GENETIC CHARACTERIZATION AND EVALUATION OF ANTIMICROBIAL RESISTANCE PATTERNS OF *SALMONELLA* TYPHI ISOLATES

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DECLARATION

I, Pauline Kwamboka Getanda, hereby declare that the work presented in this thesis is my own work and has not been submitted anywhere for the award of any degree. Where other work has been used it has been acknowledged.

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   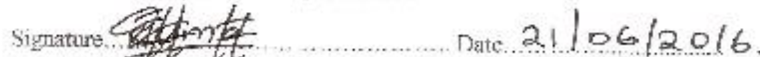
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DEDICATION

To my Family, you are God’s gift to me.

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LIST OF ABBREVIATIONS AND ACRONYMS

AMP	Ampicillin
AMC	Amino-Clavulinic Acid
AMY	Amygdalin
ARA	Arabinose
BLAST	Basic Local Alignment Search Tool
CHL	Chloramphenicol
CAZ	Ceftazidime
CDC	Centers for Disease control and Prevention
CIP	Ciprofloxacin
CIT	Citrate
CLSI	Clinical and Laboratory Standards Institute
GEN	Gentamicin
CXM	Cefuroxime
GEL	Gelatin
GLU	Glucose
H_2S	Hydrogen Sulfide
IND	Indole
INO	Inositol
LDC	Lysine decarboxylase
LPS	Lipopolysaccharide
MAN	Mannitol
MDR	Multi-Drug Resistant
MDRST	Multi-Drug Resistant *Salmonella* Typhi
MEL	Melibiose
Na^R	Nalixidic Acid Resistant

NCBI	National Center for Biotechnology Information
NTS	Non-Typhoidal Salmonellosis
ODC	Ornithine decarboxylase
RHA	Rhamnose
rRNA	ribosomal Ribonucleic Acid
STR	Streptomycin
SAC	Sucrose
SOR	Sorbitol
TDA	Tryptophan Deamination
TE	Tetracycline
TSA	Trypticase Soy Agar
URE	Urease
VP	Acetoin production
WHO	World Health Organization

ABSTRACT

Salmonella is a genus of bacteria comprising a group of closely related organisms that are pathogenic to humans and other vertebrates and pose a global health problem. Infections in humans due to *Salmonella* serotypes result in typhoid fever and gastroenteritis. There is paucity of information regarding the strains circulating and their antimicrobial resistance profiles. Reports show that *Salmonella* increasingly undergoes resistance and shows a rapidly changing nature of antibiotic resistance patterns. Continual genetic characterization and drug resistance surveillance is required to evaluate their genetic structure and resistance profiles regarding specific antibiotics. Therefore, the aim of the present study was to genetically characterize and evaluate the antimicrobial resistance profile of isolates of *Salmonella* Typhi collected from Kenyatta National Hospital and Agha Khan University Hospital. A total of 205 stool samples were collected from Kenyatta National Hospital and Agha Khan Hospital from patients suspected to be suffering from typhoid fever. The samples were cultured in nutrient broth and subcultured on Xylose Deoxycholate agar to identify *Salmonella* colonies. Fifty samples showed Salmonella morphology and were subjected to biochemical tests. Biochemical characterization identified 33% of the isolates as *Salmonella* Typhi and 67% as other *Salmonella* spp. DNA was extracted and used for polymerase chain reaction (PCR) targeting 16S rRNA gene. The amplified products were sequenced and phylogenetic analysis based on the 16S rRNA gene sequences showed that the isolates clustered close to *Salmonella* Typhi strain CT18 and its plasmid pHCM1 associated with drug resistance. Antimicrobial susceptibility tests showed that 73% of the isolates were multiply resistant to the Aminopenicillin, Sulfonamide, Phenicol and Aminoglycoside classes of drugs. A resistance of 7% to 19% and an intermediate resistance of 19% to 50% were observed in the Beta-lactamase, Fluoroquinolone and Cephalosporin classes of drugs. The current high rate of multidrug resistance and the high probability of its rise calls for continual characterization and monitoring of drug resistance as well as proper regulation in the dissemination and use of antimicrobial drugs.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background information of the study

Salmonella is a gram-negative facultative anaerobe that infects both cold and warm blooded animals causing typhoid fever, paratyphoid fever and gastroenteritis (Raffatellu *et al.,* 2008). The bacterial infections are common in poor resource areas due to inadequate sanitation and exposure to unsafe drinking water and food (Crump and Mintz, 2010). The pathogen has also been reported as a major health risk to travelers visiting highly endemic areas such as Asia, Sub-Saharan Africa and Latin America (Whitaker *et al.,* 2008). During infection with *S.* Typhi patients present with fever, headache, malaise, abdominal pain and discomfort. Diagnosis relies on laboratory confirmation for the presence of *Salmonella* Typhi in patients' blood, bone marrow or stool samples (Crump *et al.,* 2003). Although direct blood culture followed by microbiological identification is the gold standard diagnostic test for typhoid fever, its use in early diagnosis is optimal in the early phase of the disease (Sherwal *et al*., 2004). The serological widal test which is commonly used in most of the developing countries for typhoid fever diagnosis has been reported to be less sensitive, specific and of little practical value in areas where the disease is endemic (Levine *et al.,* 1999). This is because; widal test relies heavily on both the somatic (O) antigens and the flagellar (H) antigens which are widely shared by some other *Salmonella* serotypes and other members of enterobacteriaceae making the test less specific. Although less expensive and easily accessible, lack of specificity makes the role of widal test in typhoid fever unfavorable due to cross reactivity (Parry *et al.,* 2002). However nucleic acid sequence based methods have been used in the identification of *Salmonella* Typhi. Methods such as PCR have

made a significant contribution in improving the detection of *S. typh*i. Genome sequences have been widely used for the development of new diagnostic tests for the known as well as emerging pathogens (Millar *et al*., 2007) and the sequencing of 16S rRNA is a well-established method for identification of bacterial species, including *Salmonella* (Woo *et al.,* 2008).

Bacterial species are often susceptible to a number of antimicrobial agents. With this knowledge much of the antibiotics have been used indiscriminately in the treatment of both animal and human subjects. It is reported that the indiscriminate use of antibiotics caused by misdiagnosis or poor diagnosis accounts for the increased antibiotic resistance in *Salmonella* species (Omulo *et al.,* 2015). *Salmonella* spp. have shown susceptibility to the most commonly used antimicrobial agents but there has been a steady rise in the emergence of Multi-Drug Resistant *Salmonella* Typhi (MDRST), especially to the commonly used antibiotics. This has prompted for the widespread use of a number of chemotherapeutic agents such as floroquinolones, nalixidic acid and other drugs of higher potency. Unfortunately resistance to these drugs as well, has been reported in Kenya (Kariuki *et al.,* 2010).

It is therefore imperative to continually perform molecular epidemiology and antibiotic drug resistance evaluation to monitor levels of resistance. This will aid in proper disease management that involves accurate detection of *S.* Typhi and prompt and effective treatment. This will go a long way in the prevention of antimicrobial resistance resulting from misuse of antibiotics. This study was therefore aimed at genetically characterizing, identifying and determining antimicrobial susceptibility patterns of *Salmonella* Typhi isolates from Kenyatta National Hospital and Agha Khan University Hospital in Nairobi.

