Characterization of Antibiotic Resistance Plasmids in *Salmonella Enterica* *Serovar* Typhi Isolated in Nairobi, Kenya

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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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DEDICATION

My beloved parents Mr and Mrs. Mutai who bore me, raised me up and encouraged me to do my best in all matters of life. You have supported me always, taught me, and most of all loved me. It is to you that I dedicate this thesis.

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LIST OF ABBREVIATIONS AND ACRONYMS

AKUH	Aga Khan University Hospital
CDC	Centres for Disease Control and Prevention
CLSI	Clinical and Laboratory Standards Institute
CMR	Centre for Microbiology Research
DNA	Deoxyribonucleic acid
ESBL	Extended Spectrum Beta-Lactamase
JKUAT	Jomo Kenyatta University of Agriculture and Technology
KEMRI	Kenya Medical Research Institute
KNH	Kenyatta National Hospital
MDa	Mega daltons
MDR	Multi Drug Resistance
MIC	Minimum Inhibitory Concentration
mM	Millimolar
PCR	Polymerase Chain Reaction
QRDR	Quinolone resistance determining region
Rpm	Revolutions per minute
SDS	Sodium Dodecyl Sulphate
TBE	Tris-Borate ethylenediamine-tetra-acetic acid
UV	Ultraviolet
W/v	Weight per volume
WHO	World Health Organization

ABSTRACT

Typhoid fever caused by *Salmonella enterica* serotype Typhi (*S.* Typhi) is a significant cause of morbidity and mortality among children and adults in developing countries, where the disease burden is high and cost constraints prevent the widespread use of newer, more effective but more expensive agents. The emergence and global dissemination of Multiple Drug Resistant (MDR) *S.* Typhi has posed major public-health problems and is a challenge in health care in the developing countries. The aim of this study was to characterise plasmids that encode multi-drug resistance among the *S.* Typhi isolates and explore their role in the genetic diversity of *S.* Typhi strains using antibiotic susceptibility, plasmid profiling, incompatibility grouping and Polymerase Chain Reaction (PCR) of resistance genes.

A representative sample of 144 archived isolates from cultures of blood and celebrospinal fluid of patients visiting Kenyatta National Hospital and Aga Khan University Hospital in Nairobi, Kenya were tested for their susceptibilities to 10 antimicrobial agents; ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), co-amoxyclav (30 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), ceftriaxone (30 µg), and nalidixic acid (10 µg). A total of 16% of the isolates were susceptible to all the drugs tested while 84% were resistant to one or more of the 10 drugs tested. Of main concern was a high prevalence of 75% of MDR *S.* Typhi strains. A high percentage of resistance was observed for the first-line antibiotics (ampicillin (71%), chloramphenicol (72%), and tetracycline (74%) which are the main drugs used to treat infectious diseases in Kenya owing to their inexpensive nature.

Approximately 80% of the drug resistance in *S.* Typhi analysed was associated with presence of plasmid DNA. Class 1 integrons and transposon Tn21, was also detected. Chromosomal mediated resistance was also evidenced by the presence of *gyr A*, *gyrB, parE,* and *parC* genes encoding for quinolone and floroquinolone resistance. A proportion of 80% of the MDR *S.* Typhi strains selected for conjugation experiment transferred one or all their resistance phenotypes to the recipient strain *E. coli* K12 suggesting that resistance genes can easily be transferred or acquired rapidly among the bacterial population. This study revealed that the plasmids encoding antibiotic resistance genes have a high degree of genetic similarity since 95% of the MDR isolates carried plasmids of incompatibility group HI1. It appears that a single strain type containing a plasmid conferring multidrug-resistance has emerged within the *S.* Typhi bacterial population in Kenya and has been able to adapt and survive the challenge of antibiotics as they are introduced into clinical use. The other replicons identified included IncI1, IncP, and IncFIC.

Unfortunately, there are still gaps in our understanding of how new multi-resistance plasmids evolve and the genetic relatedness among them. This therefore calls for a need to carry out more studies that will address the dynamics of antibiotic resistance genes.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Salmonella is a genus of rod-shaped gram-negative enterobacteria that causes typhoid fever, paratyphoid fever, and foodborne illness (Ryan and Ray, 2004). *Salmonella enterica* serovar Typhi (*S.* Typhi) is the etiological agent, of typhoid fever and is an invasive human pathogen (Crump *et al.*, 2004). This pathogen has evolved remarkable mechanisms for persistence in its host that help to ensure its survival and transmission (Parry *et al.*, 2002). *S.* Typhi can be transmitted by ingestion of food or water contaminated by fecal or urinary carriers excreting *S.* Typhi (Gasem *et al.*, 2001). In addition, these bacteria can survive for prolonged periods in water, ice, dust and dried sewage and these may become sources of infection.

In endemic areas, peaks of transmission occur in dry weather or at the onset of rains. Risk factors for disease include eating food prepared under unhygienic conditions, drinking contaminated water and eating vegetables and salads that have been grown with human waste as fertilizer (Black *et al.*, 1985; Parry, 2006; Velema *et al.*, 1997). A close contact with a person who has recently had typhoid fever, poor housing, poor personal hygiene and recent consumption of antimicrobials are further risk factors. Transmission of typhoid has also been attributed to flies, laboratory mishaps, unsterile instruments and anal intercourse (Gasem *et al.*, 2001; Luby *et al.*, 1998; Luxemburger *et al.*, 2001).

Typhoid infections result in a clinical syndrome that varies widely in severity. In Africa, malaria is probably the most important disease from which typhoid must be distinguished (Nsutebu *et al.*, 2003). The symptoms of typhoid fever include influenza-like symptoms with chills, a dull frontal headache, malaise, anorexia, nausea, poorly localized abdominal discomfort, a dry cough, and myalgia, but with few physical signs and in some cases, a characteristic rose spot rash (Connor *et al.*, 2005; Cunha BA., 2006). A coated tongue, tender abdomen, hepatomegaly and splenomegaly are also common symptoms (Vihn *et al.*, 1996). Treatment of typhoid fever requires effective antimicrobial chemotherapy in order to reduce mortality. Historically, drugs of choice were chloramphenicol, ampicillin and co-trimoxazole(Connor and Schwartz, 2005). Indiscriminate use of antibiotics has however led to the emergence of multidrug-resistant strains of *S.* Typhi (Kariuki *et al.*, 2000; Mirza *et al.*, 2000; Parry, 2004; WHO, 1996). Antibiotic resistance is progressive, evolving from low levels through intermediate to high levels.

Bacterial resistance to antimicrobial agents is a serious problem worldwide with regard to treatment of infectious diseases. Understanding of the molecular basis of how resistance genes are acquired and transmitted may contribute to the creation of new antimicrobial strategies. The spread of antibiotic resistance is usually associated with either the clonal spread of an epidemic strain or through independent acquisition of the resistance genes on plasmids, transposons or integrons (Martínez *et al.*, 2007; Shanahan *et al.*, 2000). Naturally occurring gene expression elements called

"integrons" have been described as vehicles for the acquisition of resistance genes carried by mobile elements (Hall and Collis, 1995). These structures have also been found to be involved in the genetic reassortment of resistance determinants frequently observed in multiple-antibiotic-resistant bacterial pathogens (Collis and Hall, 1992).

Plasmid-associated resistance genes have been discovered for a majority of known antimicrobials. It is not uncommon for a single plasmid to simultaneously mediate resistance to five or more antimicrobials (Hawkey, 2003). This ability to sequester multiple resistance genes is of particular concern to modern medicine because they are responsible for emergence of drug resistance. After the multidrug-resistant (MDR) typhoid fever epidemic which occurred in Mexico in 1972 (Olarte and Galindo, 1973), large autotransferable resistance plasmids harboured by MDR serovar Typhi strains have been isolated worldwide (Rahman *et al.*, 2002; Thong *et al.*, 2000). MDR typhoid fever is worse in developing countries namely, Africa (Adeleye and Adetosoye, 1993), the Middle East (Panigrahi *et al.*, 1996), Southern Asia (Albert *et al.*, 1991; Mirza and Hart, 1993) and Southeast Asia (Ling *et al.*, 2000) where typhoid fever remains endemic.

The relatedness of resistance plasmids harbored by bacterial strains of various origins can be demonstrated by a variety of methods including incompatibility grouping, restriction fragmentation pattern analysis and identification of specific resistance determinants located on the plasmids (Carattoli, 2003). These analyses may provide a better understanding of how resistance plasmids propagate, and may help us trace their

evolution. Identification of conserved plasmids in a wide variety of pathogens isolated in different countries may help define the role of plasmids in the dissemination of antimicrobial resistance among bacterial pathogens (Carattoli, 2003).

1.2 JUSTIFICATION

The emergence and global dissemination of MDR *S.* Typhi has posed major public-health problems and a major challenge in health care in the developing countries. Based on the increases in the prevalence of the MDR phenotypes in Kenya, it appears that MDR *S.* Typhi strains have been spreading to other parts of Kenya and are gradually replacing the fully sensitive strain type.

MDR *S.* Typhi harbours a variety of plasmids. The identification of a family of related plasmids has serious public health implications since it demonstrates that broad-host-range plasmids carrying resistance to clinically relevant antibiotics can spread worldwide among bacteria. The fact that conserved plasmids can be identified in a wide variety of pathogens isolated in different countries illustrates the important role of plasmids in the dissemination of antimicrobial resistance among bacterial pathogens.

While several individual plasmids have been the subject of intensive study, few studies have addressed the dynamics of antibiotic resistance genes. Therefore, a comprehensive study is necessary to obtain a view of resistance genes possessed by strains of *S.* Typhi in order to address clinical challenges. Molecular characterization of plasmids encoding MDR in *S.* Typhi is useful in determining the genetic basis of resistance and understanding the genetic relatedness among plasmids encoding MDR in *S.* Typhi. This will allow us to understand the chain of transmission and determine the possible methods for control of emergence and spread of resistance and the

development of effective vaccines as well as the identification of new targets for novel antimicrobial agents.

1.3 HYPOTHESIS

1.3.1 Null hypothesis

The plasmids coding for MDR in *Salmonella enterica* serovar Typhi isolates do not belong to the same incompatibility group

1.4 OBJECTIVES

1.4.1 General objective

To determine the antimicrobial susceptibility patterns and the degree of genetic relatedness of plasmids encoding multi-drug resistance in *S.* Typhi isolates from patients in Nairobi.

1.4.2 Specific objectives

a) To determine antibiotic susceptibility patterns among *S.* Typhi isolates.
b) To determine the presence of plasmids encoding antibiotic resistance and their transmission potential.
c) To determine the genetic relatedness of MDR plasmids and their host incompatibility.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Characteristics of *Salmonella*

Salmonella is a genus of rod-shaped Gram-negative bacteria belonging to the family Enterobacteriaceae (Ryan and Ray, 2004; Tindall *et al.*, 2005). They are motile by peritrichous flagella and non-capsulated, and they do not ferment lactose (Kelly, 2004). The bacteria grow both aerobically and anaerobically, between 7^0C and 48^0C with an optimum temperature of 37^0C, at pH 4-8. They are resistant to both freezing and drying especially in the presence of proteins and other protectants (Baird-Parker, 1990).

2.2 Classification of *Salmonella*

Salmonella species are most commonly classified according to serology (Popoff *et al.*, 2000). Serotyping is an important epidemiological tool for determining the source of disease outbreaks (Murlin and Hickman-Brenner, 1994). There are over 2500 serovars recognized by the World Health Organisation (WHO) Collaborating Centre for Reference and Research on *Salmonella*, with 20 to 35 new serotypes described each year (WHO, 2003). Currently, the Centre for Disease Control and prevention (CDC) recognizes two species of *Salmonella* namely *S. enterica* and *S. bongori. S. bongori* contains 22 serovars, and *S. enterica* contains over 2500 serovars, divided among six subspecies: *S. enterica* subsp. *enterica* (subspecies I), *S. enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp.

diarizonae (subspecies IIIb), *S. enterica* subsp. *houtenae* (subspecies IV), and *S. enterica* subsp. *indica* (subspecies VI) (Tindall *et al.*, 2005). Most Salmonella serotypes isolated from humans belong to *Salmonella enterica* subspecies I (Cuff *et al.*, 2000; Dice, 1945).

The classification scheme instituted by White (1926) and extended and elaborated by Kauffmann (1978) used to serotype salmonellae is based on antigenic polymorphisms of the lipopolysaccharides (LPS) (O) and the flagella (H). A serotype (as well as a serovar) is determined by the O and H factor antigens present in the cell wall of *Salmonella* organisms (Popoff *et al.*, 2000). The total number of O or H factors present in each serotype varies from one to four different factors. The O factors determine the grouping, while the H factors completely define the serotype identity of a *Salmonella* strain. With several monophasic exceptions, the H antigens for each serotype are usually diphasic (e.g., *S. enterica* subsp. *enterica* [subspecies I] serotype 4, 5, 12: i: 1, 2). To fully serotype all recognized serotypes requires the availability of more than 250 O and H typing antisera (Cuff *et al.*, 2000; Dice, 1945). *S.* Typhi is serologically positive for lipopolysaccharide antigens O9 and O12, protein flagellar antigen Hd, and polysaccharide capsular antigen Vi. The Vi capsular antigen is largely restricted to *S.* Typhi, although it is shared by some strains of *S. enterica* serotypes hirschfeldii (paratyphi C) and dublin, and *Citrobacter freundii* (Parry *et al.*, 2002).

2.3 Epidemiology of typhoid fever

Typhoid fever is a significant cause of morbidity and mortality among children and adults especially in developing countries, where the infectious disease burden is high. Urbanization, poor sanitation and faecal contamination of water and food supplies, antibiotic resistance, variable efficacy of vaccine preparations, and increased regional movement of large numbers of migrant workers are significant risk factors associated with transmission of typhoid fever (Connerton *et al.*, 2000; Crump *et al.*, 2004). Cost constraints prevent the widespread application of newer, more effective yet more expensive agents (Mills-Robertson *et al.*, 2003; Wain *et al.*, 2003).

Limited surveillance systems for typhoid exist in the developing world, especially in community settings and for this reason the true burden is difficult to estimate (Bhutta, 2006). This was evident in the recent revisions in the global estimates of the true burden of typhoid. In contrast to previous estimates, which were 60% higher, investigators from the CDC estimate that there are 21.6 million typhoid cases annually, with the annual incidence varying from 100 to 1000 cases per 100, 000 population (Crump *et al.*, 2004). The global mortality estimates from typhoid have also been revised downwards from 600, 000 to 200, 000, largely on the basis of regional extrapolations (Crump *et al.*, 2004).

The main factor that affects the changing epidemiology of typhoid is the emergence of drug resistant strains (Madhulika *et al.*, 2004). After sporadic outbreaks of chloramphenicol resistant typhoid between 1970 and 1985, many strains of *S.* Typhi

developed plasmid mediated multidrug resistance to the three primary antimicrobials used (ampicillin, chloramphenicol and co-trimoxazole) (Rowe *et al.*, 1997; Senthilkumar and Prabakaran, 2005). This was countered by the advent of oral flouroquinolones, but chromosomally acquired quinolone resistance in *S.* Typhi has been described in various parts of Asia, possibly related to the widespread and indiscriminate use of flouroquinolones (Renuka *et al.*, 2005; Shirakawa *et al.*, 2006).

In pseudo-epidemic regions including the Middle East and Egypt, infection with MDR *S.* Typhi is generally related to migrant workers from the endemic zone but epidemics can also occur. Sporadic infections occur in Europe and North America, most often in immigrants who have returned to their original home country for a holiday (Mirza, 2005), with occasional point-source epidemics (Ackers *et al.*, 2000).

In developed countries, the incidence of cases and death has been greatly decreased by a combination of improved sanitation, hygiene, vaccines and effective antimicrobial chemotherapy. The first two are difficult to implement in many developing countries, and unfortunately, the effectiveness of antimicrobial chemotherapy is also being eroded by the emergence of antibiotic resistance (Mirza *et al.*, 1995; Wain *et al.*, 1997).

Multidrug-resistant *S.* Typhi showing resistance to nearly all of the commonly available "first line" antimicrobial agents including ampicillin, tetracycline, co-trimoxazole, streptomycin or chloramphenicol used for the treatment of these and

other infections have been isolated in Kenya (Kariuki *et al.*, 2000). Since the first report of MDR serovar Typhi outbreaks, which occurred in Kenya between 1997 and 1999 (Kariuki *et al.*, 2000) the prevalence of the MDR serovar Typhi has been rising steadily and that, at present, 70% to 78% of all serovar Typhi isolates from blood cultures from the main referral hospital, the Kenyatta National Hospital, in Nairobi are MDR (Kariuki *et al.*, 2004). This is raising concerns of further spread to other regions where typhoid fever is not endemic and hence it may become a global problem. Spread of MDR results from the clonal dissemination of individual MDR *S.* Typhi strains or from transfer of the resistance plasmids to multiple *S.* Typhi strains (Connerton *et al.*, 2000; Mirza *et al.*, 2000; Thong *et al.*, 2000) .

Recent population based studies from South-East Asia suggest that the incidence of typhoid is highest in children aged less than five years, with higher rates of complications and hospitalization an indication of risk and early exposure to relatively large infecting doses of the organisms in these populations (Brooks *et al.*, 2000; Siddiqui *et al.*, 2006). As with drug-susceptible typhoid fever, children are among the target population affected and constitute upto 40-50% of MDR cases during outbreaks (Mediadora, 1996). Those with MDR typhoid fever show a significantly higher incidence of complications. The overall mortality rate reported ranges between seven and 16 % higher than what is observed with susceptible typhoid fever, and is more likely due to delay in instituting effective antibiotic therapy rather than increased virulence of MDR *S.* Typhi (Slinger *et al.*, 2004).

