MOLECULAR CHARACTERIZATION OF ANTIMICROBIAL RESISTANCE IN NON-TYPHOID *SALMONELLA* FROM PATIENTS WITH BACTERAEMIA ADMITTED AT THE AGA KHAN UNIVERSITY HOSPITAL

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Molecular characterization of antimicrobial resistance in non-typhoid *Salmonella* from patients with bacteraemia admitted at the Aga Khan University Hospital

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DECLARATION

This thesis is my original work and has not been presented for a degree in any other university.

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To my family; thank you very much for being supportive every step of the way.

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"If there were no clouds, we would not enjoy the sun." - Proverb.

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ABBREVIATIONS

AIDS	Acquired Immunodeficiency Syndrome
AKUH	Aga Khan University Hospital
AMP	Ampicillin
AUG	Amoxicillin- clavulanic acid (augmentin)
BP	Base Pair
CDC	Centers for Disease Control and Prevention
CHEF- DR III	Contour-clamped Homogeneous Electric Field apparatus DR III
CIP	Ciprofloxacin
CMR	Centre for Microbiology Research
CN	Gentamicin
CRO	Ceftriaxone
CTX	Cefotaxime
CU	Cefuroxime
DNA	Deoxyribonucleic Acid
ERC	Ethical Review Committee
HAART	Highly Active Antiretroviral Therapy
HIV	Human Immunodeficiency Virus
ILRI	International Livestock Research Institute
KEMRI	Kenya Medical Research Institute
LB	Luria- Bertanii
MDR	Multidrug- resistant
NA	Nalidixic Acid

NTS	Non Typhoid *Salmonella*
PCR	Polymerase Chain Reaction
QRDR	Quinolone Resistance-Determining Region
RPM	Revolutions per minute
SSC	Scientific Review Committee
SXT	Sulphamethoxazole
TM	Trimethoprim
USA	United States of America
WHO	World Health Organization

ABSTRACT

In Africa, non-typhoid *Salmonella* (NTS) infections are common and self limiting, however, they present life-threatening complications especially in children and adults who are immunosuppressed. In these individuals, antimicrobial treatment maybe required. The increasing antimicrobial resistance in NTS contributes to its spread and threatens the use of commonly available and clinically important antimicrobial agents. Over the last decade or more, resistance to commonly available antimicrobials including ampicillin, cotrimoxazole, streptomycin, chloramphenicol and tetracycline rose remarkably.

This study used 116 culture confirmed isolates of NTS from bacteremic patients admitted in the medical ward of Aga Khan University Hospital, examined over a 12-month period, 2007. NTS isolates were identified by culture methods, and confirmed by slide agglutination tests according to Kauffmann-White scheme utilizing the Salmonella poly-O, H1 and H2 agglutination antisera. Antimicrobial susceptibility tests were done using the disk diffusion method. Conjugation experiment was done to determine genetic basis of resistance and polymerase chain reaction was done to detect presence of genes encoding the quinolone resistance-determining region.

Resistant isolates contained plasmids of various sizes. Some isolates had only one plasmid while others had up to five plasmids of varying sizes. The large plasmids extracted ranged from 90 kb to slightly over 147 kb in size; while the small size plasmids ranged from about 2.1 kb to 5.6 kb. The isolates that had

plasmids all had a 43.5kb plasmid size. Some isolates in *Salmonella* serotype group B and group C_3 had the largest plasmid size, slightly above 147 kb. The gyrB, parC and parE had 500bp products.

The resistance to ampicillin, tetracycline, cotrimoxazole and chloramphenicol was low, but there was an increase in quinolone and fluoroquinolone antimicrobials.

The study concluded that, there is a decrease in resistance to conventional drugs of choice for treatment of invasive NTS in Kenya, but there is an increase in resistance to quinolone and flouroquinolone; and a new resistance to cefotaxime and ceftriaxone.

CHAPTER ONE

1.0 INTRODUCTION

Food borne diseases caused by non typhoid *Salmonella* (NTS) are a public health problem world wide. *Salmonella* species are members of the family *Enterobacteriaceae*, are Gram-negative enteric bacilli (0.7-1.5 by 2-5μm), motile (except *S. gallinarum-pullorum*), and facultative anaerobes and non-spore formers (Cheesbrough, 2000). *Salmonella* is a single genus, named *Salmonella enterica*, and can be divided into seven distinct subspecies, namely: *enterica, salamae, arizonae, diarizonae, houtenae, bongori, and indica,* based on the DNA structure or biochemical properties (Le Minor and Popoff, 1987). The subspecies members can be divided into serotypes (serovars) based on their somatic (O) and flagellar (H) antigens and more than 2,400 are known today.

Of the large number of *Salmonella* serotypes, only a few account for the vast majority of human infections. World wide, examples of human isolates are: *S.* Enteritidis, *S.* Typhimurium, S. Virchow, *S.* Newport, *S.* Hadar, *S.* Heidelberg, *S.* Agona, and *S.* Indiana, the order of prevalence is variable according to geography and time (Threlfall, 2000). For years the dominant strain throughout the world was *S.* Typhimurium but in recent years *S.* Enteritidis has become the most prevalent serotypes in many Western countries (Ducoffre, 2003). The *Salmonella* serovars with zoonotic importance include *S.* Typhimurium, *S.* Enteritidis, and *S.* Dublin (Cooper, 1994). *S.* Dublin has been reported to be the most common *Salmonella*

serotype in cattle (Hirsh, 1990), but may also cause severe systemic infection in humans.

Most *Salmonella* infections in humans result from the ingestion of contaminated poultry, beef, eggs and milk (Gomez, *et al*., 1997). Intestinal salmonellosis usually resolves within five to seven days and does not require treatment with antimicrobials (Cheesbrough, 2000). However, bacteremia occurs in 3% to 10% of reported, culture-confirmed cases particularly in aged patients and those immunosuppressed (Gordon *et al.,* 2002). Appropriate antimicrobial therapy (e.g., ciprofloxacin in adults and ceftriaxone in children) can be lifesaving (Hohmann, 2001).

In sub-Saharan Africa, NTS are among the most common causes of invasive bacterial childhood disease, and it is problematic but not limited to immunocompromised individuals; patients with malignancy or human immunodeficiency virus or diabetes, and those receiving corticosteroid therapy or treatment with other immunotherapy agents (Cheesbrough *et al.,* 1997; Graham *et al.,* 2000). In Kenya, invasive salmonellosis has been complicated by multidrug resistant serovars, thus rendering commonly available drugs ineffective in the management of these patients (Kariuki *et al.,* 1996; Kariuki *et al*., 2000; Oundo *et al*., 2000). Isolation of drug resistant *Salmonella spp* microorganisms from both human and non human sources is a serious public health problem, primarily through the increased risk of treatment failures (Aarestrup, 1999). The resistance of nontyphoidal *Salmonella* to both fluoroquinolones and third-generation

cephalosporins has been reported (Waxmann *et al.*, 1982; Bradford *et al*., 1998), and such resistance is likely to be a therapeutic challenge in the future.

1.1 General objective

To characterize by serotyping and antimicrobial susceptibility of NTS isolated from adult patients admitted to the medical ward of Aga Khan University Hospital, and to detect genes encoding quinolone resistance.

1.2 Specific objectives

1. To characterize by serotyping NTS from adult patients admitted to the Aga Khan University Hospital.

2. To determine antimicrobial sensitivity patterns of the NTS by disk diffusion method.

3. To detect the genes encoding the quinolone resistance-determining region in the quinolone resistant isolates.

1.3 Justification for the study

Previous studies (Angulo and Swerdlow, 1995, Gordon *et al.,* 2002) have shown that cases of NTS and antimicrobial resistance to conventional drugs are on the increase especially in the HIV immunosuppressed individuals. Recent studies (Green and Cheesbrough, 1993; Cheesbrough *et al*., 1997; Lee *et al*., 2003; Berkley *et al*., 2005) have concentrated on NTS infections in children and so there is need to also study the current trends in NTS infections in adults. There are reports of increased morbidity and mortality rates in this susceptible group due to high rates of

MDR strains, which may be more virulent. Such patients also have longer hospital stay and often experience treatment failure, hence need for more expensive alternative treatments. Therefore, it is essential to determine the antimicrobial susceptibility of NTS causing bacteremia in this susceptible group of individuals to various commonly available antimicrobials in order to offer the best available treatment of choice. The study on the molecular basis of resistance in NTS will offer important epidemiological information that would be useful in developing strategies for minimizing emergence and spread of antimicrobial resistance.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Description of *Salmonellae*

DNA hybridization studies have shown all *Salmonellae* to be genetically closely related (Cheesbrough, 2000). *Salmonella* species are Gram-negative enteric bacilli (0.7-1.5 by 2-5μm), motile (except *S. gallinarum-pullorum*), facultative anaerobes and non-spore formers. They are members of the family *Enterobacteriaceae*. *Salmonella* is a single genus, named *Salmonella enterica*, and can be divided into seven distinct subspecies, namely: *enterica, salamae, arizonae, diarizonae, houtenae, bongori, and indica,* based on the DNA structure or biochemical properties (Le Minor and Popoff, 1987).

The nomenclature of the *Salmonella* genus has been updated several times hence different nomenclatures have been used by scientists in reference to *Salmonella*. Prior to the most recent update in nomenclature, *Salmonella choleraesuis* was the species name; however, this has been updated to *Salmonella enterica* (Anderson and Ziprin, 2001) with *Salmonella* bongori being a second species of *Salmonella.* An entire serovar is written thus: *Salmonella enterica* subsp. *enterica* serovar Typhimurium. Acceptable abbreviated forms of designating a *Salmonella* serovar include: *Salmonella* (ser.) Typhimurium or *Salmonella* Typhimurium (Anderson and Ziprin, 2001). For this thesis, the nomenclature for *Salmonella* serovar will be written as *Salmonella* Typhimurium. Serotyping is one

of the main characteristics used in serovar classification. The subspecies members can be divided into serotypes (serovars) based on their somatic (O) and flagellar (H) antigens and more than 2,400 are known today. The antigenic formulae (e.g. 6,7:r:1,7) represents the O antigens: the phase 1H antigens: the phase 2H antigens, respectively (Popoff and Le Minor, 1997).

The serotypes of *enterica* subspecies account for most human and warm-blooded animals infections. These serotypes are grouped on the basis of sharing of a common O antigen (Kauffman-White scheme). The commonly occurring groups of *enterica* subspecies serotypes are: Group A- *S.* Paratyphi A; Group B- *S.* Paratyphi B, *S.* Stanley, *S.* Saintpaul, *S.* Agona, *S.* Typhimurium; Group C- *S.* Paratyphi C, *S.* Cholera-suis, *S. V*irchow, *S.* Thompson; Group D- *S.* Typhi, *S.* Enteritidis, *S.* Dublin, and *S.* Gallinarum.. Serotypes *S.* Typhi and *S.* Paratyphi only colonize humans, whereas nontyphoidal *Salmonella* can infect a wide variety of animal hosts (Baumler *et al.*, 1998).

2.2 Non-typhoid Salmonellosis

2.2.1 Natural history

NTS infections in humans are the primary cause of food-borne disease in developed countries, resulting in considerable morbidity and occasionally death, especially in immunocompromised patients (Levine *et al.*, 1991; Angulo and Swerdlow, 1995). Individual cases and outbreaks in community and institutions are common.

Of the large number of *Salmonella* serotypes, only a few account for the vast majority of human infections. World wide, examples of human isolates are: *S.* Enteritidis, *S.* Typhimurium, *S.* Virchow, *S.* Newport, *S.* Hadar, *S.*Heidelberg, *S.* Agona, and *S.* Indiana, the order of prevalence is variable according to geography and time (Threlfall, 2000). For years the dominant strain throughout the world was *S.* Typhimurium but in recent years *S.* Enteritidis has become the most prevalent serotypes in many Western countries (Ducoffre, 2003). The *Salmonella* serovars with zoonotic importance include *S.* Typhimurium, *S.* Enteritidis, and *S.* Dublin (Cooper, 1994). *S.* Dublin has been reported to be the most common *Salmonella* serotype in cattle (Hirsh, 1990), but may also cause severe systemic infection in humans.