1.2 Problem statement

Salmonella Typhi the causative agent of typhoid fever is a major public health problem in developing countries especially in areas with poor sanitation and inadequate supply of clean water. Complications resulting from typhoid fever such as fever, severe headache, abdominal pain and intestinal hemorrhage lead to the hospitalization of the infected. Accurate and prompt detection of the bacteria and subsequently proper treatment are essential for patients' recovery. There is scarcity of information on the *Salmonella* Typhi strains circulating in Kenya which poses a challenge in detection of the bacteria and targeting the bacterial strains with the right combination of antibiotics. The problem is further aggravated by the emergence and spread of multi-drug resistant *Salmomella* Typhi strains and the paucity of information on the antimicrobial resistance patterns of *Salmonella* Typhi in Kenya (Kariuki *et al*., 2010). Antimicrobial profile of *Salmonella* Typhi isolates from Nairobi and indeed other regions of the country is critical in decision making as regards prescription and use of antimicrobials for the treatment of typhoid fever. It is therefore crucial to carry out molecular characterization of *Salmonella* Typhi, so as to know the circulating strains and to evaluate their antimicrobial resistant patterns. Future studies should aim at evaluating resistance genes to know whether these genes are being transferred from animals to food, to humans and between the different types of *Salmonella*.

1.3 Justification

Typhoid fever is a global health problem with an estimated 22 million cases and over 200,000 associated deaths occurring annually (Crump and Mintz, 2004). It is crucial to continually genetically characterize *Salmonella* Typhi that are in circulation in order to monitor the diagnostic capability of current detection methods to these strains, and make improvements or design new diagnostic methods depending on the strains circulating. Furthermore, the genetic profile of *Salmonella* is a key source of information in mapping the spread of bacteria in a community, enabling the implementation of control strategies (Amarantini *et al.,* 2011). There has been a steady rise in the prevalence of *Salmonella* Typhi that is resistant to some of the commonly used antimicrobial drugs. The first report of multidrug resistant *Salmonella* Typhi in Kenya, occurred between the years 1997-1999 (Kariuki *et al.,* 2000), with a prevalence of 50-65%, and is gradually rising (Kariuki *et al.,* 2004). The Multidrug resistant strains of *Salmonella* Typhi have led to the complication of the treatment with often recurring infections. The spread and continual replacement of drug sensitive strains with resistant types is also a major concern (Kariuki *et al.,* 2010). Typhoid fever also has a very high social and economic impact because of the hospitalization of patients with acute disease and the resulting loss of income attributed to the duration of the clinical illness. Continuous characterization of *Salmonella* Typhi and antibiotic resistance evaluation of typhoid fever is critical for healthcare providers in prescribing the right combination of antibiotics to target the multi-drug resistant bacterial strains and control the evolution of resistant microorganisms. The aim of this study was to genetically characterize *Salmonella* Typhi isolates and evaluate their antimicrobial resistance patterns.

1.4 OBJECTIVES

1.4.1 Main Objective

The overall objective of this study was to carry out genetic characterization and evaluation of antimicrobial resistance patterns of *Salmonella* Typhi isolates.

1.4.2 Specific Objectives

1. To genetically characterize *Salmonella* Typhi isolates from two major hospitals in Nairobi
2. To evaluate the molecular phylogeny of *Salmonella* Typhi isolates circulating within Nairobi
3. To evaluate the antimicrobial resistance patterns of the *Salmonella* Typhi isolates from the two hospitals in Nairobi

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Salmonella* spp

Salmonella, a genus of more than 2500 serological variants (serovars), includes many organisms that can cause salmonellosis. Human salmonellosis is one of the most frequently occurring food-borne zoonosis in developing and industrialized countries (Molla *et al.,* 2003; Scallan *et al.,* 2011). *Salmonella* are facultative anaerobic gram-negative bacilli in the *Enterobacteriaceae* family. Almost all warm-blooded and many cold-blooded animals serve as natural hosts of *Salmonella*, where farm animals are the major reservoir for most strains. Outbreaks of *Salmonella* infection have been associated with food-borne transmission involving water and foods contaminated with pathogenic strains of *Salmonella* (Sillankorva *et al.,* 2010).

2.1.1 *Salmonella* nomenclature

Salmonella nomenclature is complex and is still evolving (Euzeby, 1997). Currently, different nomenclatural systems are still being used. Uniformity in *Salmonella* nomenclature is necessary for identification of the genus (Brenner *et al.,* 2000). According to the World Health Organization (WHO) collaborating Centre and the Centers for Disease Control and Prevention (CDC) systems, *Salmonella* contains two species, *Salmonella enterica*, and *Salmonella bongori*, each of which contains multiple serotypes. *Salmonella enterica* is divided into six subspecies, (*I, S.enterica* subsp. enterica*; II, S. enterica* subsp. Salamae; IIIa*, S. enterica* subsp. arizonae; IIIb, *S. enterica* subsp. diarizonae; IV*, S.enterica* subsp. houtenae*; V, S.enterica* subsp. indica). In subspecies I (*S.enterica* subsp. *enterica*) serovars are assigned names depending on associated diseases, geographic origins or usual habitats (Su and Chiu, 2006).

Table 1: *Salmonella* species, subspecies, serotypes and their usual habitats Kauffmann-White Scheme (Brenner *et al.,* 2000)

***Salmonella* species and subspecies**	**No. of serotypes within Subspecies**	**Usual habitat**
S. enterica subsp. *enterica* (I)	1,454	Warm-blooded animals
S. enterica subsp**.** *salamae* (II)	489	Cold-blooded animals and the environment
S. enterica subsp. *arizonae* (IIIa)	94	Cold-blooded animals and the environment
S. enterica subsp. *diarizonae* (IIIb)	324	Cold-blooded animals and the environment
S. enterica subsp. *houtenae* (IV)	70	Cold-blooded animals and the environment
S. enterica subsp. indica (VI)	12	Cold-blooded animals and the environment
S. bongori (V)	20	Cold-blooded animals and the environment
Total	**2,463**	

2.1.2 *Salmonella* serovars

In humans *Salmonella enterica* causes both typhoidal (typhoid fever) and non-typhoidal (gastroenteritis) infections (Raffatellu *et al.,* 2008). *Salmonella* Typhi causes typhoid fever and is exclusively a human pathogen, where as other *Salmonella enterica* serovars including *Salmonella* Typhimurium and *Salmonella* Enteritidis cause non-typhoidal salmonellosis (NTS).

