Characterization of non-typhi Salmonella isolated from children admitted to hospitals with bacteremia in Kilifi and Nairobi

By

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A thesis submitted in partial fulfillment for the award of the degree of Master of Science in the School of Pure and Applied Sciences, Kenyatta University.

May 2005
DECLARATION

I declare that this thesis is my own original work and has not been presented for a degree in any other university or any other award.

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DEDICATION

Dedication to my husband Mwituria G. Mwangi for his support, encouragement and for just being there when I needed a shoulder to lean on.

O give thanks unto the Lord, for he is good: for his mercy endureth forever.

Psalms 136; 1
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ABSTRACT

Non-typhi salmonellae (NTS) are well known worldwide as causes of food borne diseases, which are characterized by gastrointestinal illnesses. Infected individuals experience mild gastrointestinal illnesses which are self-limiting and antimicrobial treatment not recommended. But among the young children, the elderly and immunosuppressed individuals the illness can lead to disease complications such as bactaeremia and other serious invasive diseases. This problem may be compounded by multidrug resistance (MDR) among the non-typhi salmonella, with resistance to four or more of the commonly available antimicrobials often being encountered. Studies in Kenya have shown that MDR among the Salmonellae is a serious problem. As Salmonella spp. acquire more resistance, they become more difficult to treat, hence leading to even greater morbidity and mortality. It is therefore important to monitor the susceptibility profiles of the prevailing NTS to commonly available antimicrobial drugs. This study aimed to characterize NTS isolated from children less than 5 years of age admitted to the hospitals with bacteremia in Nairobi and Kilifi. Characterization was done by biochemical and serological typing of the NTS, antimicrobial susceptibility of the isolates, and through DNA analysis including; plasmid analysis of both resistant and sensitive NTS by gel electrophoresis and analysis by pulsed field gel electrophoresis (PFGE) of (Xba-1) and (Spe-1) digested genomic DNA fragments. A total of 2212 blood samples were obtained from children 0-5 years of age of which 1296 (58.1%) and 916 (41.4%) samples were from Nairobi and Kilifi respectively. The number of samples positive for NTS was 148 (6.7%) out of which 118 (79.7%) were isolated from Nairobi and 30 (20.3%) from Kilifi. The main NTS serotypes identified were Salmonella enterica serotype Typhimirium 99 (66.9%), Salmonella enterica serotype Enteritidis 43 (29.1%) and other salmonella species 6(4.1%). In Nairobi 56% of Salmonella isolated were resistant to three or more drugs while in Kilifi less than 10% were resistant. Large plasmids of 98-100 kb pairs were found all ampicillin resistant and multidrug resistant Salmonella. The PFGE results of endonuclease digested DNA revealed that 70% cases had the same NTS serotypes as their contacts at home. In conclusion there were two main serotypes of Salmonella isolated from the children; Salmonella typhimurium and Salmonell enteritidis. Salmonella isolates from Nairobi were more resistant to ampicillin, cortrimoxazole, chloramphenicol and tetracycline than isolates from Kilifi. S. typhimurium was more resistant than other salmonellae PFGE though expensive is a useful tool for epidemiological studies particularly in cases of disease outbreaks. Because cases and controls had the same NTS infection; siblings and parents of the sick children may have been the sources of NTS infections. Therefore these asymptomatic carriers of NTS (as happens in typhoid) may play an important role in the epidemiology of NTS bacteremia in these children. More emphasis should be on disease prevention through public health measures and continuous antimicrobial susceptibility monitoring and surveillance.
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 Introduction

Salmonellosis is caused by gram-negative bacteria of the genus *Salmonella enterica*, family Enterobacteriaceae. However the term salmonellosis often refers to infection with non-typhi *Salmonella enterica* serotypes. Non typhi salmonellae (NTS) are major causes of food borne infections in the developed world and a common cause of gastroenteritis worldwide. Although human salmonellosis occurs world-wide it has become a major public health problem over the second half of the 20\textsuperscript{th} century in the developed world, where infections and transmission are thought to be mainly by animal to human through the food chain.

In developing countries NTS are important causes of severe invasive disease particularly in tropical Africa where *Salmonella enterica* serovar Typhimurium and *Salmonella enterica* serovar Enteritidis are the commonest causes of childhood bacteremia, meningitis, septic arthritis and pneumonia (Graham *et al*., 2000). The sources and transmission of NTS in these countries are not well documented although waterborne transmission and poor hygiene play an important role in human to human transmission. Individual cases and outbreaks in communities and in institutions are common (Seyfarth *et al*., 1997). In many of these countries, salmonellosis is an important cause of bacteremia and septicemia, often presenting as a non specific febrile illness which is difficult to distinguish clinically from malaria (Cheesbrough *et al*., 1997). Invasive NTS disease is a major problem in children living in regions of Africa
that are endemic to malaria. In Kilifi the malarial endemicity is thought to seriously complicate the health issues related to NTS (Berkley et al., 1999).

There are many *Salmonella enterica* serotypes but only a few accounts for the vast majority of human infections. Some common human isolates are *Salmonella enterica* serotype Enteritidis (*S.* enteritidis), *Salmonella enterica* serotype Typhimurium (*S.* typhimurium), *Salmonella enterica* serotype Virchow (*S.* virchow), *Salmonella enterica* serotype Newport (*S.* newport) and *Salmonella enterica* serotype Choleraesuis (*S.* cholerasuis) (Cook and Saunders, 1996). For many years, *S. typhimurium* was the dominant strain throughout the world but in recent years, *S. enteritidis* has become the most prevalent serotype in the Northern European countries. In the U.S.A, *S. typhimurium* is the most common serotype isolated from humans and animals (Bender et al., 2001).

In Kenya the commonly isolated serotypes are *S. typhimurium* and *S. enteritidis* (Kariuki et al., 1996). *Salmonellae* are widely distributed in the animal kingdom; domestic animals notably pigs, cattle and poultry are frequent excretors and many wild animals are also infected. Household pets like dogs, cats and birds are all potential sources of human infection. Human cases and convalescent carriers are also important sources. Salmonellosis is not common in adults who are immunocompetent but it is an important cause of childhood infection, often contracted in hospitals through cross infection and some times hospitals act as reservoirs for maintaining infection in the community (Mandal et al., 1986).
The emergence of multiple drug resistance (MDR) *Salmonella* spp. is an increasing problem worldwide as infections caused by these organisms increase health care costs and limit the choice of treatment (Smith *et al.*, 1984). Infections with MDR non typhi salmonella are very significant problem in developing countries, especially because options for second line antibiotics are limited, they are either unavailable or unaffordable (Graham S.M., 2002). The increasing occurrence of multiple drug resistance, of up to 4 or more of the first line antibiotics including ampicillin, co-trimoxazole, chloramphenicol and tetracycline, has had a profound effect in the developing countries in the treatment of *Salmonella* septicemia in infants and young children.

Multidrug resistance NTS in the developed world is believed to have developed as a result of use of antimicrobials in the animal husbandry i.e., use of antimicrobial drugs in food animals, for therapeutic or preventive purposes as well as growth promoters in animal feed. In the developing world MDR has developed as a result of use of antimicrobials in human medicine particularly in those countries where there is little control over their use (Cook *et al.*, 1996)

### 1.1.2 Statement of the problem

Over the last years, drug-resistant *Salmonella* serovars have emerged in the developing and developed world. In Kenya multiple drug resistance has been reported in invasive salmonellosis, rendering the commonly available drugs ineffective in the treatment of the disease. High levels of drug resistance to commonly prescribed drugs such as ampicillin, chloramphenicol, cotrimoxazole and tetracycline have been observed in
Kilifi and Nairobi (Kariuki et al., 1996). Immunosuppression caused by either malnutrition, co-infection with malaria and HIV infection further complicate Salmonella infections in both adults and children. Infection by the multidrug resistant salmonellae may lead to longer hospital stays that are a risk to other patients due to possible transmission of the multi-drug resistant Salmonella spp. In addition alternative treatment is also expensive and less readily available.

In this study, serotypes of non-typhi Salmonella (NTS) causing bacteremia in children below 5 years of age in Kilifi and Nairobi were determined, antimicrobial susceptibility profiles and the plasmid profiles for multi-drug resistant Salmonella. The two areas were chosen for purposes of comparisons, to compare MDR between urban (Nairobi) and rural (Kilifi) setup. The rural people of Kilifi are basically poor and ignorant and their access to drugs is low but in Nairobi most people are knowledgeable and can easily access antibiotics even without doctor’s prescription. The study was also part of the mandate of KEMRI in collaboration with the Ministry of Health as a Centre for Antimicrobial Susceptibility Testing and Resistance Monitoring under the World Health Organization.

1.1.3 Research questions

1. Are non-typhi Salmonella infections in children with bacteremia caused by multidrug resistant strains?

2. Which are the common serotypes and resistant types prevalent in Nairobi and Kilifi?

3. What are the genetic patterns for multidrug resistant phenotypes in NTS?
1.1.4 Null Hypothesis

Non-typhi *Salmonella* isolates from Nairobi and Kilifi are resistant to commonly used antibiotics.

1.1.5 Objectives

The broad objective of the study was to isolate and characterize non-typhi *Salmonella* from children less than five years admitted with bacteremia in Nairobi and Kilifi.

**Specific objectives**

1. Isolate and identify non-typhi *Salmonella* in blood specimens from children with bacteremia in Nairobi and Kilifi.

2. Determine the antibiotic susceptibility patterns of commonly used antibiotics by disk diffusion and minimum inhibitory concentration (MIC).

3. Determine the plasmid profiles of multidrug-resistant *Salmonella* strains.

4. Determine the sources of infections in children admitted with bacteremia and the strain-relatedness of the NTS by using pulsed field gel electrophoresis (PFGE)

1.1.6 Justification

Studies on antimicrobial resistance by enteric pathogens have demonstrated that antimicrobial resistant *Salmonella* are more virulent than susceptible strains i.e. the antimicrobial resistant strains cause more prolonged or more severe illness than the susceptible strains; this is not withstanding age and other underlying conditions (Travers *et al.*, 2002). Invasive salmonellosis is common in children in Tropical Africa.
(Cheesbrough et al., 1997), and in Kenya non-typhi *Salmonella* spp. *S. typhimurium* and *S. enteritidis* are the main causes of bacteremia, they may also be multidrug resistant. Although NTS are common in Kenya, and in particular Kilifi and Nairobi, constant monitoring of NTS is required in terms of characterization and particularly multidrug resistance so as to facilitate proper choices of antibiotics for effective treatment.
Literature Review

1.2 Salmonella classification

*Salmonellae* are flagellated non-sporing gram-negative bacilli belonging to the family Enterobactericeae. The genus *Salmonella* earlier consisted of only one species namely *Salmonella enterica* (Cook et al., 1996). Within this single species are seven subspecies *enterica, salmae, arisonae, diarizonae, hnoptenae, bongori* and *indica* based on DNA structure and biochemical tests. A new classification of *Salmonella* has grouped the genus *Salmonella* into two species, *S. bongori* and *S. enterica* and eight subspecies. Most *Salmonella* strain associated with disease in humans belong to the subspecies 1 (enterica).

In the new nomenclature, *Salmonella* serotypes in subspecies are named artificially as species. Using this classification, the formal presentation of for example, *Salmonella enterica* subspecies *enterica* serotype Typhimirium, becomes *Salmonella* serotype (ser) Typhimirium or just *Salmonella typhimurium* (Le Minor et al., 1988; Tindall et al., 2005). The subspecies can further be subdivided into serotypes or serovars based on their somatic (O) and flagella (H) antigens and more than 2,200 serovars are known to exist. The serotypes of *Salmonella enterica* account for most human and animal infections. These serotypes are grouped on the basis of sharing a common O antigen in a scheme developed by Kauffman-white (1954).

On the basis of host preference and disease manifestations in man, *Salmonella* can be placed in two broad categories; *typhi* and non-typhi. The *typhi* *Salmonella* includes *S typhi, S. paratyphi A, S. paratyphi B,* and *S paratyphi C*. These serotypes are primarily
host specific to man and cause typhoid and paratyphoid fevers which are prolonged bacteraemic illnesses with minimal diarrhoea initially. The non-typhi serotypes are adapted to animals, and infections in man are usually confined to the bowel and presents with acute diarrhea.