2.2.2 Epidemiology

The NTS organisms are widely distributed. Domestic animals, notably cattle, pigs and poultry are frequent excretors and many wild animals are also infected (Cooper, 1994; Cody *et al*., 1999; Davis *et al*., 1999; Chiu *et al*., 2002). Household pests such as dogs, cats, birds and turtles are all potential sources of human infection (Davis *et al*., 1999). Human carriers and convalescent cases are also important sources (Kariuki *et al*., 2001).

Ingestion of contaminated food products such as improperly cooked poultry, meat and eggs, and unpasteurized milk are the largest sources of human infections (Mead *et al*., 1999). Person-to-person transmission can also occur *via* the fecal-oral

route and outbreaks have occurred in hospitals and day care centers (Miller *et al.*, 1995).

S. Chorelaesuis is highly host-adapted to pigs; however, there is evidence that isolates from humans and swine have the same DNA fingerprints, which suggest that human infections were derived from pigs (Chiu *et al.*, 2002). Such infection likely arose as a result of contaminated a food or water source.

NTS are a major cause of septicaemia and bacteraemia in humans and are responsible for about 1.4 million cases annually in the United States alone, out of which 600 are fatal (Mead *et al.*, 1999). Patients with antimicrobial resistant NTS infection are more likely to have bloodstream infection and to be hospitalized than patients with pansusceptible infection (Varma *et al.*, 2005).

In the USA, it is estimated that over 95% of food-borne infections are due to NTS (Mead *et al.*, 1999). In developed countries, outbreaks of non-typhoidal *Salmonella* infection have been caused mainly by serotypes Enteritidis and Typhimurium (Threlfall, 1994). It is currently estimated that, of the 40,000 Salmonella isolates reported annually to the CDC, 8.5% are identified as serotype Typhimurium (CDC, 2004). The number of reported cases of human salmonellosis in Belgium, rose between 1987 and 1999, then began to fall, the predominant serotypes being Enteritidis and Typhimurium, the latter accounting for more than

80% of the total number of infections (Ducoffre, 2003). In Malaysian children, serovar Enteritidis was commonly isolated from 1991-2001 (Lee *et al.*, 2003).

NTS, particularly, *S.* Typhimurium and less frequently *S.* Enteritidis are common causes of bacteremia and septicemia in developing countries (Cheesbrough, 2000). In Africa, *Salmonella* species account for most paediatric bacteraemias and NTS are seen predominantly in children less than 5 years of age (Graham *et al.*, 2000; Lee *et al.*, 2003). This has been seen in children with septicemia in Rwanda (Lepage et al., 1987) and Zaire (Green & Cheesbrough, 1993). In some developing countries, including Kenya, invasive salmonellosis has been a significant cause of childhood meningitis, and may also localize in other organs such as liver causing hepatitis (Shetty *et al*., 1999). In Kenya, NTS, including *S.* Typhimurium and *S.* Enteritidis are the main causes of bacteraemia in children below the age of 3 years (Oundo *et al.*, 2000). Over a 2-year period, 1993-1994 (Kariuki *et al*., 1996), a total of 192 NTS isolates were obtained from blood and/or stool, 75% being *S* Typhimurium, with smaller numbers of *S.* Enteritidis (5%), *S.* Newport (4%), *S* .Choleraesuis (3.6%).

2.2.3 Pathogenesis

After ingestion, infection with S*almonellae* is characterized by attachment of the bacteria by fimbriae or pili to cells lining the intestinal lumen (Madigan *et al.*, 2000). *Salmonellae* selectively attach to specialized epithelial cells (M cells) of the Peyer patches (Bopp *et al*., 1999). The bacteria are then internalized by receptor-mediated endocytosis and transported within phagosomes to the lamina propria, where they are released (Cooper, 1994). Once there, *Salmonellae* induce

an influx of macrophages (typhoidal strains) or neutrophils (nontyphoidal strains). Although nontyphoid *Salmonellae* generally precipitate a localized response, *S typhi* and other especially virulent strains invade deeper tissues via lymphatics and capillaries and elicit a major immune response (Lightfoot *et al.*, 1990).

2.2.4 Virulence of the organism

The serotypes vary greatly in their potential to produce invasive illness outside the gastrointestinal tract. Although any serotype can cause invasive disease, some are more invasive than others. Of the more than 2,000 *Salmonella* serotypes, *S.* Choleraesuis is extremely invasive and usually associated with bacteremia, septicemia and metastatic illnesses in humans, and less commonly gastroenteritis (Su *et al*., 2001). Other types with increased invasiveness are *S.* Virchow, and *S.* Dublin (Gruenewald *et al.*, 1994). The multi-drug resistant *S*. Typhimurium, which have caused outbreaks in Africa, India, and the Middle East produce a high incidence of septicemia and metastatic organ involvement (Gomez *et al*., 1997). Virulence factors responsible for pathogenicity in enteric bacteria are encoded by plasmids as seen in *Escherichia coli, Yersinia* spp and *Shigella* spp (Bäumler *et al*., 1998).

In *Salmonella*, current evidence suggests that the contribution of virulence plasmids to pathogenesis is less important than in the aforementioned bacteria (Bäumler *et al*., 1998; Guiney *et al*., 1994; Gales *et al*., 2002). Virulence plasmids have been found in only a few serovars of *Salmonella,* particularly in those showing host adaptation (Guiney *et al*., 1994). These plasmids are 50-90 kb in size, and

have been called “serovar-specific plasmids”; however, not every isolate of plasmid-bearing serovar carries the virulence plasmid. The virulence plasmid of *Salmonella* is important for bacterial multiplication in the reticuloendothelial system of warm-blooded vertebrates (Gulig *et al*., 1993). Plasmids are more commonly found in *S.* Typhimurium and *S.* Enteritidis isolated from blood and other extraintestinal sources than in strains isolated from faeces (Montenegro *et al*., 1991). The virulence plasmid affects intracellular growth in macrophages, but not in non-phagocytic cells (Gulig *et al*., 1998).

The size of the infecting dose is influenced by the virulence of the organism and host factors. Although the infectious dose varies among strains, a large inoculum is thought to be necessary to overcome stomach acidity and to compete with normal intestinal flora (Bopp *et al*., 1999). Large inocula are also associated with higher rates of illness and shorter incubation periods (Cooper *et al*., 1994). However, lower infectious doses may be adequate to cause infection if these organisms are co-ingested with foods that rapidly transit the stomach (e.g., liquids) or that raise gastric pH (e.g., cheese, milk), if antacids are used concomitantly, or if these organisms are ingested by individuals with impaired immune systems (Bopp *et al*., 1999).

Risk factors for salmonellosis in man include extremes of age, alteration of the endogenous bowel flora of the intestine (e.g., as a result of antimicrobial therapy or surgery), diabetes, malignancy, rheumatological disorders, reticuloendothelial

blockade (e.g., as a result of malaria, sickle-cell disease, or bartonellosis), HIV infection, and therapeutic immunosuppression of all types (Gruenewald *et* al., 1994; Bopp *et al*., 1999; Gordon *et al*., 2002). Anatomical disruptions, including kidney stones and other urinary tract abnormalities, gallstones, atherosclerotic endovascular lesions, schistosomiasis, and prosthetic devices, may all serve as foci for persistent *Salmonella* infection (Hohmann, 2001). Gastrointestinal salmonellosis and its serious sequelae are linked to a wide variety of illnesses and therapies that affect the body's multiple defenses against enteric and intracellular pathogens (Varma *et al.*, 2005).

2.2.5 Salmonellosis and HIV

Infections with NTS have been described in patients with impaired host defenses, such as those with neoplastic disease, transplantation, cirrhosis, collagen vascular disease, renal failure requiring hemodialysis, and need for immunosuppressive drugs (Gruenewald *et al.*, 1994, Gordon *et al*., 2002). In Africa and the rest of the world, NTS bacteremia is also common in those co-infected with HIV (Hohmann, 2001; Kariuki *et al*., 2001). An increased incidence of nontyphoid salmonellosis in HIV-infected persons was originally noted in the early 1980s and nontyphoidal *Salmonella* septicemia became an AIDS-defining illness in 1987 (WHO, 2000).

Bacteremia, relapses, and severe disease are unusual in the immunocompetent host but characteristic of *Salmonella* infection in the HIV-infected population (Cheesebrough, 2000). Salmonellosis and bacteremia occur at

an increased rate in persons with HIV (Gordon, *et al.*, 2002; Murray, 1991; Salmon, *et al.*, 1991). A characteristic feature of salmonellosis in AIDS is the relapses that occur despite appropriate antimicrobial therapy (Sperber, *et al*, 1987).

Before the introduction of highly active antiretroviral therapy (HAART) for treatment of HIV-1, a decrease occurred in the incidence of *Salmonella* infections in HIV-infected patients (WHO, 2000). Both the use of zidovudine and trimethoprim/sulfamethoxazole for *Pneumocystis carinii* prophylaxis probably contributed to this decline (Hardy *et al*., 1992). Zidovudine has *in vitro* activity against Gram-negative bacteria, including *Salmonella*, and has been shown to prevent relapses of *Salmonella* bacteremia in persons with AIDS (Dellamonica *et al*, 1991; Salmon *et al*, 1991).

Persons with AIDS show a clear increase in susceptibility to infection with *Salmonella* species. Data suggest that risk for non-typhoid *Salmonella* infections is increased 20 to 100 fold among AIDS patients (Celum *et al*., 1987). Among persons infected with *Salmonella*, AIDS results in a several-fold increase in the risk for septicemia (Angulo *et al*., 1995); AIDS also results in increases in infections at other extraintestinal sites, compatible with an overall increase in risk for dissemination of the organism. This increase in risk is reflected in increases in the proportion of *Salmonella* isolated from blood. For example, for persons from ages 25 to 49 years in USA with high AIDS incidence, the percentage of *Salmonella* isolates from blood increased from 2.3% in 1978 to 17.8% in 1987 among men and from 3.1% to 8.1% among women; in contrast, no changes in blood-isolate

percentages occurred for either sex in states with low AIDS incidence (Levine, 1991; Gruenewald *et al.*, 1994). These latter studies further suggest that serotype is an important risk determinant, with increases in bacteremia in states with high AIDS incidence associated primarily with infections due to *S.* Enteritidis and *S.* Typhimurium (Gruenewald *et al.*, 1994). In a Swiss cohort of over 9,000 patients, only 22 cases of recurring salmonellosis were documented over a period of nine years (Burckhardt, 1999). In Kenya, 68.8% cases of bacteremia from blood cultures were HIV seropositive (Kariuki *et al*., 1996). The treatment of AIDS patients now includes routine prophylactic therapy with trimethoprim/sulfamethoxazole to prevent *P. carinii* pneumonia (Fishman, 1998**)**. Widespread prophylactic use of this drug in the AIDS population may have reduced the incidence of serious *Salmonella* infections, although this protective effect could be diminished in the face of increasing resistance to this antimicrobial agent among clinical isolates of *Salmonella* (Lee *et al*., 1994).

2.2.6 Clinical manifestations of *Salmonella*

Salmonellae cause a wide variety of clinical syndromes; including gastroenteritis, enteric fever, and focal infections, a bacterial disease commonly manifested by an acute enterocolitis, with sudden onset of headache, malaise, abdominal pain, diarrhea, nausea and sometimes vomiting accompanied with cramp-like abdominal pain (Baumler *et al*., 1998). Dehydration especially among infants or in the elderly, maybe severe and fever is almost always present (Gomez *et al*., 1997), anorexia and diarrhea often persists for several days. Infection may

begin as an acute enterocolitis and develop into septicemia or focal infection (Cheesbrough, 2000).