Different types of *Salmonella* serovars have been reported in blood samples of patients worldwide. For example it is reported that the most common serovars isolated from blood in the USA population, are *S.* Typhimurirm (24%), *S.* Enteritidis (19%) and *S.* Enteritidis (15%) (Jones *et al.,* 2008). *S.* Typhimurium and *S.* Enteritidis are also the most common Non-Typhoidal *Salmonella* serovars isolated from blood and other normally sterile sites from patients in Europe (Gradel, *et al.,* 2006) and the United Kingdom (Threlfall *et al.,* 1992). *S.* Enteritidis is particularly prominent in sub-Saharan Africa, where it accounts for approximately 80% of all invasive NTS in children, followed by *S.* Typhimurium at a much lower rate (Ikumapayi *et al.,* 2007). *Salmonella enterica* ser. Typhi and *Salmonella enterica* ser. Paratyphi A and B, are the typhoidal serovars which cause, typhoid and paratyphoid fevers (enteric fevers), respectively. The two serovars have also been associated with febrile illnesses (Somily *et al.,* 2011) which are characterized by infection of the gut-associated lymphoid tissue, liver, spleen, bone marrow and gall bladder and usually accompanied by a low level bacteremia (Levine *et al.,* 2004).

Non-typhoidal *Salmonella* strains generally produce a self-limiting gastroenteritis (vomiting, fever and diarrhea) in healthy humans (Voetsh *et al.,* 2004) but severe infections, including bacteremia and meningitis have also been reported (Sirinavin *et al.,* 1999). By contrast, NTS can cause severe, fatal disease in young infants, the elderly and immunocompromised hosts, in both

industrialized (Vugia *et al.,* 2004) and developing countries (Ikumapayi *et al.,* 2007). In Sub-Saharan Africa surveillance and epidemiological programs for invasive bacterial infections indicate that *Haemophilus influenzae* type b (Hib) and *Streptococcus pneumoniae* infections are widely distributed and cause severe disease in children (Berkley *et al.,* 2005). In Kenya, non-typhoidal *Salmonella* species are common causes of bacteraemia among the immunocompromised individuals, infants and the newborns (Kariuki *et al.,* 2002). However strains of the multidrug resistant (MDR) *S.* Typhimurium phage type (DT) 104, represent reservoirs in cattle and are transmitted mainly through consumption of contaminated meat, milk and milk products (Threlfall, 2000). Multidrug resistant *Salmonella* strains have steadily increased, probably due to continuous antibiotic pressure in human and veterinary medicine (Orman *et al.,* 2002).

2.1.3 Characterization of *S.* Typhi

Salmonella Typhi is a gram negative bacterium. It expresses a capsular polysaccharide (CPS) known as Vi antigen which is responsible for the mechanisms in *S.* Typhi that avoid host defenses and enhance virulence (Arya, 2002). Vi is a linear acidic homopolymer of α-1, 4 linked N-acetylgalactosaminuronate (D-GalNAcA) and variably O-acetylated at C-3. There is a link between the concentration of the O-acetyl groups on the surface and the immunogenicity of the Vi antigen (Szu and Bystricky, 2003). Vi antigen production is mediated by two chromosomal loci, Via A and Via B (Hashimoto *et al.,* 1993). The Vi polysaccharide is also a protective antigen, a vaccine based on purified Vi polysaccharide has been developed and licensed for use as a potential vaccine against typhoid (Engels *et al.,* 1998).

S. Typhi has a membranous structure covering the cytoplasmic membrane and Lipopolysaccharide referred to as the outer membrane (Kumar *et al.,* 2009). The outer membrane proteins of *S.* Typhi are immunologically important and have been shown to have potential use in diagnostics and vaccines to typhoid fever (Nandakumar *et al.,* 1993).

2.2 Typhoid fever

Typhoidal *Salmonella* serovars include *Salmonella enterica* subsp. *enterica* ser. Typhi and *Salmonella eneterica* subsp. *enterica* ser. Paratyphi A, B and C. Both serovars cause typhoid and paratyphoid fevers (enteric fevers) respectively with paratyphoid fever being a less severe (Crump and Mintz, 2010). Typhoid fever, caused by *Salmonella enterica* serovar Typhi (*S.* Typhi) is an important disease in many developing countries; however, the true global distribution of typhoid fever is not well documented. In Africa, for example the overall burden of typhoid fever remains largely unknown, mainly as a result of poor diagnosis and methodological differences (Crump *et al*., 2004) and poor infrastructure in many endemic regions. Typhoid fever is rare in industrialized countries due to the improved environmental sanitation but continues to be of significant public-health concern in developing countries.

2.2.1 Prevalence of Typhoid fever

Worldwide, Typhoid fever caused by *Salmonella* Typhi is endemic in most parts of central America (Fica *et al.,* 1996), South East Asia (Ling *et al.,* 2000) and the Indian subcontinent (Rahman *et al.,* 2002) and recently increasing numbers of cases have been reported in Africa (Kariuki *et al.,* 2000). The burden of infection by typhoid fever has been categorized by regions based on number of yearly infected individuals. Regions with high Typhoid fever (>100/100,000 cases per year) incidence include South Central Asia and South East Asia. While the rest of Asia,

Africa, Latin America and the Caribbean and Oceania except for Australia and New Zealand are regarded as regions of medium incidence (10-100/100,000 cases per year). However Europe and the rest of the developed countries are reported to have low typhoid fever incidence (<10/100,000cases per year) (Crump *et al.,* 2004).

Typhoid fever incidence in the eastern countries of the world such as China, India, Indonesia, Pakistan and Vietnam indicate high incidences particularly among children and adolescents. Moreover it is reported that there is a substantial variation in typhoid surveillance data among countries in this region (Ochiai *et al.,* 2008). In Latin America there is evidence that Typhoid fever incidence has decreased drastically in parallel with both economic transition and with water and sanitation measures earlier introduced to control cholera (Whitaker *et al.,* 2008).

2.2.2 Prevalence of Typhoid fever in Kenya

Prevalence of typhoid fever in Kenya/Eastern Africa is estimated to be 39/100,000, although this prevalence is not well documented due to limitations in facilities to carry out proper studies, especially in remote areas (Crump *et al.,* 2004). Recently, Multi-Drug Resistant (MDR) *Salmonella* Typhi showing resistance to nearly all of the commonly available “first line” antimicrobial agents used for the treatment of typhoid and other infections have been isolated in Kenya (Brown et al, 1996). Multidrug resistant *Salmonella* Typhi outbreaks in Kenya were first reported between 1997-1999 when the prevalence of the Multidrug resistant phenotype was estimated to vary from 50-65% (Kariuki *et al.,* 2000). Continuous surveillance indicate that the prevalence of MDR serovar Typhi has been rising steadily and at present between 70-78% of all serovar Typhi isolates from blood cultures from the main referral hospital in Nairobi are Multi-Drug Resistant (Kariuki *et al.,* 2004). Drug resistance by *Salmonella* Typhi should be

continuously monitored in Kenya to determine the extent of resistance to currently available drugs. Hence there is a need to improve laboratory capacity to detect multi drug resistant strains of *Salmonella* Typhi coupled with policies on antimicrobial drug administration.

2.2.3 Transmission of *Salmonella* Typhi

Humans are the primary reservoir of *Salmonella* Typhi which is largely transmitted through ingestion of food or water contaminated with the feces of an infected person (Maskalyk, 2003). Other means of *S*. Typhi transmission include ingestion of fish from sewage contaminated water beds, unwashed raw fruits or vegetables from contaminated soil or milk contaminated by carriers. The period of communicability depends on the presence of the organism in the contaminated matrix. Transmission of the pathogen is also often from asymptomatic individuals, (2-5%) of previously infected individuals who often become chronic carriers without clinical manifestation of the disease but actively shed viable organisms capable of infecting others (Heyman, 2004).