Most patients who recover from the infection are able to eliminate the bacterium completely from their bodies. However, some of them may remain as healthy or asymptomatic carriers, continuously shedding *S.* Typhi in their stool (Yichun *et al.*, 2003). An individual can asymptomatically carry the *S.* Typhi for days to years without showing any symptoms of typhoid fever (Ismail, 2006). The silent carriers are the source of *S.* Typhi for the continued episodes of infections (Senthilkumar and Prabakaran, 2005). Most infections result in self-limiting diarrhoea and do not require antimicrobial treatment. However, severe, life-threatening bacteraemia and other deep-seated infections do occur, particularly in children and immunocompromised hosts and in these cases antimicrobial treatment is recommended (Parry, 2004). In addition to improvement of sanitation and hygiene, surveillance and appropriate use of effective typhoid vaccine, control of spread of MDR *S.* Typhi should include promotion of rational antibiotic usage (Slinger *et al.*, 2004).

2.4 Pathogenesis of typhoid fever

Humans are the only natural host and reservoir of infection for *S.* Typhi. The infectious dose varies between 1000 and one million organisms (Hornick *et al.*, 1970). The Vi-negative strains of *S.* Typhi are less infectious and less virulent than Vi-positive strains (Parry *et al.*, 2002). *S.* Typhi must survive the gastric acid barrier to reach the small intestine and a low gastric pH is an important defense mechanism (Parry *et al.*, 2002). In the small intestine, the bacteria adhere to mucosal cells and then invade the mucosa (House *et al.*, 2001). The M cells, specialized epithelial cells

overlying Peyer's patches, are probably the site of the internalization of *S.* Typhi and its transport to the underlying lymphoid tissue (Parry *et al.*, 2002). After penetration, the invading microorganisms translocate to the intestinal lymphoid follicles and the draining mesenteric lymph nodes and some pass on to the reticuloendothelial cells of the liver and spleen (Parry *et al.*, 2002).

Salmonellae are able to survive and multiply within the mononuclear phagocytic cells of the lymphoid follicles, liver and spleen (House *et al.*, 2001). The incubation period is usually seven to 14 days. After the incubation period, the onset of a sustained secondary bacteraemia results in clinical disease. In the bacteremic phase, the organism is widely disseminated. The bacteraemia of typhoid fever persists for several weeks if antibiotic therapy is not given. The most common sites of secondary infection are the liver, spleen, bone marrow, gallbladder and Peyer's patches of the terminal ileum (Parry *et al.*, 2002). Organisms excreted in the bile either reinvade the intestinal wall or are excreted in the faeces.

Counts of bacteria in patients with acute typhoid fever indicate a median concentration of one bacterium per millilitre of blood (about 66 % of which are inside phagocytic cells) and about 10 bacteria per millilitre of bone marrow (Wain *et al.*, 2001). Even though *S.* Typhi produces a potent endotoxin, mortality from treated typhoid fever for patients at this stage is less than one percent. Studies have shown increased levels of circulating pro-inflammatory and anti-inflammatory cytokines in patients with typhoid and a reduced capacity of whole blood to produce inflammatory cytokines in patients

with severe disease (Butler *et al.*, 1993; Keuter *et al.*, 1994). Ulceration of Peyer's patches is seen where the inflammatory process has resulted in ischaemia and necrosis (Everest *et al.*, 2001).

Typhoid induces systemic and local humoral and cellular immune responses, but these confer incomplete protection against relapse and re-infection. The interaction of host immunologic mediators and bacterial factors in infected tissue may contribute to the necrosis of Peyer's patches in severe disease (Everest *et al.*, 2001). Major-histocompatibility-complex class II and class III alleles have been shown to be associated with typhoid fever. HLA-DRB1*0301/6/8, HLA-DQB1*0201-3 and TNFA*2(–308) alleles are found to be associated with susceptibility to typhoid fever, whereas HLA-DRB1*04, HLA-DQB1*0401/2 and TNFA*1(–308) alleles are associated with disease resistance (Dunstan *et al.*, 2001).

2.5 Clinical features of typhoid fever

S. Typhi infections result in a clinical syndrome that varies widely in severity. In Africa, malaria is probably the most important disease from which typhoid must be distinguished (Nsutebu *et al.*, 2003). Recent data from South-East Asia indicate that the presentation of typhoid may be more dramatic in children younger than five years, with higher rates of complications and hospitalisation (Siddiqui *et al.*, 2006). After the initial seven to 14 day asymptomatic phase, the clinical features of typhoid fever begin with the onset of a remitting diurnal fever, anorexia, dull frontal headache, lethargy, confusion, dry cough and abdominal pain (Caumes *et al.*, 2001). The fever, initially

low grade, rises progressively and by the second week is often high and sustained (39–40 °C) and the patient looks toxic and apathetic (Connor and Schwartz, 2005). Other symptoms include malaise, poorly localized abdominal discomfort and myalgia.

Physical signs are few, but a coated tongue, tender abdomen, hepatomegaly and/or splenomegaly may be found (Connor and Schwartz, 2005). Rose spots are reported in 5–30% of cases but are easily missed in dark-skinned patients (Parry, 2006). These rose spots are small blanching erythematous maculopapular lesions typically on the abdomen and chest. The spots are caused by bacterial immobilization (Connor and Schwartz, 2005). Relative bradycardia is common during the first two weeks. Constipation is generally more common in adults, but in young children and adults with HIV infection diarrhoea predominates (Otegbayo *et al.*, 2002). Not all patients experience classic symptoms, however, so stool and blood cultures should be performed on patients with persistent high fevers who have recently travelled to a developing country (Snow, 2006).

If untreated, the fever persists for two weeks or more and defervescence occurs slowly over the following two to three weeks (Parry, 2006). Convalescence may last for three to four months. If an appropriate antibiotic is given the fever gradually falls over three to four days. The duration of untreated illness prior to the initiation of therapy influences the severity of the disease (Parry, 2006). Those individuals infected with MDR *S.* Typhi may also suffer more severe disease with higher rates of toxicity, complications, and mortality than infections with sensitive strains (Bhutta, 1996).

Patients with typhoid fever in the second to fourth week present with accelerating weight loss, weakness, an alteration of mental state, intestinal complications and the development of complications that occur in 10–15% of hospitalized patients. Gastrointestinal bleeding occurs in approximately 10% of patients and results from erosion of a necrotic Peyer's patch through the wall of an enteric vessel. Usually the bleeding is slight and resolves without the need for blood transfusion. Intestinal perforation is the most serious complication occurring in one to three percent of hospitalized patients. Perforation may present with an acute abdomen or more covertly with simple worsening of abdominal pain, rising pulse and falling blood pressure in an already sick patient (Ugwu *et al.*, 2005).

Typhoid encephalopathy, often accompanied by shock, is associated with a high mortality. Patients may display the "typhoid" faces, a thin, flushed face with a staring, apathetic expression. Mental apathy may progress to an agitated delirium, frequently accompanied by tremor of the hands, tremulous speech, gait ataxia, muttering delirium, twitching of the fingers and wrists (*subsultus tendinum*), agitated plucking at the bedclothes (carphology) and a staring unrousable stupor (coma vigil) (Parry, 2006).

Typhoid fever in pregnancy may be complicated by miscarriage, although antimicrobial treatment has made this less common (Parry, 2004). Vertical intra-uterine transmission from a typhoid-infected mother may lead to neonatal typhoid, a rare but severe and life-threatening complication (Reed and Klugman, 1994). Relapse

occurs in five to 10% of patients, usually two to three weeks after defervescence. The illness is usually, but not invariably, milder than the original attack and the relapse *S.* Typhi isolate has the same susceptibility pattern as in the original episode.

Approximately 10% of untreated convalescent typhoid cases will excrete *S.* Typhi in faeces for one to three months and between one and four percent become chronic carriers excreting the organism for more than one year (Ismail, 2006). Approximately one and five percent of typhoid patients with acute typhoid fevers may become asymptomatic chronic carriers of *S.* Typhi (Bhan *et al.*, 2005). Faecal carriage is more frequent in individuals with gallbladder disease and is most common in women over 40 years (Caygill *et al.*, 1994). Urinary carriage evidenced by urinary excretion is associated with schistosomiasis and nephrolithiasis (Kamel, 2004). Carrier state associated with cholelithiasis is a risk for gall bladder cancer and requires cholecystectomy (Dutta *et al.*, 2000). Chronic biliary carriage may occur in two to five percent of the cases, even after treatment. Biliary carriage is defined as continued shedding of the organism for more than a year and is a public-health risk, especially for infected individuals who work in the food industry (Connor and Schwartz, 2005). Carriers require treatment with high dose quinolones; ciprofloxacin 750 mg every 12 hours for four weeks.

2.6 Diagnosis of typhoid fever

The lack of specificity of the clinical spectrum, added to the difficulty of achieving a definitive bacteriologic or serologic diagnosis, frustrates clinicians in managing

typhoid fever (Vollaard *et al.*, 2005). Culture of the infectious agent may be obtained from stool, urine, blood, bone marrow or bile. Bone marrow is the most sensitive source (80-95%) and positive blood cultures (60-80%) are facilitated by increasing the volume sampled (Wain *et al.*, 2001). Detection of *S.* Typhi DNA by polymerase chain reaction (PCR) has recently been shown to be a very sensitive index of infection (Massi *et al.*, 2003). A nested PCR using H1-d primers has been used to amplify specific genes of *S.* Typhi in the blood of patients and is a promising means of making a rapid diagnosis (Bhutta, 2006).

Serological tests for typhoid fever have been used since the late 19th century. The Widal test, useful only for infection with *S.* Typhi, detects O (surface) and H (flagellar) antigens (Parry, 2006). However, when used as a single test in endemic areas, it lacks sensitivity and specificity (House *et al.*, 2005). A four-fold rise in O, H or Vi titers provides support for the diagnosis of typhoid fever, but is not useful in the acute situation. As a result, numerous other serologic tests are being developed including ELISA, salivary IgA, a modified Widal test to detect IgM and dipstick assay (Shirakawa *et al.*, 2006; WHO, 2003).

2.7 Treatment

WHO recommends that treatment of typhoid fever begins on the basis of clinical findings prior to definitive diagnosis. However in endemic regions, facilities for definitive diagnosis, based on blood or bone marrow culture or serologic tests may be entirely lacking. In areas of endemic disease, more than 60-90% of cases of typhoid

fever are managed at home with antibiotics and bed rest. For hospitalized patients, effective antibiotics, good nursing care, adequate nutrition, careful attention to fluid and electrolyte balance, prompt recognition and treatment of complications are necessary to avert death (WHO, 2003).

Appropriate antibiotic treatment with the right drug, dose and duration is critical to curing typhoid with minimal complications. Standard treatment with chloramphenicol or amoxicillin is associated with a relapse rate of five to 15% or four to eight percent respectively, whereas the newer quinolones and third generation cephalosporins are associated with higher cure rates (WHO, 2003). The emergence of multidrug resistant typhoid in the 1990s led to widespread use of fluoroquinolones as the treatment of choice for suspected typhoid, especially in South Asia and South-East Asia where the disease was endemic (Bhutta, 2006). They are relatively inexpensive, well tolerated, have good oral absorption and are more rapidly and reliably effective than the older drugs. With reports of rapidly increasing resistance of *S.* Typhi to fluoroquinolones, it is imperative that the widespread use of these antibiotics for fever and their availability over the counter are restricted, although it may already be too late (Okeke *et al.*, 2005). However, treatment regimens must restrict as much as possible the use of further second and third line antibiotics for treating typhoid in primary care settings (Okeke *et al.*, 2005).

In recent years the emergence of resistance to quinolones has placed tremendous pressure on public health systems in developing countries as treatment options are

limited (Bhutta, 2006). Ceftriaxone or azithromycin may be preferable in treating quinolone-resistant strains (Chinh *et al.*, 2000). Third-generation cephalosporins are widely used in children with serious infections, as quinolones are not generally recommended for this age group. The older drugs chloramphenicol, ampicillin and amoxicillin and trimethoprim-sulfamethoxazole are occasionally used as alternatives (WHO, 2003).

2.8 Prevention of typhoid fever

Typhoid fever is a waterborne disease and the main preventive measure is to ensure access to safe water. Poverty, uncontrolled urbanization and inadequate infrastructure all contribute to the contamination of water supplies (Kumar *et al.*, 2002). Access to clean high quality water for domestic use is critical in prevention of typhoid outbreaks. Boiling, filtration and chlorination together are effective methods of interrupting the transmission of water-borne diseases (WHO, 2003).

Typhoid can only spread in environments where human faeces or urine come into contact with food or drinking water, therefore appropriate human waste disposal mechanisms is important in reducing transmission of diarrhoeal pathogens. This has, for example, greatly decreased the incidence of typhoid fever (due to MDR and fluoroquinolone-resistant *S.* Typhi) in Dushanbe, Tajikistan (Mermin *et al.*, 1999). Collection and treatment of sewage, especially during the rainy season, must be implemented. In areas where typhoid fever is known to be present, the use of human excreta as fertilisers must be discouraged.

Contaminated food is an important vehicle for typhoid transmission. Appropriate food handling and processing is paramount and basic hygiene measures must be implemented during epidemics (Snow, 2006; WHO, 2003). During outbreaks, food safety inspections must be reinforced in restaurants and for street food vendors activities. Typhoid can be transmitted by chronic carriers who do not apply satisfactory food-related hygiene practices. These carriers should be excluded from any activities involving food preparation and serving. Health education is paramount to raise public awareness on all the above-mentioned prevention measures. Health education should involve excellent personal, disinfection measure and proper disposal of human excreta. Community involvement is the cornerstone of behaviour change with regard to hygiene and for setting up and maintenance of the needed infrastructures (WHO, 2003).

The other approach to the control and eradication of typhoid fever has been through vaccination. Vaccination is recommended for travellers to areas where sanitation and food hygiene is likely to be poor, for household contacts of typhoid carriers and for laboratory workers who may have contact with the bacterium (Connor and Schwartz, 2005; Steffen *et al.*, 2003). There are two vaccines currently recommended by the WHO for the prevention of typhoid: these are the live, oral Ty21a vaccine and the injectable Vi capsular polysaccharide vaccine. Vi capsular polysaccharide vaccine results in local pain in 86% of children, requires 1 injection with a booster in 3 years and confers protection within seven to 10 days of inoculation. On the other hand the

Ty21a vaccine requires several doses, is only moderately immunogenic and its efficacy is reduced by simultaneous anti-malarial therapy (Faucher *et al.*, 2002). Both are between 50 to 80% protective and are recommended for travellers to areas where typhoid is endemic. There exists an older killed whole-cell vaccine that is still used in countries where the newer preparations are not available, but this vaccine is no longer recommended for use, because it has a higher rate of side effects (WHO, 2008).

The WHO advocates mass vaccination in endemic areas (WHO, 2008). However various factors have restricted mass vaccination for typhoid in these regions. A report from an urban slum community in Delhi, India showed the high costs of typhoid fever vaccinations though it still recommended more widespread vaccination (Bahl *et al.*, 2004). The other constraint is that the current Vi and Ty21a vaccines are not licensed for use in children less than 2 years, in whom its efficacy is unproven, and therefore are deemed unsuitable for expanded immunization programs which target infants in their first year of life (Levine, 2000).

2.9 Emergence of S. Typhi strains resistant to medically important antimicrobials

Antimicrobial resistance is the best-known example of rapid adaptation of bacteria to a new ecosystem (Carattoli, 2003). Resistance to multiple antimicrobial agents in bacterial pathogens is an emerging global problem that is having a serious impact on the treatment of infectious diseases (Acar *et al.*, 1997). Over the past several decades changes in the levels of antimicrobial resistance after selection pressure of the drug has reflected increased exposure of bacteria to antimicrobial compounds and due to

this resistance may emerge *in vivo* during treatment (Madhulika *et al.*, 2004). The cause appears to be increased by indiscriminate use of antibiotics in the treatment of humans and animals and the addition of growth-promoting antibiotics to the food of breeding animals (Chogle, 2002).

The body fights infection with its immune system. However HIV weakens the immune system, making the body susceptible to other infections. Many of these illnesses are very serious and they need to be treated with specific antibiotics. Extensive usage of these antibiotics in treating the opportunistic infections in HIV positive patients may predispose to the emergence of MDR.

For more than 40 years since its discovery, chloramphenicol was the drug of choice for the treatment of typhoid (Kariuki *et al.*, 2004). Plasmid-encoded chloramphenicol resistance emerged first in the early 1970s, followed by large epidemics in Central America (Olarte and Galindo, 1973). Although slightly less effective than chloramphenicol, ampicillin was used both for therapy and for elimination of the carrier state (Sanders, 1965). Again, plasmid-encoded resistance soon developed (Anderson *et al.*, 1977; Olarte and Galindo, 1973). Finally, co-trimoxazole was introduced in 1980 and plasmid-encoded resistance to trimethoprim and sulfonamides was observed shortly afterwards (Datta *et al.*, 1981). The first cases of typhoid due to *S.* Typhi carrying plasmid-encoded resistance to chloramphenicol, ampicillin and co-trimoxazole were reported from South-East Asia (Ling and Chau, 1984). Cases in this region increased exponentially. Based on the increases in the prevalence of the MDR

phenotype, it appears that MDR *S.* Typhi strains have been spreading to other regions gradually replacing the fully sensitive strain type.

The emergence of MDR *S.* Typhi isolates resistant to first-line antibiotics ampicillin, chloramphenicol and cotrimoxazole led to the use of the fluoroquinolones as alternative drugs for the treatment of typhoid fever (Chinh *et al.*, 2000; Parry *et al.*, 2002). However, widespread use of fluoroquinolones has resulted in emergence of some *S.* Typhi strains with decreased susceptibility to fluoroquinolones, particularly strains resistant to nalidixic acid (Gupta *et al.*, 2001; Shirakawa *et al.*, 2006). This reduction in susceptibility and rising minimum inhibitory concentrations (MICs) of fluoroquinolones results in a poor clinical response to treatment (Kadhiravan *et al.*, 2005). This poses a new global threat requiring debate on the optimum treatment options for enteric fever (Walia *et al.*, 2005). There are now several reports of therapeutic failure and high-level resistance to ciprofloxacin in patients with enteric fever (Aarestrup *et al.*, 2003; Harish *et al.*, 2004).