Enterocolitis may be associated with localization of pain over the left iliac fossa and development of tenderness. There is mucosal oedema, hyperaemia, petechial haemorrhages and in severe cases, friable mucosa with ulcerations (Hohmann, 2001). Histological features include dilatation congestion of capillaries in the mucosa and submucosa with focal collections of polymorphonuclear leukocytes in the lamina propia (Bäumler *et al*., 1998), in others there may also be diffuse increase in chronic inflammatory cells in the lamina propia. Crypt abscesses maybe seen with abnormal goblet cell population and there maybe pain in the liver and right lower abdomen (Cheesbrough, 2000; Hohmann, 2001). Meningitis occurs almost exclusively in neonates and children under 2 years of age (Graham *et al*., 2000). Deaths are uncommon, except in the very young, the very old and the immunosuppressed (Cheesbrough, 2000).

In the immunocompetent host, salmonellosis can be divided into four clinical syndromes: gastroenteritis, enteric fever, septicemia, and an asymptomatic carrier state. The majority of *Salmonella* infections in AIDS patients manifest as severe gastroenteritis, bacteremia, or extraintestinal focal infection (Madigan *et al*., 2000). HIV-infected patients present with diarrhea, fever, and bacteremia (Hohmann, 2001). In the normal host, bacteremia accompanies gastroenteritis approximately 5% of the time, (Gordon *et al.,* 2002) whereas in AIDS, the incidence of bacteremia is much higher. It has been noted previously that in

patients with underlying disease, bacteremia occurs more frequently, and in the majority of cases, it occurs without gastrointestinal symptoms (Albrecht *et al.*, 1992; Gordon *et al.*, 2002). This syndrome of fever and non-typhoidal *Salmonella* bacteremia without gastroenteritis has also been observed in persons with AIDS (Sperber and Schleupner, 1987). Cases of endovascular infection, lung abscess, peritonitis, septic arthritis, osteomyelitis, brain abscess, subdural empyema, and meningitis have all been reported in persons with AIDS (Albrecht *et al.*, 1992; Gordon *et al.*, 2002).

2.2.7 Diagnosis of *Salmonellae*

The diagnosis of *Salmonella* infection relies on isolation of the organism because physical examination and routine laboratory analysis are nonspecific in nontyphoidal salmonellosis (Cheesebrough, 2000). The differential diagnosis of diarrhea in an HIV-infected person is extensive. Blood cultures must be obtained in all febrile patients with diarrhea because *Salmonella* may be isolated in the blood when stool cultures are negative (Forsyth, 1998). *Salmonella* should also be considered in the HIV-infected patient presenting with sepsis. The occurrence of *Salmonella* bacteremia should prompt one to consider the diagnosis of HIV infection, keeping in mind that it can occur before other opportunistic infections (Sperber and Schleupner, 1987).

The methods of culture of the non typhoid bacillus are blood, stool and urine cultures. The diagnosis of salmonellosis requires bacteriologic isolation of the organisms from appropriate clinical specimens (Cheesbrough, 2000; Madigan *et*

al., 2000). Laboratory identification of the genus *Salmonella* is done by biochemical tests; the serologic type is confirmed by serologic testing (Madigan *et al.*, 2000; Threlfall *et al.*, 2000). Feaces, blood, or other specimens should be plated on several nonselective and selective agar media (blood, MacConkey, eosin-methylene blue, bismuth sulfite, *Salmonella-Shigella*, and brilliant green agars) as well as into enrichment broth such as selenite or tetrathionate (Cheesbrough, 2000). Any growth in enrichment broth is subsequently subcultured onto the various agars (Forsyth, 1998). The biochemical reactions of suspicious colonies are then determined on triple sugar iron agar and lysine-iron agar, and a presumptive identification is made (Cheesbrough, 2000). The presumptive biochemical identification of *Salmonella* can be confirmed by antigenic analysis of O and H antigens using polyvalent and specific antisera (Forsyth, 1998; Cheesbrough, 2000). Fortunately, approximately 95% of all clinical isolates can be identified with the available group A-E typing antisera.

2.2.8 Antimicrobial susceptibility testing for non typhoid *Salmonella*

In the treatment and control of infectious diseases, especially when caused by pathogens that are often drug resistant, sensitivity testing is used to select effective antimicrobial drugs (Cheesbrough, 2000). This is essential for the guidance of clinical management. Sensitivity tests measure antimicrobial activity against bacteria under laboratory conditions and not in the patient (Madigan *et al.*, 2000). It cannot be assumed therefore, that an antimicrobial which kills or prevents an organism from growing *in vitro* will be a successful treatment.

The antimicrobials currently in use for treatment, determination of prevalence of MDR strains and patient medical history determine the choice of antimicrobials for the test. Using the Interpretative Chart, zones sizes are interpreted reporting the organism as 'resistant', 'intermediate' or 'sensitive'.

It has been recommended that susceptibility tests should be performed against a fluoroquinolone, a third-generation cephalosporin and any other drug currently used for treatment, nalidixic acid (for determining reduced susceptibility to fluoroquinolones because of the possibility of false *in vitro* susceptibility against the fluoroquinolone used for treatment), and the previous first-line antimicrobials to which the strains could be resistant (chloramphenicol, ampicillin, trimethoprim/ sulfamethoxazole, streptomycin and tetracycline) (WHO, 2000).

2.2.9 Treatment and prevention of salmonellosis

In the immunocompetent patient with self-limited gastroenteritis, antimicrobial therapy is usually not recommended as it does not significantly improve symptoms or outcome, and may actually increase the relapse rate (Miller *et al.*, 1995). Given the increased severity, potential for extraintestinal spread, and the high relapse rate, salmonellosis requires treatment in HIV-infected persons. The classic drugs used to treat *Salmonella* infections are chloramphenicol, ampicillin, amoxicillin, and trimethoprim-sulfamethoxazole (Cheesbrough, 2000). However, there has been an increase in resistance to these antimicrobials (Kariuki *et al.*, 2000) and treatment must now be guided by antimicrobial susceptibilities. Intravenous

ceftriaxone (1 to 2 g) every 24 hours as well as oral ciprofloxacin (750 mg) twice daily have been shown to be efficacious in AIDS patients with *Salmonella* infections (Sperber *et al.*, 1987). Ciprofloxacin is effective in persons developing breakthrough bacteremia while receiving other antimicrobials and offers the advantage of oral administration (Cheesbrough, 2000).

Control of human salmonellosis is mainly by proper handling of foods of animal origin (Cody *et al*., 1999; Ahmed *et al*., 2000; Threlfall, 2000), by maintaining high standards of hygiene during processing, strict segregation of cooked and uncooked foods, and proper heat treatment of foods (Glynn *et al.,* 1998; Fey *et al*., 2000; Threlfall *et al*., 2000; Murphy *et al.,* 2001; Ahmed *et al*., 2000; Palmer *et al*., 2000; Indar-Harrinauth *et al.,* 2001 Liebena *et al.,* 2001). Undercooking contaminated foods is a major source of human infections.

2.3 Plasmid extraction

Plasmids are extra chromosomal, double-stranded circular DNA molecules generally containing 1,000 to 100,000 base pairs (Madigan *et al*., 2000). Even the largest plasmids are considerably smaller than the chromosomal DNA of a bacterium, which can contain several million base pairs. Certain plasmids replicate independent of the chromosomal DNA and can be present in hundreds of copies per cell. Some of them code for antibiotic resistance (Schlegel, 1995).

Plasmids may code for virulence factors or carry antimicrobial resistance genes (Snyder and Champness, 1997). The development of rapid and inexpensive techniques for extracting plasmid DNA and separating plasmids on the basis of

their size by agarose gel electrophoresis led to the wide spread use of plasmids in epidemiologic investigations. Because plasmids of identical sizes may vary in their nucleotide sequence, treatment of the isolated plasmid DNA with a restriction enzyme allows one to determine whether plasmids of identical size from different strains are infact the same plasmid (Montenegro *et al.*, 1991). Plasmid profiles have been useful for subtyping several bacterial species, including *Escherichia coli, Salmonella* serovars, *Shigella, Campylobacter, Vibrio cholerae, H. influenza, N. gonorrhoea, N. meningitides, S. aureus*, and *Legionella* species. Plasmid profiling may be most useful when applied in conjunction with another subtyping method and has been particularly effective in the investigation of nosocomal outbreaks (Schlegel, 1995).

2.4 Antimicrobial resistance

Antimicrobial agents represent one of the main therapeutic tools both in human and veterinary medicine to control and treat a variety of bacterial infectious diseases. There are several stages in the development of resistance. Resistance is when bacteria can both survive and duplicate when antimicrobials are present (Novak *et al.*, 1999). Resistance in *Salmonella* to quinolone is unique in that resistance is typically conferred through chromosomal mutations versus acquisition of mobile, genetic elements, although documentation of horizontal transfer of fluoroquinolone resistance is increasing (Jacoby *et al.*, 2003). This type of resistance poses a therapeutic challenge in treatment of salmonellosis.

2.4.1 Mechanisms of antimicrobial resistance

2.4.1.1 Mutation

Mutation is a change in the DNA that can sometimes cause a change in the gene product, which is the target of the antimicrobial agent (Madigan *et al*., 2000).

When a susceptible bacterium comes into contact with a therapeutic concentration of antimicrobials such as the fluroquinolones, the antimicrobial can bind to the specific enzymes, in this case, DNA gyrase (Madigan *et al*., 2000). The DNA gyrase is an essential bacterial enzyme required for DNA replication. The end result is that fluoroquinolones block bacterial DNA replication leading to cell death (Cheesbrough, 2000). However, when spontaneous mutations occur in specific areas of the genes encoding these enzymes, antimicrobials no longer bind efficiently and this allows the bacterium to continue DNA replication (Schlegel, 1995).

2.4.1.2 Destruction or Inactivation

Many bacteria possess genes which encode for enzymes that chemically degrade or deactivate the antimicrobial, rendering them ineffective against the bacterium (Madigan *et al*., 2000). Here the antimicrobial is either degraded or modified by enzymatic activity before it can reach the target site and damage the bacterial cell (Hohmann, 2001). For instance, the production of *beta*-lactamase enzymes by *Staphylococcus aureas* that destroy the beta-lactam ring of penicillin and cephalosporins; and production of acetylating, adenylating and phosphorylating

enzymes that inactivate antimicrobials such as aminiglycosides and chloramphenicol (Cheesbrough, 2000).

2.4.1.3 Efflux

Certain bacteria can often become resistant to antimicrobials through a mechanism known as efflux (Lewis, 1995). An efflux pump is essentially a channel that actively exports antimicrobial and other compounds out of the cell (Madigan *et al.*, 2000). The antimicrobial enters the bacterium through a channel termed a porin, and then is pumped back out of the bacterium by the efflux pump (Lewis, 1995). By actively pumping out antimicrobials, the efflux pumps prevent the intracellular accumulation necessary to exert their lethal activity inside the cell (Schlegel, 1995).

2.4.1.4 Genetic Transfer

Genetic material can be transferred between bacteria by several means, most often by conjugation, transformation and transduction.

2.4.1.4.1 Conjugation

Conjugation involves cell-to-cell contact and the subsequent transfer of DNA which crosses a sex pilus from donor to recipient (Madigan *et al.*, 2000). Conjugation can occur between species that are unrelated; for this reason, a large gene pool is available from which bacteria can exchange and acquire new genetic material (Guiney, 1984). Conjugation is mediated by a circular DNA called a

plasmid, which replicates independently of the chromosome (Schlegel, 1995). Many plasmids carry genes that confer resistance to antimicrobials. Once the two cell walls are in contact, this allows a mating bridge to form (Lewis, 1995). The plasmid DNA in the donor, possibly containing antimicrobial resistance genes, is nicked in one strand; this strand proceeds into the recipient cell by undergoing rolling-circle replication (Hartl *et al.*, 1998). Complementary copies of the DNA are produced in both the donor and the recipient cells. Finally, the linear plasmid in the recipient becomes circular and is ligated, and then both of the cells have a copy of the plasmid (Murray *et al.*, 1991; Madigan *et al*., 2000).