2.2.4 Symptoms of Typhoid Fever

The incubation period of typhoid fever ranges from three days to one month (Bhunia *et al.,* 2009). Individuals infected with *S*. Typhi have a wide spectrum of clinical manifestation ranging from a mild illness with low grade fever, malaise and slight dry cough to a severe infection with serious complications which can include toxic encephalopathy and intestinal perforation that leads to peritonitis (Parry *et al.,* 2002). Apathy, blanching (rose spots) on the trunk, enlargement of the liver and spleen and diarrhea may also occur after *S*. Typhi infection (Parry *et al.,* 2011).

Presentation of typhoid may be more severe in children younger than five years, with higher rates of complications and hospitalization than in adults (Siddiqui *et al.,* 2006). The clinical

presentations of the affected infants include diarrhea, toxicity, and other complications that may increase the mortality rate (Bhutta, 2006). Besides it has been reported that presentation of typhoid fever may be altered by co-existing morbidities and early administration of antibiotics. Typhoid fever is atypical infection in areas commonly infested by shistosoma and where malaria is endemic (Nsutebu *et al.,* 2003)

2.2.5 Pathogenesis of Typhoid fever

Salmonella Typhi are invasive bacteria that cause enteric fever and are transmitted via the fecal-oral route, usually through the consumption of contaminated food or water (Baker *et al.,* 2011). The disease progress is initiated following ingestion of the organisms through contaminated foods. This is followed by the colonization of the small intestine, invasion of the mucosal surface and dissemination of the pathogen throughout the body into the reticulo-endothelial system, including the liver, spleen and bone marrow (Raffatellu *et al.,* 2008). The most common sites of secondary infection are the liver, spleen, bone marrow, gall bladder and peyer's patches of the terminal ileum. The disease may be complicated by relapse and carriage in the gall bladder leading to long term chronic fecal shedding (Khatri *et al.,* 2009). These intracellular pathogens use different ways to avoid detection by the host immune system and this affects the ability to detect the organisms or signs of contact with the organism (Bäumler *et al.,* 2010).

2.3 Diagnosis of *Salmonella* Typhi infection

Various techniques have been applied for the clinical diagnosis of typhoid patients. These include the detection of *Salmonella* Typhi in culture, molecular methods such as polymerase chain reaction (PCR) and serological tests including the widal test being the most common.

Salmonella can be isolated from a number of biological materials such as cultures of bone marrow, blood, stool and intestinal secretion.

2.3.1 Culture methods

The isolation of *S.* Typhi from blood, bone marrow, rose spots and other sterile sites provides the most conclusive confirmation of enteric fever. Cultural methods are based on nutrient acquisition, biochemical characteristics and metabolic products unique to *Salmonella* species (Ricke *et al.,* 1998). Isolation of *Salmonella* Typhi from culture takes 2-7 days as it relies on the ability of *Salmonella* to multiply to visible colonies which can then be characterized by performing additional biochemical tests. This delay may prove fatal, especially for children. Blood culture technique is also limited due to the indiscriminate use of antibiotics and prior immunizations; its utility is also restricted to low numbers of bacteria in blood.

Numerous and varied bacteriological media including selective enrichment broths and selective agar plates are used to monitor for the presence of *Salmonella*. In most cases the media contains inhibitors in order to stop the growth of non-target organisms or particular substrates that only target bacteria that can degrade or that confer a particular colour to the growing colonies (Manafi, 2000). Culturing methods typically involve enrichment of the sample(s) to recover cells (Gracias and McKillip, 2004). Preliminary identification of *Salmonella* is done using classical biochemical and serological tests and positive isolates are serotyped to identify the serovar using specific antisera according to the Kauffman-white typing scheme (Shipp and Rowe, 1980).

2.3.2 Biochemical characterization of *Salmonella*

Biochemical tests distinguish different species and strains by specific biochemical profiles based on different enzyme activities and utilization of sugars (Table 2 and 3). Biochemical tests such as the API 20E kit have been used in combination with other methods for the characterization of *Salmonella enterica.*

Table 2: Common biochemical markers for *Salmonella* and some common pathogens showing different reactions and utilization of substrates (WHO, 2003)

Organism	**Slant**	**Butt**	**H_2S**	**Gas**	**Motility**	**Indole**	**Urea**	**Citrate**
***S.* Typhi**	**Alk**	**Acid**	**Wk+**	_	+	_	_	_
S. para*Typhi *A	**Alk**	**Acid**	_	+	+	_	_	_
Other *Salmonella* spp	**Alk**	**Acid**	**V**	**V**	+	_	_	**V**
E.coli	**Acid**	**Acid**	_	+	+	+	_	_
***Klebsiella* spp**	**Acid**	**Acid**	_	++	_	**V**	+	+
***Citrobacter* spp**	**V**	**Acid**	+++	+	+	**V**	_	+
***Proteus* spp**	**Alk**	**Acid**	+	+	+	**V**	++	**V**

Alk = Alkaline, Wk = Weak, V = variable result. Slant: lactose fermentation (determines the ability of the bacteria to ferment lactose), butt: glucose fermentation (determines the ability of bacteria to ferment glucose and fermentation of glucose turns the agar yellow. Motility identifies the ability of the bacteria to move. Urea, shows the ability of bacteria to produce enzyme urease that hydrolyzes urea, the accumulation of ammonia leads to arise in pH. Indole, shows the ability of the bacteria to produce enzyme tryptophanase which converts tryptophan to indole. Citrate tests for the ability of gram negative bacteria to import and utilize citrate as the sole carbon source.

Table 3: Different reactions in enzyme activities and utilization of sugars in gram negative bacteria and other non-fastidious gram negative bacteria using the API 20E kit (Biomerieux SA, 2002)

TEST	REACTION	NEGATIVE	POSITIVE
LDC	Lysine decarboxylase	Yellow	Orange or red
ODC	Ornithine decarboxylase	Yellow	Orange or red
CIT	Citrate utilisation	Light green	Blue-green or blue
H_2S	H_2S production	Colourless	Black
URE	Urea hydrolysis	Yellow	Pink
TDA	Tryptophan deamination	Yellow	Dark brown
IND	Indole production	Colourless reagent	Pink
VP	Acetoin production	Colourless	Pink or red
GEL	Gelatin hydrolysis	Colourless	Black diffuse pigment
GLU	Glucose fermentation	Blue	Yellow
MAN	Mannitol	Blue	Yellow
INO	Inositol	Blue	Yellow
SOR	Sorbitol	Blue	Yellow
RHA	Rhamnose	Blue	Yellow
SAC	Sucrose	Blue	Yellow
MEL	Melibiose	Blue	Yellow
AMY	Amygdalin	Blue	Yellow
ARA	Arabinose	Blue	Yellow
Oxidase	Cytochrome oxidase	Colourless	Purple

2.3.3 Serological identification of *Salmonella*

All serological tests for typhoid fever are based on the detection of antibodies to Lipopolysaccharide (LPS) antigens (O9 and O12 somatic antigens, Hd flagellar antigens and Vi capsular antigens) (Dutta *et al.,* 2006). *Salmonella* that cause typhoid fever and paratyphoid fever have the following antigenic compositions and belong to the indicated serogroups (Table 4)

Table 4: Antigenic compositions of *Salmonella* that cause Typhoid fever and paratyphoid fever and the serogroups they belong to (WHO, 2003).