Reduced susceptibility to ciprofloxacin has been attributed to point mutations in the *gyrA* gene, at either Ser-83 or Asp-87 and an additional mutation in *parC* (Ser-80Ile, Ser-80Arg, Asp-69Glu or Gly-78Asp) (Gaind *et al.*, 2006; Kadhiravan *et al.*, 2005; Slinger *et al.*, 2004). With the emergence of fluoroquinolone resistance, third generation cephalosporins including cefotaxime and ceftriaxone have been used in treatment of enteric fever and invasive salmonellosis. However strains resistant to third generation cephalosporins due to the production of extended-spectrum *ß*-

lactamases (ESBLs) have been reported in many parts of the world over the past few years (Jin and Ling, 2006; Walther-Rasmussen and Hoiby, 2004). These enzymes encode genes that are usually borne on plasmids, although genes on the chromosome have also been reported (Reddy *et al.*, 2005; Rodríguez *et al.*, 2004). Recently, azithromycin has being used as an alternative agent for treatment of uncomplicated typhoid fever (WHO, 2003). Aztreonam and imipenem are also potential third line drugs which have been used (Parry *et al.*, 2002).

2.10 Mechanisms of antibiotic resistance

Antibiotic resistance in bacteria may be an inherent trait of the organism or it may be acquired by means of mutation in its own DNA or acquisition of resistance-conferring DNA from another source. A significant proportion of drug resistance in bacteria is known to be associated with the acquisition of plasmid DNA, but the selective pressures that favour the maintenance of resistance are not fully defined (Gillespie, 2001). The presence of plasmids in bacteria is non-essential for their survival except in the face of a hostile external environment such as antibiotics. This factor contributes to the stability of plasmid-mediated resistance in the bacteria.

The presence of different types of antibiotic resistance genes on the plasmids and their potential for transmission suggests that plasmids are major vectors in the dissemination of resistance genes through bacterial populations (Daly *et al.*, 2000; Randall *et al.*, 2004). The dissemination of antimicrobial resistance among *Salmonella spp* is largely attributed to conjugative DNA exchange, a mechanism of horizontal

transfer of resistance genes (Carattoli *et al.*, 2005). The acquisition of a new gene may occur by genetic transformation (uptake of "naked" DNA), but when resistance genes are located on plasmids, they can in many cases be exchanged by conjugation (Guerra *et al.*, 2002).

The ability of bacteria to expand their ecological niche, also in presence of antibiotics, can be explained by accumulation of point mutations leading to the modification of existing genes or by the acquisition of resistance genes by horizontal gene transfer (Nwosu, 2001). In this process, mobile DNA molecules can be transferred from a donor to a recipient cell, as a result of contact-dependent transmission and energy-dependent processes (Daly *et al.*, 2005; Waters, 1999).

Among genetic determinants responsible for capturing and expression of resistance genes, DNA elements called integrons have been described. Since late 1980s', integrons have been recognized as naturally occurring gene expression elements responsible for the recruitment and assembly of antibiotic resistance genes in clusters (Carattoli, 2003). Integrons encode a site-specific recombinase, the integrase which efficiently promotes the acquisition of exogenous genes (Hall and Collis, 1995). Most of these integrons are located within transposons that contribute to vertical transmission, favouring their mobilisation between plasmids and the bacterial chromosome by transposition events (Carattoli, 2003).

Cebrian and co-workers (2005) reported that repeated exposure to low concentrations of all the fluoroquinolones leads to activation of the efflux pump systems and a reduction in susceptibility, even when there are no mutations in *gyrA*. Activation of these mechanisms is greatly influenced by the chemical structure of the antibiotic. The capacity of these systems to eliminate fluoroquinolones is limited and therefore, for the microorganism to acquire high-level fluoroquinolone resistance, they must be complemented by other mechanisms.

2.11 Plasmids

Plasmids are circular double-stranded deoxyribonucleic acid (DNA) molecules that replicate autonomously in a host cell. They vary in length from a few to several hundred kilobase pairs (Couturier *et al.*, 1988). Many plasmids contain genes that are useful not only to themselves but also to their host. Examples are genes controlling drug resistance, degradation of organic compounds, and virulence factors, including the production of toxins (Martinez and Baquero, 2002).

Resistance genes encoded on plasmids are often located within genetic elements called transposons. These elements include the transposase function that enables the transposon to recombine into the bacterial chromosome or plasmids (Couturier *et al.*, 1988). A resistance gene that has emerged on a plasmid, located within a transposon or an integron, may be transferred to other strains and species, enabling it to penetrate into niches not accessible to its original host strain (O'Brein, 2002). The ability of

transposons to move from one location to another in a plasmid is a major factor in dissemination of antibiotic resistance (Mayer, 1988).

2.12 Plasmid classification

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

Classification of plasmids became important at the end of the 1950s after the discovery of R plasmids and the recognition of their wide distribution (Couturier *et al.*, 1988). The first criterion to be used for plasmid classification was related to conjugal transfer. Most R plasmids are transferred at low frequencies, whereas the F plasmid is transferred at a high frequency. The phenomenon was first demonstrated in 1962 in studies with the F plasmid (F stands for fertility) of *Escherichia coli*, in which it was shown that two different autonomous F-prime (F') plasmids or an integrated and an autonomous F plasmid could not be maintained together (Echols, 1963). The former is referred to as Fa-Fa incompatibility, the latter as Fi-Fa incompatibility (DeVries *et al.*, 1974). During subsequent years, as work on plasmids progressed, it was recognized

that a given pair of plasmids either could or could not coexist stably and this led to the classification of plasmids into incompatibility groups by Datta and Hedges (1975).

Identification and classification of plasmids is based on genetic traits that are universally present and are constant. These criteria are best met by traits concerned with plasmid maintenance, especially replication control. Among plasmids, differences are found for replication control and this can be recognized by studying incompatibility relationships. Plasmids with the same replication control are incompatible, whereas plasmids with different replication controls are usually compatible (Couturier *et al.*, 1988).

2.13 Role of integrons and gene cassettes

Integrons are carried on plasmids and transposons that potentially increase the dissemination of antibiotic resistance genes among bacterial species. Integrons were defined by Hall and Collis (1995) as elements which contain the genetic determinants of the components of a site-specific recombination system that recognizes and captures mobile gene cassettes. These DNA elements mediate the integration of antibiotic resistance genes into bacteria through the site-specific recombination. Integrons consist of an integrase gene (*int*), a recombination site (*attI*), and one or two promoters responsible for expression of the inserted gene cassettes (Stokes *et al.*, 1997).

Gene cassettes are discrete genetic elements that may exist as free, circular, non-replicating DNA molecules when moving from one genetic site to another, but which are normally found as linear sequences that constitute part of a larger DNA molecule, such as a plasmid or bacterial chromosome (Collis and Hall, 1992). Gene cassettes normally contain only a single gene and an additional short sequence, called a 59 base element that functions as a specific recombination site (Hall, 1997). Accordingly, the cassettes are small, normally of the order of 500–1000 bp. The genes carried on gene cassettes usually lack promoters and are expressed from a promoter on the integron. Gene cassettes can exist as free circular molecules and are transcribed only when captured and inserted into an integron, usually at the *attI* recombination site 104 bp upstream of the *intI1* gene. Acquisition of one or more cassettes by sequential insertion at *attI* or the deletion of one or more cassettes from an existing integron creates new integrons (Hall, 1997). New cassettes are continually being discovered, and now over 60 cassettes that confer resistance to a range of antimicrobial agents have been identified (White *et al.*, 2000).

Integrons are divided into two major groups: the resistance integrons (RI) and the super-integrons (SI). RI carries mostly gene cassettes that encodes resistance against antibiotics and disinfectants, and can be located either on the chromosome or on plasmids. The large chromosomally-located integrons, which contain gene cassettes with a variety of functions, belong to the SI group. At least four classes of chromosomal and plasmid-borne RI in Gram-negative bacteria have been described

(Arakawa *et al.*, 1995; Collis and Hall, 1995). The four classes of RI are distinguished by their respective integrase (*int*) genes (Mazel *et al.*, 1998).

Class 1 integrons, mostly found as part of the Tn*21* or Tn*402* transposon family are the most widely disseminated among the members of the family Enterobacteriaceae (Leverstein-van Hall *et al.*, 2003) including many of the *S. enterica* serovars (Randall *et al.*, 2004). Integrons of this class contain a 5' conserved segment (5'-CS) with the integrase gene *intI1*, the *attI1* recombination site and the promoter, generally followed by a variable region that consists of an array of one or more resistance gene cassettes. They also contain the *sul1* gene, encoding sulphonamide resistance located in the 3'-conserved segment (3'-CS) (Hall and Collis, 1995);and two open reading frames, *orf5* and *orf6* (Hall and Collis, 1995; Partridge *et al.*, 2001).

Class 2 integrons are associated with the Tn*7* transposon family and have been identified in *Salmonella* so far only in the serovars Typhimurium and Paratyphi B (Miko *et al.*, 2003). They consist of an integrase gene followed by gene cassettes. Class 3 integrons have been described in *Pseudomonas aeruginosa*, *Serratia marcescens*, *Alcaligenes xylosoxidans*, *Pseudomonas putida* and *Klebsiella pneumoniae* isolates from Japan. The structure of Class 3 integrons is comparable to that of Class 2 integrons but containing the *blaIMP* gene cassette conferring resistance to carbapenems (Arakawa *et al.*, 1995). Class 4 is a distinctive class of integrons located in the *Vibrio cholerae* genome and is not known to be associated with antibiotic resistance (Rowe-Magnus *et al.*, 1999).

2.14 Role of integrons in facilitating antibiotic resistance transfer

Until 1999 only a few reports had described the epidemiology of Class 1 integrons, but it was concluded from these reports that integrons are common among the family Enterobacteriaceae, and that they contributed to antibiotic resistance (Fluit and Schmitz, 2004). The integron-carrying isolates are usually multiresistant, whereas the integron-lacking isolates are more susceptible to antibiotics. Although only identified as discrete, defined structures relatively recently, integrons and their associated components, gene cassettes, are known to have been constituents of the first resistance plasmids reported in the mid 1960s (Bennett, 1999).

A resistance gene that has emerged on a plasmid, located within a transposon or an integron, may be transferred to other strains and species, enabling it to penetrate into niches not accessible to its original host strain thus facilitating resistance transfer (O'Brein, 2002). The spread of resistance genes is greatly enhanced when they form part of a mobile gene cassette, since this provides for horizontal transfer by several mechanisms. These mechanisms include; first the mobilization of individual cassettes by the integron-encoded integrase, secondly by the movement when the integron containing the cassette relocates probably by targeted transposition, thirdly by dissemination of larger transposons such as Tn*21* carrying integrons, and fourthly the movement of conjugative plasmids containing integrons among different bacterial species (Peter *et al.*, 2001)**.** It is therefore not surprising that many of the antibiotic

resistance genes found in clinical isolates of gram-negative microorganisms are part of a gene cassette inserted into an integron (Hall and Collis, 1995).

The DNA exchanges mediated by conjugative plasmids and the integration of resistance genes into specialised genetic elements play a major role in the acquisition and dissemination of resistance genes (Carattoli, 2003). A very efficient genetic mechanism by which bacteria can acquire resistance genes is the integrase-mediated site-specific recombination. The most frequently reported are Class 1 and Class 2 integrons, which have been shown to contribute to the spread of antimicrobial resistance genes (Fluit and Schmitz, 2004). Class 1 integrons are the most commonly studied and largely implicated type in the dissemination of antibiotic resistance among clinical isolates (Hall and Collis, 1995). Having the ability of horizontal transfer of antibiotic resistance genes between interspecies of bacteria, integrons play a dominant role in the development of multiresistance in Enterobacteriaceae (Fluit and Schmitz, 2004).

Class 1 integrons promote the capture of one or more gene cassettes within a specific attachment site (*attI1*), thereby forming composite clusters of antibiotic resistance genes. Their essential components are the integrase gene (*intI1*) and the promoter, which drives the expression of any suitably, oriented array of gene cassettes integrated at the *attI1* site. These functions are contained in the so-called 5'-conserved segment (5'-CS) of the integron (Hall and Collis, 1995).

Integrons act as receptors of antibiotic resistance cassettes. More than 60 cassettes conferring resistance to β-lactams, aminoglycosides, trimethoprim, chloramphenicol, streptomycin, and other antibiotics are known (Kovalevskaya, 2002). For instance, twenty-nine multiple-drug-resistant *S.* Typhimurium strains isolated in 1997 in Albania from sporadic cases of infantile gastroenteritis carried the IncFI plasmids (Tosini *et al.*, 1998). These strains were resistant to ampicillin, chloramphenicol, kanamycin, streptomycin, spectinomycin, sulfonamides, tetracycline and trimethoprim. Four different resistance genes were located within two Class 1 integrons, both located in the IncFI plasmid: In-t1 carrying the *aadB* and *catB3* genes, conferring kanamycin and chloramphenicol resistance, and In-t2 carrying the *oxa1* and *aadA1* genes, conferring beta-lactams and streptomycin-spectinomycin resistance (Tosini *et al.*, 1998). Recently, plasmids carrying In-t1 and In-t2 have been identified in *S.* Typhimurium from Europe and Africa, suggesting that the IncFI plasmid could have re-emerged. The integration site of In-t1 within the IncFI/97 plasmids showed a close physical link between the In-t1 and In-t2 integrons (Lindstedt *et al.*, 2003).

Integrons are themselves translocatable, though most are defective transposon derivatives. Integron movement allows transfer of the cassette-associated resistance genes from one replicon to another or into another active transposon which facilitates spread of integrons that are transposition defective. Horizontal transfer of the resistance genes can be achieved when an integron containing one or more such genes is incorporated into a broad-host-range plasmid. Likewise, single cassettes integrated

at secondary sites in a broad-host-range plasmid can also move across species boundaries (Hall, 1997).

2.15 Mode of transmission of plasmids

One of the most peculiar characteristics of plasmids is their capacity to be mobilized by the conjugative transfer. In this process, plasmid DNA can be transferred from a donor to a recipient cell via a contact-dependent transmission mechanism. Bacterial conjugation is the unidirectional transfer of genetic material between bacteria through direct cell-to-cell contact (Holmes and Jobling, 1996). Conjugation is a mechanism of horizontal gene transfer as are transformation and transduction although these mechanisms do not involve cell-to-cell contact (Griffiths *et al.*, 2000). The prototype for conjugative plasmids is the F-plasmid, also called the F-factor (Holmes and Jobling, 1996). The F-plasmid is an episome that is a plasmid that can integrate itself into the bacterial chromosome by genetic recombination. It carries its own origin of replication, the *oriV*, as well as an origin of transfer, or *oriT* (Ryan and Ray, 2004). There can only be one copy of the F-plasmid in a given bacterium, either free or integrated.

The host bacterium is called F-positive or F-plus (denoted F^+). Strains that lack F plasmids are called F-negative or F-minus (F^-). Among other genetic information, the F-plasmid carries a *tra* and a *trb* locus, which together are about 33 kb long and consist of about 40 genes. The *tra* locus includes the *pilin* gene and regulatory genes, which together form pili on the cell surface, polymeric proteins that can attach

themselves to the surface of F^- bacteria and initiate the conjugation. Though there is some debate on the exact mechanism, the pili themselves do not seem to be the structures through which the actual exchange of DNA takes place; rather, some proteins coded in the *tra* or *trb* loci seem to open a channel between the bacteria.

When conjugation is initiated, through a mating signal, a relaxase enzyme creates a nick in one plasmid DNA strand at the origin of transfer, or *oriT*. The relaxase may work alone or in a complex of over a dozen proteins, known collectively as a relaxosome. In the F-plasmid system, the relaxase enzyme is called *TraI* and the relaxosome consists of TraI, TraY, TraM, and the integrated host factor, IHF. The transferred, or *T-strand*, is unwound from the duplex plasmid and transferred into the recipient bacterium in a 5'-terminus to 3'-terminus direction. If the DNA being transferred is a plasmid, it is made double-stranded and circularized in the recipient, whereupon it can presumably replicate. The remaining strand is replicated, either independent of conjugative action or in concert with conjugation. Conjugative replication may necessitate a second nick before successful transfer can occur. A recent report claims to have inhibited conjugation with chemicals that mimic an intermediate step of this second nicking event (Lujan *et al.*, 2007).

If the F-plasmid becomes integrated into the host genome, donor chromosomal DNA may be transferred along with plasmid DNA. If the transfer DNA is chromosomal, circularization cannot occur, but in some way a complementary strand is generated and homologous recombination with the chromosome can occur (Griffiths *et al.*,

2000). The conjugation assay is used to test the transferability of antimicrobial resistance from a *Salmonella* isolate, used as donor strain, to a K12 *Escherichia coli* strain. The *E. coli* strain should be recognizable from the donor strain, by the presence of a resistance marker located on the chromosome. The transconjugant strains will be screened on plates containing antibiotics that selected the resistance markers of the donor and the recipient strains.

2.16 Incompatibility grouping for characterisation of plasmids

A property which is universally inherited by plasmids and which is more suitable for classification is incompatibility (Inc) groups. Incompatibility is a manifestation of relatedness, sharing common replication controls or equipartitioning elements (Couturier *et al.*, 1988). Plasmids incompatible with each other are assigned to the same incompatibility group. About 30 incompatibility groups have been recognized among plasmids of enteric bacteria and 7 are recognized among staphylococcal plasmids (Couturier *et al.*, 1988; Frost *et al.*, 2005). Four major incompatibility groups have been defined on the basis of genetic relatedness and pilus structure: the IncF group, including IncF, IncS, IncC, IncD, IncJ, the IncP group, including IncP, IncU, IncM, IncW, the Ti plasmid group, including IncX, IncH, IncN, IncT and the IncI group, including IncI, IncB and IncK (Waters, 1999).

Incompatibility grouping ensures that the cell contains a wider selection of plasmids, since only plasmids not closely related will live in the same cell. It has been discovered that incompatible plasmids share more sequence homology than

compatible plasmids, and in the case of conjugative plasmids, usually specify similar transfer systems, hence incompatibility between independently isolated heterologous plasmids reflects their similarity or evolutionary relatedness. For purpose of characterizing plasmids, incompatibility provides a feature of distinction that applies to both conjugative and non-conjugative plasmids (Couturier *et al.*, 1988).