2.4.1.4.2 Transformation

In transformation, DNA is acquired directly from the environment, having been released from another cell. Genetic recombination can follow the transfer of DNA from one cell to another leading to the emergence of a new genotype (recombinant). It is common for DNA to be transferred as plasmids between mating bacteria. Since bacteria usually develop their genes for drug resistance on plasmids, they are able to spread drug resistance to other strains and species during genetic exchange processes (Maiden, 1998). During this process, genes are transferred from one bacterium to another as “naked” DNA (Schlegel, 1995).

When cells die and break apart, DNA can be released into the surrounding environment. Other bacteria in close proximity can scavenge this free-floating DNA, and incorporate it into their own DNA (Maiden, 1998). This DNA may

contain advantageous genes, such as antimicrobial resistant genes and benefit the recipient cell (Madigan *et al.*, 2000).

2.4.1.4.3 Transduction

Transduction occurs when a bacteriophage carries DNA between mating bacteria (Madigan *et al*., 2000). During this process, bacterial DNA may inadvertently be incorporated into the new phage DNA (Jones, 1997). Upon bacterial death and lysis, these new phage go on to infect other bacteria, it may carry pieces of chromosomal DNA or plasmids from the previous host (Madigan *et al*., 2000). An occasional phage may carry some bacterial DNA (Jones, 1997). Recombination can then occur between the phage (carrying bacterial DNA) and the new host's bacterial DNA (Lacey, 1984 and Hartl and Jones, 1998).

2.5 Antimicrobial resistance in *Salmonella*

Over the past several decades, the prevalence of antimicrobial-resistant *Salmonella* has increased (CDC, 2004). This disturbing trend has been noted in many parts of the world (Gales *et al*., 2002). Ling and Wang from Singapore in 2001 reported that 75% of *Salmonella* serovar Enteritidis isolated were resistant to sulphonamide. The emergence of multi-drug resistant *Salmonella* is also widespread in Africa (Graham *et al*., 2000). Resistance of NTS to potent but expensive antimicrobials, such as quinolones and third generation cephalosporins have been noted as well (Chiu *et al*., 2002; Dunne *et al*., 2000). In 1980, 13% of *Salmonella* serotype Typhimurium isolates were the most common *Salmonella* serotype isolated from humans in the USA. They were resistant to more than 1 of 9

antimicrobial agents; by 2001, and this proportion has increased to 51% (CDC, 2004).

It is currently estimated that, of the 40,000 *Salmonella* isolates reported annually to the Centers for Disease Control and Prevention, 8.5% are identified as serotype Typhimurium (CDC, 2004). These organisms, *Salmonella* Typhimurium, are often resistant to five or more antimicrobial agents, including ampicillin (A), chloramphenical (C), streptomycin (S), sulfonamides (Su), and tetracycline (T) - the characteristic resistance (R) type ACSSuT (Bolton *et al.*, 1999). A recent example is the global spread of a multidrug-resistant *S. Typhimurium* phage type DT104 (MR-DT104) in animals and humans (Davis *et al.*, 1999). While the spread of DT104 may have been facilitated by the use of antimicrobials, international and national trade of infected animals is thought to play a major role in international spread (WHO, 2000).

The emergence of MDR *Salmonella* strains with resistance to fluoroquinolones and third-generation cephalosporins is a serious development, which results in severe limitation of the possibilities for effective treatment of human infections (Glynn *et al.*, 1998). MDR strains of *Salmonella* are now encountered frequently and the rates of multidrug-resistance have increased considerably in recent years. Even worse, some variants of *Salmonella* have developed multidrug-resistance as an integral part of the genetic material of the organism, and are therefore likely to retain their drug-resistant genes even when

antimicrobial drugs are no longer used, a situation where other resistant strains would typically lose their resistance (WHO, 2000). Use of antimicrobial agents in aquaculture in Asia may have contributed to the emergence of MR-DT104 (Davis *et al.*, 1999). The incidence of MR-DT104 in Japan was detected as 1.9% (Ahmed *et al.*, 2005), similar as that reported in by Izumiya *et al.*, 1999, at 2.2%. In the 2001, it accounted for 7% of nontyphoidal *Salmonella* isolates tested in US national public health surveillance (CDC, 2004). The resistant determinants of MR-DT104 reside on the chromosome, within a transferable element (Ridley *et al*, 1998). The genes encoding the five antimicrobial resistance, $bla_{\text{PSE-1}}$ and *floR-aadA2-sul1-tet* (G), are located within a chromosomal island (SGI1), with different variants reported over time, which include two class 1 integrons described as In4-type integrons (Boyd *et al.*, 2002; Threlfall, 2002). Chloramphenicol resistance in MR-DT104 is due to *floR*, a florfenicol resistance gene (Bolton *et al.*, 1999).

Acquisition and dissemination of genetic determinants of antimicrobial resistance are accounted for in part by R plasmids and transposons (Daly *et al.*, 2000). Often, the frequency with which resistance to sulphonamide is encountered suggests the involvement of a third mechanism linked to a novel group of naturally occurring mobile genetic elements, integrons (Falbo *et al.*, 1999).

The number of nontyphoid *Salmonella* isolates resistant to nalidixic acid has increased with a concomitant decrease in the level of susceptibility to ciprofloxacin, a strong relationship between specific *Salmonella* serovars and phage types, and

resistance to quinolones has been observed (Hakanen *et al*., 1999). The types most frequent described as resistant to quinolones are *S. enterica* serovars Hadar, Virchow, Enteritidis, and Typhimurium DT104 (Prats *et al*., 2000). *S.* Choleraesuis was susceptible to fluoroquinolones before 1999 (Su *et al*., 2001). Since 2000, there has been rapid increasing resistance to ciprofloxacin in *S.* Choleraesuis isolated from both human and swine sources in Taiwan (Chiu *et al*., 2002). The resistant strains of *S.* Choleraesuis found in Taiwan carried mutations that gave rise to the substitution of phenylalanine for serine at position 83 and asparagine for aspartic acid at position 87 in GyrA (Chiu *et al*., 2002). In addition, mutations leading to an amino acid change from serine to isoleucine at position 80 in ParC were demonstrated in the ciprofloxacin-resistant *S.* Choleraesuis isolates (Chiu *et al*., 2004). The emergence of fluoroquinolone resistance in *S.* Choleraesuis was mainly due to the dissemination of an endemic, resistant clone (Chiu *et al*., 2002; Hsueh *et al*., 2004). This finding is a cause for concern because fluoroquinolones are first-line drugs to treat systemic, nontyphoid salmonellosis.

Different phenotypes of S Typhimurium strains isolated from humans and animals, especially cattle, confer antimicrobial resistance genes encoding for β-lactamase (Lee *et al*., 1994). Different types of β-lactamase have already been identified in *Salmonella*: TEM-1, TEM-2, OXA-1 types are the commonest; and SHV-1 type is predominant in Africa (Wegener *et al*., 1997). Resistance to expanded-spectrum oxyimino-cephalosporins among *Salmonella* strains is mostly due to acquisition of plasmids encoding various class A extended-spectrum β-

lactamases (Bradford *et al*., 1998). Production of plasmid-mediated class C β-lactamases by *Salmonella* isolates has also been described previously (Fey *et al*., 2000). The emergence of such strains may have serious implications because of the limitation of therapeutic choices for patients with invasive *Salmonella* infections and by facilitation of the spread of *bla* genes in the community. Investigation of a 4.9-fold increase in *Salmonella* Newport isolations from Californians in 1985 showed that 87 percent of the isolates had an unusual antimicrobial-resistance pattern (including chloramphenicol resistance) and a single, identical plasmid (Spika *et al.,* 1987).

In Kenya, invasive salmonellosis has been complicated by multidrug resistant serovars, thus rendering commonly available drugs ineffective in the management of these patients (Kariuki *et al.,* 1996; Kariuki *et al,* 2005; Oundo *et al*., 2000). Multidrug-resistant *S* Typhimurium is the predominate isolate in children with salmonellae bacteraemia (Kariuki *et al*., 2002). In the study by Kariuki *et al* 2000, isolates from Nairobi (56.3%) and Kilifi (33%) were multiple resistant to ampicillin, chloramphenicol, co-trimoxazole and streptomycin; and a plasmid of ca. 100kb was present in each of the MDR *S* Typhimurium strains from Nairobi and Kilifi, in addition to other plasmids ranging from 5-42 kb. Previous studies have shown availability of these transferable plasmids in circulation within the population (Kariuki *et al*., 2005), posing a challenge in managing salmonellosis with antimicrobials.

CHAPTER THREE

3.0 METHODOLOGY

3.1 Study design and population

This was a cross sectional descriptive study over a period of one year involving the use of NTS isolates already stored in freezers at Aga Khan University Hospital and later transferred to Center for Medical Research, KEMRI.

3.1.1 Bacterial isolates

NTS isolates were obtained from stock cultures at CMR. These were originally from blood cultures of patients with suspected cases of sepsis/bacteremia admitted at AKUH. Consent was sought from both the Ethical Review Committee of AKUH to transfer and use the NTS isolates to CMR; and Scientific Steering Committee of CMR to undertake the study in the laboratories.

3.1.2 Sample size determination

Sample size was calculated using this formula (Glynn *et al.*, 1998)

$$N = \frac{Z^2\ P(1-P)}{D^2} = \frac{1.96^2 \times 0.08(0.92)}{0.005^2} = 113$$

N= Minimum sample size

Z= 1.96 standard error

P= Expected prevalence of condition of interest

D= 0.05 (inverse of 95% allowable error)

Therefore, a minimum sample of 116 NTS isolates was sought randomly from a total of 140 NTS isolates already stored in freezers.

3.2 Study site

This study concentrated on characterizing NTS previously isolated from adult patients with bacteremia. Thus no blood cultures were done. The laboratory work involving the NTS isolates were performed at the Center for Microbiology Research-KEMRI and International Livestock Institute, (Biotechnology Theme) Laboratory 5 (five).

3.2.1 Inclusion criteria for bacterial isolates

The NTS previously isolated from blood cultures only. These were NTS isolated from adult patients i.e. above 18 years only.

3.2.2 Exclusion criteria for bacterial isolates

The NTS isolates from persons under the age of 18 years, and isolates from stool samples.

3.3 Ethical Consideration

3.3.1 Permission to carry out the study

This study did not involve sampling patients from the hospital directly.

NTS previously isolated from adult patients, were obtained from AKUH, during the Year 2006; all data given by the hospital only contained patient number and corresponding laboratory number; no names of patients were contained in these records. Permission to carry out the study was obtained from the graduate school, JKUAT; SSC and ERC of KEMRI, and AKUH Scientific and Ethical Review Committees (Appendix 1).

3.3.2 Confidentiality

All information obtained about the patients were handled with utmost confidentiality and only used for intended purposes.

3.3.3 Risks

There were no risks to patients directly attributable to this study as only isolates of NTS strains were obtained from the hospital. The laboratory work on the isolates was done in a biosafety level 2 laboratory.

3.3.4 Benefits

The patient through the clinician in-charge at AKUH got results for all the diagnostic and antimicrobial susceptibility tests undertaken for management of the patients.

3.4 *Salmonella* identification

NTS isolates from bacteremic adults hospitalized at the AKUH were identified by culture methods and confirmed using slide agglutination tests according to the Kauffmann-White scheme (Kauffmann, 1954) utilizing *Salmonella*

the poly-O, H1 and H2 agglutination antisera (Remel, Dartford., UK). The 116 organisms were serotyped using the following poly O and poly H (phase 1 and phase 2) antisera: *Salmonella* 9-O, *Salmonella* 3,10,15,19-O, poly H phase 2, *Salmonella* f,g- H, *Salmonella* 4-O, *Salmonella* 8-O, poly O Group A-G, *Salmonella* d-H, *Salmonella* Vi, Salmonella Group C1(6,7), *Salmonella* 7-O, *Salmonella* i-H, *Salmonella* g,m-H, Salmonella 1,2-H, *Salmonella* 1,5-H, and *Salmonella* 1,7-H. NTS isolates that were confirmed by serotyping were stored at $^{-}$ 80° C until further analysis.

