanness of the diggers. High coliform number in wells in this study could also be as a result of the presence of domestic animals and lizards which are usually found near the wells as coliforms have been isolated from their intestines. Lizards are known to introduce faecal coliform into well water when such wells are uncovered, and at the level with the soil surface. (Ijostem *et al*., 1997).

Other factors affecting contamination of wells may be structural defects of the well which may allow seepage of pollutants from nearby sewage into wells (Ciravolo *et al*., 1979). Where wastewater can drain down through macropores such as root channels, rodent burrows and structural voids, the ground water may become significantly polluted with faecal coliforms and streptococci (Allen, 1979; Amadi *et al*., 2014).

In Nigeria majority of users are illiterates who may not be aware of the hazards or risks of unhygienic well environment. In many instances, household sewage is not properly disposed, some sewage pits close to the well contribute to pollution of well water if the effluent flow to wells. It is not uncommon to also find wells close to septic tanks. The location of septic tanks close to wells could also result in leakages or seepage of faecal material into the wells resulting in contamination of the ground water. This was clearly illustrated in a study done in Palm bay Florida, USA which examined the seasonal correlation of well contamination and septic tank distance, and found a significant correlation between increasing coliform and decreasing the distance between wells and septic tanks (Arnade, 1990). Containers used to draw water from wells constitute another source of danger to health as this maybe dirty and improperly kept after use (Tukur and Amadi, 2014).

Lack of enforcement of the regulations guiding the digging of wells and or drilling of boreholes in Nigeria by the relevant agencies also contribute to the menace of water contamination. Wells are not properly planned before drilling and are often located near unlined septic tanks or pit latrines with majority not properly covered. In the rural communities in Nigeria, hygiene is least considered when designing a well, since the primary objective is to supply water for drinking, washing, cooking and construction. Wells therefore stand the risk of microbial and faecal contamination (Tukur and Amadi, 2014). The isolation of *Salmonella* species from two well water samples indicate the potential health risk associated with drinking well water. However, Garba *et al.* (2007) found a higher prevalence for *Salmonella* in Zamfara State. The difference in prevalence

could be due to differences in the study design as wells located near sewage effluents were targeted.

The results of total coliform counts and faecal coliform counts in this study agrees with findings by Garba *et al.* (2007). High coliform counts were also reported by Idowu *et al.* (2011) from Shagamu; Yabaya and Aliyu (2012) from Kaduna, Tukur and Amadi (2014) from Katsina. Aboh *et al.* (2015) reported that all 10 well water samples analysed from Samaru, Zaria, had 100% total coliform counts in excess of 180cfu/100ml.

The result of coliform count in sachet water showed that 55.6% had total coliform counts above the required standard limits for drinking water both by WHO and the Nigerian Standard for Drinking Water Quality, and 37.0% contained faecal coliforms as against the standard for drinking water.

In water packaging, the most significant factor responsible for contamination is non adherence of manufacturers to Good Manufacturing Practice (GMP). GMP is defined as part of quality assurance which ensures that products are consistently produced and controlled to the quality standards appropriate to their intended use and as required by product specifications (NAFDAC, 2001). GMP guidelines are used to eliminate the risks inherent in any production that cannot be prevented completely from contamination through the testing of final products. Under GMP, quality is built into a product and not just tested for in the finished product (NAFDAC, 2001). Other factors that could be responsible for the contamination observed in the sachet water include poor state of the manufacturing environment, dirty filling equipment, contaminated packaging materials, unhygienic handling of the products and lack of microbiological in-house controls.

The failure of the various tiers of government to provide clean, clear and potable water for the populace has led to the commercial sachet water producers to fill the vacuum. This study established that a significant proportion of water being sold as sachet or 'pure' water are not potable. Similar reports were published by Mendie (2002), who found contamination of 5-200 coliforms/100ml from 10 producers. Oni and Olayeni (2003) reported 90% faecal coliform contamination in Lagos and 50% contamination in Kaduna. Garba *et al.* (2007) reported similar contamination levels from Zamfara State.

Water samples from borehole showed relatively low bacterial counts, less than any of the other sources of water. This may be due to the enclosed nature of boreholes which leaves little or no room for extrinsic contamination. Their location, mostly in open areas, a distance away from domestic refuse sites, septic tanks and pit latrines also play a part in preventing contamination due to seepage. Water is usually obtained from deep aquifers where seepage from land surface may not easily reach, and since no distribution channels are mostly involved in its operation, chances of contamination along broken pipes are also eliminated. However, these factors are also limited in certain situations hence the observed contamination in some of the samples. This high total coliform count observed in the water, however, does not necessarily indicate recent water contamination by faecal waste, since total coliforms includes both environmental and thermotolerant (faecal) coliforms. A study in Taraba State by Oko *et al.* (2014) to assess the water quality index of boreholes and well water also indicated that borehole water in Wukari was of better quality than well water.