1.3 Pathogenesis and clinical manifestations

Pathogenesis by *Salmonella* occurs once the bacteria evade the hostile environment of the stomach and upper small intestines, and attach to the epithelial cells of the ileum and to a lesser extent the colon. They then penetrate and migrate to the Lamina propria, causing inflammation characterized by local leucocyte infiltration, congestion, and oedema; moreover blood stream infections may also occur from these sites. *Salmonella* also produces two types of toxins, an enterotoxin and a cytotoxin which enhance its virulence.

The infecting dose is important to the outcome of *Salmonella* infections; an infecting doze of $10^5$ organisms has been found to initiate infection but as few as 17 organisms have been shown to cause infections (Cowden J. M. 1990). The size of the dose necessary to cause infection is influenced by the virulence of the organisms, host factors such as age, immune status, and the physiological state of the stomach and the upper intestines at the time of ingestion of the organisms. Gastric acidity is a significant barrier to enteric infection and hypoacidity or increased transit time increases the susceptibility to infection (Gianella et al., 1973). Malnutrition and presence of virulence plasmids may also be important in the pathogenesis of bacteremia in humans (Fierer et al., 1992). Virulence plasmids are required to trigger systemic
disease and are important for the bacterial multiplication in the reticular endothelial system of warm blooded animals. Both *S. typhimurium* and *S. enteritidis* which are the most common NTS possess the virulent plasmid (50-100kb). Plasmids are also more frequently found in *S. typhimurium* and *S. enteritidis* strains isolated from blood specimens and other extra intestinal sources than in strains from faeces (Rutgers *et al.*, 1999).

Two clinical manifestations of salmonellosis are often seen, acute enterocolitis which is the most common and invasive salmonellosis with septicaemia (bacteremia) or metastatic extra-intestinal localization of infection. The incubation period is usually 12-48 hours but longer incubation periods have been reported (Cowden *et al.*, 1989).

**1.3.1 Acute enterocolitis**

This term refers to the acute diarrhea of salmonellosis; both small and large intestines are involved in the disease process. The illness begins with nausea and vomiting often associated with malaise, headache and fever, followed by cramp-like abdominal pains and diarrhea. Initially, the stools are of large volume and watery without visible blood or mucus, but may decrease as blood and mucus appear indicating development of colitis. The severity of diarrhea may vary from a mild attack of several loose stools per day to voluminous watery stools every half hour. The elderly, particularly those with debilitating illness and those with gastro hypoacidity, are likely to develop severe diarrhea. Occasionally colitis may dominate the clinical picture with the passage of blood stained stool-containing pus (Cook *et al.*, 1996).
1.3.2 Invasive salmonellosis

NTS serotypes vary greatly in their potential to cause invasive illness outside the gastrointestinal tract. Although many serotypes cause invasive disease some are more invasive than others; Serotypes with increased invasiveness are *Salmonella virchow* and *Salmonella dublin*. Bacteraemia is common in *Salmonella* infection even in previously healthy individuals and its frequency depends on the serotype and the host factors. Overall bacteremia rates of 8% have been observed, with higher rates for some serotypes e.g. *Salmonella cholerasuis, Salmonella virchow* and *Salmonella dublin*.

The incidence of bacteraemia is higher in the elderly and in the very young (Mandal *et al.*, 1998, Meadow *et al.*, 1985) because in these two age groups the immune status is compromised and young age is also a risk factor for mortality. In healthy individuals, bacteraemia is transient but in minority of patients, particularly those with risk factors, such as immunosuppression, gastric hypoacidity and sickle cell disease, bacteremia may be significant and characterized by septicemia illness. *Salmonella* bacteremia is one manifestation of immunosuppression in patients with human immunodeficiency virus (HIV) infection (Levin *et al.*, 1991) and septicemia is a frequent cause of death in HIV-infected adults in developing countries (Ssali *et al.*, 1998).

Invasive salmonellosis is common among children in tropical Africa, typically presenting as non-specific febrile illness with non specific localizing signs, that is difficult to distinguish clinically from malaria (Cheesbrough *et al.*, 1997). In a study done in Kenya by Gilks *et al.* (1990) it was observed that HIV-seropositive patients
with bacteremia due to non-typhi *Salmonella typhimurium* had a higher mortality rate than HIV-seronegative.

### 1.3.3 Clinical and laboratory diagnosis

Clinical diagnosis is based on the clinical symptoms associated with enterocolitis, but the symptoms are however not unique to non-typhi *Salmonella* as infections by other Enterobacteria such as *Shigella, Vibrio cholerae* and even *Escherichia coli* may show similar symptoms. The definitive diagnosis is the laboratory demonstration of the specific non-typhi *Salmonella* in the fecal specimens. For bacteremia the symptoms will resemble those of any other microorganisms that cause fever. Blood cultures are recommended in all severely ill patients for a positive diagnosis of any of the causative organisms. Bacteremia/septicemia is an important cause of fever which can be caused by various other diseases including malaria, so a differential diagnosis that includes ruling out malaria in endemic areas is important. In Kilifi Kenya, children infected with malaria have been found to be co-infected with non-typhi *Salmonella* (Berkley et al., 1999) complicating the disease outcome.

### 1.4 Treatment

Most patients with *Salmonella* enterocolitis have a short, self-limiting illness and require only increased fluid intake. Chloramphenicol, co-trimoxazole and amoxycillin are effective against invasive illness if the infecting organism is sensitive. However, the incidence of infection due to *Salmonella* organisms resistant to one or more of these drugs has increased in many parts of the world (Zouk.K.1988). In Kenya the drugs available in public hospitals for the treatment of salmonellosis are ampicillin,
tetracyline, contrimozale, gentamicin, chloramphenical and augumentin (Personal communication) These are first line antibiotics available in most public hospitals. The second line antimicrobials such as ciprofloxacin, ceftazidime are too expensive and not available in most public hospitals.

1.5 Drug Resistance and associated mechanism

Development of resistance in bacteria previously susceptible to antimicrobial agents can be due to (i), alteration in the cells physiology or structure, also referred to as biological resistance or (ii) environmental resistance, defined as the resistance that directly results from physical or chemical characteristics of the environment that either directly alter the antimicrobial agent or alter the organism’s normal physiological response to the drug (Forbes et al., 1996). Microorganism mediated resistance refers to resistance that is due to genetically encoded traits of the organism and can be divided into two categories: (i) intrinsic or inherent and (ii) acquired. Plasmid mediated resistance is an example of such acquired resistance where there is acquisition of genes from other organisms through transfer mechanisms. Most bacteria carry plasmids that can also carry genes that code for virulence (Forbes et al., 1996). Plasmids also carry one or more genes that can code for resistance to one or several drugs. Plasmid resistance genes often code for enzymes that destroy or modify drugs e.g. hydrolysis of penicillin or the acetylation of chloramphenicol and aminoglycoside drugs thus rendering them ineffective.

Previous studies on non-typhi Salmonella bacteremia in children in Kenya have shown that multidrug resistance is common and that resistance was due to the presence of
large self transmissible plasmids 100 kb (Kariuki et al., 1996). In a study of isolates from patients in Kilifi it was observed that 56% of all Salmonella enterica serovars, S. enteritidis and S. typhimurium were resistant to three or more of the following antimicrobials tested: ampicillin, gentamicin, chloramphenicol, amoxycillin/clavulanic acid, tobramycin, cefotaxim, co-trimoxazole, and ciprofloxacin (Oundo et al., 2000). In Western Kenya Dougle et al. (1997) observed multi-resistance between two commonly isolated strains of Salmonella, S. typhimurium and S. enteritidis. This resistance is of great concern owing to the high morbidity and mortality associated with multidrug resistant non-typhi salmonella (NTS) infection due to their increased virulence (Graham et al., 2000, Hadsfield et al., 1985 and Helms et al., 2002) and regular surveys to monitor antimicrobial resistance patterns and spread among the NTS is of great importance.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Study areas

The study was carried out in Kilifi District hospital and three hospitals in Nairobi. Kilifi hospital is located in Kilifi town which is 60km north of Mombasa town. This hospital serves a rural population of 180 000 people who are mainly peasant farmers and keep cattle and goats. In Nairobi three hospitals were chosen, Kenyatta National Hospital (KNH), Gertrude’s Garden Hospital and Aga Khan Hospital. The KNH is the country’s referral hospital that serves the poor and middle income groups of Nairobi and its environs. Most of the patients are referred from Nairobi City Council and private clinics in the city, there are also referrals from other hospitals in the country. The Aga Khan and Gertrude’s Garden hospitals serve the middle and high income groups.

2.2 Specimen collection and processing

Blood samples for culture were obtained from children below 5 years of age admitted to these hospitals with febrile illness. About 5 mL of blood was drawn from each patient before treatment and inoculated directly into 25 mL of brain heart infusion broth (OXOID: Basingstoke, UK) containing paraminobenzoic acid. Blood specimens were taken to the laboratory where they were incubated at 37°C aerobically for 18 to 24 hours in 5% carbon dioxide. Positive blood cultures, those showing turbidity or gas production were subcultured onto 5% sheep blood agar, chocolate blood agar and MacConkey agar plates (OXOID,
Basingstoke, UK) and incubated for 18 to 24 hours. Blood and chocolate agars were included to capture other bacteria that cause bacteremia but cannot grow on MacConkey, such as *Streptococcus pneumoniae*.

After overnight incubation the MacConkey plates were examined for non-lactose fermenters (NLFs). The non lactose fermenters were picked from the MacConkey plates and inoculated onto biochemical test media including Urea, Triple sugar iron (TSI), indole and citrate tests (Appendix 1). Non lactose fermenters that were suspected to be *Salmonella* spp were further identified biochemically by use of Analytical Profile Index (API 20E) (Appendix 2) (API System Montalieu varcieu, France) and were serotyped using *Salmonella* agglutinating antisera (Murex Diagnostic, Dartford, UK) and stored in trypticase soy broth/glycerol media at -70°C for further analysis.

### 2.2.1 Serological typing of NTS

Fresh colonies of suspected NTS were emulsified on a clean glass slide in 0.85% physiological saline; a drop of polyvalent somatic 0 antiserum was added to identify *Salmonella* antigens in the sample. The contents were mixed for one minute and observed for agglutination. If a strong and visible agglutination was seen the test was considered positive for the presence of *Salmonella* antigens. The isolate was further typed with various monovalent (specific) O antisera for the presence of specific *Salmonella* antigens. And if agglutination was observed with any of the specific antisera the isolate was named according to the specific antiserum it agglutinated with, e.g. *Salmonella typhimurium* and this is according to the Kauffman-White scheme of
Salmonellae classification and identification. If there was no agglutination with polyvalent somatic O antiserum the isolate did not have Salmonella antigens and was therefore negative. However if agglutination occurred with polyvalent somatic O antiserum only and there was no reaction with the monovalent antiserum the isolate was classified as a Salmonella species.

2.2.2 Specimens from siblings and environment

Children (cases) from whom NTS were isolated were followed to their homes where possible, upon discharge from hospitals and stool specimens from siblings and parents, household pets such as dogs, cats: domestic animals such as goats, and pigs collected. From parents and siblings; dry- leak proof stool containers were supplied a day before with instructions to put stool specimens, approximately a spoonful or pea size into the container and cap immediately and firmly. The specimens were picked the following day and transported to the laboratory. Where it was not possible to get a stool specimen, a rectal specimen was taken by use of a sterile cotton swab on an applicator stick; the swab was put into sterile transport media (Cary Blair). From pets and domestic animals, fresh stool droppings were collected and put in peptone water (Appendix 3). Samples of drinking water, raw milk where possible were also collected. NTS isolated from these specimens from the patients’ environment were thereafter referred to as contacts.