3.5 Antimicrobial susceptibility testing

Antimicrobial susceptibility tests for commonly available drugs including ampicillin 10 microgram (µg), tetracycline 30µg, trimethoprim 5µg, chloramphenicol 30µg, gentamicin 10µg, cefotaxime 30µg, ceftriaxone 30µg, cefuroxime 30µg, ciprofloxacin 30µg, amoxicillin-clavulanic acid 30µg, sulphamethoxazole 25µg and nalidixic acid 30µg, was done using the Kirby Bauer disk diffusion technique (Cheesbrough, 2000). Antimicrobial disks (all from Hi media, Maharashtra, India) were placed onto Mueller Hinton agar plates (Oxoid), containing NTS test bacteria conforming to 0.5 McFarland's standard. The plates were incubated at 37^{o}C for 18 h and the susceptibility results interpreted according to the National Committee for Clinical Laboratory and Standard (NCCLS) Guidelines (2000). *Escherichia coli* ATCC 25922 was used as a control for potency of antimicrobials.

3.6 Minimum inhibitory concentrations (MIC) determination

The minimum inhibitory concentration was determined by the E-test strips technique (AB BIODISK, Solna, Sweden) by a plate incorporation method according to manufacturer's instructions using Mueller Hinton agar. Overnight bacterial culture of each test organism was suspended in 0.85% normal saline, conforming to 0.5 McFarland's standard, and plated onto Mueller Hinton agar plate using a sterile swab. After the plates dried, Etest strips were placed. The following antimicrobials were used: Nalidixic acid, chloramphenicol, ampicillin, sulphamethoxazole and ciprofloxacin. *E. coli* ATCC 25922 of known MIC was included for each antimicrobial as control. The plates were incubated at 37˚C in air for 18 h. MIC results were read from where the edge of the inhibition ellipse intersects the side of the strip.

3.7 Plasmid DNA extraction and analysis

Plasmid DNA was extracted from 76 NTS isolates, which were resistant to one or more antimicrobials, using Plasmid Mini Prep-spin kit (Qiagen, West Sussex, UK) with the following modifications: After alkaline lysis of bacteria and neutralization of the lysate, the DNA was precipitated by adding 1 volume of isopropanol and centrifuging immediately at 14,000 rpm for 10 min at room temperature. DNA pellet was washed twice with 1 ml 70% ethanol and then air-dried as described (Sambrook *et al*., 1989). The DNA was then redissolved in TE buffer (10mM Tris-HCl, 5mM EDTA pH 7.8) and stored at -20˚C. Plasmid DNA was separated by electrophoresis on 1% horizontal agarose gel, after staining with

ethidium bromide, DNA bands visualized and photographed with an ultraviolet transilluminator (UVP Inc. San Gabriel, Calif).

Plasmid molecular sizes were determined by coelectrophoresis with *E. coli* strains 39R861 (NCTC 50192) (147, 63, 43.5, and 6.9 kb) and V517 (NCTC 50193) (53.7, 7.2, 5.6, 3.9, 3.0, 2.7 and 2.1 kb) on 1% horizontal agarose gels.

3.8 Conjugation experiments

In vitro conjugation tests for transferable plasmids from NTS, the donor, (ampicillin resistant but nalidixic acid sensitive) were done using *E coli* K12 as the recipient (nalidixic acid resistant but ampicillin sensitive) using the following protocol: Overnight cultures on LB agar (Oxoid), of donor and recipient were subcultured into 5 ml of LB broth for four hours on a shaker at 37°C. The donor and recipient were then mixed in the ratio of 1:10 (donor to recipient), then incubated overnight at 37°C without shaking. The bacterial mixture was then subcultured onto MacCkonkey media containing Ampicillin (30µg/ml), Nalidixic Acid (30µg/ml) and both Ampicillin with Nalidixic Acid. The cultures were incubated overnight at 37°C. NTS and *E coli* K12, and plates containing no antimicrobial were included as controls. Transconjugants were identified on MacCkonkey plates containing both antimicrobials. Antimicrobial sensitivity tests and plasmid extraction was done as previously described in sub sections 3.5 and 3.7, to obtain information on the transferable resistance-encoding plasmids. Plasmid DNA was extracted from both donor and transconjugant strains. *E coli* K12 and NTS were used as positive controls for the MacCkonkey plates.

Plasmid molecular sizes were determined as described above in section 3.7.

3.9 Detection of genes encoding quinolone resistance-determining region (QRDR) by polymerase chain reaction

PCR allows for the production of more than 10 million copies of a target DNA sequence or gene from only a few molecules. PCR of *gyrA***,** *gyrB***,** *parC***,** and *parE* genes in the QRDR of *Salmonella*, was performed on 38 isolates resistant to quinolone antimicrobials i.e. ciprofloxacin and nalidixic acid (appendix 2). A single colony from an overnight culture on MacCkonkey of test isolate was used. Total DNA was prepared by boiling NTS isolates suspended in sterile triple distilled water for ten minutes, followed by centrifugation at 13,000 rpm for two minutes to obtain supernatant. PCR was performed on PTC 100 programmable thermal controller (MJ Research, Inc. USA). Reaction conditions were as follows: The four set of primers used are shown in table 1. Reaction conditions consisted of 2 µl of total DNA, 0.25 µl of GoTaq DNA polymerase (Promega), 5 µl of 5X Green GoTaq Flexi buffer at pH 8.5 (Promega), 0.25 µl of forward primer, 0.25 µl of reverse primer (shown in table 1), 2.5 µl of 25mM deoxynucleoside triphosphate mixture, 0.2 µl of 25mM $MgCl_2$, and 14.55 µl PCR grade water, to make 25 µl per reaction mixture in a 0.5 ml Eppendorf tube. The mixture was overlaid with a drop of mineral oil and the microtubes inserted into the thermocycler and the DNA amplified using the following thermal cycling conditions: initial denaturation at 94°C for 5 min followed by 34 cycles of denaturation at 94˚C for 20 seconds, annealing at 55˚C for 20 seconds, and extension at 72°C for 30seconds; followed by

a final extension step at 72°C for 10 minutes. The PCR products were analyzed on a 1.5% agarose gel electrophoresis.

Table 1. List of primers used in this study

geneName	Primer name	Primer sequence	Ref
gyrA	gyrA1	5'-ATGAGCGACCTTGCGAGAGAAATTACACCG-3'	Brown *et al.*, 1996
	gyrA2	5'-CTTCTGTAGTCGTAACTTCCCGACTACCTT-3'	
gyrB	gyrB1	5'-AAGCGCGATGGCAAAGAAG-3	Hirose *et al.*, 2002
	gyrB2	5'-AACGGTCTGCTCATCAGAAAGG-3	
parC	parC1	5'-ATGAGCGATATGGCAGAGCG-3'	Giraud *et al.*, 1999
	parC2	5'-TGACCGAGTTCGCTTAACAG-3'	
parE	parE1	5'-GACCGAGCTGTTCCTTGTGG-3'	Giraud *et al.*, 1999
	parE2	5'-GCGTAACTGCATCGGGTTCA-3'	

CHAPTER FOUR

4.0 RESULTS

4.1. Identification of NTS isolates by serotyping

A total of 116 NTS were used in this study. All the isolates were identified to be NTS by serological methods. The *Salmonella* group B was dominant (39/116) followed by *Salmonella* group D (33/116) (Table 2). There were 2 isolates identified in group E and as untypable (Table 2). There were 30 *Salmonella* group C_3 and only 10 group C_1. A total of 21 *Salmonella* Enteritidis, 1 *Salmonella* Chincol, 4 *Salmonella* Typhimurium, 1 *Salmonella* Derby, 2 *Salmonella* Kenturcky, and 1 *Salmonella* Rissen were identified using the Kauffmann-White scheme (Kauffmann, 1954). The rest, which were 86 isolates, could not be identified to the serotype level in our laboratories.

Table 2: O group *Salmonella* serotyping results

O Group of *Salmonella*	Frequency	Percentage
Group B	39	33.6
Group C_1	10	8.6
Group C_3	30	25.9
Group D	33	28.4
Group E	2	1.7
Untypable	2	1.7
Total N	**116**	**100.0**

4.2 Antimicrobial sensitivity tests

A total of 116 isolates were tested for their susceptibility to 12 antimicrobials (Appendix 2). A total of 40 (34.48%) isolates were susceptible to all the 12 antimicrobials tested while 76 NTS isolates (65.52%) were resistant to one or more antimicrobials; MDR isolates were 42/116 (36.21%) (MDR are isolates resistant to three or more antimicrobials). The highest resistance observed to tetracycline at 36.2% (42/116), followed by nalidixic acid at 32.8% (38/116) (Table 3).

The 19.8% (23/116) isolates were resistant to one antimicrobial (Fig. 1) and 1/116 resistant to 9 out of 12 antimicrobials tested. The highest resistance in combination of antimicrobials was observed in trimethoprim and sulphamethoxazole at 28.4% (33/116 isolates), followed by ampicillin and augmentin at 27.6% (32/116). The lowest resistance in combination of antimicrobials was observed in cefuroxime and ciprofloxacin at 1.7% (2/116). The resistance to the following antimicrobials: Nalidixic acid, chloramphenicol, ampicillin, sulphamethoxazole and ciprofloxacin, was confirmed by doing the MIC of each organism resistant to the antimicrobials; 38, 15, 36, 36, and 12 respectively (Appendix 2). In Kenya, the drugs of choice for treatment of salmonellosis are ampicillin, chloramphenicol or trimethoprim-sulfamethoxazole hence the importance to know their MICs. Nalidixic acid and ciprofloxacin were included for MIC profile because they are second drugs of choice for treatment of salmonellosis.

There was a confluent growth on the MIC plates on all the isolates confirming their resistance profile against these drugs.

Table 3. Antimicrobial sensitivity testing by disc diffusion of non typhoid *Salmonella* isolates

Antimicrobial Susceptibility				Total
Antimicrobial	Resistance	Intermediate	Sensitive	
Nalidixic acid	38 (32.8%)	4 (3.4%)	74 (63.8%)	116
Ceftriaxone	3 (2.6%)	1 (0.9%)	112 (96.6%)	116
Cefotaxime	3 (2.6%)	2 (1.7%)	111 (95.7%)	116
Chloramphenicol	15 (12.9%)	4 (3.4%)	97 (83.6%)	116
Trimethoprim	33 (28.4%)	1 (0.95)	82 (70.7%)	116

Cefuroxime	9 (7.8%)	11 (9.5%)	96 (82.850	116
Tetracycline	42 (36.2%)	20 (17.2%)	54 (46.6%)	116
Sulphamethoxazole	36 (31.0%)	0	80 (69.0%)	116
Gentamicin	15 (12.95)	12 (10.3%)	89 (76.7%)	116
Ciprofloxacin	12 (10.3%)	30 (25.95)	74 (63.8%)	116
Augmentin	32 (27.6%)	2 (1.7%)	82 (70.7%)	116
Ampicillin	36 (31.0%)	6 (5.2%)	74 (63.8%)	116