A high level of contamination was observed from tap water samples analysed in this study compared to borehole water. This could be due to improper water treatment or post treatment contamination along distribution channels. In several instances water samples obtained from far distances from the treatment plant were more contaminated than those obtained at the treatment plant. This suggests that there are leakages or broken pipes along distribution channels or biofilms built up along the channels.

Overall, there was no significant difference in water quality among the three states studied. The total coliform and faecal coliform counts of well, borehole, sachet and tap water respectively followed similar trend among the states. This could be attributed to similarity in behavioral patterns and cultural background of people within the study area.

The use of antimicrobial agents in the treatment of diarrhoea cannot be overemphasized, because specific antimicrobial treatment may be required to supplement supportive anti-dehydration treatment which is the cornerstone of therapy of acute infantile diarrhoea. However, inadequate health services, inadequate drug supplies, non-adherence to treatment strategies and dubious drug quality all favour the emergence of microbial resistance (WHO, 2002). This was clearly evident from this study where over 90% resistance to three antibiotics (Ampicillin, Amoxicilin, and Amoxicilin Clavulanic acid) by *Salmonella* species was observed.

It is noteworthy that most of the diarrhoeal patients enrolled in this study first engaged in self-medication before seeking physician's attention. This act may be responsible for the large number of the multiple antibiotic resistance patterns observed among the isolates in

this study. It has become increasingly important to monitor patterns of resistance as the antibacterial susceptibility of bacterial pathogens which contribute significantly to the burden of infantile diarrhoea is declining.

The resistance of the *Salmonella* isolates to the antibiotics tested in this study calls for great concern, as it depicts a high prevalence of antibiotic resistance by *Salmonella* strains to the β-lactam class of antibiotics (Ampicillin, Amoxycillin, Amoxycillin-clavulanic acid, and Cefotaxime) which are frequently used empirically for the treatment of diarrhoea and a number of infectious diseases . However, all the strains had varying percentage susceptibility to Nalidixic acid (72.4%), Ciprofloxacin (89.7%) and Sulphamethoxazole Trimethoprim (72.4%). This means that these chemotherapeutic agents are fairly effective in the treatment of diarrhoea caused by these pathogens. The use of Ciprofloxacin in young children however, has grave risks as complications involving troubled breathing, swelling of the face, lips, tongue, mouth, or throat, irregular or uneven heartbeat, fainting or seizures can develop as side effects.

Resistance, particularly to the commonly available antibiotics poses major health concerns. This is because the most effective chemotherapeutic agents such as Ciprofloxacin, are often expensive and not readily available in most rural and urban communities. The high susceptibility of *Salmonella* to ciprofloxacin as shown in this study is probably due to the reasons mentioned, and therefore, are less abused.

Salmonella strains in this study generally showed 100% susceptibility to the aminoglycoside gentamicin, however, according Clinical Laboratory Standard Institute (CLSI), *in vitro* susceptibility of *Salmonella* and *Shigella* species to aminoglycosides may not correlate with clinical efficacy and therefore, should not be reported as susceptible (CLSI, 2014).

Resistance of *Salmonella* spp. to various antibiotics has been demonstrated in previous studies. Ehinmidu (2003); Duru *et al.* (2014) and Moses *et al.* (2014) reported a high resistance by *Salmonella* to beta-lactam antibiotics, namely; ampicillin, augmentin and ceftriazone. Olowe *et al.* (2007), similarly reported a high level resistance to amoxycillin and ampicllin in Osogbo. Ajibade (2013), reported a progressive rise in ampicillin resistance from 20% to 100% from 2009 to 2011 in Ikole Ekiti. In a related study, Ifeanyi *et al.,* 2010 reported that strains of *Salmonella species* showed substantial resistance to Amoxycillin, Amoxycilin-clavulanic acid, Cephalexin and Cefuroxime. Sule *et al.* (2012) also observed 100% resistance to augmentin, gentamicin, co-trimoxazole and ampicillin in Anyigba.