2.2.3 Stool Samples – processing

These were inoculated directly onto MacConkey, Salmonella-Shigella (SS) agar plates and into selenite F broth for enrichment and incubated at 37°C overnight. After
overnight incubation the MacConkey and SS agar plates were examined for non lactose fermenters, Selenite F broth culture was plated onto MacConkey agar, SS agar and incubated at 37°C overnight. None lactose fomenters were picked out and tested for NTS using biochemical methods including API 20E as in the blood cultures. They were further identified serologically by use of *Salmonella* agglutinating antisera (Murex diagnostics, Dart ford, UK)

### 2.2.4 Environmental sample- processing

Water samples were first enriched in sterile phosphate buffered peptone water and MacConkey broth, and then incubated overnight at 37°C. From peptone water the specimens were sub cultured onto Rappaport Vasilliades Soya broth (RVS) and incubated overnight at 37°C, followed by plating onto Xylose Lysine Deoxycholate (XLD), MacConkey, and SS agars. Non-lactose fermenters were identified as *Salmonella* species by biochemical tests including API 20E system and by serotyping with *Salmonella* agglutinating antisera. Soils and stool droppings from the chicken, ducks and goats were processed like water samples but the MacConkey broth was not included. Milk specimens were inoculated directly onto peptone broth and incubated overnight. They were subcultured onto MacConkey and SS agar after the first 24 hours and again after 48 hours respectively. Non-lactose fermenters were selected and identified for NTS as in the other specimens.
2.3 Antimicrobial Susceptibility testing

2.3.1 Disk diffusion susceptibility testing method

Antimicrobial susceptibility tests for the commonly available antimicrobials were carried out on a total 148 NTS isolates by the Kirby Bauer disk diffusion technique (Bauer et al, 1966) using commercially available (OXOID) antimicrobial sensitivity disks. The disk is a piece of blotting paper that is impregnated with known volume and concentration of an antimicrobial agent, and placed on a plate of sensitivity testing agar uniformly inoculated with test organism. The basis of the test is that the antimicrobial diffuses from the disk to the medium and the growth of test organism is inhibited at a distance from the disk that is related to the sensitivity of the organism. Strains sensitive to the antimicrobial are inhibited at a distance from the disk whereas resistant strains have smaller zones of inhibition or grow up to the edge of the disk.

The drugs tested were ampicillin (AMP) 10μg, co-trimoxazole (trimethoprim – sulphamethoxazole) (SXT/TS) 19/1 μg, tetracycline (TC) 30μg, chloramphenical (CL) 10μg, gentamicin (GN) 10μg, nalidixic acid (NA) 30μg, augumentin (amoxicillin-clavulanic acid) (XL/AMC) 30μg, ceftriaxone (CRO/TXL) 30μg and ciprofloxacin (CIP) 5 μg (All from OXOID Basingstoke, UK).

Fresh colonies of NTS isolates were emulsified in sterile distilled water to conform to 0.5 McFarland Turbidity Standards (Appendix 4) and then diluted further to give a concentration of approximately $10^5$ colony forming units per ml (cfu). A sterile cotton swab was dipped into the emulsified bacterial isolates, squeezed on the sides of the bottle to remove excess broth and the test organisms were spread uniformly onto Mueller Hinton agar (OXOID) plate. The plates were allowed to dry for 15 minutes and
the antimicrobial disks added aseptically. Fresh *Escherichia coli* ATCC 25922, of known zones of inhibition was included as control for bacterial growth and for potency of the antimicrobial disks. The plates were incubated at 37°C aerobically for 18 hours; they were then examined for presence or absence of inhibition zones. Inhibition or no growth of bacteria around the disk and depending on the zone size was considered as sensitive (Figure 3.5 A). Total growth or small zone of inhibition and depending on the zone size and the interpretation criteria was considered resistant (Figure 3.4 A).

The diameters of the zones of growth inhibition of the tests and control plates were measured in millimetres using a zone reader and recorded. The zone diameter sizes for each antibiotic on the control plate had to be within the ranges given in the NCCLS manual, otherwise both the test and the control were considered invalid and were repeated. Susceptibility results of the organisms were interpreted as sensitive or resistant, according to National Committee for Clinical Laboratory Standards (NCCLS 2002) manual.

2.3.2. Minimum Inhibitory Concentration (MIC) by E-test method

MIC is the lowest concentration of antimicrobial agent showing complete inhibition of bacterial growth and is used to quantitatively measure the in vitro activity of an antimicrobial agent against a bacterial isolate.

The MIC was carried out on the 148 NTS isolates which were used for disk diffusion method and the same antimicrobials were used. The antimicrobials and the MIC ranges were, ampicillin, (0.016-256μg), co-trimoxazole, (0.002-32μg), tetracycline, (0.016-256μg), chlamphenical, (0.016-256μg), gentamicin, (0.016-256μg), augmentin (0.016-
256\(\mu\)g nalidixic acid (0.16-256\(\mu\)g) ceftriaxone (0.002-32\(\mu\)g) ciprofloxacin (0.002-32\(\mu\)g). The MICs were determined by the Etest (AB-BIODISK Solna, Sweden) method. Etest consists of a non-porous plastic strip calibrated with MIC values covering two-fold dilutions. A predefined antibiotic gradient is immobilized on the surface opposite the MIC scale with minimum and maximum concentration at each end of strip. When a strip is placed on inoculated agar surface, there is immediate and effective transfer of the preformed antibiotic from the plastic carrier to the medium beneath.

Briefly, the overnight growth of organisms were suspended in 0.85\% sterile normal saline to a McFarland Standard of 0.5 and then diluted further to get a concentration of approximately \(10^5\) colony forming units per ml. By use of sterile cotton swabs the emulsified organisms were aseptically spread uniformly onto Mueller Hinton agar plates, were swabbed and the plates allowed to dry for not more than 15 minutes. Control \textit{E. coli} ATCC 25922 with known MIC range was included for control of growth and the potency of the E-test strips. The plates were incubated aerobically at 37\(^\circ\)C for 18 hours after which the MIC was read and recorded.

The MIC value was read in micrograms per millilitre (\(\mu\)g/ml) from the scale at the point where the inhibition zone stopped and growth of microorganisms on the strip started. Where there was no zone of inhibition the isolate was considered resistant (Figures 3.4 B and 3.5 B). The results were interpreted according to the NCCLS (2002) guidelines.
2.4 Extraction of Plasmid DNA and analysis by gel electrophoresis

A total of 42 multidrug resistant (MDR) strains were randomly selected for plasmid analysis. MDR isolates were defined as those that were resistant to 3 or more antimicrobials. Another 40 isolates that were sensitive to all the antibiotics were also selected for comparison of plasmid profiles. Plasmid DNA was extracted following the protocol described by Birnboin and Doly (1979) and modified by Sambrook et al. (1989). NTS isolates were first inoculated on MacConkey agar, incubated at 37°C aerobically for 18 hours. A single colony of fresh cultures was then inoculated into 3mls Luria-Bertani (LB) broth (Appendix 5), incubated at 37°C for 18 hours with vigorous shaking. 1.5 mls of broth culture was transferred to sterile eppendorf tubes, capped and centrifuged at 13000 rpm for 60 seconds at 4°C.

The supernatant was aspirated off leaving a cell pellet, 100 micro liters of cold solution 1(Appendix 6) was added to resuspend the bacteria cells and mixed by vigorous vortexing. About 200 microliters of freshly prepared alkaline detergent solution II (Appendix 7) was added to lyse the bacteria and destroy the chromosomal DNA. The tubes were tightly closed and the contents gently mixed by inverting the tubes rapidly 5 times and making sure the entire surface of the tubes come into contact with the lysis solution 11. The tubes were kept in ice, 150 micro litres of ice cold neutralizing acidic solution III (Appendix 8) was added and tubes closed tightly, vortexed gently in an inverted position for 10 seconds to disperse solution III through the entire bacterial lysate. The tubes were again kept in ice for 5 minutes before being centrifuged at 4°C for 10 minutes at 15,000 rpm to precipitate chromosomal DNA and other cellular
debris. The supernatant which was rich in plasmid DNA was transferred to fresh sterile tubes for purification.

Plasmid DNA was precipitated in 2 volumes of 95% ethyl alcohol (Analytical grade) at room temperature, mixed by vortexing and allowed to stand at room temperature for 5 minutes. The DNA/Alcohol mixture was then centrifuged at 4° C for 10 minutes at 15,000 rpm to obtain the DNA pellet. The supernatant was removed by gentle aspiration, care taken not to touch the DNA pellet and the tubes put in an inverted position on a paper towel for the fluid to drain, any drops of adhering fluids were carefully removed. About 1ml of cold 70 % ethyl alcohol was then added to the DNA pellet mixed and centrifuged in a refrigerated micro centrifuge (Tomy Seiko, corporation, Tokyo, Japan.) at 4° C for 5 minutes at a speed of 15,000 rpm. The supernatant was again removed and the DNA left to dry in an inverted position at room temperature for 10 minutes. Purified plasmid DNA was resuspended in TE buffer (Ph 8.0) (Appendix 9) containing DNAase free pancreatic RNAase (20 microgram/ml). The DNA was now ready for agarose gel electrophoresis.

Briefly 0.9 % agarose was prepared in 0.5 TBE (Tris-Borate-EDTA ) buffer (Appendix 10) and the gel allowed to cool up to 50°C, was then poured into the gel slab and left to solidify. About 24.1 of the extracted plasmids were mixed with 12 micro litres of loading buffer (Appendix11). The plasmid/buffer mixture was loaded onto the wells in the horizontal gel, plasmid DNA of Escherichia coli strains with known molecular sizes 39R861 (147, 63, 43.5, 6.9kb) and V517 (53.7, 7.2, 5.6, 3.9, 3.0, 2.7, 2.1kb) were run alongside the tests as standards to determine the sizes of the unknown DNA.
Electrophoresis was performed at 100V for 2 hours and the gel stained in Ethidium Bromide at a concentration of 0.5 microgrammes per ml. The DNA bands were visualized on a UV illuminator (UVP Inc. CA, U.S.A.) and photographed using a Polaroid MP-3 camera (Polaroid, MS, U.S.A)

2.5 Strain relatedness by Pulsed Field Gel Electrophoresis (PFGE)

Pulsed-field gel electrophoresis was used to compare the genetic relatedness of the DNA fragments of NTS isolates from cases and their controls and also to compare the DNA fragments of the different serotypes from Nairobi and Kilifi. Chromosomal DNA was prepared in agarose plugs by the method described by Thong et al. (1995).

Bacteria from a single colony of a fresh (18-24 hours) NTS culture were inoculated into Luria Bertani (LB) broth incubated at 37°C for 18 hours in a shaking incubator thereafter 100 microliters of bacterial broth (per sample) was transferred to a sterile eppindorf tube and centrifuged for 60 seconds at 12,000 rpm to get a bacterial cell pellet. The supernatant was aspirated off with a sterile micropipette; the cells were resuspended in 100 microliters cold cell suspension buffer, made up of Tris Base, NaCL, and EDTA at pH 7.2 (Appendix 12).

To the cell suspension, 10 microlitres of lysozyme (25 mg/ml) was added and mixed gently to help in the digestion of bacterial cell wall. About 100 micro liters of 2 % Clean Cut Agarose (PFGE) previously melted in sterile distilled water and cooled to 50°C was mixed gently with the cell suspension. The mixture was poured into mould plugs care being taken not to create air bubbles, 100 micro liters per well, (two wells
per sample) for the two restriction enzymes to be used and the agarose left to solidify at room temperature for 45 minutes.

The solidified agarose plugs were transferred to 5ml sterile culture tubes, one for each sample, one ml of lysis buffer (Tris Base, NaCL sodium deoxycholate, sordium Lauryl Sarcosine) (Appendix 13) and 60 micoliters of lysozyme (25mg/ml) was added to each tube and mixed gently by inversion and incubated at 37°C for 3 hours. The lysis buffer was aspirated, using a sterile disposable pipette and the plugs washed in 1.5 ml, x 1 Wash Buffer (Tris Base pH 8 and EDTA (Appendix 14). The Wash Buffer was aspirated off, 1 ml Proteinase K Buffer (EDTA, Sodium deoxycholate, Sodium lauryl Sarcosine) (Appendix 15) and 40 microliters of Proteinase K enzyme at a concentration of 25mg per ml of buffer was added to digest cell proteins and the plugs incubated at 37°C overnight.