Serotype	O-antigen	H-antigen	Serogroup Phase 1:2
S. Typhi	9,12 (Vi)	D	Group D1
S. paraTyphi A	1,2,12	9: (1,5)	Group A
S. paratyph B	1,4,(5),12	b: (1,2)	Group B
S. paraTyphi C	6,7,(Vi)	a: (1,5)	Group C1

Antigens in parentheses are either weak or absent in some isolates

The O antigen of *Salmonella* is actually determined by means of slide agglutination test with group-specific antiserum followed by agglutination with factor antiserum (Table 4).

Growth from non-selective agar or kligers iron agar can be used for the determination of O-antigen. Strains of *S.* Typhi and *S.* Paratyphi may possess Vi antigen that render the strains non-agglutinable in O antisera. These cultures agglutinate in Vi antiserum. They will agglutinate in O-antiserum but after destruction of the Vi antigen by boiling the culture for ten minutes. The specific O-antigen is confirmed by slide agglutination with factor antiserum. H antigen *Salmonella* is usually determined by means of the tube agglutination test. Determination of the O antigen and the phase 1H antigen is usually sufficient for the identification of typhoid fever organisms and paratyphoid fever organisms (Table 5)

Table 5: Specific O-antigens and 1H antigens for typhoid fever organisms (WHO, 2003)

Organism	Specific O antigen	Factor phase 1H antigen
S.typh	9	D
S.paratyph A	2	A
S.paratyph B	4	B
S. paratyph C	6/7	C

2.3.3.1 Felix-Widal Test

Widal test is a presumptive serological test for enteric fever. The technique utilizes a suspension of killed *Salmonella* Typhi as an antigen to detect typhoid fever in serum from suspected *Salmonella* Typhi patients who present with febrile illness. Widal test detects agglutinating antibodies to O and H antigens of *Salmonella* Typhi (Ley *et al.,* 2010). However the rise in antibodies to detection levels needed by this technique takes between 7-14 days, limiting its applicability in early diagnosis of Typhid fever. Similarly a single widal test may not be valid due to the number of cross-reacting febrile infections.

Blood infections may be caused by a wide range of other gram negative and positive bacteria, viruses and parasites, including malaria (Zimmerman *et al.,* 2008). Hence the definitive diagnosis of typhoid fever depends on isolation of *Salmonella* Typhi from blood, stool, urine, or other body fluids.

Usually O antibodies appear on days 6-8 and H antibodies on days 10-12 after the onset of the disease. False negative results are reported to occur when the blood is collected too early in the disease indicating that negative results do not rule out S. Typhi infection (House *et al.,* 2005).

However these results are often used as a baseline for subsequent comparative titrations. Similarly false positive results are often associated with past immunizations for typhoid fever, cross reacting antibodies (House *et al.,* 2001) and other possible related infections.

As a confirmatory test, widal test has been greatly hampered by a lack of standardization of reagents and inappropriate result interpretation (Nsutebu *et al.,* 2002). For example a positive result is usually confirmed by a fourfold increase in antibody titers. However the antibody titers in infected patients often rise before the clinical onset, making it difficult to demonstrate the required fourfold rise between initial and subsequent samples for a confirmatory diagnosis (House *et al.,* 2005). With this hurdle in hand, this has called for the need to look for other sensitive and rapid detection techniques such as the molecular based techniques

2.3.4 Molecular based methods for *Salmonella* identification

Molecular techniques have been considered, as an improvement of blood culture in the diagnosis of typhoid fever due to its rapidity and sensitivity. One of the techniques that have been explored and seems quite promising in *Salmonella* identification is the polymerase chain reaction (PCR) and the small number of bacilli present in clinical samples may be amplified. Conventional or real time PCR has been explored, but not exhaustively for the detection of S. Typhi from several samples (Baker *et al.,* 2011).

Targets for *S.* Typhi PCR-based amplification assays have included the Hd flagellar gene, fliC-d (Song *et al.,* 1993) and the Ha flagellar gene, fliC-a, the Vi capsular gene Via B (Bhutta, 2006) and other genes. Further analysis and understanding of the genome of *S.* Typhi and *S.* Paratyphi A may lead to new and better targets for nucleic acid amplification tests (Ou *et al.,* 2007).

2.3.4.1 Polymerase chain reaction

Polymerase chain reaction (PCR) is a technique based on the amplification of a short target DNA sequence. Each new double stranded DNA is then targeted during a new thermal cycle, and the exponential amplification of the specific DNA sequence is achieved. Amplification of specific genes can be utilized to differentiate *Salmonella* species, such as differentiating *Salmonella* Typhi from *Salmonella* Typhimurium (Jordan *et al*., 2009). PCR has been used to show evolutionary relationships of bacteria using nucleotide sequences such as 16S rRNA which has been used to show phylogeny (Chang *et al.,* 1997) as well as identify pathogenic bacteria. Specific PCR detection methods for *Salmonella enterica* based on the modification of 16S rRNA primer pairs has led to greater specificity in identification of the species (Trkov and Avgustine,2003).

2.4 Antibiotic resistance in *Salmonella* Typhi

Antimicrobial therapy is crucial for the treatment and management of typhoid fever, however the reported emergence and spread of antibiotic resistant strains of *Salmonella* Typhi has complicated the treatment of typhoid infections. The occurrence of the resistant *S*. Typhi phenotypes has been largely attributed to the indiscriminate use of antibiotics or to a high acquisition of resistant genes from resistant serotypes. It has been reported that the link between mutations in *S*. Typhi resistant phenotypes and selective pressure is due to antibiotic use in humans (Holt *et al.,* 2008). Use of antibiotics in domestic animals is also a probable contribution to this antibiotic resistance in humans (Omulo *et al.,* 2015).

Antibiotic resistance involves mechanisms whereby the bacteria inactivates the antimicrobial agent, modifies the drug target site or mediates efflux of the antimicrobial drug (Ugboko and De,

2014). Antimicrobials are classified based on their mode of action and potency (Table 6). The development of high resistance to Chloramphenicol, ampicillin and cotrimoxazole (Mirza *et al.,* 2000) as well amoxicillin and Sulfamethoxazole has led to their replacement with the more potent fluroquinolones (WHO, 2003; Bhan *et al.,* 2005). However, there has been occurrence and spread of *Salmonella* Typhi that is resistant to Nalixidic acid (Nal^R) and shows reduced susceptibility to fluoroquinolones (Parry *et al.,* 2011). Rapid detection of Nal^R *Salmonella* Typhi as well as S. Typhi that is resistant to multiple drugs is critical in clinical practice for proper antibiotic therapy as well as controlling the emergence of resistance strains due to indiscriminate use of antimicrobial agents (Song *et al.,* 2010).