In 1988, Couturier and co-workers developed a hybridization method for the comprehensive typing of bacterial plasmids according to replicon type (Couturier *et al.*, 1988). From this study, a bank of Rep probes corresponding to 19 different Inc groups in the Enterobacteriaceae was developed (Couturier *et al.*, 1988). Although this procedure represented a significant advance in plasmid typing, the method was time-consuming, labour-intensive, and incompatible with current high-throughput approaches. Recognizing a need for a streamlined procedure, Carattoli and co-workers demonstrated that a PCR-based replicon typing protocol could be used to detect 18 plasmid replicons frequently found among the Enterobacteriaceae (Carattoli *et al.*, 2005). Although their procedure is greatly simplified, specific and very sensitive, it still requires several steps, including five multiplex and three simplex PCR procedures. Therefore, it would be tedious and costly to apply this procedure to large collections of isolates.

2.17 Importance of plasmids in the dissemination of resistance in Enterobacteriaceae

Antimicrobial resistance arises from a complex multifactorial process supported by panoply of mobile genetic elements that contain and transfer resistance determinants. Resistance genes located on plasmids move from one bacterium to another, conferring phenotypic characteristics. Several studies on molecular characterization of Enterobacteriaceae outbreak strains have revealed that resistance to commonly used antimicrobial agents is encoded by plasmids of the different incompatibility groups. Recently a PCR-based replicon typing method was developed that allowed the rapid identification of major plasmid incompatibility groups among Enterobacteriaceae, i.e. FIA, FIB, FIC, HI1, HI2, I1-Iγ, N, P, W, T, A/C, K, B/O, X, Y, F, and FIIA (Carattoli *et al.*, 2005). This PCR-based method has been used to monitor the dissemination and evolution of resistance plasmids.

Resistance to both apramycin and gentamicin has been detected in different *Salmonella* serotypes. In *S.* Typhimurium DT204c the gentamicin resistance has been shown to be specified by three types of plasmids of the I1 incompatibility group, which also conferred resistance to apramycin. In the 1990s, the increasing frequency of *Salmonella* with plasmids of the I1 incompatibility group conferring resistance to gentamicin and apramycin was reported in other European countries (Pohl *et al.*, 1993; Threlfall *et al.*, 2003). Plasmids belonging to incompatibility group I1 (IncI1) are widespread in Enterobacteriaceae and are characterized by the presence of a cluster of genes encoding the type IV pili, contributing to the virulence of Shiga-toxigenic

Escherichia coli. Recently, IncI1 plasmids were identified in *E. coli* and *Salmonella* strains of animal origin as responsible for the dissemination of β-lactamase genes.

As in MDR *S.* Typhi outbreaks reported from many developing countries, MDR in Kenyan serovar Typhi was shown to be encoded on large self-transferable IncHI1 plasmids of ~110 kb (Kariuki *et al.*, 2000; Kariuki *et al.*, 2004; Mills-Robertson *et al.*, 2002). The genes that are responsible for chloramphenicol, trimethoprim and ampicillin resistance are chloramphenicol acetyltransferase type 1, dihydrofolate reductase type VII, and TEM-1 beta lactamase respectively (Shanahan *et al.*, 1998). It is likely that resistance was acquired through the horizontal transfer of plasmids to previously sensitive strains irrespective of susceptibility to the quinolones commonly in use in Kenya: nalidixic acid, norfloxacin, and ciprofloxacin (Kariuki *et al.*, 2004). *S.* Typhi CT18 harbors two plasmids. The larger conjugative plasmid, pHCM1, is 218 kb in length and shares approximately 168 kb of DNA with the plasmid R27, with more than 99 percent sequence identity (Sherburne *et al.*, 2000), R27 is an incHI1 plasmid, first isolated in the 1960s from *S. enterica,* that is closely related to the chloramphenicol-resistance plasmids detected in *S.* Typhi in the 1970s (Sherburne *et al.*, 2000).

The pHCM1 plasmid encodes resistance to chloramphenicol (*catI*), ampicillin (TEM-1, *bla*), trimethoprim (*dhfr*1b), sulfonamides (*sul* II), and streptomycin (*str*AB) (Ploy *et al.*, 2003). The smaller plasmid, pHCM2, is 106.5 kb in length and is phenotypically cryptic, but it has striking homology with the pMT1 virulence-associated plasmid of

Yersinia pestis (Parkhill *et al.*, 2001). The HI2 replicon was previously detected on CTX-M-9-positive *Salmonella* and *E. coli* strains of human origin from Spain (Garcia *et al.*, 2005).

Recent reports also indicate that a variety of widely disseminated conjugative plasmids are associated with the spread of CTX-M and Amp-C-type enzymes. Some of the plasmids belong to the so-called broad-host-range plasmid groups, such as IncN (Carattoli *et al.*, 2006; Hopkins *et al.*, 2006; Novais *et al.*, 2007), IncP1-α (Novais *et al.*, 2006), IncL/M (Novais *et al.*, 2007), and IncA/C2 (Carattoli *et al.*, 2006; Novais *et al.*, 2007), and thus their further spread to other members of Enterobacteriaceae and other bacterial species is a cause for concern.

In *E. coli* and *Salmonella*, CMY-2-type A and CMY-2-type B plasmids have been shown to belong to the A/C_2 and I1 incompatibility groups, respectively (Carattoli *et al.*, 2006; Hopkins *et al.*, 2006). These plasmids have spread in the United States and in Europe. Depending on the $bla_{CTX\text{-}M}$ gene, different CTX-M plasmids have recently been identified and found to be of the I1, FII, HI2, K, and N incompatibility groups (Hopkins *et al.*, 2006). These results demonstrate the association of certain β-lactamase genes with specific plasmid backbones. Very recently, the dissemination and persistence of $bla_{CTX\text{-}M\text{-}9}$ were shown to be linked to CR1-containing Class 1 integrons linked to defective transposon derivatives from Tn*402* located in old antibiotic resistance plasmids of the HI2, P1-α, and FI incompatibility groups (Novais *et al.*, 2006). It was suggested that the presence of this ESBL gene on broad-host-

range IncP1-α plasmids might contribute to its dissemination to hosts that are not members of the family Enterobacteriaceae.

High level fluoroquinolone resistance has been reported in *S.* Typhi - this resistance has been shown to be associated with mutations in the *gyr*A gene (Mehta *et al.*, 2001). Notably, the presence of a plasmid-borne integron in ciprofloxacin-resistant *S.* Typhi may lead to a situation of untreatable enteric fever (Gaind *et al.*, 2006). *S.* Typhi harbouring a 50 kb transferable plasmid carrying a Class 1 integron (*dfrA15/aadA1*) that confers resistance to co-trimoxazole and tetracycline has also been reported.

2.18 Molecular methods in characterization of bacterial strains

Epidemiological studies of pathogens are of great importance in controlling their dissemination. The capability to strain type pathogens is a critical tool in epidemiological investigations. A number of typing systems have been used to determine whether a set of bacterial isolates represents a single strain or more than one strain. These genotyping methods include plasmid analysis, restriction endonuclease analysis, PCR assays, multilocus enzyme electrophoresis (MLEE), multilocus sequence typing (MLST), pulsed-field gel electrophoresis (PFGE), DNA sequencing, ribotyping, PCR ribotyping, restriction fragment length polymorphism studies, randomly amplified polymorphic DNA (RAPD), amplified fragment length polymorphism (AFLP), and repetitive sequence-based PCR (rep-PCR) (Van, 2003). All of these genotyping methods have increasingly become an integral part of both

clinical and research microbiology laboratories in understanding the natural history of bacterial diseases and in distinguishing bacterial strains or clones.

Plasmid profile analysis has been used as a rapid method and has shown some success in the discrimination of *Salmonella* strains for several of these serotypes (Crichton *et al.*, 1996)**.** The approach to using plasmid profiles as an epidemiological typing system is similar to that of other typing schemes. The ideal situation would be to show that all of the outbreak associated strains have the same plasmid profile and are the same by any other typing system used (Mayer, 1988). The presence or absence of plasmids does not by itself determine whether plasmid profiles can be done. For any typing system to be effective, it must differentiate case from noncase isolates. When plasmid profiles of groups of isolates are compared, there must be sufficient differences in the patterns to allow them to be readily separated.

In some bacterial genera, such as *Salmonella* or *Shigella*, plasmids occur so frequently that multiple isolates of a plasmid-free strain from an outbreak setting would be an epidemiologically significant finding. The lack of plasmids itself may be used as a marker for some isolates in some situations (Mayer, 1988). Analysis of bacterial plasmids technique consists in the extraction of plasmid DNA and subsequent separation of this DNA by electrophoresis in agarose gels. It is an easily executed and interpreted technique, however it has several limitations, especially inherent in the fact that plasmids are mobile extrachromosomal elements that can be spontaneously lost or readily acquired by bacteria. Consequently, epidemiologically related isolates can

display different plasmid profiles. Moreover, many plasmids carry resistance determinants contained in transposons that can be readily lost or acquired, quickly altering the composition of plasmid DNA. The reproducibility of the generated profiles can be affected by the fact that plasmids exist in different spatial conformations (supercoiled, nicked, and linear), which possess different migration velocities when subjected to agarose gel electrophoresis (Trindade *et al.*, 2003).

Further analysis by PCR and DNA sequencing of transposon content by insertion sequence evaluation have been useful to establish the potential of antibiotic resistant genes. PCR requires template DNA from the organism being typed, two complementary oligonucleotide primers that are designed to flank the sequence on the template DNA to be amplified, and a heat-stable DNA polymerase. The PCR primers serve as the starting point for the polymerase to add the bases that make up a strand that is complementary to the template. A growing number of organisms have been studied using this approach (Grimm *et al.*, 2004). Each amplification cycle consists of a heat denaturation phase in which double-stranded DNA is melted into single strands, an annealing phase where the primers bind to the single-stranded target sequences, and an extension phase. It is during the extension phase, in which the copy number of the DNA is doubled, that the DNA synthesis proceeds from the primers along the template strands, generating copies of the original double-stranded DNA molecule (Aparajita *et al.*, 2006).

One potential way to overcome the lack of diversity of resistant genes is through the sequencing of these genes, which will likely be under greater selective pressure to mutate. DNA sequencing is the process of determining the nucleotide order of a given DNA fragment. The genome sequencing is revolutionizing all aspects of the biological sciences of bacterial pathogens. This has facilitated the discovery of many previously unidentified determinants of pathogenicity and has provided novel insights into what creates a pathogen and showed the role of the horizontal gene transfer in the evolution of the prokaryotic organisms (Field *et al.*, 2004). This technique uses sequence-specific termination of an *in vitro* DNA synthesis reaction using modified nucleotide substrates. Sequence data for specific loci (such as genes for virulence, pathogenicity and, drug resistance) from different strains of the same species have revealed variability in a specific gene, such as single-nucleotide polymorphisms and areas with repetitive sequence that demonstrate potential for epidemiologic application (Aparajita *et al.*, 2006).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study area

The study was carried out at Kenya Medical Research Institute's (KEMRI) Centre for Microbiology Research (CMR), Nairobi. The *S.* Typhi isolates were obtained from blood specimens from individuals diagnosed with bacteremia due to *S.* Typhi at the Kenyatta National Hospital (KNH) and Aga Khan University Hospital in Nairobi (AKUH) from 2004 to 2007. The KNH is a tertiary national referral institution that provides subsidized services to patients from the neighbouring towns and those referred from peripheral hospitals. AKUH on the other hand serves a wide range of patients from all over the country and beyond Kenyan borders. It is one of the private, tertiary health facility that receives referrals from all over the region. The isolates used in this study were from routine microbiological investigations of specimens from KNH and AKUH requiring microbiological and molecular analysis which is offered at CMR.

3.2 Study design

The study design was a laboratory based retrospective-cohort study involving the characterization of multi-resistance plasmids in clinical isolates of *S.* Typhi.

3.3 Bacterial isolates

The study involved the use of stored isolates (2004-2007) of *S.* Typhi at the Enteric Bacteriology Laboratories at CMR. The isolates were obtained for a period of four years from blood and celebrospinal fluid specimens from individuals who presented with bacteremia due to *S.* Typhi in KNH and AKUH. The isolates were subcultured in MacConkey agar to check for purity. The current study was cleared by both the KEMRI Scientific Steering Committee and the National Ethical Review Committee and has been assigned SSC No. 1320.

3.3.1 Inclusion criteria

S. enterica serotype Typhi isolates collected between 2004 and 2007 from blood and celebrospinal specimens.

3.3.2 Exclusion criteria

Cultures obtained from stool specimens.

3.4 Sample size

Going by the reports (Kariuki *et al.*, 2004) on typhoid fever in Nairobi the estimated prevalence of MDR *S.* Typhi is 75%. Therefore using this prevalence the minimum sample size was determined using the following formula (Fishers *et al.*, 1998);

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

Where;

N is the minimum sample size

P is the estimated prevalence (75%)

δ is the level of significance set (5%)

Z is the standard normal deviate that corresponds to 95% confidence interval (1.96)

$$\text{Therefore, } n = \frac{(1.96)^2 \times 0.75(1-0.75)}{(0.05)^2} = 288$$

Therefore the minimum sample size expected was 288 however, for this study the number of isolates available between 2004 and 2007 were 144 which were anaysed in this study.

3.5 Laboratory Procedures

3.5.1 Antibiotic susceptibility testing

A total of 144 *S.* Typhi isolates were tested for susceptibility to various antimicrobials agents by quality controlled disk diffusion technique (Bauer *et al.*, 1966). Few distinct colonies from nutrient agar plates were aseptically inoculated into 5ml nutrient broth tubes with sterile wire loop. Inoculated broth tubes were incubated at 37^{o}C for one

hour. The isolates were suspended in sterile water to conform to 0.5 McFarland turbidity standards.

Mueller-Hinton agar was used for susceptibility testing. The standardized broth cultures were spread evenly on the surface of pre-dried Mueller-Hinton agar plates with the aid of sterile cotton swab. Inoculated plates were left for 15 minutes at room temperature to make the surface dry. The following antibiotic discs were placed on the inoculated surface with the aid of sterile pairs of forceps keeping equal distance between the discs; ampicillin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg), gentamicin (10 µg), co-amoxyclav (30 µg), ciprofloxacin (5 µg), cefuroxime (30 µg), ceftriaxone (30 µg) and nalidixic acid (10 µg). The plates were then incubated at 37ºC for 18hrs.

After incubation, zones of inhibition were measured and recorded in millimetres. The results were interpreted as sensitive, intermediate or resistant according to the guidelines provided by the Clinical and Laboratory Standards Institute (CLSI, 2006). *Escherichia coli* ATCC 25922 was used as the control.

3.5.2 Determination of minimum inhibitory concentrations

The MICs of the test antibiotics were determined using the E-test strips. E-test directly quantifies antimicrobial susceptibility in terms of discrete MIC values. The MIC values correlate with the ability of an antibiotic to inhibit a given bacterial culture under specified laboratory conditions. It gives a gauge to the susceptibility of an isolated pathogen and these values form the basis for antimicrobial selection. Thirty *S.*

Typhi isolates that were resistant to ciprofloxacin, cefuroxime, amoxy-clavulin, chloramphenicol, cefotaxime and nalidixic acid by disc diffusion technique were selected randomly for the MIC test.

Well isolated colonies of *S.* Typhi from an overnight culture plate were homogenized in 0.85% NaCl to achieve 0.5 McFarland turbidity. The standardized inoculum suspensions were then swabbed evenly into the entire surface of Mueller-Hinton agar. Excess moisture was allowed to absorb for about 10-15 minutes for the agar surface to completely dry. E-test strips were applied to the inoculated agar surface. Air pockets underneath the strips were removed by pressing gently on the strip with a pair of forceps. The agar plates were incubated at 37ºC for 18 hours. After the incubation period the MIC values were read at the point of intersection between the zone edge and the E-strip. The cut off MIC provided by CLSI guidelines was used to interpret the results. *Escherichia coli* ATCC 25922 (with known MICs) was used as the control.

3.5.3 Plasmid profile analysis

Plasmid extraction and profiling for the antibiotic resistant strains was done by the alkaline lysis technique as described by (Birnboim and Doly, 1979). Discrete colonies representing each strain were inoculated into 3ml of Luria broth and incubated at 37°C overnight on a shaker (250 rpm). After 18 h incubation, 1.5 ml of culture was transferred to a 1.5 ml Eppendorf tube for plasmid extraction, and the remainder stored

at ⁻20°C after the addition of glycerol. All manipulations were carried out at room temperature.

The tube was centrifuged at room temperature for five minutes at 13,000 xg in a microcentrifuge. The supernatant was carefully removed with a fine-tip aspirator and discarded and the cell pellet thoroughly suspended in 100 ul of lysis solution I (25 mM Tris-HCl (pH 8.0), 50 mM glucose, 10 mM EDTA) to wash off any broth residue and mucopolysaccharide on the cells. After a 30 minutes of incubation at room temperature, 200 ml of solution II (0.2 M NaOH (pH 12.5), 1% sodium dodecyl sulphate (SDS) was added to digest bacterial cell wall and the tube gently vortexed. The tubes were maintained for five minutes at 0°C and then, 150 µl of solution III (3M sodium acetate pH 5.0) was added to precipitate the cell wall debris and chromosomal DNA. The contents of the tube were gently mixed by inversion for a few seconds to allow the formation of DNA pellet. The tubes were maintained at 0°C for 60 minutes to allow most of the protein, high molecular weight RNA and chromosomal DNA to precipitate. Centrifugation at 13000xg for five minutes yielded almost clear supernatant.