The influence of antimicrobial resistance in *Salmonella* species is quite extensive, reaching many areas. Acquisition of resistance genes adds complexity to laboratory diagnosis and complicates therapeutic outcomes. Antimicrobial resistance also affects the therapeutic regimen, leading to considerable public health concerns and substantial economic burden.

Among the 234 multiple-antibiotic-resistant *Salmonella* isolates (defined as isolates that were resistant to two or more antibacterial agents), 3 genes conferring resistance to three categories of antibiotics, including β-lactams, aminoglycosides, phenicols, were identified. The PCR results were consistent with the antibiotic susceptibility phenotypes (Table 6). The chloramphenicol acetyltransferase genes, *cat*1 was detected in the chloramphenicol-resistant *Salmonella* isolates. *bla*TEM-1, a β -lactamase gene (conferring resistance to β-lactam antibiotics was detected in 8 isolates all of which were resistant to ampicillin, amoxicillin and amoxicillin clavulanic acid. Similar findings were made by Ling *et al.* (1991) and Chen *et al.* (2004).

However, several other *Salmonella* strains with similar resistance pattern were negative for *bla*TEM-1 gene in this study, this could be due to the fact that there are several other kinds of β-lactamase genes such as *bla*CMY-2 gene, *bla*PSE-1gene or *bla*CTX genes which were not covered in this study but have been known to confer similar resistance in *Salmonella* (Chen *et al.,* 2004). In a similar study, Ajibade (2013), reported *Salmonella* strains which contained *qnrB*2, *blaCMY*-2 and *blaCMY*-23, the mechanisms for extended-spectrum cephalosporin, Aminoglycosides and Quinolone resistance respectively.

In a peculiar situation, a chloramphenicol sensitive phenotype in this study was observed to be carrying *cat*1b gene which is expected to confer resistance to chloramphenicol. This is probably a case of unexpressed gene.

Integron analysis of the 23 multiple antibiotic resistant isolates showed that 18(78.3%) of the isolates harboured *integron class1* gene. Integrons are known as natural cloning and expression vectors that mediate the integration or excision of gene cassettes (Carattoli,

2001). The multiple antibiotic resistance of the *Salmonella* isolates in this study may also be due to possession of *integrons*. It has been noted that the development of multiple resistance is based on the capacity of *integrons* to cluster the gene cassettes and to express antimicrobial resistance genes (Carattoli, 2003; Schwarz and Kadleck, 2008).

The result of sequence analysis using 16S ribosomal RNA sequence, which is a highly conserved region among organisms when compared with established sequences in the data base using the National Center for Biotechnology Information (NCBI) Basic Local Alignment Search Tool (BLAST), revealed 11 *Salmonella enterica* subspecies enterica serovar Typhi and 7 *Salmonella enterica* subspecies enterica serovar Typhimurium. These represent the most prevalent *Salmonella* serovars within the study population, as no other serovars were detected. *S.* Typhi and *S*.Typhimurium have both been reported among the study population in a previous study by Abdullahi *et al*. (2010).

The result of multiple alignment of sequences of the isolates showed a high level of homology in the sequences between isolates. A close look at the alignment shows numerous conserved regions among the strains thus pointing to their common ancestry. Significant homology has been seen among genomes of *S*. Typhi-CT18 and *S*. Typhimurium LT2 (McClelland *et al*., 2001) genomes, indicating a common evolutionary origin and sequence comparison between these two organisms revealed that 89% of *S*.Typhimurium LT2 CDSs were homologous to *S*.Typhi CT18 at the nucleotide level (McClelland *et al*., 2001, de Jong *et al.,* 2012).

The phylogenetic tree constructed from the sequences by multiple sequence alignment using maximum likelihood analysis, with a goal to assemble a phylogenetic tree representing a hypothesis about the evolutionary ancestry of strains of *Salmonella* isolates

in the study. The clustering observed on the tree may affirm the notion that *Salmonella* is a monophyletic group and are closely related with limited sequence diversity. Maximum likelihood method uses standard statistical techniques for inferring probability distributions and assign probability values to particular possible phylogenetic trees. Therefore, the probability values observed in the phylogenetic tree is an indication of reliability of the tree. Organisms considered in the study therefore are regarded as having close ancestry (Felsenstein, 1981).

To determine the consistency of the data obtained, bootstrapping was also carried out. This gives values which indicate how consistent the data from which a phylogenetic tree constructed are. In this study, values observed indicate a moderate consistency in the evolutionary relationship among the isolates.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

These research findings show that, though there are a number of causative agents of diarrhoeal diseases*,* *Salmonella* still remains one of the major and most important bacterial pathogen of diarrhoea among children in the study area with a prevalence of 4.1%. Age of children and source of drinking water play significant roles in both their exposure and susceptibility of the study subjects to *Salmonella* infections.