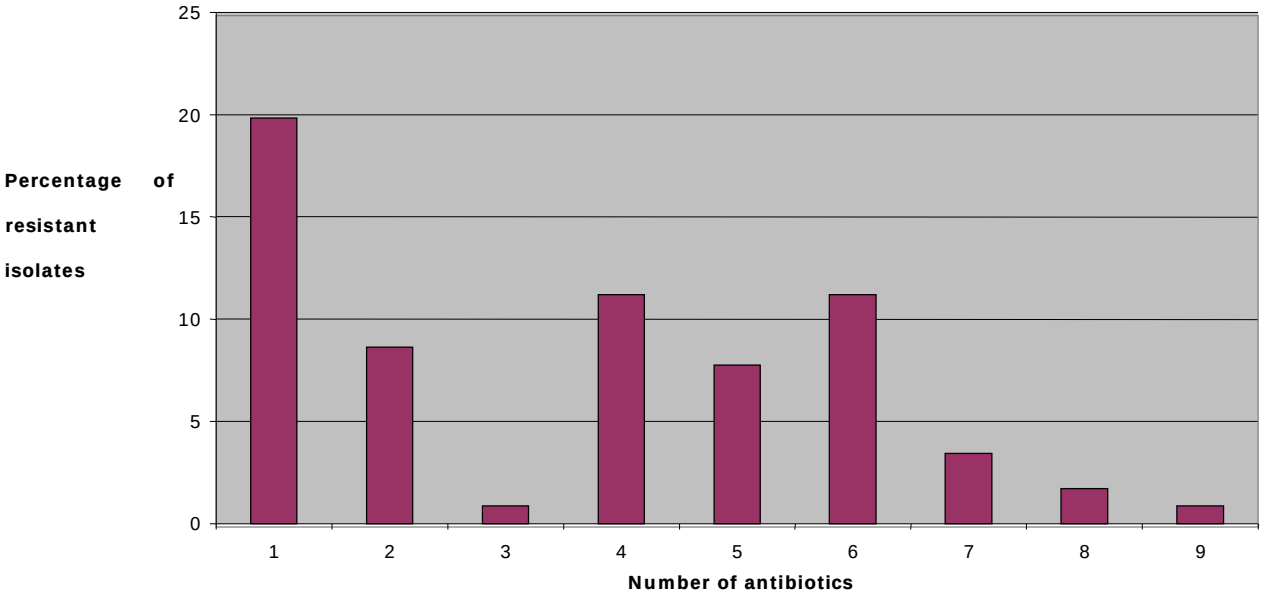

Figure 1. Percentage of isolates resistant to one or more antimicrobials

4.3 Plasmid extraction and analysis

Plasmids extraction was done on a total of 76 NTS isolates. These were isolates resistant to one or more antimicrobial tested (Appendix 2). The plasmid profile of selected study isolates is shown in Figure 2. Not all isolates subjected to plasmid extraction had plasmids. Resistant isolates contained plasmids of various sizes. Some isolates had only one plasmid while others had up to five plasmids of varying sizes. The large plasmids extracted ranged from 90 kb to slightly over 147 kb in size; while the small size plasmids ranged from about 2.1 kb to 5.6 kb. The isolates that had plasmids all had a 43.5kb plasmid size. Some isolates in

Salmonella serotype group B and group C_3 had the largest plasmid size, slightly above 147 kb.

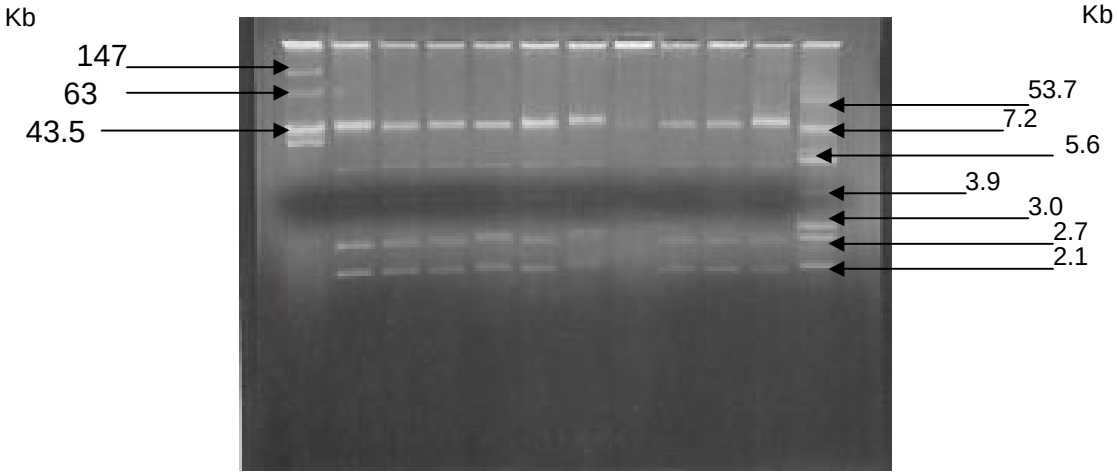

Figure 2. Plasmid DNA extraction profile of NTS resistant to more than 1 antimicrobial

4.4 Conjugation experiments

A total 20 isolates were subjected to mating experiments. These were isolates resistant to ampicillin but sensitive to nalidixic acid (appendix 2). Conjugation occurred in 16/20 (80%) of the isolates subjected to mating experiments. All transconjugants contained resistant plasmid of 90 to 100 kb molecular weight.

Eighty percent of transconjugants showed resistance to ampicillin. There was transferable resistance to augmentin (80%), cotrimoxazole (40%), tetracycline

(45%), chloramphenicol (35%), trimethoprim (35%). Resistance to ceftriaxone and cefotaxime was not transferred.

4.5 Detection of genes encoding quinolone resistance-determining region (QRDR) by polymerase chain reaction

A total of 38 isolates were tested for genes encoding QRDRs, these were resistant to either ciprofloxacin or nalidixic acid or both (Appendix 2). The size of PCR product was estimated using a bench top PCR marker (Promega, USA) consisting of six DNA fragments with sizes of 50, 150, 300, 500, 750, and 1000bp. A 598bp product was sought for the QRDR. Out of the 38 isolates subjected to PCR, only 23 had either/or gyrB, parC or parE genes or all. The primer for gyrA gene did not yield any product.

The gyrB, the parC and parE primers had 500bp products. The group C serotypes of NTS isolates had only parC and parE genes detected. Group B and D NTS serotypes had gyrB, parC and parE genes detected. The four isolates that had both parC and parE genes were resistant to both nalidixic acid and ciprofloxacin. Three of these isolates belonged to *Salmonella* serotype C_3 and one to group B.

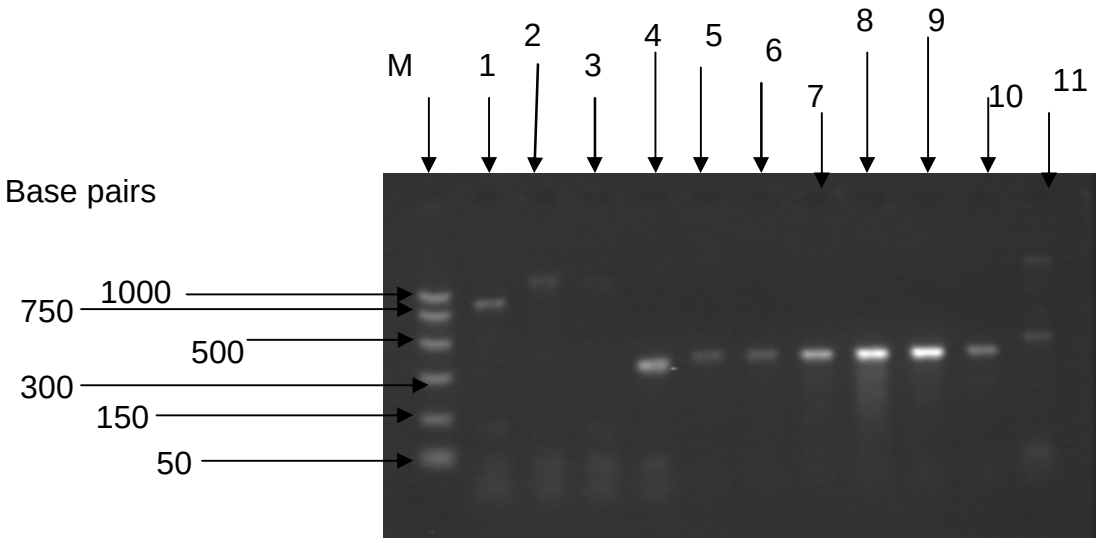

Figure 3. PCR analysis of the gyrB and parC genes.

The fig.4 is a representation of the PCR products. The lane M is the PCR top bench marker (Promega, USA) consisting of six DNA fragments with sizes of 50, 150, 300, 500, 750, and 1000bp. Lane 1, 2 and 4 are products of gyrB gene, lane 5, to 10 are products of parC gene and lane 11 is a contaminant.

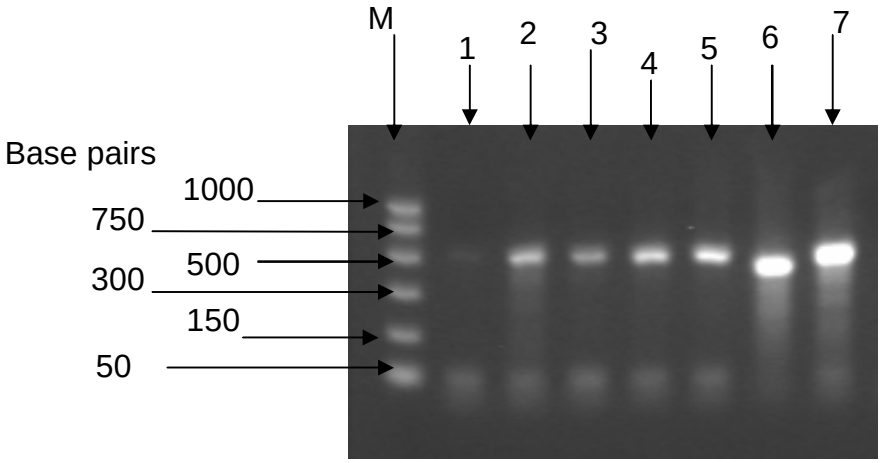

Figure 4. PCR analysis of the parE gene

Lane M2 is the PCR top bench marker (Promega, USA) consisting of six DNA fragments with sizes of 50, 150, 300, 500, 750, and 1000bp.

Lanes 1 to 7 are parE gene products of about 500bp.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSIONS AND RECOMMENDATIONS

5.1 Discussion

Non typhoid salmonellosis presents a serious health problem world wide. Salmonellosis is usually a self-limiting diarrhoeal disease requiring little or no medical intervention (Fey *et al*., 2000). However, in cases of invasive disease or infections with added complications, such as at the extremities of age or in the presence of underlying disease, antimicrobial treatment maybe required. An infection with nontyphoidal salmonella causes illnesses in approximately 1.4 million patients in the United States (Mead *et al*., 1999). The development of multidrug resistance in *Salmonella* presents a serious challenge in treating salmonellosis (Thielman *et al*., 2004).

In this study, a total of 116 isolates were tested for their antimicrobial sensitivity profile. The predominant serotype in this study was *S*. Enteritidis. This is unlike other studies where *S* Typhimurium was predominant (Lepage *et al*., 1987; Green and Cheesbrough, 1993; Kariuki *et al*., 2005; Kariuki *et al.,* 2006). In the late 1990s, *S.* Typhimurium serogroup B and *S.* Enteritidis serogroup D were the most frequently isolated serotypes, accounting for ~50% of isolates from patients in the United States (CDC, 2004). MDR *S* Typhimurium was the predominant cause of community-acquired bacteraemic illness in both children and adults (Kassa-Kalembho, *et al*., 2003). A total of 23/116 (19.83%) isolates were fully susceptible

to all the 12 antimicrobials tested, compared to the study in Kariuki *et al*., (2005), whereby, from 1994-97 only 16% of tested NTS were fully susceptible to 11 antimicrobials; 1997-2000, and 2001-2003, fully susceptible isolates were 21.4% and 18% respectively. This shows a decline in susceptibility previous to this study.