The supernatant was removed and transferred to a second Eppendorf tube. To purify the plasmid DNA extract solution mixture of phenol: chloroform: iso-amyl alcohol in the ratio of 25:24:1 was added and the tube inverted for a few seconds. This step further denatures proteins and removes debris from the aqueous cell lysate. The aqueous phase was collected in a new 1.5 ml eppendorf tube. To pellet the plasmid

DNA one ml of cold 95% ethanol was added to the aqueous cell lysate and gently mixed by inversion. This was held at -70°C for 30 minutes and then centrifuged for 10 minutes at 13000xg to pellet the DNA.

The pellet was then washed with 500 µl of 70% ethanol and the precipitate collected by centrifugation at 13000xg for five minutes. The supernatant was removed by aspiration. The pellet was resuspended in 40 µl of nuclease-free water and 10 µl of buffer and stored at -20°C until used.

3.5.4 Agarose gel electrophoresis

A volume of 10-20 µl of the plasmid DNA was loaded to an agarose gel for electrophoretic analysis. The DNA was removed from ⁻20°C and allowed to thaw at room temperature. A 1% agarose gel was prepared by dissolving 1g of agarose in 100 ml of 1X Tris-borate-EDTA buffer and boiling in a microwave oven. The agarose was cooled to about 45ºC and then poured into the gel container with the combs in place. The gel was left to set for 30 minutes.

A 1:10 solution of the Tris-Borate ethylenediamine-tetra-acetic acid (TBE) electrophoresis buffer was prepared and poured into the electrophoresis tank. This was followed by loading into each well of the gel a mixture of 10 µl plasmid DNA solution and five µl of stop mix. The first Lane in the gel was loaded with plasmids of *E. coli* strain 39R861 (NCTC 50192) which has a molecular size of 98, 42 and 24 MDa as size markers. The gel was allowed to run for approximately four hours at 150 V. After electrophoresis, the gel was stained for 20 minutes in ethidium bromide solution (0.5

µg/ml). The gel was then washed in distilled water at room temperature for 30 minutes and transferred onto a UV transilluminator (ultra-violet products), and photographed using transmitted illumination camera fitted with a Polaroid film (Gelcam UK).

3.5.5 Transferable resistance plasmids

3.5.5.1 Preparation of plates for the selection of trans-conjugant

Drug sensitivity testing agar was prepared by suspending 20 g of the agar in 500 ml of distilled water in a conical flask. The agar was then boiled to dissolve. Upon complete dissolution of the MacConkey agar, it was sterilized by autoclaving at 121^{o} C for 15 minutes and allowed to cool to 50^{o} C in a water bath maintained at this temperature.

Replicate pairs of disposable 20 ml round Petri dishes were labelled with the concentration of antimicrobial agents to be tested. Two milliliter of the antimicrobial solution was then added to the Petri dish at ten times the intended concentration. To each Petri dish 18 ml of the agar equilibrated at 50^{o} C was added using a 20 ml disposable pipette. The antimicrobial solution was mixed thoroughly with the molten agar and left to solidify on a flat bench top at room temperature. For the plates that were used to select the transconjugants 2ml of each of the drug and 16 ml of the agar was added.

Control plates were prepared alongside the test plates by mixing 2 ml of sterile distilled water with 18 ml of molten agar. The plates was then stored at 4^{o} C and inoculated with the isolates to be tested. One plate contains 32 µg/ ml of nalidixic acid, another 32 µg/ml of ampicillin and to another both 32 µg of ampicillin and

nalidixic acid. Quality control strains (*E. coli* R39 (NCTC 50192) containing known molecular weight plasmids were loaded in the gel to estimate the size of the trasconjugants plasmids.

3.5.5.2 Conjugation experiment

In-vitro conjugation tests on transferable antimicrobial resistance were performed according to the method of Walia *et al.*, (1987). Isolates of the donor were resistant to ampicillin (MIC = or > 32 µg/ml) and the recipient isolates were resistant to nalidixic acid. A total of sixty-eight MDR *S.* Typhi resistant to ampicillin and sensitive to nalidixic acid were selected for this experiment. Isolates of each donor *Salmonella* strain and the recipient *E. coli* K12F strain were inoculated in 3 ml of tryptic soy broth and allowed to grow to the logarithmic phase (10^8 cells) by incubating on a shaker (250 rpm) at 35°C for three hours. The donor and the recipient bacterial broth cultures were diluted in a ratio of 1:10 (i.e. 0.5 ml of the culture to 4.5 ml of freshly prepared and pre-warmed tryptone soy broth at 37° C) to make a five ml broth culture. The donor and recipient broth cultures were then mixed in equal proportions in four ml volumes. The mixture was then incubated at 37° C for 18 hours and conjugation allowed to take place overnight without shaking. The culture was centrifuged (14000xg) for five minutes and the media discarded. The pellet was then washed with normal saline (0.85 sodium chloride solution in distilled water) to wash off any enzyme that might have leaked into the media from the bacterial cells.

To select transconjugants, three µl of the mixture was drawn from overnight culture and plated onto three sets of MacConkey agar plates, containing ampicillin, nalidixic acid and a third set containing a mixture of ampicillin and nalidixic acid to select the donor strain, the recipient strain and the transconjugant. The agar plates were incubated at 37°C overnight. To determine the resistance profiles conferred by the plasmids, the transconjugant cells were screened for sensitivity to the nalidixic acid and ampicillin.

To determine the transferable resistance-encoding plasmids, plasmid DNA was extracted from both donor and transconjugant strains and compared on a gel. The presence of plasmids was determined by electrophoresis on one percent horizontal agarose gels. The sizes of the plasmids were estimated by comparing with plasmids of known molecular sizes from *E. coli* strains 39R861 (NCTC 50192) with molecular sizes of 98, 42, 24, and 4.6MDa. The plasmids were observed on a UV transilluminator, and photographed using a transmitted illumination camera fitted with a Polaroid film (Gelcam UK).

3.5.6 Incompatibility grouping using PCR

For plasmid incompatibility group identification the recently developed PCR based replicon typing method consisting of 18 pairs of primers **(Table 1)** was used (Carattoli *et al.*, 2005). These primers **(Table1)** are a representative of the major plasmid incompatibility groups found among the Enterobacteriaceae. The replicon typing procedure used in this study was a modified version of that described by Carattoli *et al.* (2005). Changes in this procedure included (i) use of a boiling lysis preparation rather than a commercial genomic DNA purification kit; and (ii) use of a universal PCR procedure for all the primer set. These changes resulted in an overall decrease in the time, materials and costs to type these isolates.

Five multiplex- and three simplex-PCRs were performed to recognize the FIA, FIB, FIC, HI1, HI2, I1-Iγ, L/M, N, P, W, T, A/C, K, B/O, X, Y, F and FIIA replicons. PCR procedure consisted of five different multiplex-PCRs recognizing three different replicon types (HI1-, HI2-, I1/Iγ-carrying plasmids), and three simplex-PCRs for F, K and B/O. Primers were used at a two µl final concentration in a reaction volume of 28 µl containing 25 µl of PCR water and one µl of DNA as the template added to a *Taq* polymerase master mix.

All PCR amplifications were performed with the following amplification scheme: one cycle of denaturation at 94°C for five minutes, 30 cycles of denaturation at 94 °C for one minute, annealing at 60°C for 30 seconds, elongation at 72°C for one minute and

final extension cycle at 72°C for five minutes. 20 µl of each of the amplified products were separated on 1.5% agarose gels containing 0.5 µl of ethidium bromide for one hour at 120 V. After electrophoresis the gels were visualized under UV transilluminator, and photographed using a transmitted illumination camera fitted with a Polaroid film (Gelcam UK).

Table 1: Primers used for replicon typing

Name	DNA sequence	Target site	Accession No.	Amplicon (bp)
HI1	FW 5′-GGAGCGATGGATTACTTCAGTAC-3′	ParA-ParB	AF250878	471
HI1	RV 5′-TGCCGTTTCACCTCGTGAGTA-3′			
HI2	FW 5′-TTTCTCCTGAGTCACCTGTTAACAC-3′	Iterons	BX664015	644
HI2	RV 5′-GGCTCACTACCGTTGTCATCCT-3′			
I1	FW 5′-CGAAAGCCGGACGGCAGAA-3′	RNAI	M20413	139
I1	RV 5′-TCGTCGTTCCGCCAAGTTCGT-3′			
X	FW 5′-AACCTTAGAGGCTATTTAAGTTGCTGAT-3′	ori γ	Y00768	376
X	RV 5′-TGAGAGTCAATTTTTATCTCATGTTTTAGC-3′			
L/M	FW 5′-GGATGAAAACTATCAGCATCTGAAG-3′	Rep A, B, C	U27345	785
L/M	RV 5′-CTGCAGGGGCGATTCTTTAGG-3′			
N	FW 5′-GTCTAACGAGCTTACCGAAG-3′	RepA	NC_003292	559
N	RV 5′-GTTTCAACTCTGCCAAGTTC-3′			
FIA	FW 5′-CCATGCTGGTTCTAGAGAAGGTG-3′	Iterons	J01724	462
FIA	RV 5′-GTATATCCTTACTGGCTTCCGCAG-3′			
FIB	FW 5′-GGAGTTCTGACACACGATTTTCTG-3′	RepA	M26308	702
FIB	RV 5′-CTCCCGTCGCTTCAGGGCATT-3′			
W	FW 5′-CCTAAGAACAACAAAGCCCCCG-3′	RepA	U12441	242
W	RV 5′-GGTGCGCGGCATAGAACCGT-3′			
Y	FW 5′-AATTCAAACAACACTGTGCAGCCTG-3′	RepA	K02380	765
Y	RV 5′-GCGAGAATGGACGATTACAAAACTTT-3′			
P	FW 5′-CTATGGCCCTGCAAACGCGCCAGAAA-3′	Iterons	M20134	534

P	RV 5′-TCACGCGCCAGGGCGCAGCC-3′			
FIC	FW 5′-GTGAACTGGCAGATGAGGAAGG-3′	RepA2	AH003523	262
FIC	RV 5′-TTCTCCTCGTCGCCAAACTAGAT-3′			
A/C	FW 5′-GAGAACCAAAGACAAAGACCTGGA-3′	RepA	X73674	465
A/C	RV 5′-ACGACAAACCTGAATTGCCTCCTT-3′			
T	FW 5′-TTGGCCTGTTTGTGCCTAAACCAT-3′	RepA	K00053	750
T	RV 5′-CGTTGATTACACTTAGCTTTGGAC-3′			
FIIS	FW 5’-CTGTCGTAAGCTGATGGC-3′	RepA	AE006471	270
FIIS	RV 5′-CTCTGCCACAAACTTCAGC-3′			
FREPB	FW 5′-TGATCGTTTAAGGAATTTTG-3′	RNAI/RepA	AY234375	270
FREPB	RV 5′-GAAGATCAGTCACACCATCC-3′			
K/B	FW 5′-GCGGTCCGGAAAGCCAGAAAAC-3′	RNAI	M93063	160
K/B	RV 5′-TCTTTCACGAGCCCGCCAAA-3′			
B/O	RV 5′-TCTGCGTTCCGCCAAGTTCGA-3′	RNAI	M28718	159

3.5.7 Detection of quinolone resistance determining region (QRDR) resistance genes by PCR

DNA isolated from *S.* Typhi was subjected to PCR to determine any variation in resistance genes encoding resistance in quinolones and fluoroquinolones including, nalidixic acid and ciprofloxacin, respectively. DNA was extracted from bacteria by boiling the cells at 99° C Primers designed to detect the specific antimicrobial resistance genes *gyr A, gyr B, par C* and *par E* were used to amplify *S.* Typhi plasmid DNA by PCR. The sequence of the primers is indicated in **Table 2**. Reaction conditions consisted of one µl of plasmid DNA and 1.5 µl concentrations of each primer in five µl of buffer, six µl of $MgCl_2$, eight µl of deoxynucleoside triphosphate mixture, 12 µl of PCR water and 0.25 µl of *Taq* polymerase in a final volume of 35.25 µl.

PCR amplification was conducted in a thermal cycler using the following program: an initial denaturation at 95.0° C for five minutes; 30 cycles of 95.0° C for 30 seconds, 55° C for one minute and 72.0° C for one minute with a final extension of 72.0° C for five minutes. The amplified PCR products were analysed by electrophoresis on 1.5 % agarose gels using TBE (Tris-borate/EDTA) as running buffer at 120 V for one hour(Sambrook and Russell, 2001). Gels were stained with ethidium bromide and visualized with UV transilluminator, and photographed using a transmitted illumination camera fitted with a Polaroid film (Gelcam UK).

Table 2: Sequence of primers to detect *gyrA, gyrB, parC and parE*

Target gene	Nucleotide sequence (5' -3')	Product size (bp)
Gyr A	F 5' -ATGAGCGACCTTGCGAGAGAGAAATACACCG	632
	R 5' - TTCCATCAGCCCTTCAATGCTGAGTCTTC	
Gyr B	F 5' - AAGCGCGATGGCAAAGAAG	1500
	R 5' - AACGGTCTGCTCATCAGAAAGG	
Par C	F 5'- CTATGCGATGTCAGAGCTGG	294
	R 5'- TAACAGCAGCTCGGCGTATT	
Par E	F 5'- TCTCTTCCGATGAAGTGCTG	191
	R 5'- ATACGGTATAGCGGCGGTAG	

3.5.8 PCR detection of Class 1 integron and trasposon 21

Whole cell DNA was extracted from 108 MDR *S.* Typhi isolates by boiling the cells at 99° C. Integron Class1 and transposon 21 were detected by PCR with the degenerate primers. The sequences for the reverse and forward integrons Class 1 primers used were 5'GGTGTGGCGGGCTCGTG3' and 5'GCATCCTCGGTTTTCTGG3', respectively. For the Tn*21* the sequences for the reverse and forward primer used were GGAGGTGGTAGCCGAGG and TCAACCTGACGGCGGCGA, respectively. The

amplifications cycle for both Class1 Integron and transposon 21 consisted of 35 repetitions of one minute at 94° C, one minute at 60° C, and five minutes at 74° C. Amplification products were analyzed by agarose gel electrophoresis.

CHAPTER FOUR

4.0 RESULTS

4.1 Antibiotic sensitivity pattern

High level of antimicrobial resistance was observed with ampicillin (71%), chloramphenicol (72%) and tetracyline (74%). Less than 10% of the isolates were resistant to cefotaxime (5%), ceftriaxone (2%), ciprofloxacin (1%), cefuroxime (4%), gentamicin (4%) and nalidixic acid (6%) **(Table 3)**. The most effective drugs recorded were gentamicin, with 137 (95%) isolates being susceptible; this was followed by ceftriaxone in which 136 (94%) isolates were susceptible. The proportion of isolates sensitivity to other antimicrobials was 131 (91%) to ciprofloxacin, 134 (93%) to cefotaxime, 118 (82%) to Amoxycillin-clavulinate, 81 (81%) to cefuroxime and 99 (69%) to nalidixic acid **(Table 3)**.

Table 3: Antibiotic susceptibility patterns among the 144 *S.* Typhi isolates

Drug	Sensitive		Intermediate		Resistant	
	Frequency	**%**	**Frequency**	**%**	**Frequency**	**%**
Amoxycillin-clavulinate	118	82	6	4	20	14
Ampicillin	34	24	7	5	103	71
Cefotaxine	134	93	3	2	7	5
Ceftriaxone	136	94	4	3	3	2
Chloramphenicol	39	27	1	1	104	72
Cefuroxime	81	56	57	37	6	4
Ciprofloxacin	131	91	11	8	2	1
Gentamicin	137	95	1	1	6	4
Nalidixic acid	99	69	36	25	9	6
Tetracycline	29	20	8	6	107	74

4.2 Multi- drug resistant S. Typhi

Twenty three isolates (16%) were susceptible to all the ten drugs **(Table 4)**. One hundred and eight of the isolates (75%) had Multiple Drug Resistance (MDR) (resistant to more than three antibiotics). The most frequently observed multiresistance profiles included ampicillin, chloramphenicol and tetracycline.

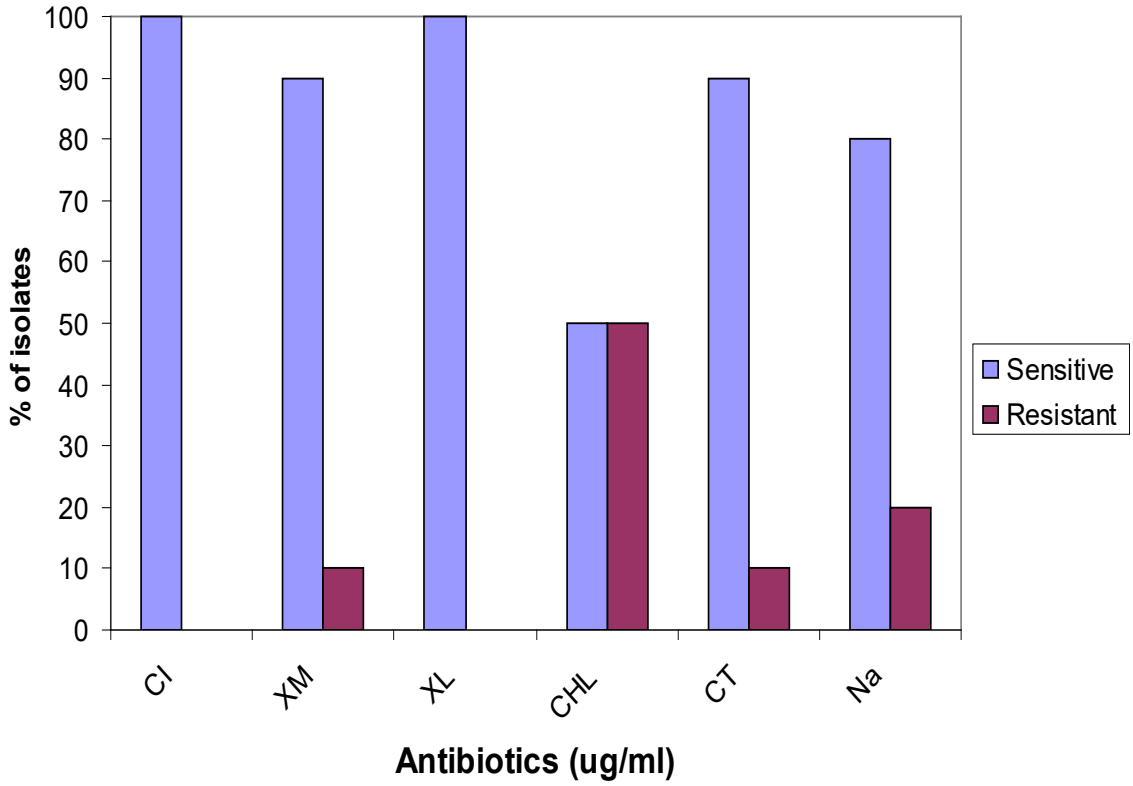
Table 4: Proportion of isolates sensitive and resistant to the ten drugs tested.