Table 6: Critically important antimicrobials for human medicine (WHO, 2011).

Classification	Antimicrobials	Antimicrobial Therapy
Critically important Antimicrobials	**Aminoglycosides** Gentamicin Kanamycin Streptomycin **Cephalosporins (3rd and 4th generation)** Cefotaxime Ceftazidime Ceftriaxone **Quinolones/ Fluoroquinolones** Ciprofloxacin Norfloxacin Nalixidic acid	Sole therapy or one of few alternatives to treat serious human disease. Treatment of diseases caused by organisms that maybe transmitted through non-human sources or by organisms that may acquire resistance genes such as *Salmonella*
Highly important Antimicrobials	**Amphenicol** Chloramphenicol Thiamphenicol **Cepholosporins (1st and 2nd generation** Cefoxitin Cefuroxime **Sulfonamides** Sulfamethoxazole Trimethoprim **Tetracyclines** Tetracycline	Treat diseases caused by organisms that may be transmitted through non-human sources or by organisms that may acquire resistance.

CHAPTER 3

3.0 MATERIALS AND METHODS

3.1 Study design

A cross-sectional study design was used in the. The isolates were obtained from stool samples collected from patients with symptoms of typhoid fever including fever, headache, abdominal pain, muscle aches, weakness and fatigue.

3.2 Collection of samples

A total of 205 stool samples were collected from Kenyatta National Hospital and the Aga Khan hospital during the period of January 2010 - April 2015, from patients suspected to be suffering from typhoid fever. Kenyatta National Hospital is a referral hospital that attends to patients of different socio-economic status from different parts of Nairobi. It is fairly affordable and hence would have a reasonable representation of people from different parts of Nairobi. Agha Khan University Hospital is presumed to attend to middle income to high income patients in Nairobi.

Sample size calculation:

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

n = sample size,

Z = Z statistic for a level of confidence,

P= expected prevalence or proportion (in proportion of one; i.e. if 20%, P= 0.2)

d = precision (in proportion of one; i.e. if 5%, d = 0.05).

For *Salmonella*:

Z = 1.96 (for a level of confidence of 95%) (Lwanga and Lemeshow, 1991)

P = 0.0065 (or 0.65%) (Kariuki, 2008)

d =0.011 (1.1%) (Naing *et al.*2006).

Therefore

$$205 = \frac{1.96^2(0.0065)(1-0.0065)}{0.011^2}$$

=205 samples were collected for this study.

3.3 Culturing and identification of *Salmonella* isolates

The samples were cultured in nutrient broth and incubated for 20 hrs at 37 °C. After 20 hrs of incubation, a loopful of broth from each isolate was streaked on the surface of XLD (Xylose Lysine Deoxycholate) agar plates and then incubated at 37 °C for 20 hrs. Fifty isolates out of the 205 showed *Salmonella* morphology and were characterized in the study. Glycerol stocks of the 50 *Salmonella* Typhi isolates were prepared by mixing 500 µl of the overnight cultures with 500 µl of 80% sterile glycerol in sterile micro centrifuge tubes and the cells were dispersed by inverting the tubes up and down for a few seconds. They were stored at -20 °C for future use.

3.3.1 Morphological characterization

A bacterial smear was prepared for the fifty isolates by picking out a clear distinct colony from the overnight growth of *Salmonella* colonies using a sterile cotton swab. It was then dissolved in 5 ml of 0.85% normal saline and a drop of the mixture was heat fixed on a clean glass slide and placed on a staining mesh. The fixed smear was flooded with crystal violet for 1 minute then rinsed off with tap water. It was then flooded with iodine solution for 1 minute and rinsed off with tap water, flooded with acetone and immediately rinsed off with tap water. It was finally flooded with phenol red solution for 1 minute and rinsed off with distilled water. The slide was allowed to dry and covered with immersion oil then viewed at 100x magnification under Leica ICC 50 microscope (Leica microsystems, Germany).

3.4. Biochemical characterization

Biochemical characterization was done using the API 20E Kit (Biomerieux, Marcy, France). Briefly, after the overnight growth of the *Salmonella* isolates on XLD agar (37 °C), the center of a well isolated colony was gently touched with a sterile cotton swab and inserted into an ampule

of 5 ml of 0.85% saline solution with the tip of the swab at the center of the ampule and was rotated in a vortex like action and the ampule was recapped and labeled for each isolate. Strips were prepared by setting up the incubation trays and lids and recording the culture numbers on each tray. Subsequently 5 ml of distilled water was dispensed into the incubation tray to provide a humid atmosphere during incubation. The API strips, with 20 micro tubes containing different substrates, were unsealed and each placed into the labeled, humid incubation trays. The ampule containing the bacterial suspension was uncapped and using a 5 ml Pasteur pipette the bacterial suspension was dispensed into each micro tube, while ensuring that the API strip was slightly bent. Micro tubes containing the substrates Arginine Dehydrolase (ADH), Lysine Decarboxylase (LDC), Ornithine Decarboxylase (ODC), Hydrogen Sulfide (H_2S) and Urease (UREA) were slightly filled to ensure that reactions were well read. After inoculation the ADH, LDC, ODC, H_2S and UREA cupule sections were completely filled with mineral oil. The plastic lid was placed on the trays and the strips were incubated for 20 hrs. After incubation all reactions not requiring addition of reagents were recorded. The remaining strips were read 5 minutes after the addition of reagents to the rest of the tubes, by referring to the reading table and values were given depending on the reactions for all substrates and these were recorded on the record sheets.

3.5 DNA Extraction

The *Salmonella* spp. colonies that were identified morphologically were subsequently cultured in nutrient broth and incubated for 20 hrs in a thermo shaker (GallenKamp, London) at 37 °C. Total genomic DNA was extracted by the Qiagen DNA extraction kit (QIAamp DNA Mini Kit) following the manufacturer’s instructions. The bacterial cells in the broth were pelleted by centrifugation for 10 minutes at 4402 x *g*. The bacterial pellet was resuspended in 250 µl buffer P1 (Resuspension buffer) (with RNase A) and transferred to a 1.5 ml microcentrifuge tube.

About 250 µl buffer P2 (Lysis buffer) was added and the eppendorf tube was gently inverted 6 times to mix. Then 350 µl of buffer N3 (Neutralization buffer) was added and the eppendorf tube was immediately inverted 6 times. This was then centrifuged for 10 minutes at 13,226 x *g*. The supernatant was applied to the spin column, centrifuged for 2 minutes and the flow through discarded. The spin column was washed by adding 0.5 ml PB buffer to remove trace nuclease activity, and was centrifuged for 2 minutes and the flow through discarded. The spin column was washed again by adding 0.75 ml PE buffer and centrifuged for 2 minutes. The flow through was then discarded and the column centrifuged for an additional 2 minutes to remove residual wash buffer. The spin column was placed in a sterile 1.5 ml micro centrifuge tube and 50 µl elution buffer (EB) was added at the center of the spin column to elute the DNA. It was left to stand for 1 minute then centrifuged for 2 minutes at 13,226 x *g*. The eluted DNA was stored at -20 °C until use.