Proteinase K Buffer was aspirated off and the plugs washed in x 1 wash buffer for 1 hour at room temperature with gentle shaking followed by a second wash in 1.5 ml of x1 Wash Buffer and 40 microliters of 100 mM PMSF (Appendix 16) for 1 hour at room temperature with gentle shaking repeated twice. The wash buffer was aspirated off, the plugs transferred to sterile micro centrifuge tubes and further washed in 0.5 ml of x 0.1 Wash Buffer and were finally ready for enzyme digestion.

The wash buffer was aspirated and 0.5ml of sure Cut buffer added, incubated at room temperature for 1 hour with gentle shaking. The buffer was aspirated; the plugs were then digested overnight with XbaI and SpeI restriction enzymes, 25 units of each
enzyme per plug in 300 microliters of *sure cut* restriction Buffer (Roche Diagnostics, GmbH, Germany).

The restricted DNA fragments were separated by PFGE; briefly thin slices of the agarose plugs were cut and loaded onto horizontal 1% agarose (PFGE) gel in distilled water. PFGE was performed in a CHEF-DR III system, Bio-Rad, (Richmond, CA, USA) for 30 hours in 0.5 TBE (Tris Borate EDTA) buffer, field angle 120° temperature 4° C, voltage 6v/cm, starting pulse time 4 seconds and ending at 40 seconds. Lambda PFGE DNA markers (50-1000 kb) were used as DNA size markers. The gel was stained in Ethidium bromide 0.5mg/ml, visualized and digitally recorded with Gel DOC 2,000 (Bio-Rad) Digest patterns were analyzed by visual comparison of the printed gel images. The restriction enzyme digest patterns were interpreted by considering migration distance, intensity of all visible bands and by following the guidelines described by Tenover *et al.* (1995).
2.5 Data analysis

Descriptive data such as variation among Salmonella species, prevalence of Salmonella among different age groups, sexes and locations and the sensitivity profiles were analysed using chi-square SPSS (Statistical Package for the Social Sciences) version 11.5. Quantitative data such as variation in minimum inhibitory concentrations were analysed using ANOVA in SSPS. The degree of polymorphism was quantified using Shannons's index of phenotypic diversity. Genetic relationships were inferred using dendrogram based on Nei's UPGMA modified from NEIGHBOR procedure of PHYLIP version 3.5. The phenetic tree were visualized using program MEGA (Molecular Evolutionary Genetic Analysis) version 2.1 of Kumar et al., (2001)
CHAPTER THREE

RESULTS

3.1 Prevalence of *Salmonella* species in Kilifi and Nairobi

The prevalence of *Salmonella* species was assessed in 2212 children with ages ranging from 1 day to 5 years sampled from Nairobi and Kilifi. A total of 1296 (58.1 %) were sampled from Nairobi while 916 (41.4 %) from Kilifi. One hundred and forty eight (6.7 %) children were found infected by *Salmonella* species (NTS). Three different non typhi *salmonella* (NTS) species were identified namely; *S. typhimurium*, 99 (66.9 %), *S. enteritidis* 43 (29.1 %) and other *Salmonella* spp 6 (4.1 %). Of the 148 NTS isolates identified, 118 (79.7 %) were found in Nairobi while 30 NTS (20.3 %) were isolated from Kilifi. Out of the 118 *Salmonella* species isolated in Nairobi 83 (70.3 %) were *S. typhimurium*, 31 (26.2 %) were *S. enteritidis* while other *Salmonella* spp accounted for 4 (3.4 %) of the total isolates. Similarly out of the 30 *Salmonella* species isolated in Kilifi 16 (53.3 %) were *S. typhimurium*, 12 (40 %) were *S. enteritidis* while other *Salmonella* spp accounted for 2 (6 %) of the total isolates (Fig 3.1). There were no significant differences in the prevalence of the three salmonella species isolated in Nairobi and Kilifi ($\chi^2 = 1.406; \text{df} = 2; P = 0.493$).
Figure 3.1: The prevalence of *Salmonella* species in Nairobi and Kilifi

3.1.1 Prevalence of *Salmonella* infections among different age groups

Children from the two locations were classified into 5 different age groups; group 1 (1 day – 1 year), group 2 (1 – 2 years), group 3 (2 – 3 years), group 4 (3 – 4 years) and group 5 (4 – 5 years). Out of the 148 children infected, 60 (40.5 %) were in group 1 while 38 (25.7 %), 21 (14.2 %), 23 (15.5 %) and 6 (4.1 %) were in groups 2, 3, 4, and 5 respectively.
Seventy percent of the children in group 1 were infected with *S. typhimurium*, 26.7% with *S. enteritidis* and (3.3%) with other *Salmonella spp.* Among the children in group 2, (73.7%) were infected with *S. typhimurium* and (26.3%) with *S. enteritidis*, there was no *Salmonella spp* infection in this group. Children in group 3 were infected with *S. typhimurium*, (76.2%), *S. enteritidis* (19.1%) and other *Salmonella spp* (4.8%). Infection cases in group 4 were *S. typhimurium* (43.5%), *S. enteritidis* (43.5%) and *Salmonella spp* (13%) while group 5 had *S. typhimurium* (50%), *S. enteritidis* (50%) and infections with no *Salmonella spp*. (Fig.3.2).

There was significant difference in the prevalence of *Salmonella* infections between the ages (0-1 years) and ages (4-5 years), ($\chi^2 = 14.08; \text{ df } = 8; P = 0.04$). Children in age group 0-1 year had higher prevalence of non typhi *Salmonella* infections. There was however no significant difference in the prevalence among the other age groups.
Figure 3.2: Variation in prevalence of salmonella infections among children’s age groups

3.1.2 Prevalence of *Salmonella* infections between different sexes

Out of the 148 infected children 81 (54.7 %) were males and 67 (44.3 %) were females. Out of 99 *S.typhimurium* infections 51 (51.5 %) were found in males while 48 (48.5 %) females were infected. Similarly 26 (60.5 %) infections out of 43 *S. enteritidis* isolated were found in males and 17 (39.5 %) in females. However 2 (33.3 %) out of 6 other *Salmonella spp* infected males while 4 (66.7 %) infected females (Fig 3.3). The infection prevalence between males and females were not significantly different ($\chi^2 = 3.605; df = 2; P = 0.265$).
3.2 Susceptibility profile of *Salmonella* isolates in the study areas

The 148 *Salmonella* isolates, 118 from Nairobi and 30 from Kilifi were analyzed for their antimicrobial susceptibility (Table 3.1) against ampicillin, co-trimoxazole tetracycline, chloramphenical, gentamicin, nalidixic acid, amoxycillin-clavulanic acid, ceftriaxone, and ciprofloxacin, using the disk diffusion method. A total of 67 isolates from both study areas were sensitive to ampicillin, of which 39 (33.1 %) and 28 (93.3
% were from Nairobi and Kilifi respectively while 81 were resistant to ampicillin, where 79 (85.9 %) and 2 (6.7 %) were from Nairobi and Kilifi respectively. More isolates from Kilifi were sensitive to ampicillin compared to those from Nairobi ($\chi^2 = 19.195; \text{df} = 1; P = 0.0001$). Similarly, 73 NTS isolates were sensitive to co-trimoxazole, of which 45 (38.1 %) and 28 (93.3 %) were from Nairobi and Kilifi respectively while 75 were resistant, of which 73 (61.9 %) and 2 (6.7 %) from Nairobi and Kilifi respectively.

*Salmonella* species isolated from Kilifi were more sensitive to co-trimoxazole than those from Nairobi ($\chi^2 = 20.485; \text{df} = 1; P = 0.0001$). One hundred and thirty bacterial isolates were sensitive to tetracycline of which 101 (85.6 %) and 29 (96.7 %) were from Nairobi and Kilifi respectively while 18 were resistant out of which 17 (14.4 %) and 1 (3.3 %) from Nairobi and Kilifi respectively but there was no significant difference in sensitivity patterns between the isolates. ($\chi^2 = 3.14; \text{df} = 1; P = 0.076$). One hundred and five NTS were sensitive to chloramphenical of which 75 (63.6 %) and 29 (96.7 %) were isolated from Nairobi and Kilifi respectively while 43 were resistant of which 42 (36.4 %) and 1 (3.3%) from Nairobi and Kilifi respectively. The difference in sensitivity patterns between the two locations was significant ($\chi^2 = 5.278; \text{df} = 1; P = 0.022$). NTS from Kilifi were more sensitive to chloramphenical.

One hundred and twenty eight NTS were sensitive to gentamicin of which 98 (83.1 %) and 30 (100 %) from Nairobi and Kilifi respectively while 20 (16.9 %) all from Nairobi were resistant. There was no significant difference in sensitivity profiles between the two locations ($\chi^2 = 2.842; \text{df} = 1; P = 0.092$). Similarly one hundred and forty six
isolates were sensitive to nalidixic acid of which 116 (98.3 %) and 30 (100 %) were from Nairobi and Kilifi respectively while 2 (1.7 %) all from Nairobi were resistant, but difference in sensitivity profiles was not significant ($\chi^2 = 0.772$; df = 1; P = 0.38). One hundred and forty four isolates were sensitive to amoxycillin-clavulanic acid of which 114 (96.6 %) and 30 (100 %) were from Nairobi and Kilifi respectively while 4 (3.4 %) all from Nairobi were resistant.

There was no significant difference in sensitivity profiles between the two locations ($\chi^2 = 0.102$; df = 1; P = 0.749). One hundred and forty five isolates were sensitive to ceftriaxone of which 115 (97.5 %) and 30 (100 %) from Nairobi and Kilifi while 3 (2.5 %) all from Nairobi were resistant, and there was no significant difference in sensitivity profiles between the two locations ($\chi^2 = 0.014$; df = 1; P = 0.906). All the 148 isolates from the two locations were sensitive to ciprofloxacin.
Table 3.1: Proportion of resistant NTS isolates (in percentages) from Nairobi and Kilifi.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Antibiotic resistance %</th>
<th>Total %</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Nairobi %</td>
<td>Kilifi %</td>
<td></td>
</tr>
<tr>
<td>Ampicilin</td>
<td>66.9</td>
<td>6.7</td>
<td>54.7</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>61.8</td>
<td>6.7</td>
<td>50.7</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>14.4</td>
<td>3.3</td>
<td>12.2</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>36.4</td>
<td>3.3</td>
<td>29.1</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16.9</td>
<td>0</td>
<td>13.5</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>1.7</td>
<td>0</td>
<td>1.4</td>
</tr>
<tr>
<td>Amoxy-clavulanic Acid</td>
<td>3.4</td>
<td>0</td>
<td>2.7</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>2.5</td>
<td>0</td>
<td>2.1</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>
Figures 3.4 A & B, Multi-drug resistance shown by NTS, *S. typhimurium* by Disk diffusion method, figure 4 A, and by Minimum Inhibitory Concentration (MIC) figure 4 B. Growth around the disk/strip means resistance, and clear area means sensitive.
Figures 3. 5 A & B: full sensitivity shown by NTS, *S. enteritidis* by disk diffusion figure 5A and by Minimum inhibitory concentration (M IC) Figure 5B, clear zones around the disk/strip means sensitivity.
3.2.2 Susceptibility profiles of *Salmonella* isolates among the different age groups

The sensitivity patterns of the NTS species isolated from the 5 age groups are shown in Table 3.2. In general 67 (45.3 %) and 81 (54.7 %) were sensitive and resistant respectively to ampicillin, but there were no significant difference in sensitivity patterns among the age groups ($\chi^2 = 6.233; \text{df} = 4; P = 0.182$). Of the 148 *Salmonella* species 73 (49.3 %) and 75 (50.7 %) were sensitive and resistant respectively to co-trimoxazole but there was no significant difference ($\chi^2 = 5.089; \text{df} = 4; P = 0.278$).