Sensitivity to all drugs	Frequency	Percent
Yes	23	16
No	121	84
Total	144	100

4.3 Minimum inhibitory concentration

All the 30 (100%) isolates selected for MIC test were susceptible to amoxycillin-clavulanic acid and ciprofloxacin. Three isolates were resistant to cefotaxime (10%), three were resistant to cefuroxime (10%), six were resistant to nalidixic acid (20%) and 15 (50%) isolates were resistant to chloramphenicol (**Figure 1**).

Figure 1: MICs of selected antibiotics for 30 selected multi-drug resistant *S.* Typhi isolates

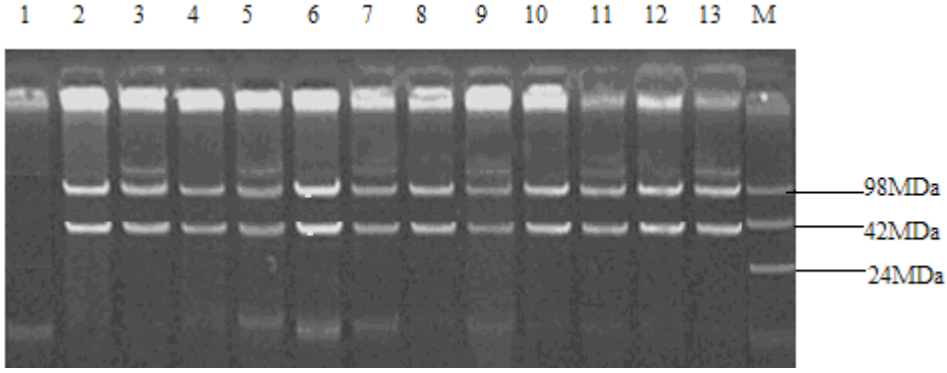


Key: CI - Ciprofloxacin, XM- Cefuroxime, XL- Amoxycillin-clavulanic acid, CHL- Chloramphenicol, CT- Cefotaxime and Na - Nalidixic acid

4.4 Plasmid Analysis

Plasmid DNA was extracted from a total of 108 MDR *S.* Typhi isolates that were resistant to three or more antibiotics **(Figure 2)**. The plasmid DNA from each strain was separated by electrophoresis on agarose gels, and the plasmid profiles were compared. Eighty percent of the 108 MDR *S.* Typhi strains contained a common 98 MDa plasmid and a smaller 42 MDa plasmid **(Figure 2).** Isolates sensitive to all the drugs tested did not have a plasmid **(Figure 2, lane 1).**

Figure 2: Plasmid profile from selected multi-drug resistant isolates



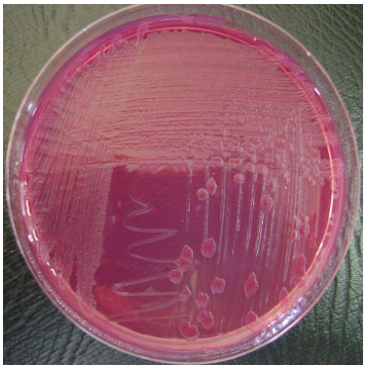
Electrophoresis on a 1% agarose gel of plasmid DNA extracted from MDR *S.* Typhi isolates. Lane M represents the plasmids of the reference strain *E. coli* 39 R861 (NCTC 50192); lane 1 represents isolate sensitive to all the drugs tested. Lanes 2-13 represent plasmids of MDR *S.* Typhi isolates with molecular sizes of 98 MDa and 42 MDa.

4.5 Conjugation experiments

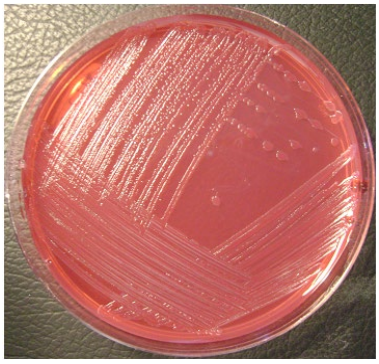
Sixty eight MDR isolates selected for conjugation were resistant to atleast ampicillin and sensitive to nalidixic acid. *S.* Typhi grew in the plates containing ampicillin only **(Figure 3b)** while *E. coli* K12 grew in the MacConkey plates containing nalidixic acid only **(Figure 3a)**. The transconjugants grew on the plates containing both ampicillin and nalidixic acid **(Figure 3c)**. Using disk diffusion sensitivity test to confirm transfer of resistance genes, 54 (80%) of the MDR *S.* Typhi selected for conjugation transferred resistance of one or more antimicrobials to recipient *E. coli* K12 **(Table 5)**. Forty two (62%) MDR *S.* Typhi transferred their full resistance phenotype. Ampicillin resistance was transferred from 46 (68%) of the resistant *S.* Typhi. Chloramphenicol resistance was transferable from 45 (66%) of the resistant *S.* Typhi and tetracycline resistance from 51 (75%). Amoxycillin-clavulanic acid resistance was transferable from 6 (9%) of the resistant isolates. Resistance to gentamicin was not transferable. In each case that resistance was transferred whatever the phenotype, it was associated with the large (98 MDa) plasmid **(Figure 4)**. Plasmid DNAs isolated from the transconjugants co-migrated with the plasmid isolated from their corresponding donor strains. The antibiotic sensitive strains of *S.* Typhi did not show any plasmid band in the gel **(Figure 4, lane 1).**

Figure 3: Conjugation experiment

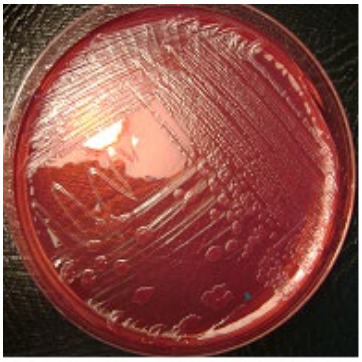
a



b



c



F**igure 3a**: MacConkey plates containing nalidixic acid with colonies of the recipient strain *E. coli* K12

Figure 3b: MacConkey plate containing ampicillin with colonies of the donor strain *S.* Typhi

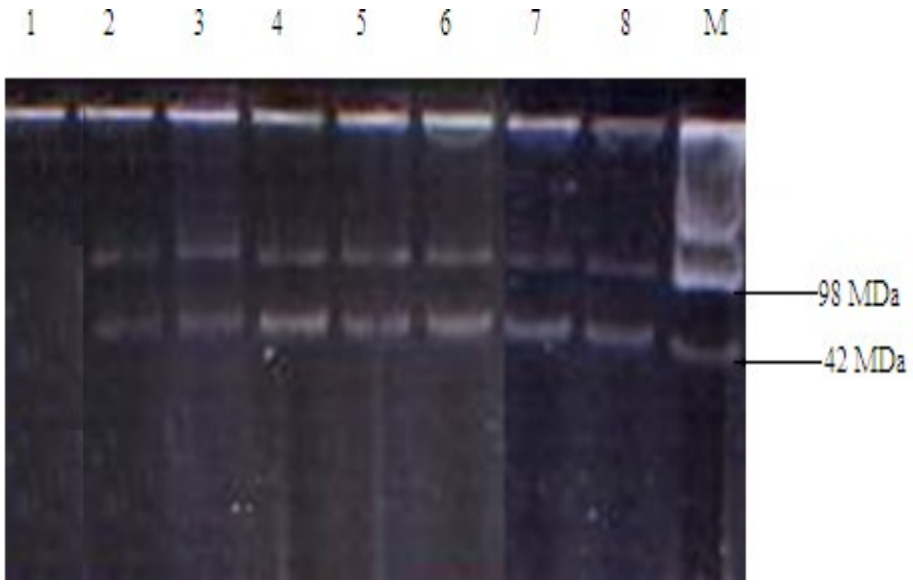
Figure 3c: MacConkey plate containing both ampicillin and nalidixic acid with colonies of transconjugants

Table 5: Patterns of antibiotic resistance transferred from multi-drug resistance *S.* Typhi isolates by in-vitro conjugation

Isolate designation		**Antimicrobial resistance pattern**	
	Phenotypes of the donor	**No. of successful transfers**	**Phenotype transferred to *E. coli* K-12**
Group 1	Amc, Te, C, Ax	6 (9%)	Amc,Te, C, Ax
Group 2	Te, C, Ax	36 (53%)	Te, C, Ax
Group 3	Te, C, Ax	5 (7%)	Te
Group 4	Te, C, Ax	3 (45%)	C
Group 5	Te, C, Ax	4 (6%)	Te, Ax
Group 6	Te, C, Ax	14 (21%)	NT

Key: Amc= Amoxy-clavulin, **C** = Chloramphenicol**, Ax** = Ampicillin**, Te** = Tetracycline, **NT=** Not transferable

Figure 4: Plasmid analysis for isolates selected from transconjugants

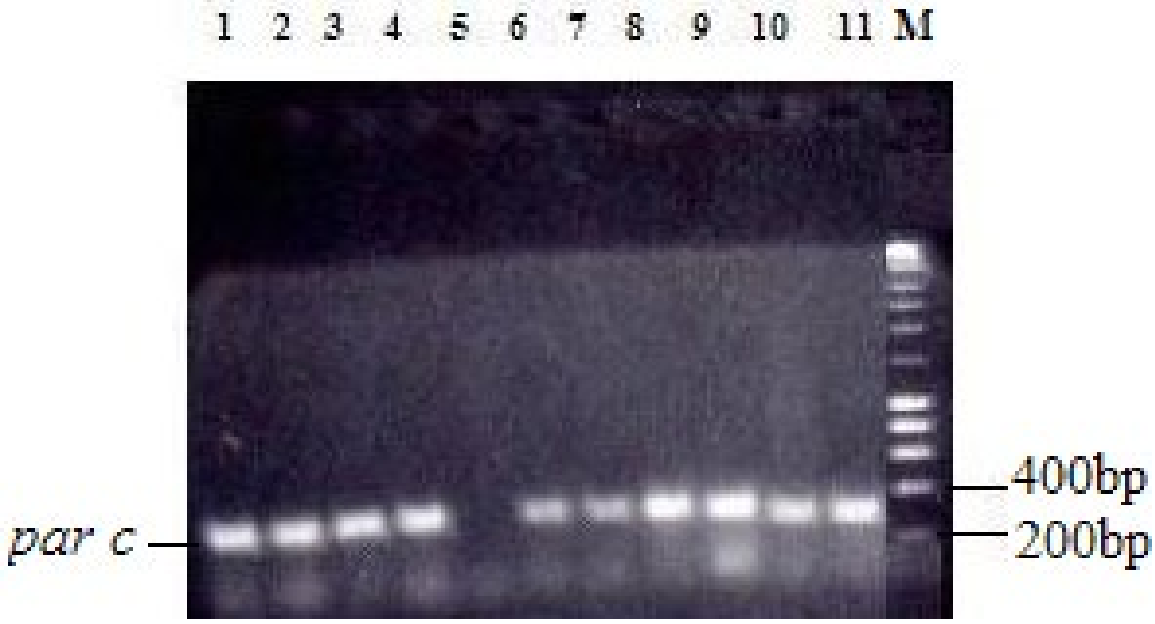


Lanes 2-8 transconjugants resistant to chloramphenicol, tetracycline and ampicillin; Lane 1 was loaded with a control strain of *S*. Typhi that was sensitive to all the antibiotics tested; Lane M shows plasmids from the marker *E. coli* 39 R861 (NCTC 50192).

4.6 PCR of the quinolone resistance determining region

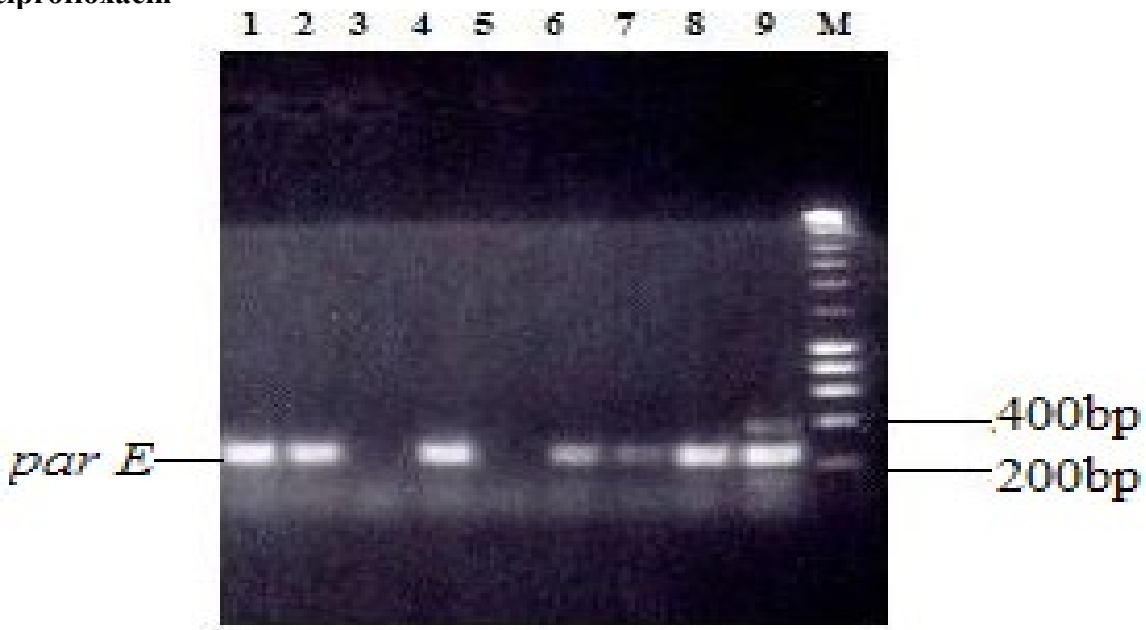
Eleven quinolone resistant *S.* Typhi isolates were amplified by PCR to detect the presence of the *par C*, *par E*, *and gyrase A*, *gyrase B* genes. PCR screening results showed that 7 isolates were positive for *par* E, 10 for *par C*, 7 for *gyr A* and 4 for *gyr B* as shown in figure 5, 6, 7 and 8.

Figure 5: Amplification of *par C* genes of *S.* Typhi resistant to nalidixic acid and ciprofloxacin



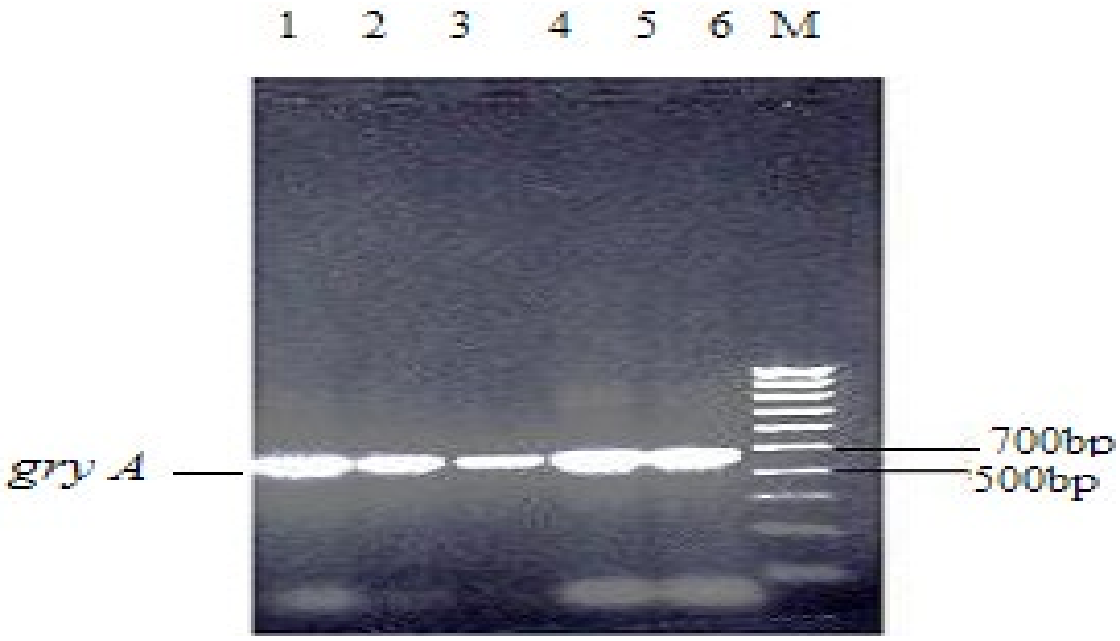
Lanes 1, 2, 3, 4, 6, 7, 8, 9, 10 and 11 represent the *S.* Typhi that were resistant to nalidixic acid and ciprofloxacin. Lane 5 represents *S.* Typhi isolate that was resistant to nalidixic acid and did not contain the *par C* genes. Lane M contains the 200 bp molecular weight ladder (200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000 and 6000 bp).