The present study showed contamination of all the well water (i.e 100%) samples with faecal coliform thus, making the water unsafe for human consumption and potential health risk. A good number of the other water sources were equaly contaminated with TCC and FCC in this other:borehole (74.4%; 24.3%), sachet water (55.6%; 37.0%) and tap water (73.3; 63.3%) against the maximum allowable limits for both total coliform and faecal coliform counts in drinking water

The results of antibiotic susceptibility in this study shows a high resistance rate among isolates especially to the β-lactam group of antibiotics making them completely unreliable in the management of *Salmonella* infections. Multiple antibiotic resistance was observed in over 80% of the isolates, this defines the level of significance antibiotic resistance has become to public health especially in child health.

The situation of antibiotic resistance is further worsen with many strains of the organism harbouring genetic element that provide near permanent resistance to the agents. In this study a few antibiotic resistant genes were observed in the genomic DNA of the isolates

meaning that resistance to antibiotics is not only horizontally acquired but is also inherent in many of the strains of the organism.

Phylogenetic studies of the organisms indicate a high probability of common ancestry of the organisms.

6.2 Recommendations

1. Diarrhoeal diseases among children can be minimized by observing strict personal hygiene, quality of drinking water, quick isolation and treatment of infected cases as well as encouragement of breast feeding of infants.
2. Government should endeavour to provide adequate potable water to the community. Improving the sanitary awareness through basic health education, careful surveillance, monitoring incidence and spread of diarrhoeal diseases, may help to reduce the disease burden in children.
3. Appropriate interventions such as hand washing with soap, household water treatment and safe storage, avoidance of community-wide open defaecation and oral rehydration therapy given to children by mothers must be taught to reduce the debilitating effect of diarrhoeal disease .
4. As a public health measure to reduce the disease burden, an integrated package of immunization services and other childcare programmes need to be implemented in addition to well focused health education messages to improve treatment–seeking behaviour for childhood diarrhoea as well as improved personal and environmental hygiene.

5. It is recommendable that wells should be well constructed. The distance between wells and possible source of contamination should be considered when locating site for well construction.
6. Periodic examination of wells to assess their quality and safety or otherwise of the water. Government regulatory agencies such as NAFDAC and SON should extend their periodic inspection to private water sources and if need be, enlighten the residence on control and preventive measures of contaminations.
7. Sachet water production needs supervision, control and regulation. Quality control is imperative to avoid backyard and one room producers holding sway at the expense of public health. The issuance of NAFDAC registration number for a product should only be a first step towards compliance, other factors such as periodic change of filters, quality control, sanitation and hygiene, qualified personnel, documentation and clean operating equipment must be ensured by NAFDAC inspectors for appropriate corrective action.
8. Strict regulation on the purchase, sales and the use of antibiotic should be adopted to control the abuse of antibiotics in order to curtail the menace of antibiotic resistance.
9. More research is encouraged into molecular characterization of *Salmonella* strains in circulation within our community and the molecular detection of important genetic markers like antibiotic resistance genes.
10. The establishment of a systems for controlling of *Salmonella* infections and the development of national and local guidelines for antibiotic treatment, Continued

epidemiological surveillance and timely monitoring for the emergence and re-emergence of multi-drug resistance (MDR), are encouraged.

6.3 Limitations of the Study

The inability to carry out an extensive molecular detection of all antibiotic resistance genes among the *Salmonella* isolates due to some some challenges including poor primer optimization was a limitation of this study.

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APPENDICES

APPENDIX I: ETHICAL APPROVAL BY STATE MINISTRIES OF HEALTH

MINISTRY OF HEALTH, KADUNA STATE

All Communications to be Addressed to:
THE HON. COMMISSIONER
Quoting Reference and Date
Tel: (062) 248084
(062) 248252

Independence Way,
P.M.B. 2014,
Kaduna.
Kaduna State, Nigeria

MOH/ADM/744/VOL.I/

30th May, 2013

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....
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PERMISSION TO CONDUCT RESEARCH

I have been directed to convey the Ministry's approval to STEP-B PROJECT TEAM who are students from Department of Microbiology, Faculty of Science Ahmadu Bello University, Zaria Kaduna State Nigeria. To conduct a research on the Topic: **"DIARRHOEAL AGENTS AND THE NUTRITIONAL STATUS OF CHILDREN IN NORTH-WESTERN NIGERIA"** for the award of, Ph.D /M.Sc in the Department of Microbiology Faculty of Science Ahmadu Bello University, Zaria.