3.5.1 Analysis of genomic DNA by gel electrophoresis

Presence and quality of extracted DNA was analysed in a 1% (w/v) agarose gel in 1x Tria-acetate-EDTA (TAE) buffer. The 1% agarose gel was prepared by dissolving 1g agarose powder in 100 ml of 1x TAE, boiled and allowed to cool to about 60 °C prior to the addition of 0.05 µg/ml ethidium bromide. A comb was placed in position in a gel casting chamber and the gel solution poured in. After around 30 minutes of polymerization the gel was transferred into an electrophoresis chamber containing 1x TAE buffer brought up to a level that covers the gel. DNA samples, premixed with 6x loading dye in the ratio of 5:1 were loaded onto the wells in the gel. The samples were electrophoresed at 100 V for 45 minutes. The bands were then visualized under a UV Transilluminator (Herolad, Germany).

3.5.2 PCR amplification of 16S rRNA

Polymerase chain reaction was performed in a TProfessional thermocycler (Biometra® Germany) for the amplification of 16S rRNA gene using *Salmonella*-specific primers (Table 7).

Table 7: 16S rRNA PCR primers

Primer		Primer Sequence 5'-3'	Target size (bp)	Reference
Minf	Forward	ACGGTAACAGGAAGCAG	402	(Trkov and Avgustine, 2003)
Minr	Reverse	TATTAACCACAACACCT		

The PCR reaction was a total volume of 50 µl performed in a PCR tube containing 25 µl master mix, 15 µl deionized H_20, 2.5 µl foward primer, 2.5 µl reverse primer, 5 µl template DNA. The thermo cycler conditions were as follows: An initial denaturation step at 95 °C for 2 minutes, followed by 30 cycles of 95 °C for 30seconds, annealing at 42 °C for 30 seconds, extension at 72 °C for 45 sec and a final extension step of 72 °C for 10 minutes. The PCR products were electrophoresed on a 1% agarose gel in 1x TAE buffer with 0.5 µg/ml ethidium bromide and visualized under a UV transilluminator (Herolab, Germany) to determine consensus, sizes and quality. The amplified PCR products were gel extracted and purified using a gel extraction kit (Qiagen) following manufacturer's instructions. The DNA fragment from agarose gel was excised using a clean scalpel and its size was minimized by removing extra agarose, making sure not to cut the DNA fragment. The gel slice was then weighed in a colorless micro centrifuge tube and 3 volumes (300 µl for 100 mg gel slice) of solubilization buffer (QG) was added. It was incubated at 50 °C for 10 minutes while mixing by inverting the tube in an up and down motion for 2 minutes during the incubation to help dissolve the gel. An aliquot of 1 gel volume of

Isopropanol (100 µl for 100 mg gel slice) was added to the dissolved gel mixture. A spin column was placed in a 2 ml collection tube and the sample was applied to this column and centrifuged at 13,000 revolutions per minute (rpm) for 2 minutes. The flow through was discarded and spin column placed back on the same collection tube. 0.5 ml of buffer QG was added to the column and this was centrifuged for 2 minutes (13,000 rpm). An aliquot of 0.75 ml of buffer PE (wash buffer) was added to the column and was left to stand for 4 minutes before centrifuging for 2 minutes (13,000 rpm). The flow through was discarded and the column was centrifuged for an additional 2 minutes at 13,000 rpm. The column was placed in a sterile micro centrifuge tube. An aliquot (30 µl) of buffer EB (Elution buffer) was added to the center of the spin column membrane to elute the DNA. It was left to stand for 1 minute and then centrifuged for 2 minutes (13,000 rpm) and the eluted DNA was stored at -20 $^{\circ}$C. The gel extracted and purified PCR products for each isolate was sent to Macrogen (The Netherlands) for sequencing.

3.5.3 Phylogenetic analysis

A consensus sequence for each of the isolates 16S rRNA was generated using Boiedit software. The 16S rRNA sequences obtained from sequencing results were compared with known 16S rRNA sequences using Basic Local Alignment Search Tool (BLAST) algorithm form the National Centre for Biotechnology Information (NCBI) database. All the sequences were aligned using CLUSTAL W algorithm. Phylogenetic trees were constructed based on these nucleotide sequences with the Bayesian phylogenetic method, using Mr. Bayes program for the Bayesian influence of phylogeny based on Markov Chain Monte Carlo (MCMC). The resulting trees were visualized using Fig tree software.

3.6 Evaluation of antimicrobial resistance

Antimicrobial susceptibility tests were performed according to the Kirby-Bauer Disc Diffusion .Susceptibility test protocol (1966). The *Salmonella* isolates were grown on Trypticase Soy Agar (TSA) as the enrichment agar and incubated at 37 °C for 20 hrs. Four well isolated colonies of each isolate were subsequently picked out, using sterile cotton swabs and suspended in 5 ml of sterile 0.85% normal saline solution and then vortexed to create a smooth suspension. The turbidity of the suspension was adjusted to a 0.5 Mcfarland standard. Sterile swabs were dipped into each inoculum tube containing the bacterial suspensions and these were streaked on the surface of dried Mueller Hinton agar in plates. The streaking was done three times over the entire agar surface while rotating the plate appropriately to ensure even distribution of the inoculum. The plates were left to stand for five minutes at room temperature. The appropriate antimicrobial impregnated discs were carefully placed on the inoculated plates at marked spots based on a template. This was done using sterile forceps, one disc at a time, and was gently pressed with the forceps to ensure complete contact with the agar surface. This was done for all the isolates using a panel of 12 antimicrobial drugs including: Streptomycin (S), Gentamicin (G), Chloramphenicol (C), Nalixidic acid (NA), Tetracycline (TE), Ciprofloxacin (C), Trimethoprim-Sulfamethoxazole (SXT), Amoxicillin/Clavulinic acid (AMC), Ampicillin (AMP), Ceftazidime (CAZ), Ceftriaxone (CRO), Cefuroxime (CXM). The plates were left to stand for 20 minutes at room temperature then incubated for 18 hrs (37 °C). The diameter of the zone of inhibition was measured for each drug from the underside of the plate, in millimeters using a ruler. *Escherichia coli* ATCC 25922 was used as the standard for potency of the antibiotic discs. Antimicrobial activity of each drug for all isolates was interpreted according to the guide lines provided for antimicrobial disc susceptibility tests by Clinical and Laboratory Standards Institute (CLSI, 2013).

Table 8: Zone diameter interpretive standards for Enterobacteriaceae antimicrobial susceptibility tests (CLSI, 2013).