Similarly 130 (87.8 %) and 18 (12.2 %) bacterial isolates were sensitive and resistant respectively to tetracycline but difference was not significant ($\chi^2 = 4.763; \text{df} = 4; P = 0.312$). A total of 105 (70.9 %) and 43 (29.1 %) *Salmonella* isolates were sensitive and resistant respectively to chloramphenical, however the difference was not significant ($\chi^2 = 8.906; \text{df} = 4; P = 0.063$). There was no significant difference between the resistant and sensitive non typhi salmonella isolates to gentamicin, nalidixic acid amoxy-clavulanic acid ceftrixone and ciprofloxacin among the different age groups.
Table 3.2: Proportion of NTS isolates presented as percentage (%) resistant to various antibiotics among the different age groups.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>0 - 1</th>
<th>1 - 2</th>
<th>2 - 3</th>
<th>3 - 4</th>
<th>4 - 5</th>
<th>Total</th>
<th>P.Value</th>
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<tbody>
<tr>
<td>Ampicillin</td>
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<td>50.0</td>
<td>50</td>
<td>40.9</td>
<td>50</td>
<td>54.7</td>
<td>0.182</td>
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<tr>
<td>Co-trimoxazole</td>
<td>63.8</td>
<td>34.2</td>
<td>41.7</td>
<td>50</td>
<td>50</td>
<td>50.7</td>
<td>0.278</td>
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<tr>
<td>Tetracycline</td>
<td>13.8</td>
<td>13.2</td>
<td>8.3</td>
<td>9.1</td>
<td>16.7</td>
<td>12.2</td>
<td>0.312</td>
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<tr>
<td>Chloramphenical</td>
<td>37.9</td>
<td>28.9</td>
<td>33.3</td>
<td>9.1</td>
<td>0</td>
<td>29.1</td>
<td>0.063</td>
</tr>
<tr>
<td>Gentamicin</td>
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<td>7.9</td>
<td>8.3</td>
<td>13.6</td>
<td>4.3</td>
<td>13.5</td>
<td>0.776</td>
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<td>Nalidixic Acid</td>
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<td>Amoxy-clavulanic acid</td>
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<td>2.1</td>
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<tr>
<td>Ciprofloxcin</td>
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<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1000</td>
</tr>
</tbody>
</table>
3.2.3 Susceptibility profiles of *Salmonella* isolates between sexes

The 80 NTS isolated from males and 68 from females were analyzed to determine their susceptibility to different antimicrobials. (Table 3.3). There was significant difference in susceptibility profiles against amoxy-clavulanic acid ($\chi^2=4.929$; df; =1; P =0.026), females were more sensitive (100%) than males (95%) but there was no significant difference to the other antimicrobials tested.

Table 3.3: Proportion of resistant *Salmonella* isolates between the sexes presented as percentage (%)

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sex and the % resistance</th>
<th></th>
<th></th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Male</td>
<td>Female</td>
<td>Total</td>
<td></td>
</tr>
<tr>
<td>Ampicillin</td>
<td>53.7</td>
<td>55.9</td>
<td>54.7</td>
<td>0.908</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>51.2</td>
<td>50</td>
<td>50.7</td>
<td>0.461</td>
</tr>
<tr>
<td>Tetracyline</td>
<td>10</td>
<td>14.7</td>
<td>12.2</td>
<td>0.430</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>31.2</td>
<td>29.4</td>
<td>29.1</td>
<td>0.858</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>16.2</td>
<td>10.3</td>
<td>13.5</td>
<td>0.152</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>1.5</td>
<td>1.5</td>
<td>1.4</td>
<td>0.011</td>
</tr>
<tr>
<td>Amoxy-clavulanic</td>
<td>5</td>
<td>0</td>
<td>2.7</td>
<td>0.026</td>
</tr>
<tr>
<td>Ceftiaxone</td>
<td>2.5</td>
<td>1.5</td>
<td>2.1</td>
<td>0.434</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1000</td>
</tr>
</tbody>
</table>
3.2.4 Variation in susceptibility profiles of *Salmonella* species

The variation in susceptibility profiles among the three NTS species, (99 *S. typhimurium*, 43 *S. enteritidis* and 6 other *Salmonella species*) isolated from both study areas was assessed (Table 3.4). Generally 67 (45.3 %) and 81 (54.7 %) were sensitive and resistant respectively to ampicillin. *S. enteritidis* and other *Salmonella species* were more sensitive to ampicillin than *S. typhimurium* ($\chi^2 = 6.206$; df = 2; $P = 0.045$). Seventy three 73 (49.3 %) bacterial isolates were sensitive while 75 (50.7 %) were resistant to co-trimoxazole *S. enteritidis* and other *Salmonella spp.* were more sensitive than *S. typhimurium* to co-trimoxazole ($\chi^2 = 8.343$; df = 2; $P = 0.015$). Similarly 130 (87.8 %) and 18 (12.2 %) bacterial isolates were sensitive and resistant respectively to tetracycline.

*Salmonella enteritidis* and other *Salmonella species* were more sensitive to tetracycline than *S. typhimurium* ($\chi^2 = 10.279$; df = 2; $P = 0.006$). One hundred and five (70.9 %) NTS were sensitive to chloramphenicol but with no significant difference in their sensitivity patterns among the *Salmonella* species ($\chi^2 = 1.836$; df = 2; $P = 0.399$). One hundred and twenty eight (86.5 %) NTS and 20 (13.5 %) were sensitive and resistant respectively to gentamicin. *S. enteritidis* and other *Salmonella species* were more sensitive to gentamicin than *S. typhimurium* ($\chi^2 = 6.737$; df = 2; $P = 0.034$). There were no significant differences in susceptibility profiles among the NTS to nalidixic acid, amoxy-clavulanic acid, ceftriaxone and ciprofloxacin.
Table 3.4: Resistance profiles in percentages (%) among the three *Salmonella* species

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th><em>S. typhimurium</em></th>
<th><em>S. enteritidis</em></th>
<th><em>Salmonella</em> spp</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>62.6</td>
<td>39.5</td>
<td>54.3</td>
<td>54.7</td>
<td>0.045</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>57.6</td>
<td>37.2</td>
<td>33.3</td>
<td>50.7</td>
<td>0.015</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>7.1</td>
<td>23.3</td>
<td>16.7</td>
<td>12.2</td>
<td>0.006</td>
</tr>
<tr>
<td>Chloramphenical</td>
<td>32.3</td>
<td>23.3</td>
<td>16.7</td>
<td>29.1</td>
<td>0.399</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>17.2</td>
<td>4.7</td>
<td>0</td>
<td>13.5</td>
<td>0.034</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2.1</td>
<td>0</td>
<td>0</td>
<td>1.4</td>
<td>0.254</td>
</tr>
<tr>
<td>Amoxy-clavulanic acid</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>2.7</td>
<td>0.254</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>3.6</td>
<td>0</td>
<td>0</td>
<td>2.1</td>
<td>0.383</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.1000</td>
</tr>
</tbody>
</table>
3.3.1 Variation in Minimum Inhibition Concentration (MIC) by study areas

The Minimum inhibitory concentration values for all the 118 non typhi salmonella isolated from Nairobi and 30 from Kilifi were determined against ampicillin, co-trimoxazole, tetracycline, chloramphenicol, gentamicin, amoxycillin-clavulanic acid, ceftriaxone, nalidixic acid and ciprofloxacin (Table3. 5). The MIC values, in micrograms per milliliter(µg/mL) for the bacterial isolates from the two locations varied significantly for ampicillin, [(F = 46.597; DF = 1; P < 0.0001), co-trimoxazole (F = 40.613; DF = 1; P < 0.0001) and chloramphenical. (F = 14.405; df = 1; P < 0.0001) Isolates from Nairobi had higher MIC values. However there were no significant variations in MIC values against tetracycline, gentamicin, amoxycillin-clavulanic acid, ceftriaxone, nalidixic acid and ciprofloxacin.
Table 3.5: Minimum Inhibition Concentration (MIC) range values in μg/mL, among bacteria isolated from two locations

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Location and MIC ranges</th>
<th>Nairobi</th>
<th>Kilifi</th>
<th>p.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td></td>
<td>1 – 256</td>
<td>1 - 256</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td></td>
<td>0.047 – 32</td>
<td>0.047 - 32</td>
<td>&lt; 0.0001</td>
</tr>
<tr>
<td>Tetracycline</td>
<td></td>
<td>0.75 – 256</td>
<td>1.5 – 256</td>
<td>0.051</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td></td>
<td>1.5 – 256</td>
<td>1.5 – 4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Gentamicin</td>
<td></td>
<td>0.38 – 256</td>
<td>0.094 – 0.38</td>
<td>0.144</td>
</tr>
<tr>
<td>Amoxy-clavulanic Acid</td>
<td></td>
<td>0.5 – 256</td>
<td>0.5 – 6</td>
<td>0.194</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td></td>
<td>0.023 – 32</td>
<td>0.032 – 0.125</td>
<td>0.179</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td></td>
<td>2 – 256</td>
<td>2 - 6</td>
<td>0.392</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td></td>
<td>0.006 – 0.25</td>
<td>0.008 – 0.023</td>
<td>0.206</td>
</tr>
</tbody>
</table>
3.3.2 Variation in Minimum Inhibition Concentration (MIC) with sexes

The MIC values of the 81 and 67 NTS isolated from males and females respectively were compared and the results are shown in Table 3.6. There were significant differences in MIC values among NTS isolated from the two sexes against tetracycline ($F = 6.376; \text{df} = 1; P = 0.013$). NTS isolated female patients had higher MIC values against tetracycline and therefore more resistant than those from male patients. However there was no significant variation in the MIC of the NTS against all the other antimicrobial drugs tested.

Table 3.6: Minimum Inhibition Concentration (MIC) range values in $\mu$g/mL, of *Salmonella* isolated from male and female children.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Male</th>
<th>Female</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>0.25 – 256</td>
<td>1 - 256</td>
<td>0.538</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>0.023 – 256</td>
<td>0.094 - 32</td>
<td>0.455</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>0.75 – 256</td>
<td>2 - 256</td>
<td>0.013</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.5 – 256</td>
<td>1.5 – 256</td>
<td>0.965</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.038 – 256</td>
<td>0.125 – 256</td>
<td>0.958</td>
</tr>
<tr>
<td>Amoxy-clavulanic Acid</td>
<td>0.038 – 256</td>
<td>0.5 – 24</td>
<td>0.158</td>
</tr>
<tr>
<td>Ceftriaxone</td>
<td>0.047 – 32</td>
<td>0.032 – 32</td>
<td>0.915</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2 – 30</td>
<td>2 - 256</td>
<td>0.133</td>
</tr>
<tr>
<td>Ciorofloxacin</td>
<td>0.008 – 0.064</td>
<td>0.008 – 0.25</td>
<td>0.1000</td>
</tr>
</tbody>
</table>
3.3.3 Variation in Minimum Inhibition Concentration (MIC) of the three \textit{Salmonella} species

The MIC values for 99 \textit{S. Typhimurium}, 43 \textit{S. Enteritidis} and 6 (4.1 \%) \textit{Salmonella species} were determined and results are shown in Table 3.7. There were significant differences in MIC values among the three species against ampicillin \{(F = 3.026; df = 2; P = 0.031), co-trimoxazole (F = 4.200; df = 2; P = 0.007), and tetracycline (F = 4.715; df = 2; P = 0.004). However there were no significant difference in the MIC values among the three isolates against chloramphenical, gentamicin, augmenting ceftriaxone, nalidixic and ciprofloxacin.