It is expected that necessary assistance be accorded to any of the step-B Project team members in the process of the research, please.

However, it is mandatory for the researcher to submit a copy of his/her research finding(s) to the office of the Honourable Commissioner, Ministry of Health, as soon as it is concluded.

Accept the assurances of the Honourable Commissioner's highest regards.

F. A. KURAH (MRS)
SECRETARY, ETHICAL COMMITTEE

MINISTRY OF HEALTH

BLOCK 14 & 16, SHEHU KANGIWA SECRETARIAT
P.M.B. 2113, SOKOTO STATE, NIGERIA.
TEL: 060-232856, 232425, 232172.

No.: Date:

Ref: No. SMH/962B/Vol.I **10th June, 2013**

Dr. (Mrs) M. Aminu Muktar
Faculty of Science
Biological Sciences Step-B Diarrhoea Project,
Ahmadu Bello University,
Zaria – Nigeria.

ETHICL CLEARANCE FOR RESEARCH ON "DIARRHOEA AGENTS AND THE NUTRITIONAL STATUS OF CHILDREN IN NORTH-WESTERN NIGERIA"

I am directed to refer to your application on the above and to inform you that, the proposal submitted was reviewed by State Health Research Ethics Committee and found the protocol and other documents related to the survey satisfactorily.

In the light of the above, I am further directed to convey the approval of the Committee for the conduct of the above survey. It is however, expected that the results of the survey will be sent to the Committee for documentation as soon as it's concluded.

Please, accept the congratulations of the committee on behalf of the Honourable Commissioner.

Thank you.

(DADI ADARE) Dallatun Gobir
Director HPRS/Co-chairman HREC
For: Honourable Commissioner

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MINISTRY OF HEALTH

KATSINA STATE

TEL" Hon. Commissioner 065-434537(DL). State secretariatComplex, 434518(DL)
I.B.B. Way Dandagoro
P.M.B.2075,Katsina
Permanent Secretary 065-35554

Our Ref

17th June, 2013

The STEP-B Project Group,
Dept of Microbiology,
A.B.U. ZARIA.

APPROVAL OF THE DIARRHEA RESEARCH

I have been directed to convey to you the approval of the honorable commissioner on your research proposal "**Diarrhoea Agents and Nutritional Status of Children in Northwestern Nigeria**". That was after the state ethics subcommittee had gone through the proposal and recommended it to the state ORAC which deliberated on it and finally recommended it for approval by the commissioner.

Kindly adhere to all principles guiding ethical conduct of research in Nigeria and we wish to inform you that ORAC will as usual require your logistic support in monitoring this activity. We also would like to share the findings.

Please accept the Hon. Commissioner's highest regards.

Dr Idriss A. H
For
Hon Commissioner

"Home of Heritage and Hospital"

APPENDIX II: INFORMED CONSENT FORM

DEPARTMENT OF MICROBIOLOGY
FACULTY OF SCIENCE
AHMADU BELLO UNIVERSITY, ZARIA, NIGERIA

Head of Department: Prof. S.A. Ado; B.Sc., M.Sc., Ph.D. E-mail: microbiology@abu.edu.ng

INFORMED CONCENT FORM (ICF)

Serial No.......... Hospital No.......... Age........... Phone No.......................................

This Informed Consent Form is basically for mothers or caregivers who have children within the ages of 5 years and less, attending some selected Hospitals in Kaduna, Katsina, and Sokoto States. We are inviting you to participate in this research work titled " TYPING OF SALMONELLA STRAINS ISOLATED FROM WATER AND DIARRHOEIC STOOLS OF CHILDREN UNDER FIVE YEARS IN PARTS OF NORTHWESTERN NIGERIA" . Diarrhoea is defined as the passage of three or more loose or liquid stools per day (or more frequent than is normal for the individual). It is usually a symptom of an infection in the intestinal tract, which can be caused by a variety of bacterial, viral and parasitic organisms. The research will involve the collection of stool, urine and blood samples of participants. The results obtained thereby may be used in any way to improve the understanding and management of the disease in our community. Individual data will be treated with strict confidentiality. Participation is strictly voluntary and refusal to participate in the research study will not in any way affect your right and benefit in this clinic/hospital.