Figure 6: Amplification of *par E* genes of *S.* Typhi resistant to nalidixic acid and ciprofloxacin



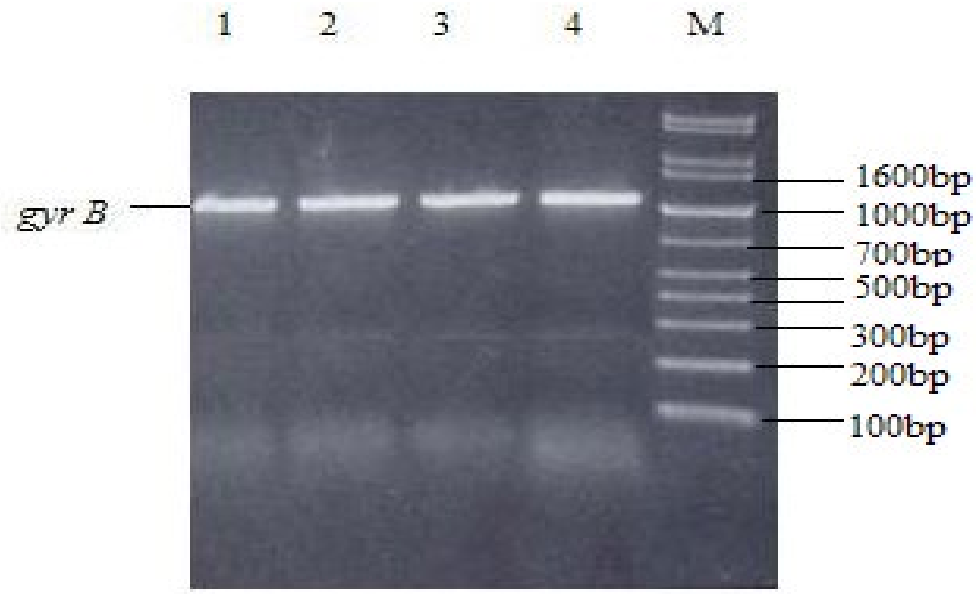
Lanes 1, 2, 4, 6, 7, 8 and 9 represent the *S.* Typhi that were resistant to nalidixic acid and ciprofloxacin. Lane 3 and 5 represents *S.* Typhi isolates that were resistant to nalidixic acid and ciprofloxacin and did not contain the *par E* genes. Lane M represents the marker with 200 bp MW ladder (200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000 and 6000 bp).

Figure 7: Amplification of *gyrA* from *S.* Typhi resistant to nalidixic acid and ciprofloxacin.



Lanes 1, 2, 3, 4, 5 and 6 represents *S.* Typhi isolates that were resistant to nalidixic acid and ciprofloxacin positive for *gyrA* genes. Lane M represents the marker with 100 bp ladder (200, 400, 600, 800, 1000, 1500, 2000, 2500, 3000, 4000, 5000 and 6000 bp).

Figure 8: Amplification of *gyr B* from *S.* Typhi resistant to nalidixic acid and ciprofloxacin.

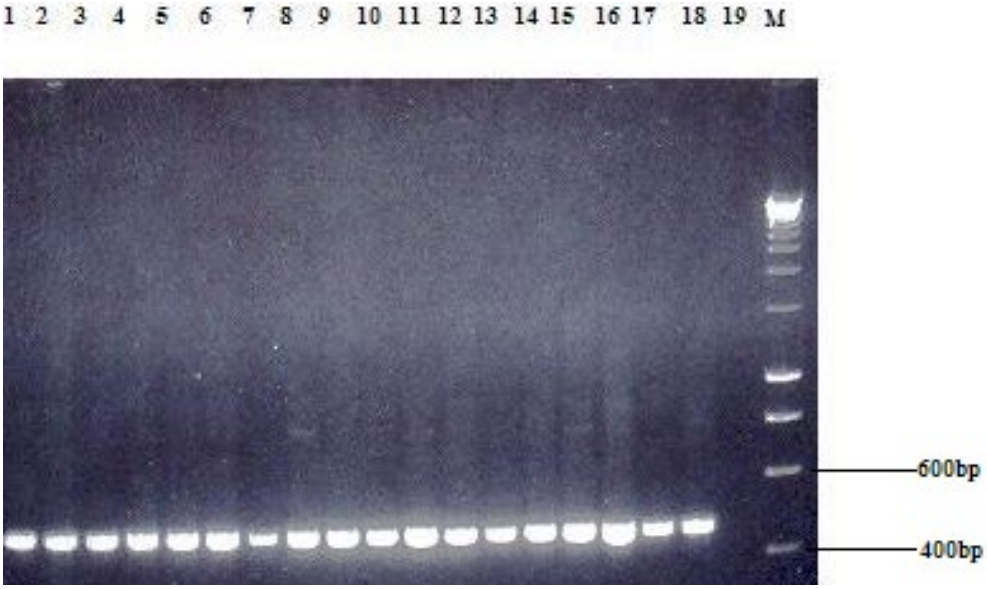


Lanes 1, 2, 3 and 4 represent bands for the *S.* Typhi that were resistant to nalidixic acid and ciprofloxacin and positive for *gyrB*. Lane M represents the size marker containing a 100 bp ladder.

4.7 Incompatibility Grouping using PCR

The replicons produced bands of expected sizes for their respective replicon types. One hundred and three (95%) of the MDR *S.* Typhi isolates were positive for the 471-bp PCR product corresponding to IncHI1 plasmid recognition site **(Figure 9)**, 13 (12%) isolates were positive for the 262-bp PCR product corresponding to IncFIC recognition site **(Figure 10c)** and one (1%) isolate was positive for IncI1 139-bp **(Figure 10a)**. One isolate (1%) was also positive for 543-bp IncP plasmid **(Figure 10b)**. No amplicons were observed in the negative control fully sensitive *S.* Typhi **(Figure 9, lane 19)**.

Figure 9: Incompatibility grouping for IncHI1 plasmid by PCR



Lanes 1-18 represent a 471-bp IncHI1 PCR Product; Lane 19 represents antibiotic sensitive *S.* Typhi; Lane M represent 200 bp MW ladder (400, 600, 800, 1000, 1500, 2000, 2500, 3000 and 4000)

Figure 10: PCR analysis of IncI1, IncP and IncFIC

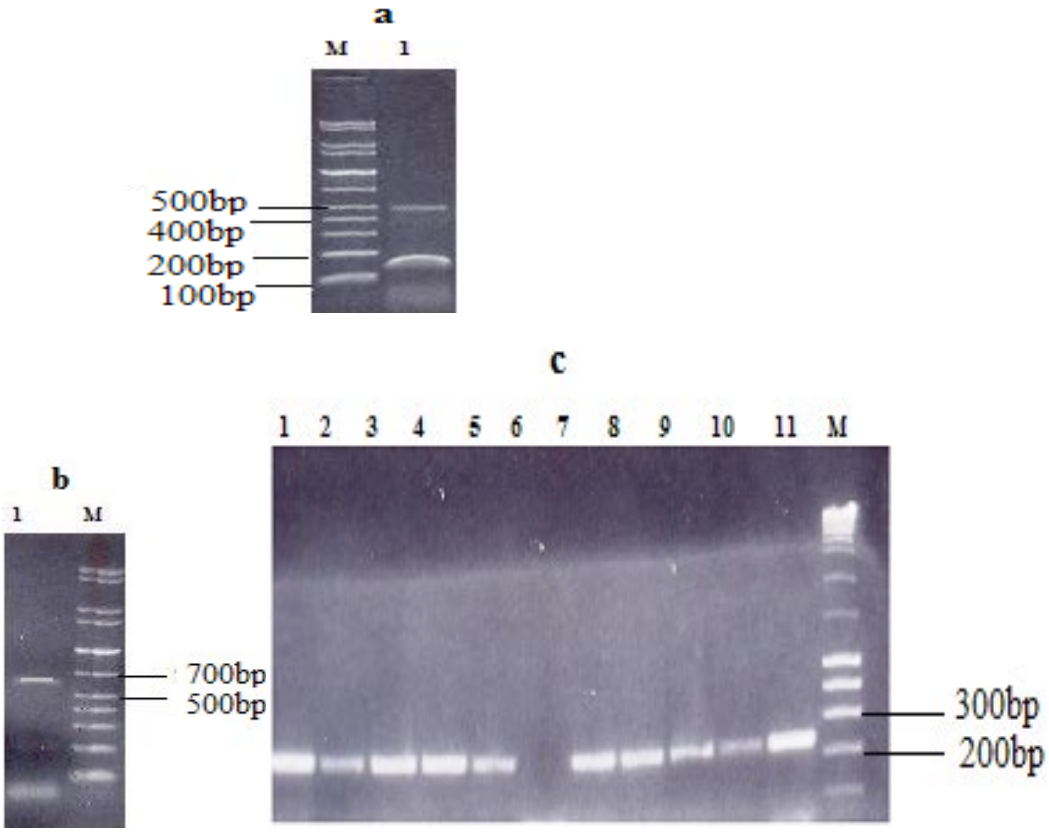


Figure 10 a -lane 1 PCR analysis of IncI1 with molecular size of 139bp; lane M 100bp marker

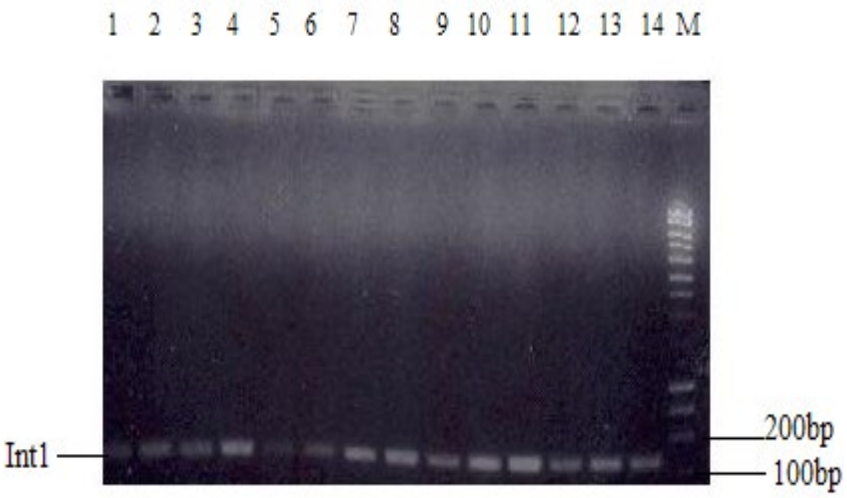
Figure 10 b- lane 1 PCR analysis of IncP with molecular size of 534bp; lane M 100bp marker

Figure 10 c- lane 1, 2, 3, 4, 5, 7, 8, 9, 10, 11 PCR analysis of IncFIC with molecular size of 262bp; lane 6 MDR *S.* Typhi that did not contain plasmids of IncFIC. Lane M shows a 100bp ladder

4.8 PCR for detection of Class 1 integrons and transposon 21

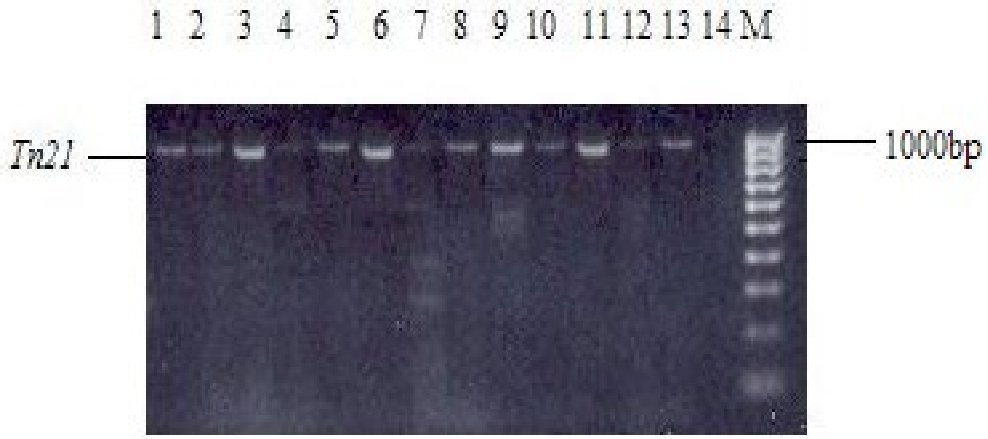
Detection of integron and trasposons was performed with a PCR assay. The results of these amplifications and the molecular sizes of the amplicons are summarized in **Figures 11** and **12**. The Class 1 integrons and transposon 21 among multiresistant plasmids carrying *S*. Typhi strains were similar in size.

Figure 11: PCR products of integrons carried by multi-drug resistant *S.* Typhi



Lanes 1-14 represent PCR analysis of Int-1 of 120bp. lane M shows the molecular size standards in basepairs (100, 200, 300, 400, 500, 600, 700, 800, 900 and 1000).

Figure 12: PCR products of transposons found on plasmids carried by *S.* Typhi



Lanes 1-13 represent PCR analysis of MDR *S.* Typhi isolates that contained transposon *Tn21*. Lane 14 represents MDR *S.* Typhi isolates that did not contain *Tn21.* Lane M shows the molecular size standards in basepairs (100, 200, 300,400, 500, 600, 700, 800, 900, and 1000).

CHAPTER FIVE

5.0 DISCUSSION

Typhoid fever is a serious systemic infection that causes high morbidity in many tropical countries. In developed countries typhoid is sporadic affecting mainly travellers returning home from endemic regions, with occasional point-source epidemics (Ackers *et al.*, 2000). Typhoid fever requires proper antimicrobial therapy in order to reduce mortality rates. Previously, various types of antimicrobial agents have been effective for the treatment of typhoid fever, but options for treatment of this disease are now becoming limited due to the emergence of multidrug-resistant strains of *S.* Typhi.

The trends in susceptibility pattern of *S.* Typhi from this study showed that only a small percentage (16%) of the isolates were susceptible to all the ten drugs tested while 84% were resistant to one or more of the ten antibiotics tested; 3% were resistant to one drug, 6% resistant to two drugs and 75% were considered MDR. The high level of MDR strains observed raises a concern over treatment options for typhoid fever in Kenya, hence there is an urgent need to advocate for the frequent monitoring of any changes in antibiotic response to these strains. It is also important that prescriptions made to the patients be changed from time to time to reduce the frequency of the emergence of resistant strains.

The first report of MDR *S.* Typhi was reported in Kenya in 1997-1999 when the prevalence of the MDR phenotype was 50 to 65% (Kariuki *et al*. 2000). A continuous surveillance of *S.* Typhi in Kenya has shown that the prevalence of MDR *S.* Typhi has been rising steadily. This is evident by a study conducted in 2001 and 2002 which recorded a prevalence of 70 to 78% of MDR *S.* Typhi isolated from blood cultures from the main referral hospital in Nairobi (Kariuki *et al*. 2004). This figure is similar to the one obtained in this study where 75% of the isolates were MDR**.** This figure is much higher than the 52% prevalence for MDR *S*. Typhi reported in outbreaks of typhoid in Ghana (Mills-Robertson *et al.*, 2002) and in Egypt where MDR *S*. Typhi (resistant to chloramphenicol, ampicillin and cotrimoxazole) was isolated from 29% patients (Srikantiah *et al.*, 2006), but is close to the high prevalence of MDR *S*. Typhi noted in Nigeria (Akinyemi *et al.*, 2005). Based on the increase in the prevalence of the MDR phenotype, it appears that prevalence of MDR *S*. Typhi strains have been on the increase in Kenya and are gradually replacing the fully sensitive strain types, probably due to their survival advantage over sensitive strains. This is also supported by Kariuki *et al*. (2000) in a study where they reported that the MDR phenotype seemed to be replacing the sensitive strain of *S.* Typhi. Indeed there is an urgent need to examine the status of resistant *S*. Typhi so that a rational approach to therapy may be adopted.

There has been an increasing concern about the prevalence of MDR *S*. Typhi strains that are resistant to chloramphenicol, ampicillin and tetracycline. In Kenya, as in most

developing countries, the 'first-line' antibiotics for treating most bacterial infections are ampicillin, tetracycline, or chloramphenicol. It is worrying that a high proportion of MDR *S.* Typhi strains isolated in this study were resistant to the three most commonly used 'first-line' antibiotics including ampicillin (71%), tetracycline (74%) and chloramphenicol (72%). These proportions are too high for physicians and other health care givers to rely on these antibiotics for empirical therapy. These findings are in agreement with studies by Kariuki *et al*. (2000) and Mirza *et al.* (2000) who found out that the *S.* Typhi that had been isolated in previous years were resistant to ampicillin, tetracycline and chloramphenicol. Ampicillin, chloramphenicol and tetracycline are inexpensive antibiotics and this explains why they have been used extensively for the treatment of most bacterial infections in developing countries.

MDR *S.* Typhi strains mainly resistant to the three first-line antibiotics have become a serious problem globally and have been reported worldwide. As a result, the potential of other antimicrobial agents including Second-line agents that is, the broad-spectrum cephalosporins and fluoroquinolones for the treatment of typhoid fever have been investigated. In this study ciprofloxacin (91%), cefotaxime (93%), amoxyclav (82%), ceftriaxone (95%) and gentamicin (95%) were uniformly effective *in vitro* against majority of the isolates. The reason could be that the strains were not frequently exposed to these antibiotics. These drugs have proven to be very effective but are difficult to obtain because they are expensive.

The resistance to amoxy-clavulate observed in this study may be due to a combination of mechanisms that could not be inferred directly from the results of the current study. However of concern in this study was the occurrence of isolates that were resistant and those that showed an intermediate susceptibility to nalidixic acid (6% and 36%), newer quinolones [ciprofloxacin (1% and 8%)] and the second generation cephalosporins namely, cefuroxime (4% and 37%) and third generation cephalosporins including ceftriaxone (2% and 3%) and cefotaxime (5% and 2%), respectively. This result is surprising as these drugs are not frequently prescribed by physicians in most clinics and hospitals in Kenya. However, the emergence of *S.* Typhi with low susceptibility and resistance to nalidixic acid, ciprofloxacin, ceftriaxone and cefotaxime is probably attributed to increasing rate of over-the-counter sales without prescriptions and indeed all other first-line antibiotics for typhoid fever particularly as a result of poor diagnostic procedures for suspected cases of typhoid in numerous clinics in the poor informal settlements. Several studies in developed countries have shown that the use of quinolones in food animals have led to the rapid emergence of resistant *Salmonella* infections in humans (Threlfall, 2002).