In developing world, MDR, particularly to commonly available antimicrobials, remains a major challenge for the healthcare system (Bonfiglio *et al*., 2002; Kariuki *et al*., 2005; WHO, 2000). In this study, MDR isolates were 42/116 (36.21%), (MDR are isolates resistant to three or more antimicrobials). This is similar to previous studies conducted in Kenya by Kariuki *et al*, 2005. The highest resistance was observed in tetracycline at 36.2% (42/116), followed by nalidixic acid at 32.8% (38/116). The latter may indicate wide use of this antimicrobial in recent years, it is not a first line antimicrobial in treatment of invasive salmonelosis in Kenya, hence the low resistance level compared to studies by Kariuki *et al*., 2002; Cabrera *et al*., 2004 and Chiu *et al*., 2002 . Highest MDR was observed in the group B *Salmonella* serotype (19/39) followed by group C_3 *Salmonella*. Both serotypes were resistant to mostly augmentin, ampicillin and tetracycline.

In Kenya, the drugs of choice for treatment of salmonellosis are ampicillin, chloramphenicol or trimethoprim-sulfamethoxazole. In this study, the resistance of these were 31.0% (36/116), 12.9% (15/116) and 31.0% (36/116) respectively. This shows that resistance to chloramphenicol is still low and the antimicrobial is still

considerably effective for treatment of salmonellosis, as compared to ampicillin and trimethoprim-sulfamethoxazole. In mating experiments, resistance plasmids conferring resistance to ampicillin and augmentin were transferable. These drugs are readily available over the counter in Kenya. Previous studies have shown availability of these transferable plasmids in circulation within the population (Kariuki *et al*., 2005), posing a challenge in managing salmonellosis with these antimicrobials. A 90kb plasmid was transferable; the smaller plasmids were not transfered. Eighty percent of transconjugants showed resistance to ampicillin. There was transferable resistance to augmentin (80%), cotrimoxazole (40%), tetracycline (45%), chloramphenicol (35%), trimethoprim (35%). Resistance to ceftriaxone and cefotaxime was not transferred. This could mean their resistance could be chromosomal mediated rather than plasmid mediated.

In Gram negative bacteria the principal target of quinolone/ fluoroquinolone activity is the type II topoisomerase, DNA gyrase, and less frequently, quinolone resistance is associated with point mutations in the type IV topoisomerase (Snyder *et al*., 1997). Nalidixic acid resistance has been on the rise in various parts of the world (WHO, 2000). There were 38 isolates (32.8%) resistant to nalidixic acid, in this study, compared to 11% (Kariuki *et al*., 2002). This shows a remarkable increase in resistance to nalidixic acid. PCR studies showed that *gyrB*, *parC* and *parE* genes were detected. There was no *gyrA* gene detected. A 598bp product was sought for the QRDR. Out of the 38 isolates subjected to PCR, only 23 had either/or gyrB, parC or parE genes or all. The parC and parE had 500bp products.

The group C serotypes of NTS isolates had only parC and parE genes detected. Group B and D NTS serotypes had gyrB, parC and parE genes detected. The four isolates that had both parC and parE genes were resistant to both nalidixic acid and ciprofloxacin. Three of these isolates belonged to *Salmonella* serotype C_3 and one to group B. PCR products were not sequenced for further studies to determine mutation points within the genes detected. However, the marked increase in quinolone/ fluoroquinolone antimicrobials could indicate the changing lifestyles of the Kenyan population. In animal husbandry practices, antimicrobial agents are used for treatment and prevention of animal diseases, as well as for growth promotion (Tollefson *et al.,* 1999). Hence this type of resistance can be transferred from animals to humans in the food chain. Previous studies in Kenya, showed that there was no significant association between NTS isolates from humans and those from animals living in close contact (Kariuki *et al.*, 2002); however, in Taiwan, genotypic studies showed that sources of resistance to ciprofloxacin observed in two hospitals were herds of pigs (Chui *et al.*, 2002).

In Kariuki *et al.*, (2005), the level of resistance for some commonly available antimicrobials including ampicillin, cotrimoxazole, chloramphenicol, ciprofloxacin and nalidixic acid rose from 48%, 46%, 26%, 0% and 0% respectively in 1994 to 62%, 68%, 50%, 0% and 11% respectively in 2003. In this study resistance to these drugs is at ampicillin 31.0%, cotrimoxazole 31.0% and chloramphenicol 12.9%, showing a decline, but an increase in resistance in nalidixic acid and ciprofloxacin at 32.8% and 10.3% respectively. This could imply

that prescription of quinolone and fluoroquionolone has been preferred after an increase in resistance to drugs of choice for treatment of invasive NTS infection in Kenya; these include ampicillin, chloramphenicol or trimethoprim-sulfamethoxazole. For most patients these are the only drugs available.

A new phenomenon found in this study was the presence of resistance to ceftriaxone and cefotaxime both at 3/116 (2.6%). Two of these resistant isolates belong to *Salmonella* serotype group D and one to group B serotype. These isolates were also resistant to cefotaxime, cefuroxime, tetracycline, gentamicin, augmentin and ampicillin. This corresponds to studies done in Russia (Gazouli *et al*., 1998), whereby, a cefotaxim-resistant *Salmonella* Typhimurium was also resistant to ceftriaxone, penicillin, gentamicin, trimethoprim, tetracycline and chloramphenicol. This unique phenomenon has not been observed in Kenya in previous studies. Therefore, resistance to ceftriaxone and cefotaxime could possibly mean that, this resistance could be of external source. This is because, other than resistance to ampicillin, the three ceftriaxone-cefotaxime resistant isolates were not resistant to chloramphenicol or trimethoprim-sulfamethoxazole, which are the drugs of choice in Kenya. Although in developing countries, including Kenya, unlike in the developed countries, there is scanty data on the likely sources of NTS that cause human infections and reservoirs of NTS are not clearly understood (Kariuki *et al*, 2001).

5.2 Conclusions

The NTS MDR isolates were 36.21%; these were isolates resistant to three or more antimicrobials. Highest MDR was observed in the group B *Salmonella* serotype.

There is a decrease in resistance to conventional drugs of choice for treatment of invasive NTS in Kenya, but there increase to quinolone and flouroquinolone; and a new resistance to cefotaxime and ceftriaxone.

There was no gyrA gene detected, however, gyrB, parC and parE were detected in the fluoroquinolone resistant isolates.

5.3 Recommendations

1. Further analysis of antimicrobial resistance should be done on the quinolone and fluoroquinolone resistant isolates. These include DNA sequencing of the PCR products in order to determine mutation points in the QRDR genes detected and continued monitoring for susceptibility.
2. Surveillance/reporting system should be put in place for confirmed quinolone resistant NTS isolates in Kenya. This also applies to all resistant isolates to other antimicrobials. This will create a data bank, which will help in treatment and management of salmonellosis in Kenya.
3. There should be further analysis on the cephalosporin resistant isolates in this study.

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APPENDICES

Appendix 1. Consent seeking to transfer isolates from Aga Khan University Hospital to KEMRI-CMR

The purpose of this research

As a partial fulfillment for award of a Master's degree in Medical Microbiology at the Institute of Tropical Medicine and Infectious Diseases, KEMRI/JKUAT, through the Centre for Microbiology Research (CMR) I am carrying out a study of molecular characterization of antimicrobial resistance in non-typhoid *Salmonella* from adult patients with bacteraemia. This is a disease that is common in persons with immune suppression diseases and which may cause severe illness and admission to hospital. This disease is caused by bacteria called *Salmonella*, which may spread through handling food with unwashed hands, contamination with dirt from animals and drinking contaminated water.

We seek permission to transfer NTS isolates for further laboratory work in laboratories outside AKH i.e. CMR and ILRI. We also seek to obtain patient information from hospital records about their HIV status in order to do comparison studies in the two populations of HIV status.

We require permission from you to store these bacteria in our freezer in order to do more tests in future to better understand this disease and to test if these bacteria may

be carrying antimicrobial resistance. This information is useful in investigating potential transmission of drug resistance to bacteria that may cause severe illness and to come up with efficient treatment protocols.

How many isolates will be required for the study and how will they be selected?

We are expecting to obtain 113 isolates over duration of 1 year. Stock cultures from CMR will also be used in the study. This will be from culture confirmed NTS isolates from patients with bacteraemia.

What will happen after the study?

After the study we will be able to establish a database on the best drugs to use for treatment of blood poisoning infections affecting adults. These data will be available to caregivers and the Ministry of Health for the general improvement of treatment of these infections in this susceptible group.

In case of need for further information about this study, please contact the lead investigators; Dr G. Revathi, Dr Ann Muigai or Dr S. Kariuki on 2718247. P.O. Box 54840, Nairobi, and **the Chairman Ethical Review Committee, KEMRI, on 2722541**, P. O. Box 54840, 00200.

Appendix 2. Antimicrobial sensitivity profile per isolate

Isolate #	NA	CRO	CTX	C	TM	CU	T	SXT	CN	CIP	AUG	AMP	No of R
6479	R	S	S	S	S	S	S	S	S	I	S	S	1
6480	R	S	S	S	S	S	R	S	R	R	R	R	6
6481	R	S	S	I	S	R	R	S	S	R	R	R	6

6482	R	S	S	S	S	S	R	S	R	R	S	S	4
6483	S	S	S	R	R	S	S	R	S	S	R	R	5
6484	R	S	S	S	S	S	R	S	R	R	S	S	4
6632	R	S	S	S	S	S	S	S	S	I	S	S	1
6633	S	S	S	S	S	S	S	S	S	S	S	S	0
6634	S	S	S	S	S	S	R	S	S	S	S	S	1
6635	R	S	S	S	R	S	R	R	I	I	S	S	4
6637	S	S	S	S	S	I	S	S	I	S	S	S	0
6638	S	S	S	S	S	S	S	S	I	I	S	S	0
6639	S	S	S	S	S	S	S	S	S	S	S	S	0
6640	S	S	S	S	S	S	R	S	S	S	S	S	1
6641	S	S	S	S	S	S	S	S	I	S	S	S	0
6643	S	S	S	S	S	S	S	S	S	S	S	S	0
6647	S	S	S	S	S	S	S	S	S	S	S	S	0
6652	S	S	S	S	S	S	S	S	S	S	S	S	0
6776	S	S	S	S	S	S	S	S	S	S	S	S	0
6777	S	S	S	R	R	S	S	R	S	S	R	R	5
6779	S	S	S	R	R	S	R	R	S	S	R	R	6
6780	I	S	I	I	S	I	I	S	S	I	R	R	2
6784	S	S	S	S	S	S	S	S	S	S	S	S	0
6785	R	S	S	S	S	S	S	S	S	I	S	S	1
6786	R	S	S	S	R	R	R	R	R	R	R	R	9
6787	S	S	S	R	R	S	S	R	S	S	R	R	5
6788	R	S	S	S	S	S	S	S	S	I	S	S	1
6783	R	S	S	S	R	S	R	R	S	I	S	S	4
6793	S	S	S	S	S	S	S	S	S	S	S	S	0
6794	S	S	S	S	S	S	S	S	S	S	S	S	0
6795	S	S	S	S	S	S	S	S	S	S	S	S	0

6840	S	S	S	S	S	S	R	S	S	S	S	S	1
6789	S	S	S	S	S	S	S	S	R	S	S	S	1
6790	S	S	S	S	S	S	S	S	S	S	S	S	0
6791	S	S	S	S	S	S	S	S	S	S	S	S	0
6792	I	S	S	S	R	S	S	R	I	S	S	S	2
6800	S	S	S	S	S	S	R	S	S	S	S	S	1
6802	S	S	S	R	R	S	S	R	S	S	R	R	5
6805	S	S	S	S	S	I	I	S	S	S	S	S	0
6806	R	S	S	S	S	S	S	S	S	I	S	S	1
6807	S	S	S	S	S	S	I	S	S	S	S	S	0
6808	S	S	S	S	R	S	S	R	I	S	S	S	2
6809	S	S	S	S	R	S	S	R	S	S	S	S	2
6822	S	S	S	S	R	S	R	R	S	S	R	R	5
6823	S	R	R	S	S	R	R	S	R	S	R	R	7
6825	S	S	S	S	S	S	I	S	S	S	S	S	0
6814	S	S	S	S	S	S	S	S	S	S	S	S	0
6815	S	S	S	S	S	S	I	S	S	S	S	S	0
6817	S	S	S	S	S	S	S	S	S	S	S	S	0
6819	S	S	S	S	S	S	S	S	S	S	S	S	0
6818	S	R	R	S	S	R	R	S	R	S	R	R	7
6820	S	S	S	S	S	S	I	S	S	S	S	S	0
6866	I	S	S	R	R	S	R	R	S	I	R	R	6
6867	S	R	R	S	S	R	R	S	R	S	R	R	7
6871	S	S	S	S	R	I	S	R	S	S	R	R	4
6872	S	S	S	S	S	S	I	S	S	S	S	S	0
6869	S	S	S	S	S	S	I	S	S	S	S	S	0
6870	S	S	S	S	S	S	S	S	S	S	S	S	0
6873	S	S	S	S	S	S	S	S	S	S	S	S	0