CERTIFICATE OF CONSENT

I .... of .... do hereby consent to participate in this study. The full procedures of the test/study have been explained to me by the investigator. I understand that stool, urine and blood samples will be taken for the test. I therefore, give this consent voluntarily without being subjected to any pressure.

Name of participant ....
Signature/thumb print .... Date ....

Statement by witness:

I have witnessed the accurate reading of the consent form and detail explanation of the study to the potential participant, and the individual has had the opportunity to ask questions. I consent that the individual has given consent freely.

Name of witness .... Signature............................ Date ....

Statement by Researcher/ Person Taking Consent:

I confirm that sufficient information, including risks and benefits, to make an informed decision have been fully explained to the participant. The participant was given an opportunity to ask questions about the study and all questions asked by the participant have been answered correctly and to the best of my ability. I confirm that the individual has not been coerced into giving consent, and the consent has been given freely and voluntarily.

Name of Researcher .... Signature .... Date ....

APPENDIX III: STRUCTURED QUESTIONNAIRE

DEPARTMENT OF MICROBIOLOGY
FACULTY OF SCIENCES
AHMADU BELLO UNIVERSITY, ZARIA

Typing of *Salmonella* Strains Isolated from Water and Diarrhoeic Stools of Children Under Five Years in Parts of Northwestern Nigeria.

Instruction: Please kindly respond to the questions below by ticking the appropriate boxes provided. The information obtained will be used strictly for academic purpose and absolute confidentiality will be ensured.

1. Background information

LGA..Name/Address of Health facility............................

Name of child...Sex(M/F) .....Age(months)................

2. Socio-economic Data

Occupation of father: Civil servant [ ] Trader [ ] Artisan [ ] Farmer [ ]Unemployed [ ]

Occupation of mother: Civil servant [ ] Trader [ ] Artisan [ ] Farmer [ ] Unemployed [ ]

3. Main source of drinking water

Pipe-borne [ ] Private well [ ] Public well [ ] Pond [ ] Borehole [ ] River/stream [ ]

4. Treatment of drinking water

Boiling [ ] Filtering [ ] None/raw [ ] Others ....

5. Type of toilet

Pit latrine [ ] Water closet [ ] Open field [ ] Others ....

6. Assessment of diarrhoea

How do you know your child has diarrhoea?..

How many times has your child passed loose stools in the last 24 hours?..............................

For how long has the child passed loose stool?...

How do you treat diarrhoea?...

Have you used any drugs to treat the child before coming to health centre?...........................

Have used ORT/ORS to treat the child?..

What do you think is the cause of diarrhoea?..

7. Physical examination of the child

What is the child's general condition?

Well and alert [ ] Restless and irritable [ ] Lethargic and unconscious [ ] Malnourished [ ]

Normal eyes [ ] Sunken eyes [ ] Oedema [ ] Other ailments....................................

8. Feeding practice

When your child has diarrhoea how does it affect his/her appetite?

Increase [ ] Decrease [ ] No effect [ ]

Are you still breast feeding the child? ....

At what age did you introduce foods other than breast milk to the child?...............................

Less than a month [ ] 1 -2 months [ ] 3 -4 months [ ]5 - 6 months [ ] Above 6 months [ ]

How do you normally feed the child? Personal plate [ ] Feeding bottle [ ] Group plate [ ]