\textbf{Table 3.7: MIC range values in \(\mu g /ml\) of the three species of \textit{Salmonella} isolates}

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>\textit{S. typhimurium}</th>
<th>\textit{S. enteritidis}</th>
<th>\textit{Salmonella spp}</th>
<th>P.value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1 - 256</td>
<td>1 - 256</td>
<td>1 - 256</td>
<td>0.031</td>
</tr>
<tr>
<td>Co-trimoxazole</td>
<td>0.094 - 32</td>
<td>0.0094 - 32</td>
<td>0.19 - 32</td>
<td>0.007</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>1 - 256</td>
<td>1 - 256</td>
<td>2 - 256</td>
<td>0.004</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>1.5 - 256</td>
<td>0.38 - 256</td>
<td>1.5 - 256</td>
<td>0.575</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.019 - 256</td>
<td>0.094 - 8</td>
<td>0.125 - 3</td>
<td>0.940</td>
</tr>
<tr>
<td>Amoxy-clavulanic acid</td>
<td>0.5 - 256</td>
<td>0.038 - 12</td>
<td>0.75 - 12</td>
<td>0.480</td>
</tr>
<tr>
<td>Ceftrixone</td>
<td>0.047 - 32</td>
<td>0.047 - 16</td>
<td>0.032 - 0.094</td>
<td>0.576</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2 - 256</td>
<td>2 - 256</td>
<td>3 - 6</td>
<td>0.931</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.008 - 0.025</td>
<td>0.008 - 0.064</td>
<td>0.012 - 0.023</td>
<td>0.872</td>
</tr>
</tbody>
</table>
3.3.4 Variation in Minimum Inhibition Concentration (MIC) with age groups

The MIC values of the NTS isolated from the 5 age groups were determined (Table 3.8) among the 60 NTS isolated from age group 1, 38 NTS from age group 2, 21 NTS from age group 3, 23 NTS from age group 4, and 6 NTS from age group 5. There were no significant differences in the MIC values among bacteria isolated from the five age groups against all the antimicrobials tested.

Table 3.8: Minimum Inhibition Concentration (MIC) ranges in μg/mL among Salmonella species isolated from different age groups.

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>0 – 1</th>
<th>1 – 2</th>
<th>2 – 3</th>
<th>3 – 4</th>
<th>4 – 5</th>
<th>P. value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ampicillin</td>
<td>1 – 256</td>
<td>1.5 – 256</td>
<td>0.25 – 256</td>
<td>0.75 – 256</td>
<td>1 – 256</td>
<td>0.419</td>
</tr>
<tr>
<td>Cotrimoxazole</td>
<td>0.047 – 32</td>
<td>0.047 – 32</td>
<td>0.023 – 32</td>
<td>0.064 – 32</td>
<td>0.125 – 32</td>
<td>0.441</td>
</tr>
<tr>
<td>Tetracycline</td>
<td>2 – 256</td>
<td>1.5 – 256</td>
<td>0.75 – 256</td>
<td>1 – 256</td>
<td>2 – 8</td>
<td>0.068</td>
</tr>
<tr>
<td>Clorampenicol</td>
<td>1.5 – 256</td>
<td>1.5 – 256</td>
<td>1.5 – 256</td>
<td>1 – 256</td>
<td>2 – 256</td>
<td>0.364</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>0.094 – 256</td>
<td>0.0125 – 1</td>
<td>0.038 – 16</td>
<td>0.019 – 256</td>
<td>0.38 – 8</td>
<td>0.304</td>
</tr>
<tr>
<td>Ceftiaxone</td>
<td>0.38 – 4</td>
<td>0.5 – 16</td>
<td>0.5 – 256</td>
<td>0.75 – 256</td>
<td>1 – 12</td>
<td>0.326</td>
</tr>
<tr>
<td>Amoxy-clavulanic acid</td>
<td>0.023 – 34</td>
<td>0.0147 – 0.19</td>
<td>0.047 – 32</td>
<td>0.047 – 24</td>
<td>0.047 – 0.125</td>
<td>0.549</td>
</tr>
<tr>
<td>Nalidixic acid</td>
<td>2 – 256</td>
<td>2 – 256</td>
<td>2 – 12</td>
<td>3 – 8</td>
<td>2 – 6</td>
<td>0.896</td>
</tr>
<tr>
<td>Ciprofloxacin</td>
<td>0.008 – 0.047</td>
<td>0.008 – 0.25</td>
<td>0.012 – 0.064</td>
<td>0.008 – 0.064</td>
<td>0.012 – 0.023</td>
<td>0.955</td>
</tr>
</tbody>
</table>
3.4 Plasmid profiles

Fifty non-typhi salmonellae isolates that were resistant to 3 or more antimicrobials from Nairobi and 40 fully sensitive NTS from Nairobi and Kilifi were selected for plasmid profiling. Large (147 - 63 kbp) and small (43.5 - 6.9 kbp) plasmids were found in the resistant and sensitive NTS isolates. Results were as given on figures 3.6 and 3.7. From the 50 multidrug resistant NTS isolates in Nairobi, twenty had 63-147 kbp plasmid and other smaller plasmids of 6.9-43.5 kbp, 22 had the 43.5-63 kbp plasmids and 5 had no plasmids at all. Twenty fully sensitive NTS isolates from Nairobi had both large and small plasmids, 3 NTS isolates had the 63-147 kbp plasmid and other small plasmids. Ten isolates had the 43.5-63 kb plasmid and smaller plasmids, 5 isolates had no plasmids at all. The plasmid profiles of 20 fully sensitive NTS isolates from Kilifi were; 3 had the 43.5-63 kbp plasmid and other small plasmids of less than 6.9kb, 3 NTS had the 6.9-43.5 kbp plasmid but 10 NTS had no plasmids at all. None of the NTS isolates from Kilifi had the large plasmid, 63-147 kbp. It was not possible to carry out conjugation tests to determine if any of these large plasmids from the multidrug resistant NTS were transmissible and also confer resistance to sensitive recipient bacteria, usually *Escherichia coli* K12.
Figure 3.6: Plasmid profile of sensitive non-typhi salmonella, lanes 1 -11, lanes 12, DNA size markers I (39R86) with corresponding sizes.

Figure 3.7: Plasmid profile of multidrug resistant non-typhi salmonella, lanes 1-11 and molecular size marker R39 in lane 12.
3.5 Genetic relationship using PFGE

3.5.1 PFGE amplification products

The PFGE profiles of some *Salmonella typhimurium* and *Salmonella enteritidis* samples are shown in figures 3.8, 3.9, 3.10 and 3.11 below. Figure 8 shows the PFGE patterns of NTS isolates from children (cases) and their environment (contacts). Similar banding patterns were observed among the cases and contact groups. In this figure one banding pattern was observed for *S. typhimurium*, case and contact and one for *S. enteritidis*, within the internal standard DNA size marker.

![Figure 3.8: Gel electrophoresis of Restriction Fragment Length Polymorphism analysis of *Salmonella* isolate (case and contact) DNA using *spel* separated on PFGE. Lanes 1 through 9 represents isolates Lane 1, 5598- *S. typhimurium* from child in Nairobi and Lane 2, 5598c- *S. typhimurium* from family member of child 5598. Lane 3, 5563- *S. typhimurium* from child in Nairobi and lane 4, 5563c- *S. typhimurium* from family member of child 5563. Lane 5, 5703 *S. enteritidis* from child in Nairobi and lane 6, 5703c, *S. Enteritidis* of family member of child 5703. Lane 7, 5884- *S. enteritidis* of child from Nairobi and lane 8, 5884c- *S. enteritidis* of family member of child 5884. MW is the molecular size marker (PFGE marker 1. λ ladder).
Figure 3.9 shows PFGE fragment digest patterns of NTS isolated from samples collected from children and their siblings. The majority case and contact samples had similar banding profiles as seen in lanes 5, 6, 7, 8, 9, 12 and 13 for *S. typhimurium*. There were also similar banding profiles among *S. enteritidis*, (lanes 3, 4, 10, 7&11, cases and contacts). However lane 1 and 2, though both *S. typhimurium* differed in their banding profiles from other *S. typhimurium*. In this figure there was one banding pattern for *S. enteritidis*, and two banding patterns for *S. typhimurium* within the internal standard DNA size marker.

![PFGE gel electrophoresis of *Salmonella* isolate (case and contact) DNA using *speI* separated on PFGE. Lanes 1 through 13 represent isolates Lane 1, 4105-*S. typhimurium* from child in Nairobi and lane 2, 4105c-also *S. typhimurium* from family member of child 4105. Lane 3, 4122-*S. enteritidis* from Child in Nairobi and lane 4, 4122c-*S. enteritidis* from family member of child 4122. Lane 5, 5314- *S. typhimurium* from child in Nairobi and lane 6, 5314c-*S. typhimurium* from family member of child 5314. Lane 7, 4672-*S. typhimurium* from child in Nairobi and lanes 8 4672c and lane 9, 4672cc, all *S. typhimurium* from family members of child 4622. Lane 10, 5107- *S. enteritidis* from child in Nairobi and lane 10, 5107-*S. enteritidis* of family member of child 5107. Lane 12, 5114-*S. typhimurium* from child in Nairobi and lane 13, 5314c-*S. typhimurium* of family member of child 5314. MW is the molecular weight marker (PFGE marker 1.λ ladder).
Figure 3.10 shows PFGE patterns of NTS, *S. typhimurium* and *S. enteritidis* from different children in Nairobi. Lanes 1, 3, 4, 5 and 10 are all *S. enteritidis*, and have similar banding profiles. Lanes 2, 6, 7, 8, and 9 are all *S. typhimurium*, lanes 7 and 8 have identical banding patterns, lanes 2 and 9 have similar banding patterns. Lane 6 is closely related to the other *S. typhimurium* by its banding pattern. One banding pattern for *S. enteritidis* was observed in this picture while three banding patterns for *S. typhimurium* were observed.

*Figure 3.10: Gel electrophoresis of Restriction Fragment Length Polymorphism analysis of Non typhi salmonella isolate DNA using *speI* separated on PFGE in lanes 1 through 10. Lanes 1, 3, 4, 5, and 10 are all *S. enteritidis* isolated from children in Nairobi. Lanes 2, 6, 7, 8, and 9 are all *S. typhimurium* isolated from children in Nairobi. MW molecular weight marker (PFGE marker 1. λ ladder*
Figure 3.11 shows PFGE fragment digest patterns of *S. typhimurium* and *S. enteritidis* isolated from different children in Kilifi. Majority had identical banding patterns as seen in lanes 1, 2, 5, 9, 10, 11 and 12); lanes 3 and 4 have similar banding patterns and are all *S. typhimurium*. Lanes 6, 7 and 8 are *S. enteritidis*, lanes 6 and 8 have similar banding patterns but lane 7 differs slightly from them and has a different pattern. There were two banding patterns for *S. enteritidis* and three-banding patterns for *S. typhimurium*.

Figure 3.11: Gel electrophoresis of Restriction Fragment Length Polymorphism analysis of Non typhi *Salmonellae* DNA using *SphI* separated on PFGE in Lanes 1 through 13. Lanes 1, 2, 3, 4 and 5 are all *S. typhimurium* from children in Kilifi, lanes 6, 7 and 8 are *S. enteritidis* from children in Kilifi and lanes 9, 10, 11, 12, and 13 are *S. typhimurium* from children in Kilifi. MW is the molecular weight marker (PFGE marker 1. λ ladder).
CHAPTER FOUR

4. Discussion, Conclusion and Recommendations

In this study a total of 2212 blood samples were drawn from children under 5 years of age, admitted with bacteremia to hospitals in Nairobi and Kilifi. The overall prevalence of non typhi salmonellae (NTS) in the two study areas was 6.7%. The prevalence did not differ significantly by study area and sex; it however differed significantly between age group 1 (1 day -1 year) and age group 5 (4-5 years). Children in age group 1 had a much higher prevalence of NTS infection than age group 5. Younger children less than 12 months, by virtue of their age, and also by the fact that they are handled more are believed to be at higher risk of infection with not only NTS bacteremia but many other bacterial infections. Children at 6 months and 24 months are at the highest risk of NTS infection with a peak age of 10-14 months. Indeed age is a risk factor for mortality (Graham et al.; 2000, Green et al., 1993). There is also the risk of chronically or recently infected mothers infecting their neonates and infants (Wilson et al., 1982). Health care workers who are themselves healthy carriers of NTS can also be the sources of infections for the very young and overcrowding in hospitals can facilitate nosocomial transmission (Kumar et al., 1995).