6874	S	S	S	R	R	S	S	R	S	S	R	R	5
6875	S	S	S	S	S	S	S	S	S	S	S	S	0
6876	R	S	S	S	R	S	R	R	S	I	S	S	4
6877	S	S	S	S	S	S	I	S	S	S	S	S	0
6878	R	S	S	S	R	S	R	R	S	I	S	S	4
6718	R	S	S	S	R	S	R	R	S	I	S	S	4
6719	R	S	S	S	S	S	S	S	S	I	S	S	1
6720	R	S	S	S	R	S	R	R	S	I	S	S	4
6711	S	S	S	S	S	S	S	S	S	S	S	S	0
6712	R	S	S	S	S	S	S	S	S	I	S	S	1
6713	R	S	S	S	S	S	S	S	S	I	S	R	2
6714	S	S	S	S	S	S	I	S	S	S	S	S	0
6716	S	S	S	S	S	S	I	S	S	S	S	S	0
6717	R	S	I	S	S	S	R	S	R	R	R	R	6
6721	R	S	S	R	R	S	S	R	S	I	R	R	6
6722	R	S	S	S	S	I	R	S	R	R	R	R	6
6723	R	S	S	S	R	S	R	R	R	R	R	R	8
6724	R	S	S	S	S	S	R	S	R	R	R	R	6
6725	R	S	S	S	S	S	S	S	S	I	S	S	1
6726	R	S	S	S	S	S	I	R	I	I	S	S	2
6727	S	S	S	S	S	S	R	S	S	S	S	S	1
6729	R	S	S	R	R	S	R	R	S	R	R	R	8
6731	R	S	S	S	S	S	R	S	R	I	R	R	5
6732	S	S	S	R	R	S	R	R	S	S	R	R	6
6735	R	S	S	S	S	S	S	S	S	R	S	S	2
6733	S	S	S	R	R	S	S	R	S	S	R	R	5
6734	R	S	S	S	R	S	R	R	S	I	S	S	4
6736	R	S	S	S	R	S	R	R	S	I	S	S	4

6738	R	S	S	R	R	S	S	R	S	I	R	R	6
6740	S	S	S	S	S	I	R	S	S	S	S	S	1
6715	I	S	S	S	S	S	S	S	S	I	S	S	0
6730	S	S	S	S	S	S	R	S	S	S	S	S	1
6695	S	S	S	S	S	I	I	S	R	S	S	I	1
6693	R	S	S	S	R	S	R	R	S	I	S	S	4
6671	S	S	S	S	S	S	R	S	S	S	S	R	2
6694	S	S	S	S	S	I	I	S	I	S	S	I	0
6886	S	S	S	S	S	S	S	S	S	S	S	S	0
6887	S	S	S	S	S	S	R	S	S	S	S	R	2
6888	S	S	S	R	R	S	R	R	S	S	R	R	6
6889	S	S	S	S	S	S	R	S	S	S	S	S	1
6891	S	S	S	S	S	S	S	S	S	S	S	S	0
6892	R	S	S	S	I	R	S	S	S	I	R	R	4
6893	S	S	S	S	S	S	S	S	S	S	S	S	0
6894	S	S	S	S	S	S	S	S	S	S	S	S	0
6326	S	S	S	S	S	S	S	S	S	S	S	I	0
6327	R	S	S	S	S	S	I	S	S	I	I	S	1
6328	S	S	S	S	S	S	I	S	S	S	S	S	0
6329	R	S	S	S	S	S	I	S	S	I	S	I	1
6447	S	S	S	R	R	R	I	R	I	S	R	R	6
6448	S	S	S	S	S	S	R	S	I	S	S	S	1
6445	S	S	S	S	S	S	S	R	S	S	S	I	1
6446	S	S	S	I	R	R	R	R	R	S	R	R	7
6450	S	S	S	S	S	I	R	S	S	S	S	S	1
6451	S	S	S	R	R	I	I	R	S	S	R	R	5
6926	R	S	S	S	R	S	R	R	I	R	S	R	6
6928	S	S	S	I	S	I	I	S	S	S	R	R	2

6929 R I S S S R S R I I I I 3

Appendix 3: Media preparation

MacConkey Agar

Formula

- Peptic digest of animal tissue 20g/l
- Lactose 10g/l
- Sodium taurocholate 5g/l
- Neutral red 0.04g/l
- Agar 20g/l

Preparation

Suspend 50 g in one liter of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving ay 121^{o} C for 15 minutes. Cool to 45 to 50^{o} C and pour in 20ml amounts in Petri dishes.

Use: Enteric bacteria

Mueller Hinton agar

Formula

- Beef infusion
- Starch
- Acid hydrolysate of casein
- Agar

Preparation

Suspend 55 g in one liter of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121^{o} C for 15 minutes, cool to 50^{o} C and pour 20ml of media into the plate.

Use: For sensitivity testing.

Luria- Bertani (LB) broth

Formula

- Tryptone 10g/l
- Yeast extract 5g/l
- Sodium chloride 5g/l
- 10N NaOH 1ml/l

Preparation

Heat gently, to thoroughly dissolve all the ingredients. Avoid boiling. Dispense 3ml into screw capped tubes and autoclave at 121^{o}C for 15minutes.

Appendix 4: Serotyping for non-typhoid *Salmonella*

Principle

The Kauffman-White classification system is used to classify salmonellae by serological methods. This scheme differentiates isolates by determining which surface antigens are produced by the bacterium, i.e. the O (polysaccharides associated with the lipopolysaccharide of the bacteria outer membrane) and H (proteins associated with the flagellar) antigens. Different "H" antigens are produced depending on the phase in which the S*almonella* is found, motile or non motile phase. Pathogenic strains of *Salmonella typhi* carry an additional antigen, "Vi", so-called because of the enhanced virulence of strains that produce this antigen, which is associated with a bacterial capsule.

O antigens: these are cell wall, heat-stable antigens. Salmonellae are grouped by there O antigens. The groups are designated A to Z, 51 to 61, and 64-66. Many of the medically important salmonellae belong to the groups A-Z.

Each group has what is called a group factor. This is an O antigen, common to all members of the group and not possessed by salmonellae belonging to other groups.

H antigens: these are flagella, heat labile antigens. Salmonellae are serotyped by their H antigens. Many salmonellae are diphasic, that is, they can occur in two antigenic forms referred as phase 1 and phase 11.phase 1 are given alphabetic letters, and phase 11 antigens are either numbered or given a letter if known to occur in both phases.

Phase 1 antigens are specific and therefore an organism can be identifies as if it is in phase 1 (or is single phase salmonellae such as *S*. Typhi).

Vi antigens: this surface (K) antigen can be found on S. Typhi, s. Paratyphi C, and a few other salmonellae. It is associated with virulence and can be detected using Vi antiserum. Vi antigen can interfere with O antigen testing. If therefore an isolate agglutinates Vi antiserum but not an O antiserum, interference from Vi should be suspected. A saline suspension of the organism should be heated in a container of boiling water for 20 minutes, and after being allowed to cool, the bacterial cells should be retested with the O antiserum.

Procedure

Grown organisms on MacConkey agar from a single selected colony and re-grown on Mueller Hinton agar plates. The plates were incubated at 37^{o} C for 24 hours. Using a sterile loop, pick a single colony from the agar surface. Emulsify the colony in three drops of normal saline and mixed thoroughly on to a glass slide. Put a small drop each antisera to each bacterial suspension; the following antisera were used: poly O and poly H (phase 1 and phase 2) antisera: *Salmonella* 9-O, *Salmonella* 3,10,15,19-O, poly H phase 2, *Salmonella* f,g- H, *Salmonella* 4-O, *Salmonella* 8-O, poly O Group A-G, *Salmonella* d-H, *Salmonella* Vi, Salmonella Group C1(6,7), *Salmonella* 7-O, *Salmonella* i-H, *Salmonella* g,m-H, Salmonella 1,2-H, *Salmonella* 1,5-H, and *Salmonella* 1,7-H. Mixed the suspensions thoroughly and the slide was tilted back and forth to observe for agglutination.

Appendix 5: Antimicrobial sensitivity testing

Reporting Results:

A susceptible, intermediate, or resistant interpretation of zone diameter measurements are reported and defined as follows:

1. **Susceptible (S)**

The "susceptible" category implies that an infection due to the strain may be appropriately treated with the dosage of antimicrobial agent recommended for that type of infection and infecting species, unless otherwise contraindicated.

2. **Intermediate (I)**

The "intermediate" category includes isolates with antimicrobial agent MICs that approach usually attainable blood and tissue levels and for which response rates may be lower than for susceptible isolates. The "intermediate" category implies clinical applicability in body sites where the drugs are physiologically concentrated (e.g., quinolones and ß-lactams in urine) or when a high dosage of a drug can be used (e.g., ßlactams). The "intermediate" category also includes a "buffer zone" which should prevent small, uncontrolled technical factors from causing major discrepancies in interpretations, especially for drugs with narrow pharmacotoxicity margins.

3. **Resistant (R)**

Resistant strains are not inhibited by the usually achievable systemic concentrations of the agent with normal dosage schedules and/or fall in the range where specific microbial resistance mechanisms are likely (e.g., ß-lactamases) and clinical efficacy has not been reliable in treatment studies.

Appendix 6. Zone diameter interpretive standards and equivalent minimal inhibitory concentration (MIC) breakpoints for Enterobacteriaceae

Test/Report Group	**Antimicrobial Agent**	**Disk Content**	**Zone Diameter, Nearest Whole mm**			**Equivalent** MIC (µg/mL)	
			R	I	S	R	S
PENICILLINS							
A	Ampicillin	10 µg	≤13	14-16	≥17	≥32	≤8
B-LACTAM/β-LACTAMASE INHIBITOR COMBINATIONS							
B	Amoxillin-clavulanic acid	20/10µg	≤13	14-17	≥18	≥32/16	≤8/4
CEPHEMS(PARENTERAL)							
B	Cefuroxime sodium (parenteral)	30µg	≤14	15-17	≥18	≥32	≤8
B	Cefotaxime	30µg	≤14	15-22	≥23	≥64	≤8
B	Ceftriaxone	30µg	≤13	14-	≥21	≥64	≤8

				20			
AMINOGLYCOSIDES							
A	Gentamicin	10µg	≤12	13-14	≥15	≥8	≤4
TETRACYCLINES							
C	Tetracycline	30µg	≤14	15-18	≥19	≥16	≤4
FLUOROQUINOLONES							
B	Ciprofloxacin	5µg	≤15	16-20	≥21	≥4	≤1
O	Nalidixic Acid	30µg	≤13	14-18	≥19	≥32	≤8
FOLATE PATHWAY INHIBITORS							
B	Trimethoprim-sulfamethoxazole	1.25/23.75µg	≤10	11-15	≥16	≥8/152	≤2/38
U	Trimethoprim	5µg	≤10	10-15	≥16	≥16	≤4
PHENICOLS							
C	Chloramphenicol	30µg	≤12	13-17	≥18	≥32	≤8