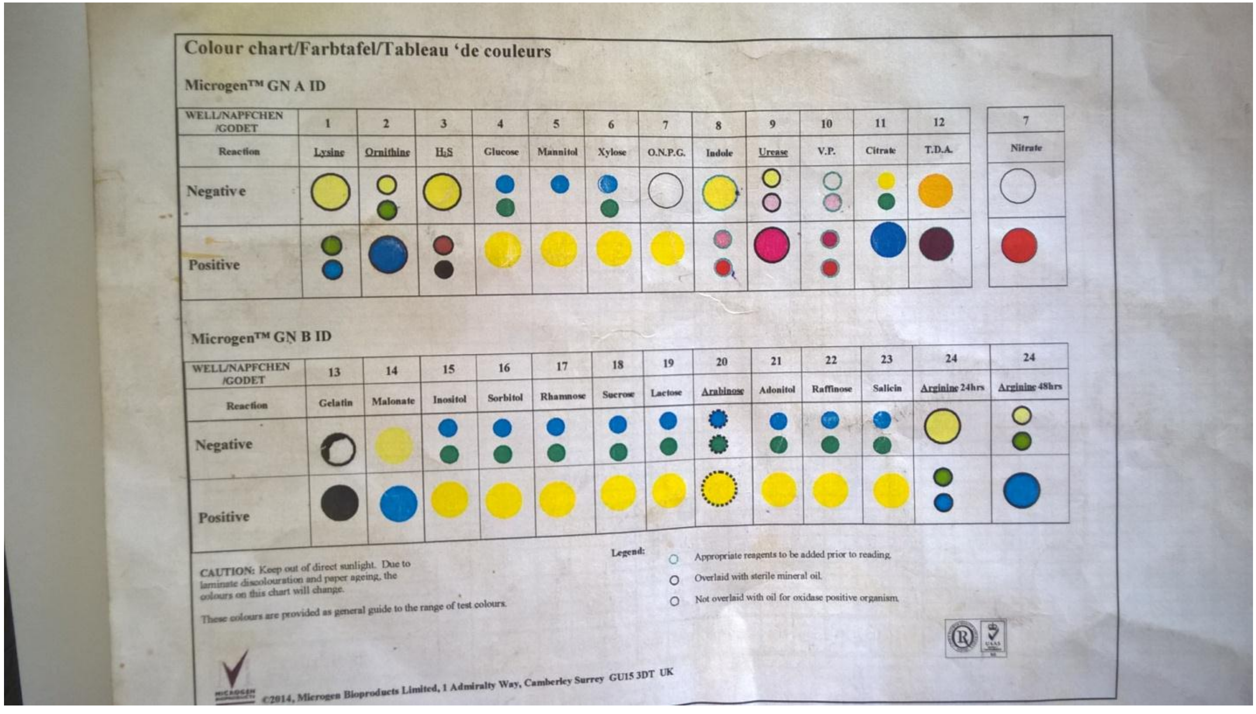
List what you have given to your child to eat in the last 24 hours ....

9. Respondent: Mother [ ] Father [ ] Others...

APPENDIX IV: COMPOSITION OF MEDIA USED FOR ISOLATION AND CULTURE OF BACTERIA IN THE STUDY

Medium	Composition	Expiry date
Nutrient Agar (NA)	Lab - Lemco powder 1.0g ; Yeast extract 2.0g; Peptone 5.0g; NaCl -5.0g; Ager 15.0g	2017/04
Salmonella Shigella Agar (SSA)	Lab-Lemco powder 5.0g; Peptone 5.0g; Lactose 10.0g; Bile salt 8.5g; Sodium citrate 10.0g; Sodium thiosulphate 8.5g; Ferric citrate 1.0g; Brilliant green 0.00033g; Neutral red 0.025g; Agar 15.0g.	2018/04
Selenite F Broth	Peptone 5.0g; Lactose 4.0g; Sodium phosphate 10.0g and Sodium biselenite	2018/04
Eosin Methylene Blue (EMB)	Peptone 10.0g; Di - Potassium hydrogen phosphate 2.0g; Eosin Y. 0.4g; Methylene blue 0.06g; ager 15.0g	2017/08
Mueller Hinton Ager	Beef infusion from 300g/l; casein hydrolysate 17.5g; Ager 17.0g; Starch 1.5g	2017/01
Xylose Lysine Desoxycholate (XLD) agar	Yeast extract - 3.0g; L - Lysine - 5.0g; Xylose 3.5g; Lactose - 7.5g; Sucrose - 7.5g Sodium chlorode - 5.0g; Sodium thiosulphate 6.8g; Sodium desoxycholate 2.5g; Ferric Ammonium citrate 0.8g; Phenol red 0.08g; Ager - 13.5g	2016 /05