This prevalence is lower than reported in other studies, but it is close to that of 11.5% reported by Kariuki et al, (1996) in Nairobi while a prevalence of 27.8% was reported in Kilifi by Oundo (2000). In Malawi an NTS prevalence of 38 % was reported during a 1 year period (Walsh et al., 2000). The actual prevalence of NTS in Nairobi and Kilifi may actually be higher than the one recorded in this study for several reasons; Firstly
Kilifi is a malaria endemic zone and therefore any non specific febrile illness in the presence of malaria parasite will be treated as malaria. Secondly it is possible that the children were treated with antimicrobial drugs before they were brought to hospitals, or depending on the condition of the child upon admission, antimicrobial treatment was instituted before blood specimen for culture was taken, resulting in lower prevalence values for NTS. It is recommended by the World Health Organization (Vandepitte et al., 1991) that where possible blood for culture should be taken before antimicrobial treatment is started. Blood cultures are positive for NTS during the peak infective stage, i.e. the first week of infection and it is possible that some children were brought to hospitals after this mid first stage of bacteremia hence they were blood culture negative.

Two main serotypes of salmonella were isolated from children in the two study areas; *S. typhimurium* *S. enteritidis* and a small number of other *Salmonella species*. Similar observations have been made in studies in other countries in Africa (Lappage et al., 1987; Green and Cheesbrough, 1993). In previous work done in Kenya by Kariuki et. al (2004), *S. typhimurium* was the most prevalent in the years 1994 to 1996, constituting 78% of all the NTS isolated and between 1997 and 2003 *S. enteritidis* increased steadily to almost same prevalence as *S.typhimurium*. Although it appears like the isolation rate of *S. enteritidis* is increasing, in this study *S. typhimurium* still constituted the majority of NTS isolates from both areas, with the isolation rate of *S. typhimurium* twice that of *S. enteritidis*. In other reports *S. typhimurium* and *S. enteritidis* constituted majority of human cases of non typhi salmonella infections reported in the Centers for Disease Control (CDC) in USA. In Germany the same isolates accounted for more than
80% of human infections reported to the reference centre at the Robert Koch institute (Rabsch et al., 2001). Between 1996 and 1998 *S. typhimurium* was the most prevalent NTS (78%) followed by *S. enteritidis* (13%) in a study done on Malawian children (Graham et al., 2000). Similar findings have been reported elsewhere (Biendo et al., 2003). *S. typhimurium* was the most common serotype of *Salmonellae* isolated from humans and animals (Bender et al., 2001). It has been speculated that *S. typhimurium* maintains itself through human to human transmission while the other serotypes are introduced into the community through food vehicles and perhaps due to poor sanitary conditions and low levels of personal hygiene among the communities studied which would explain why the prevalence was higher than the rest. This study confirms that *S. typhimurium* is the most common cause of NTS bacteremia in Kenya and is also associated with a more severe form of invasive disease.

The antimicrobial susceptibility profiles of NTS isolated from Kilifi and Nairobi differed significantly. This study has demonstrated that in Nairobi the resistance to commonly available antimicrobials especially to ampicilin, chloramphenical, co-trimoxaxole and gentamicin is high. The corresponding minimum inhibitory concentrations against these antimicrobials were also high. In this study 59% of all the NTS from Nairobi were resistant to three or more antimicrobial agents. Lower levels of resistance to the other antimicrobials were also reported but there was no resistance to ciprofloxacin. The fact that there was no resistance to ciprofloxacin is an indication this drug has not been misused and that it can be used when the other drugs fail. The reason why it has not been abused is because it is not readily available over the counters
without a prescription and it is also expensive. These high levels of resistance have been reported in other studies in Kenya (Kariuki et al., 1997) and other countries (Lin-Hui Sui et al., 2004, Yang Jin Soo, 2001). Although the number of NTS isolates in Kilifi was small, lower levels of resistance was observed with a reduction from previously reported 56 %, (Oundo et al., 2000) to 6.7 % for ampicillin, co-trimoxazole (6.7 %), chloramphenical (3.3 %) and tetracycline (3.3 %). In Nairobi S. typhimurium were more resistant than S. enteritidis to ampicillin, co-trimoxazole and tetracycline. Similar findings have been reported elsewhere (Graham et al. 2000).

Increased prevalence and resistance of S. typhimurium presents a grave situation for Kenyan children as increased resistance may lead to more virulence, which has been associated with more morbidity and mortality (Helms et al., 2002). In a study in Rwanda, bacteremia due to S. typhimurium had higher hospital admission rates and higher case fatalities than those with bacteremia due to S. enteritidis (Lapange et al., 1987). typhimurium was also found to be more resistant than S. enteritidis. The high levels of MDR in Nairobi may be due to improper use of antimicrobials which are readily available over the counters in pharmacies even without a physician’s prescription. It also shows that people from Nairobi misuse the drugs more than the Kilifi people It is postulated that this misuse of drugs has led to excessive exposure of bacteria to antimicrobials rendering even otherwise sensitive bacteria resistant. The high levels of MDR in Nairobi are of grave concern as this may complicate treatment of NTS infections with adverse consequences such as treatment failure (Vassalo et al., 1998). Effective alternate treatment with second line antimicrobials, such as
ceftriaxone, amoxy-clavulanic acid, gentamicin and ciprofloxacin are often not available, not affordable or of limited availability to most patients and this may lead to treatment failure.

In Kenya it has been shown that there has been a marked rise in antimicrobial resistance among the non typhi salmonellae in the last ten years (Kariuki et al., 2004). In the developed countries the use of antibiotics for therapeutic or preventive purposes in veterinary medicine and as growth promoters in animal feed has been associated with antimicrobial résistance. There is evidence to suggest that the use of antimicrobial agents in food animals contributes to the development of antimicrobial resistant salmonella that cause human infections. However Kariuki et al. (2002) did not find any significant association between the NTS isolated from humans and animals, infact NTS from animals were not only different from the human isolates but they were also more susceptible to commonly available antimicrobials.

Results of plasmid analysis indicate that both multidrug resistant and fully sensitive NTS isolates from Nairobi had both large and small plasmids, 43-143 kb for large and 6.9-43.5 kb for the small plasmids. The large plasmids were however more than the small plasmids. In Kenya it has been reported that plasmids with a molecular size 98-100 kb are responsible for high levels of resistance in non typhi salmonella serotypes to several antimicrobials, such as ampicillin co-trimoxazole, chloramphenical and tetracycline. This resistance was also transferable to E. coli K 12 (Kariuki et al., 1996).
NTS isolates from Kilifi did not have the large plasmid of 63-143 kb, being a correlation to the low number of resistant *Salmonellae* found in this region.

PFGE patterns of *Xba* I and *Spe* I digested chromosomal DNA fragments revealed a close strain relationship between cases and their contacts. The 62 NTS isolates and their controls of each serotype produced indistinguishable PFGE restriction patterns with the same number of bands and the same molecular size in kilo base pairs (kbp). These were therefore considered to represent the same strain of NTS suggesting that they came from a common source. The fact that the cases and contacts had the same NTS would imply there was a common source of these infections, suggesting the presence of a reservoir within the child’s environment. The source could perhaps be a sibling, a parent or an asymptomatic carrier of NTS, domestic animals such as goats and chicken or even household pets such as cats and dogs. PFGE of digested chromosomal DNA of 37 *S. typhimurium* isolates from unrelated sources demonstrated that they were closely related with 2-3 band differences suggesting that they were subtypes of the same epidemiological strains.

4.2 Conclusions

- The prevalence of non typhi salmonellae from Nairobi and Kilifi was the same in the two geographically different areas;

- Two major serotypes of *Salmonella* i.e. *Salmonella enterica* serotype *Typhimurium* and *Salmonella enterica* serotype *Enteritidis* were the most prevalent NTS infections in Kilifi and Nairobi; and that *S. typhimurium* was the more prevalent NTS isolate from Kilifi and also from Nairobi.
• NTS isolates from Nairobi were highly resistant to commonly available antimicrobials, (ampicillin, co-trimoxazole, tetracycline and chloramphenical) as has been previously reported with corresponding levels of minimum inhibitory concentration.

• *Salmonella typhimurium* was not only the most prevalent but the most resistant to the antimicrobials.

• Isolates from children and their siblings shared similar serotypes and indistinguishable PFGE patterns, suggesting that they were from the same source and epidemiologically represented the same strain.

### 4.3 Recommendations

1. There is need for continuous surveillance of antimicrobial susceptibility for the emergence and spread of not only MDR serotypes of *Salmonella* but also for other pathogenic bacteria. More tests should be done to determine if the large plasmids were responsible for the high levels of resistance seen in NTS isolates from Nairobi.

2. Rapid investigation and identification of the NTS pathogens and antimicrobial susceptibility testing should be used to guide in the antimicrobial choice for treatment to reduce the morbidity and mortality associated with NTS infections.

3. Improper use of antimicrobials may have contributed to the development of
resistance and there is need therefore to educate people on the problems associated with misuse of any medication.

4. The close relatedness between NTS isolates from sick children and their contacts at home suggests that NTS infections were acquired and spread through food and water contaminated with human or animal faeces mainly through poor hygiene practices. Thus control of the infections should be through prevention and control by applying public health measures and provision of clean drinking water in these areas.

5. Malaria is believed to be a risk factor for NTS infection and it also presents the same symptoms as bacteremia, making it difficult to distinguish the two infections clinically. This calls for differential diagnosis, with malaria tests being done alongside other tests for bacteremia in Kilifi.
REFERENCES


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Failure of ciprofloxacin therapy in invasive non typhoidal Salmonellosis.

*Clinical Infectious Diseases*, **26**, 535-536.


*Plasmid* **17**, 3-12


APPENDICES

1. Biochemical tests

i) Urease Test

The test is used to determine the ability of an organism to produce the enzyme urease, which hydrolyses urea. The urease test helps in the identification of certain species of Enterobacteraceae. Urease enzyme hydrolyses substrate urea into ammonia, water, and carbon dioxide. The presence of the enzyme is determined by inoculating an organism to broth or agar that contains urea as the primary carbon source and detecting the production of ammonia. Ammonia increases the pH of the medium so its presence is readily detected using a common indicator (such as phenol red) of metabolic process and, the Ph shift is detected by the color change of indicator from light orange at pH 6.8 to magenta Ph 8.1.

Procedure

1) Streak the surface of a urea agar slant with a portion of a well isolated colony or inoculate slant with 1 to 2 drops from an overnight brain heart infusion broth culture.

2) Leave the cap on loosely and incubate tube at 35-37°C in ambient air for 48 hours to 7 days.

Expected results

Positive: Change in colour of slant from light orange to magenta

Negative: No colour change in media.
Quality control:

Known urea positive, *Proteus vulgaris* and urea negative *Escherichia coli* should always be included when doing the test.

Both *S.typhimurium* and *S.enteritidis* are urease negative.

ii) Citrate utilization test

This test is used to determine the ability of an organism to utilize sodium citrate as its only carbon source and inorganic ammonium salts as its nitrogen source. Bacteria that can grow on this medium turn bromothymol blue indicator from green to blue.

**Method**

Inoculate Simmons citrate agar lightly on the slant by touching the tip of a needle to a colony that is 18-24 hours old. Broth culture is not recommended as the inoculum will be too heavy.

Incubate at 35-37°C for upto 7 days.

Observe for development of blue color, denoting alkalinization.

**Expected Results**

Positive: Growth on the medium, with or without change in color of the indicator

The color change of the indicator is due to acid or alkali production by the test organism as it grows on the medium. Growth usually results in the bromothymol blue indicator, turning from green to blue.
Quality control:

Known citrate positive, *Klebsiella pneumoniae* and citrate negative, *Escherichia coli*.

Both *S.enteritidis* and *S.typhimurium* do not utilize citrate as their source of carbon hence they do not grow in this medium.

**Indole test**

Bacteria that produce the enzyme tryptophanase are able to degrade the amino acid tryptophan into pyruvic acid, ammonia and indole. Indole is detected by combining with an indicator, aldehyde (1 % paradimethylaminoaldehyde), that results in a red/pink color formation. This test is used in many identification schemes, especially to presumptively detect *Escherichia coli*, and many other gram negative bacilli most commonly encountered in diagnostic bacteriology. This test is used to determine the ability of an organism to split tryptophan to form the compound indole.