Some of the isolates resistant to these first line and second line agents showed an increased MIC range (>256µg/ml) for cefuroxime, chloramphenicol, cefotaxime and nalidixic acid. Three (10%) isolates were resistant to cefotaxime, three (10%) were resistant to cefuroxime, six (20%) were resistant to nalidixic acid and 15 (50%) isolates were resistant to chloramphenicol. The high prevalence of MDR *S.* Typhi

strains for which the MIC's for these reserve drugs were high means that soon resistance may emerge that renders these drugs ineffective, as has happened in Southeast Asia, where even moderate rises in MIC's has led to clinical treatment failure (Chinh *et al.*, 2000; Threlfall and Ward, 2001).

This study reported 11 isolates that were resistant to nalidixic acid and ciprofloxacin by disk diffusion. PCR was used to determine the presence of genes that code for resistance to nalidixic acid and ciprofloxacin. The presence of *gyr A* was detected in 7 isolates*, gyr B* in 4 isolates, *par C* in 10 isolates and *par E* in 7 isolates that were resistant to nalidixic acid and ciprofloxacin. Strains of *S.* Typhi showing complete resistance to ciprofloxacin were reported in 2006, first in Karachi (Pakistan) (Siddiqui *et al.*, 2006) and more recently in India (Chau *et al.*, 2007). Selective pressure on the bacterial populations resulting from the uncontrolled use of quinolones and fluoroquinolones has likely led to emergence of resistance to this group of antimicrobials. There may be single or multi-mutations in the quinolones resistance-determining region (QRDR) of either DNA gyrase (*gyr A* or *gyr B* or both) or DNA topoimerase IV (*parC* and *parE* or both) or both enzymes, or may be also plasmid mediated (Jacoby *et al.*, 2006), which cause resistance of *S.* Typhi strains to fluoroquinolone (Kariuki *et al.,* 2004). However, other mechanisms of resistance such as decreased permeability and active efflux of the antimicrobial agent may be involved.

In vitro evidence suggests that the selection of a *gyr A* mutation coupled with a *par C* change is often sufficient to increase the MIC's of newer quinolones beyond the susceptible breakpoint (Gaind *et al.*, 2006). In *Salmonella*, the most common mutations leading to quinolone resistance have been Ser-83 and Asp-87 in the *gyr A* gene, either alone or together (Brown *et al.*, 1996). Mutation of the topoisomerase gene *gyr* A, characteristically occurring at position 83 of the DNA gyrase enzyme (changing serine to phenylalanine) and position 87 (changing aspartate to tyrosine or glycine) (Brown *et al.*, 1996). Therefore, the introduction of more potent agents into clinical practice may be necessary if *S.* Typhi strains with multiple QRDR mutations begin to emerge.

In this study, there was co-existence of antibiotic sensitive and MDR strains of *S.* Typhi, but unlike MDR strains the sensitive strains did not contain any plasmid. The source of resistance plasmids remains unclear but it is tempting to speculate that they are derived from commensal enterobacteria of other humans as has been suggested from a study done in Kolkata, India (Mandal *et al.*, 2004) based on the fact that most of the enteric isolates (*Escherichia coli*, *Proteus vulgaris, Klebsiella pneumoniae*) showed a common resistance pattern (ampicillin, chloramphenicol, cotrimoxazole and tetracycline) and that the resistance pattern was transferred to the drug sensitive *S.* Typhi with the same degree of resistance. Thus, the present findings suggest that the MDR *S.* Typhi may arise from sensitive isolates by acquisition of multidrug resistance plasmid from antibiotic-resistant enteric bacteria.

Another possible source of resistance plasmids is the enteric flora of domestic animals which can be disseminated to pathogenic bacteria by various genetic processes (Threlfall, 2002). The selection exerted by antibiotic treatment of enteric fever may be the cause of acquisition of resistant plasmid. Through the acquisition of a plasmid conferring multidrug resistance, the strain undergoes the necessary and appropriate adaptation for survival in the changing antibiotic environment. Thus, it appears that the already existing sensitive strain, by the acquisition of a resistant plasmid, have been able to adapt to the challenge of antibiotics as they are introduced into clinical medicine. As in the case in Kenya the study conducted between 1997 to 1999 indicated a prevalence of 50% - 65% of MDR *S.* Typhi. This study and other studies done from 2000 have however indicated an increased prevalence of MDR *S.* Typhi of up to between 70% -78%. This inceasing trend of resistant S. Typhi clearly shows that more sensitive strains might have acquired the resistance plasmid leading to the emergence of more MDR *S.* Typhi, replacing the sensitive strain population, a case that was not reported a decade ago.

In the current study among the replicon profiles identified, 95% of the MDR *S.* Typhi contained large self-transmissible plasmids 98 MDa of incompatibility group HI1. It appears that this plasmid has been maintained over the entire study period and may be a contributing factor to the fitness and persistence of the MDR *S.* Typhi phenotype in Kenya. Similarly large MDR plasmids of incompatibility group HI1 carrying resistance to nearly all commonly available antibiotics have been characterized in *S.*

Typhi strains from endemic areas of Vietnam (Le *et al.*, 2007), India (Wain *et al.*, 2003) and Pakistan (Mirza *et al.*, 2000). Plasmids belonging to the incompatibility group HI1 (IncHI1) are known to encode multiple antibiotic resistances.

Several studies suggest that these plasmids, which belong to the IncHI group, frequently encode resistance to chloramphenicol, trimethoprim, ampicillin, sulfonamides and tetracyclines and have been estimated as being between 110 and 120 MDa (165 and 180 kb) (Threlfall *et al.*, 1992). Similarly in the current investigation plasmids encoding MDR resistance mainly to chloramphenicol, ampicillin and tetracycline among the *S.* Typhi were found to belong to the IncHI group, specifically IncHI1 and were estimated to be 98MDa. From this study it is likely that the plasmids encoding the antibiotic resistance genes among the *S.* Typhi have a high degree of relatedness indicating that the MDR isolates were more homogeneous. This is in agreement with previous studies on the molecular epidemiology of *S.* Typhi strains from Asia which showed that MDR isolates were more homogeneous (Thong *et al.*, 2000).

The other replicons identified include IncI1, IncP and IncFIC. A survey conducted between 1987 and 1994 in France, demonstrated a dramatic increase from 0 to 42.5% in the prevalence of β-lactam resistance among *Salmonella* isolates. Several types of β-lactamases were found on plasmids belonging to different incompatibility groups Q, P, F and HI(Llanes *et al.*, 1999). Plasmids belonging to the IncI1 family are considered as virulence plasmids since they are characterized by the presence of a

cluster encoding the type IV pili. Many type IV pili are known to play important roles in the attachment of bacterial pathogens to membranes of eukaryotic host cells. These peculiar pili are a virulence factor and the association of virulence and resistance determinants may favour the positive selection of plasmids belonging to the IncI1 family (Carattoli, 2003). IncI1 plasmids are reported in different countries and they are associated with the dissemination of a great assortment of resistance genes such as *bla*CTX-M, *bla*CMY-2 and *blaSHV*. IncI1 plasmids carrying extended-spectrum and AmpC β-lactamase genes isolated from animals and humans have been described in *E. coli* and *Salmonella* (García-Fernández *et al.,* 2008). IncI plasmids have been shown to contain Class 1 integrons and transposases similar to those of Tn*21*. IncP plasmids have been isolated from community isolates and, unlike those previously described, contain *merA* mostly associated with Tn*21*-like structures. Detailed analyses of antibiotic-resistance plasmids isolated from clinical and environmental bacteria revealed that plasmids belonging to the IncP incompatibility group serve as vectors for the horizontal mobility of the encoded accessory genes (Dröge *et al.*, 2000; Heuer *et al.*, 2002).

In the present study, 90% of the MDR *S.* Typhi isolates contained Class1 integrons indicative of their high frequency of occurrence in *S.* Typhi. Class1-like integron have been shown to carry from one to four antibiotic resistance genes. The integron-carrying isolates are usually multiresistant, whereas the integron-lacking isolates are more susceptible to antibiotics. In previous studies (Miko *et al.*, 2005; White *et al.*,

2001), a strong association of presence of Class1 integrons and resistance to antibiotics has been demonstrated and attributed in part to the existence of resistance genes (*aadA* for streptomycin and spectinomycin, *aadB* for gentamicin and kanamycin, *beta-lactamase* for ampicillin) within these integrons.

Horizontal and vertical transfer of antibiotic resistance genes in members of the family Enterobacteriaceae is mostly due to large broad-host range of plasmids and to the transposons they carry and share with other replicons (Davies, 1997). Many transposons encoding multiple antibiotic resistances in Gram-negative members of the Enterobacteriaceae belong to the Tn*21* subgroup of the Tn*3* family. Transposon Tn*21* carry within them integrons. Tn*21*-like elements are carried on large conjugative plasmids and are known to contribute to the ready mobility of the bacterial strain.

In vitro conjugation experiments showed that 80% of the MDR selected for conjugation transferred some or all of the resistance to *E. coli* K12. Conjugal transfer of the resistance genes was conferred by the plasmid having a molecular mass of ≥ 98 MDa. The analysis for presence and conjugal transfer of plasmids clearly indicate that plasmids found in *S.* Typhi have independently acquired a single composite transposon encoding MDR. This provides a mechanism for the acquisition of a large number of drug resistance genes in a single transfer and should serve as a warning that MDR can be acquired rapidly by human pathogens in a single step. It also suggests that selection for resistance to one antibiotic may lead to the proliferation of resistance to many antibiotics. Moreover, if MDR can be transferred rapidly from one serovar to the other,

whether by horizontal gene acquisition or plasmid transfer, then the impact of selection for resistance in one serovar can affect resistance in the other. This is an important consideration in a clinical environment as it suggests that treatment choices for *S.* Typhi can impact on treatment options for *E. coli* K12 infection.

In the present study, results of conjugation experiments showed that resistance to amoxycilin-clavulanate, ampicillin, chloramphenicol, and tetracyline of *S.* Typhi isolates was transferable. The common resistance pattern (AX, C, and T) was the most commonly transferable. These are the most commonly used drugs in developing countries for treating infectious diseases. Plasmid transfer via conjugation has shown potential for rapid spread through populations. This is an important mechanism for DNA exchange or dissemination, and this presumably accelerates the evolution of bacterial pathotypes, contributing towards the plasticity of bacterial genomes and the diverse combinations of virulence and resistance factors that continue to be reported.

CHAPTER SIX

6.0 CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

1. Majority (75%) of the *S*. Typhi isolates were MDR.
2. High level of resistance of *S*. Typhi to chloramphenicol, ampicillin and tetracycline was recorded.
3. The effective antibiotics including ciprofloxacin ceftriaxone and cefixime would go a long way in stemming the prevalence of persons with chronic infections.
4. The presence of *gyrA, gyrB, parC* and *parE* genes in *S*. Typhi resistant to quinolones and fluoroquinolones suggest that the resistance was probably chromosomal mediated.
5. The presence of 98MDa and 46MDa size plasmids in MDR isolates and none in susceptible isolates clearly suggests that the spread of antibiotic resistance in *S*. Typhi is associated with independent acquisition of the resistance genes on plasmids DNA.
6. The plasmids encoding antibiotic resistance genes have a high degree of genetic similarity since 95% of the MDR isolates carried plasmids of incompatibility group HI1
7. Antibiotic resistance can be transferred rapidly from one serovar or organism to the other, by horizontal gene acquisition or plasmid transfer.

6.2 RECOMMENDATIONS

6.2.1 Scope for further studies

These data indicate that MDR *S.* Typhi is becoming an important problem, especially taking into account the limited choice of antimicrobial agents for treatment of enteric fever and the possibility of transfer of resistance to other enteric organisms. However, additional investigations are needed to ascertain the role of plasmid in the mediation of multidrug resistance among enteric bacteria. Further research is necessary to determine the role of plasmids in characterizing *S.* Typhi and understanding epidemiology of typhoid outbreaks. Replicon identification may provide useful clues to the evolution of these resistant plasmids. The ability to trace and screen plasmids by PCR may facilitate further understanding of the horizontal transfer of antimicrobial drug resistance. Knowledge of the regulation mechanism of plasmid transfer could be used to develop new antibacterial agents targeting development of plasmid-mediated resistance.

6.2.2 Surveillance

Constant collection and monitoring of data that document antibiotic resistance patterns is equally advocated. Antimicrobial resistance is a global problem. Hence a nationwide or a worldwide network for surveillance and response to emerging antibiotic resistant organisms such as the WHONET is required. These networks enhance monitoring of drug resistance in hospitals and communities and helps in merging files into national, regional, and global networks for surveillance of drug

resistance thus providing the professionals with all the useful information on the situation of the diseases at national and international level. Thousands of laboratories are distributed worldwide and need to be interlinked for proper data integration and dissemination on the most clinically relevant organism on a daily basis to ensure enormous sharing of information on infectious disease outbreaks and other health related issues.

WHONET has been established in Kenya and should be used in tracking the genetic lineage and the clonal relationship of isolates in the country. This database would facilitate tracking down the origin of MDR isolates during disease outbreaks and thus enhance control and preventive measures. The surveillance data will allow adjustment of treatment strategies, national drug policies and assessment of intervention strategies.

Research is essential in efforts to understand, prevent, control and respond to new and re-emerging infectious diseases. Innovations in biotechnology is making it easier to identify and track strains of infectious organisms and to determine the causes and sources of outbreaks as well as the source of disease transmission and how they respond to antibiotics. More in-depth genetic analysis of bacterial isolates also allows tracing back the origin of these infections, sometimes avoiding their diffusion from a common source. Introduction and application of these techniques in Kenya will make diagnosis more rapidly available with a higher accuracy and precision.

6.2.3 Policy issues

A national guideline on the proper usage of antibiotics is required for urgent implementation in Kenya. Restriction and/or withdrawal for a while of chloramphenicol and ampicillin for the treatment of typhoid fever should be initiated as this has been found to reverse resistance trends for plasmid-mediated resistance.

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APPENDICES

Appendix 1: Media preparation

1. MacConkey Agar

Formula

Gelatin peptone	17.0g
Lactose	10.0g
Sodium chloride	5.0g
Bacteriological agar	13.5g
Bile salts no.3	1.5g
Neutral red	0.03g
Peptone mixture	3.0g

Preparation

Suspend 50g in 1 litre of distilled water. Boil to dissolve the medium completely. Sterilize by autoclaving at 121^{o} C for 15 minutes. Cool to 45 to 50^{o} C and pour 15-20 ml amounts into petri dishes.

Use: for selection of enteric bacteria

2. Mueller Hinton agar

Formula

Beef infusion	300.0g
Starch	1.5g
Acid hydrolysate of casein	17.5g
Agar	17.0g

Preparation

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

Use: for sensitivity testing.

3. 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° C for 15minutes.

4. Tryptone Soy broth

Formula

Pancreatic digest	17.0 g/l
Papaic digest of soy bean meal	3.0 g/l
Sodium chloride	5.0 g/l
Di-basic potassium phosphate	2.5 g/l
Glucose	2.5 g/l

Preparation

Dissolve 30 g in 1 liter of distilled water. Dispense in to the required proportions into universal tubes and sterilize by autoclaving at 121^{o}C for 15 minutes.

Use: General-purpose media broth.

Appendix 2: Plasmid extraction reagents

1. 10N NaOH

80g of sodium hydroxide pellets was dissolved in 200ml deionized water.

2. 20% SDS

40g of SDS was dissolved in 160ml deionized water and filled up to 200ml with deionized water and stored at room temperature.

3. 0.5M EDTA, pH8.0

37.22g of Na EDTA HO was added to 160 deionized water and the Ph adjusted to 8.0 with sodium hydroxide pellets and filled upto 200ml with deionized water and sterilized by autoclaving and stored at room temperature.

4. 20% Glucose

40g dextrose was dissolved in 200ml in deionized water and stored at 4^{o}C.

5. 1M Tris Cl (pH8.0)

121.1g Tris base was dissolved in 800ml deionized water. The pH was adjusted to 8.0 with HCl and filled upto 1 litre and sterilized by autoclaving.

6. 20 X TE buffer pH8.0

24.11g of Tris base and 7.45g NaEDTA.2H_2O were dissolved in 800ml distilled water. The pH was adjusted to 8.0 with HCL and filled up to 1000ml with distilled water

7. Stop mix (0.07% BPB, 7% SDS and 20% Ficol)

70 mg of bromophenol blue, 7 g SDS and 20 g Ficol type 400 were dissolved in 80ml of distilled water. This was then warmed to dissolve, and topped up to 100 ml with distilled water.

8. 5X TBE electrophoresis buffer (used as 1:10 solution in the bath) 1X is 89mM Tris, 89mM boric acid and 2.8mM EDTA)

This was made by dissolving 53.89 g Tris base, 27.51ml boric acid and 5.21 ml $Na_2EDTA.2H_2O$ in 1000 ml distilled water.

9. 10mg/ml ethidium bromide

0.5g of ethidium bromide was dissolved in 50ml distilled water in a pre-sterile bottle. The mix was stored at 4^{o}C until ready for use.

10. 70% Ethanol

This was made by mixing 170 ml ethanol with 30 ml sterile distilled water in a pre-sterilized bottle.

11. Solution I

50 mMol glucose

25 mMol Tris Cl (pH8.0)

10 mMol EDTA (pH 8.0)

Solution I can be prepared in batches of approximately 100ml, autoclaved for 15 min at 10 1b/sq. in. on liquid cycle and stored at 4 ^{o} C.

12. Solution II

0.2 N NaOH (freshly diluted from a 2 N stock)

1% SDS

13. Solution III

3M Sodium acetate, pH 5.2 (B-III) was made by dissolving 204.12 sodium acetate. $3H_2O$ IN 400 distilled water. pH was adjusted to 5.2 with about 100ml acetic acid and filled to about 500ml with distilled water and sterilized by autoclaving.

Appendix 3: MIC strip and antibiotic ranges used

Antibiotic agent	**MIC range (ug/ml)**
Ciprofloxacin	0.002-32
Cefuroxime	0.016-256
Amoxy-clavulin	0.016-256
Chloramphenicol	0.016-256
Cefotaxime	0.016-256
Nalidixic acid	0.016-256