APPENDIX V: MICROGEN™ COLOUR CHART: GENERAL GUIDE TO THE RANGE OF TESTS COLOURS

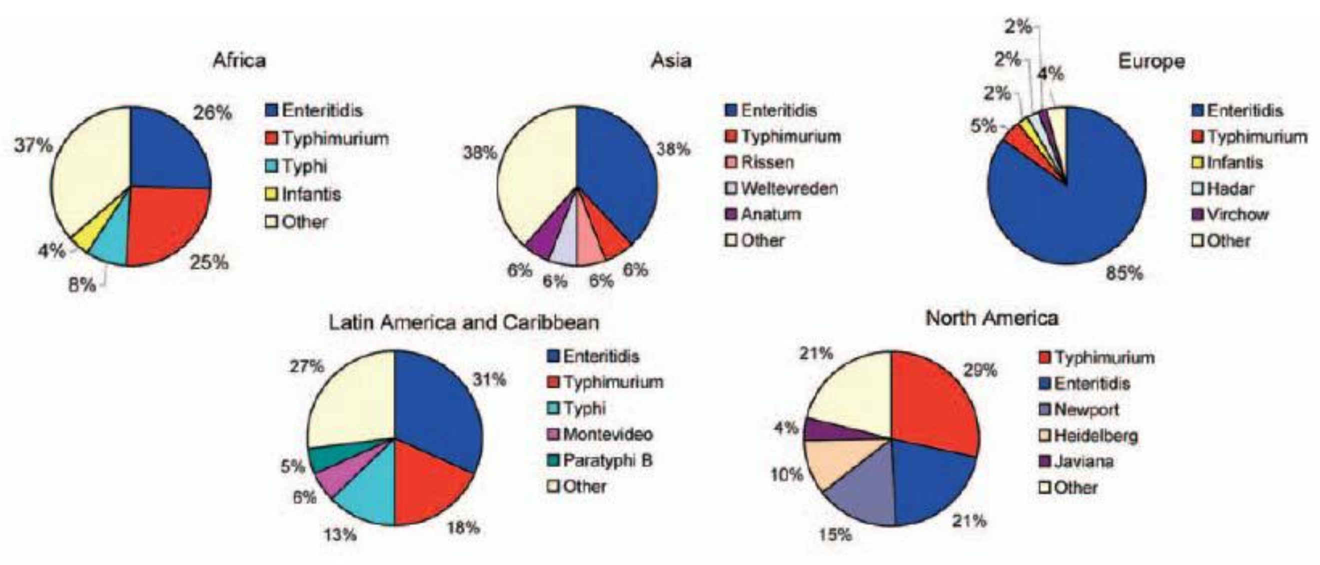


APPENDIX VI: PRELIMINARY IDENTIFICATION RESULTS FOR *SALMONELLA* SPECIES ISOLATED FROM DIARRHOEA STOOLS AND WATER SAMPLES.

			Biochemical Characteristics															
No of Isolates	**Growth on SSA**	**Microscopy**	**Motility**	**Lysine**	**Ornithine**	**H_2S**	**Glucose**	**Mannitol**	**Xylose**	**ONPG**	**Indole**	**Urease**	**V.P.**	**Citrate**	**TDA**	**Octal code**	**Agglutination with Polyvalent (O/H) antisera**	**Presumptive identification**
7	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	+	-	-	+	-	7722	+	*Salmonella* sp.
4	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	-	-	-	-	+	7701	+	*Salmonella* sp.
2	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	+	-	+	+	-	7726	+	*Salmonella* sp.
7	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	-	-	-	+	-	7702	+	*Salmonella* sp.
8	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	-	-	+	+	-	7706	+	*Salmonella* sp.
1	Colourless with black centre	Slender rods	+	+	+	+	+	+	+	-	-	-	-	-	-	5740	+	*Salmonella* sp.

Key: SSA - *Salmonella Shigella* agar, O- somatic antigen, H- flagella antigen

APPENDIX VII



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

APPENDIX VIII: BREAKPOINTS AND INTERPRETIVE CRITERIA FOR ANTIBIOTICS USED IN THE STUDY

Antibiotic	**Disk potency**	**Zone Diameter Interpretive Criteria (Nearest whole mm)**		
		Sensitive	**Intermediate**	**Resistant**
Ampicillin	10µg	≥17	14 - 16	≤13
Amoxicillin	30µg	≥17	14 - 16	≤13
Amoxicillin-clavulanate	10µg	≥18	14 -17	≤13
Cefotaxime	30µg	≥26	23 - 25	≤22
Gentamicin	10µg	≥15	13 - 14	≤12
Tetracycline	30µg	≥15	12 - 14	≤11
Ciprofloxacin	5µg	≥21	16 - 20	≤15
Nalidixic acid	30µg	≥19	14 - 18	≤13
Trimethoprim-sulfamethoxazole	25µg	≥16	11 -15	≤10
Chloramphenicol	30µg	≥18	13 - 17	≤12

Adapted from Performance Standards for Antibiotic Susceptibility Testing (CLSI, 2014)

APPENDIX IX: ADDITIONAL GENOTYPING/SEQUENCE BASED TECHNIQUES APPLICABLE FOR *SALMONELLA* SPECIES (ADOPTED 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 IS200multicopy 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 thewhole 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.