**Method**

A) Enterobacteriaceae

Inoculate tryptophane broth with a drop from a 24 hour brain heart infusion broth culture.

Incubate at 35°C in ambient air for 24-48 hours.

Add 0.5 mls of Kovacs reagent

*Expected results*

Positive: Pink to wine colored red ring after addition of appropriate reagent

Negative: No color change after addition of the appropriate reagent.
S. typhimurium and S. enteritidis are indole negative

iv) Triple Sugar Iron (TSI)

Principle

TSI is used to determine whether a gram negative bacillus utilizes glucose and lactose or sucrose fermentatively and forms hydrogen sulphide (H₂S). TSI contains 10 parts lactose: 10 parts sucrose: 1 part glucose and peptone. Phenol red and ferrous sulphate serve as indicators of acidification and H₂S formation respectively. When glucose is utilized by a fermentative organism, the entire medium becomes acidic (yellow) in 12 to 18 hours. The butt remains acidic after the recommended 18 to 24 hours incubation period because of the presence of organic acids resulting from the fermentation of glucose under anaerobic conditions in the butt of the tube. The slant however reverts to the alkaline (red) state because of oxidation of the fermentation products under aerobic conditions in the slant.

This change is a result of the formation of carbon dioxide and water and the oxidation of peptones in the medium to alkaline amines. When in addition to glucose, lactose and/or sucrose are fermented, the large amount of fermentation products formed on the slant will more than neutralize the alkaline amines and render the slant acidic (yellow), provided the reaction is read in 18 to 24 hours. Reactions in TSI should not be read beyond 24 hours of incubation, because aerobic oxidation of the fermentation products from lactose and/or sucrose will not proceed and the slant will eventually revert to the alkaline state. The
formation of carbon dioxide and water (hydrogen gas) is indicated by the presence of bubbles or cracks in the agar or by separation in the tube. The production of H$_2$S requires an acidic environment and is manifested by blackening of the butt of the medium.

**Method**

1) With a straight inoculation needle, touch the top of a well isolated colony.

2) Inoculate TSI by first stabbing through the center of the medium the bottom of the tube and then streaking the surface of the agar slant.

3) Leave the cap on loosely and incubate the tube at 35° -37°C in ambient air for 18 -24 hours.

**Expected Results**

Alkaline slant/no change in the butt (K/NC=glucose, lactose, and sucrose non utilizor: this may also be recorded as K/K (alkaline slant /alkaline butt).

Alkaline slant/acid butt (K/A)=glucose fermentation only.

Acid slant/acid butt (A/A) =glucose, sucrose, and /or lactose fermenter.

Note: A black precipitate in the butt indicates production of ferrous sulfide and H2S gas (H2S +)

Bubbles or cracks in the tube indicate the production of carbon dioxide or hydrogen. Drawing the circle around the A for acid butt, this is A/A, usually this means the organism ferments glucose and sucrose, glucose and lactose, or sucrose and lactose, with the production of gas.
Quality control

A: *Escherichia coli*

K/A H2S+: *Salmonella typhi, S. typhimurium*

K/NC: *Pseudomonas aeruginosa*

*S. typhimurium* produces H2S while *S. enteritidis* does not

2. **Analytical Profile Index (API 20E)**

API 20 E is an identification system for *Enterobacteriaceae* and other non fastidious gram negative bacteria which uses 21 standardized and miniaturized biochemical tests and a database.

The API 20 E strip consists of a plastic strip with 20 micro tubes containing dehydrated biochemical substrates. These biochemical substrates in the 20 micro tubes are inoculated with a pure culture of bacteria evenly suspended in sterile physiological saline.

During incubation, metabolism produces color changes that are either spontaneous or revealed by addition of reagents. The reactions are read according to the reading table and the 20 test results are converted to seven or nine digit profile. This profile is used with the computer or API profile index book to find the name of the bacteria. Identification is obtained by referring to the Analytical Profile Index or using identification software.
3 Peptone water

Distilled water 200m
Tryptone 2 gm
Sodium chloride 1 gm

Dissolve and adjust to Ph 7.4-7.6,
Distribute 2.5 ml in bijou bottles and autoclave at 121°C for 15 minutes.

4 Turbidity standard equivalent to McFarland 0.5.

This is a barium sulphate standard against which the turbidity of the test and control inocula can be compared. When matched with the standard, the inocula should give confluent almost confluent growth. Shake the standard immediately before use.

Preparation of turbidity Standard

1) Prepare a 1% v/v solution sulphuric acid by adding 1 ml of concentrated sulphuric acid to 99 ml of water. Mix well

Caution: Concentrated sulphuric acid is hygroscopic and highly corrosive, therefore do not mouth pipette, and never add the water to the acid.

2) Prepare 1% w/v solution of barium chloride by dissolving 0.5 g of dehydrated barium chloride

(BaCl2.2H2O) in 50 ml of distilled water.

3) Add 0.6 ml of the barium chloride solution to 99.4 ml of the sulphuric acid solution and mix.

4) Transfer a small volume of the turbid solution to a capped tube or screw-cap
bottle of the same type as used for preparing the test and control inocula.

When stored in a well-sealed container in the dart at room temperature (20-28°C), the standard can be kept for up to 6 months.

5. **Luria Bertani Broth**

Tryptone, 10g

Yeast extract, 5g

NaCl, 5 g

10 N NaOH, 1 ml = 0.4 g

Distilled water. q.s to 1 L.

Heat gently to thoroughly dissolve all ingredients, avoid boiling. Dispense in 3 mls

Quantities in screw cap tubes or bottles and autoclave.

**Plasmid DNA Extraction**

**Reagents**

0.5 M Glucose (dextrose)

Distilled 18.02 g

Distilled water, q.s to 200 ml

Dissolve glucose in water. Filter, sterilize and store at 4°C
6. Lysis buffer (Solution 1)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5M Glucose</td>
<td>0.5 ml</td>
<td>50 mM</td>
</tr>
<tr>
<td>0.1 M EDTA, pH 8.0</td>
<td>0.5 ml</td>
<td>10 mM</td>
</tr>
<tr>
<td>0.25 M Tris-HCl, pH 8.0</td>
<td>0.5 ml</td>
<td>25 mM</td>
</tr>
<tr>
<td>Distilled water, q.s. to 5 ml</td>
<td>0.5 ml</td>
<td>lysozyme</td>
</tr>
</tbody>
</table>

Prepare fresh immediately before use and keep on ice until use.

0.1 M EDTA (ethylene diamine tetracetic acid) pH 8.0

Note: Either the disodium or trisodium salt of EDTA may be used with adjustment for formula weight. The disodium salt will not dissolve until the pH is adjusted to approximately 8.0 with NaOH.

Disodium EDTA 2H₂O, 7.44 g

Distilled water, 800 ml

Stir vigorously on magnetic stirrer. Verify that pH is 8.0. Adjust volume 200 ml. Autoclave.

0.5 M EDTA pH 8.0

Disodium EDTA 2H₂O, 186.1 g

Distilled water 800 ml

Stir vigorously on a magnetic stirrer. Verify that pH is 8.0. Adjust volume to 1 L. Autoclave.
0.25 M Tris-HCl ("Tris" tris (hydroxymethyl) aminomethane, pH 8.0. Tris base may be obtained from several commercial sources. Follow manufacturer’s instructions for preparing a 0.25 M solution. Adjust pH to 8.0 with concentrated HCL as directed. Autoclave.

1 M Tris-HCl, pH 8.0 (7.5)
Follow manufacturer’s instructions for preparing 1 M solution. Adjust pH to 8.0 (7.5) with concentrated HCl as directed. Autoclave

7 Alkaline detergent solution (Solution II)

<table>
<thead>
<tr>
<th></th>
<th>Quantity</th>
<th>Final Conc.</th>
</tr>
</thead>
<tbody>
<tr>
<td>NaOH pellets</td>
<td>0.4 g</td>
<td>0.2 N</td>
</tr>
<tr>
<td>OR 10 N NaOH</td>
<td>(1 ml)</td>
<td>(0.2 N)</td>
</tr>
<tr>
<td>Sodium dodecyl sulfate (SDS)</td>
<td>0.5 g</td>
<td>1%</td>
</tr>
</tbody>
</table>

(Also known as sodium lauryl sulfate or SLS)

Distilled water q.s to 50 ml in polypropylene bottle. (Avoid storing alkali in glass bottles.) Prepare fresh every few days. (Maximum shelf life is one week.)

8 Acidic salt solution (3 M sodium Acetate, pH 4.8) (solution 111)

Sodium acetate, anhydrous, 24.6 g

Distilled water, 45 ml
Dissolve sodium acetate in water. Adjust pH to 4.8 with glacial acetic acid (25-40 ml).

Bring to 100 ml final volume with distilled water. Autoclave.

**9 TE (Tris hydromethyl aminomethane EDTA(ethylene diamine tetraacetic acid**

For the 100 ml TE

- 10 mM Tris-HCl pH 8.0
- 1 mM EDTA pH 8.0
- 1 mL 1 M Tris-HCl, pH 8.0
- 0.2 ml 0.5M EDTA pH 8.0
- Q.S to 100 ml with H2O
- Filter sterilize, store at RT

**10 5x TBE Buffer (Tris Borate EDTA)**

- 27.5 boric acid
- 54.0 g Tris base
- 20.0 mL 0.5M EDTA pH 8.0
- Q.S with distilled H2O to 100 mL

**11 Alkaline loading buffer**

- 300 mM NaOH
- 6 mM EDTA
- 18% Ficoll (Type 400; Pharmacia) in water
- 0.15% bromocresol green
- 0.25% xylene cyanol FF
Pulsed field gel electrophoresis (PFGE) reagents

12 Cell Suspension Buffer

10 mM Tris base pH 7.2
20 mM NaCl
50 mM EDTA

Make up 100 ml as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol. wt.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>121.1</td>
<td>0.1211g</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.4</td>
<td>1.862g</td>
</tr>
<tr>
<td>EDTA</td>
<td>373.24</td>
<td>0.11688g</td>
</tr>
</tbody>
</table>

Add 75ml distilled water, pH to 7.2 and make up to 100ml autoclave

13 Lysozyme Solution Buffer

10 mM Tris base
50 mM NaCl
0.2% sodium deoxycholate
0.5% sodium lauryl sarcosine (sarkosyl)

Make up 1 litre as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Mol. wt.</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris</td>
<td>121.1</td>
<td>1.211g</td>
</tr>
<tr>
<td>NaCl</td>
<td>58.4</td>
<td>2.92g</td>
</tr>
</tbody>
</table>
Na deoxycholate 2.0g
Sarkosyl 5.0g
Add 750ml distilled water pH to 7.2 and make up to 1 litre. Autoclave

14 Wash Buffer x 1.0

20 mM Tris base pH 8.0
50 mM EDTA
Make up 1 litre as follows:-
Tris (mol. wt. 121.1) 2.422g
EDTA (mol. wt. 372.24) 18.62g
Add 750ml distilled water, pH to 8.0 (needs large volume of concentrated NaOH) and make up to 1 litre

15 Proteinase K Reaction Buffer

100 mM EDTA
0.2% sodium deoxycholate
1.0% sodium lauryl sarcosine (sarkosyl)
Make up 1 litre as follows:-
EDTA (mol. Wt. 373.24) 37.224g
Na deoxycholate 2.0g
Sarkosyl 10.0g
Add 750ml distilled water, pH to 8.0 and make up to 1 litre. Autoclave
16 Phenylmethylsufonylfluoride (PMSF)

Iso-propanol 10.0ml

PMSF 0.174g

Mix the contents well; PMSF is not stable in aqueous solutions. Activity lasts approximately 30-60 minutes at room temperature, pH to 8.0 store at room temperature. Stable for 1 year.

Caution; Gloves and face mask must be worn when weighing PMSF, the room should be quiet and not full of traffic. If spilt it must be cleaned with lots of water.