Antimicrobial class	Antimicrobial agent	Disk content	Zone diameter interpretive criteria (mm)		
			Susceptible	Intermediate	Resistant
Aminopenicillin	Ampicillin	10µg	≥17	14-16	≤13
Aminopenicillin plus Betalactamase	Amino-Clavulanate	20µg	≥18	14-17	≤13
3rd generation Cephalosporin	Ceftriaxone	30µg	≥23	20-22	≤19
3rd generation Cephalosporin	Ceftazidime	30µg	≥21	18-20	≤17
2nd generation Cephalosporin	Cefuroxime	30µg	≥18	15-17	≤14
Aminoglycoside	Gentamicin	10µg	≥15	13-14	≤12
Aminoglycoside	Streptomycin	10µg	≥15	12-14	≤11
Tetracycline	Tetracycline	30µg	≥15	12-14	≤11
2nd generation Fluoroquinolone	Ciprofloxacin	5µg	≥31	21-30	≤20
1st generation Fluoroquinolone	Nalixidic acid	30µg	≥19	14-18	≤13
Amphenicol	Chloramphenicol	30µg	≥18	13-17	≤12
Sulphonamide	Trimethoprime-Suphamethoxazole	1.25/23.75µg	≥16	11-15	≤10

3.7 Data analysis

Biochemical identification of the isolates was done using the database (V.40) with the analytical profile index; the recorded numerical profile was matched up with the list of profiles in the software to identify the organism. Data on the different identification methods was analyzed using Chi square (Fishers exact test) using Statistical Package for Social Science (SPSS V.20).

CHAPTER 4

4.0: RESULTS

4.1 Culturing and morphological characterization of *Salmonella* isolates

Forty eight (n = 48) out of fifty (96%) *Salmonella* isolates from Kenyatta National Hospital and the Aga Khan hospital showed colonies characteristic of *Salmonella* on XLD agar. The morphological appearance was pink colonies with blank centers (Figure 1A). All the 48 isolates were subjected to gram staining whereby they absorbed the phenol red stain showing rods with a pinkish appearance, implying that they were all gram negative rods (Figure 1B)

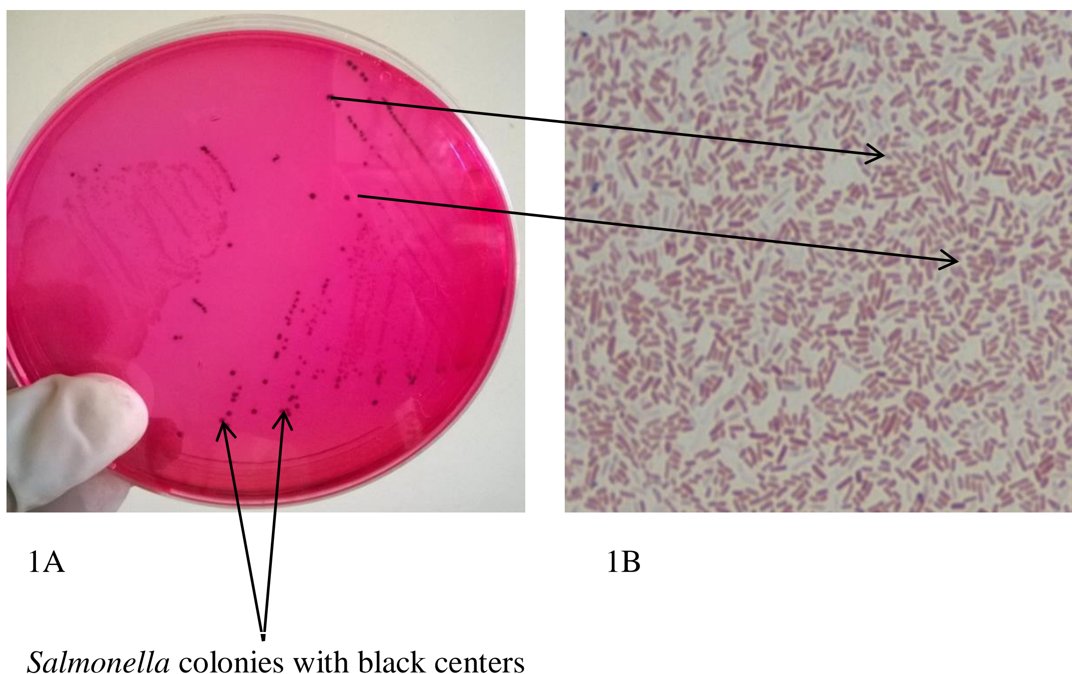

Figure 1: Inoculated XLD agar plate (1A) of isolate 22a from Kenyatta National Hospital showing hydrogen sulfide production of *Salmonella* colonies (pink colonies with black centers). A gram stain of isolate 22a (1B) from Kenyatta National Hospital, showing gram negative rods of *Salmonella*.

4.2 Biochemical characterization

The representative isolates that were positive by culture (21) were subjected to biochemical testing using the API 20E Biochemical test (Biomerieux, Marcy, France) (Figure 2). All the isolates tested positive of which 33% and 67% were identified as *Salmonella* Typhi and other *Salmonella* spp, respectively. Citrobacter braakii was used as a negative control (Table 8)

Figure 2: Biochemical test strips with 20 micro tubes containing different substrates after inoculation with bacteria and incubated overnight at 37 °C.

Table 9: Biochemical tests showing positive and negative reactions to different substrates for isolates 16 (Negative control), 40a and 45a (*S*. Typhi isolates)

Isolate	ONPG	ADH	LDC	ODC	CIT	H_2S	**URE**	TDA	IND	VP	GEL	GLU	MAN	INO	SOR	RHA	SAC	MEL	AMY	ARA	**Identity**
16	+	-	-	+	+	+	**-**	-	-	-	-	+	+	-	+	+	-	-	+	+	Citrobacter braakii
40a	-	-	+	-	-	+	**-**	-	-	-	+	+	+	-	+	-	-	+	-	-	*Salmonella* Typhi
45a	-	+	+	+	+	+	**-**	-	-	+	-	+	+	+	+	+	-	+	-	+	*Salmonella* sp.

+ (Positive reaction), – (Negative reaction

4.3 Molecular characterization

The expected product size of 402 bp was obtained (Figure 3) in 45 (90%) out of the 50 isolates during PCR amplification (Table 10).

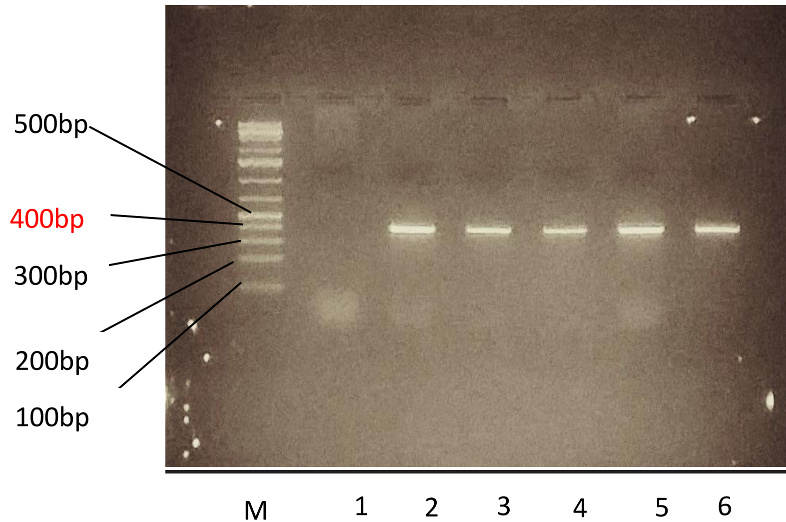

Figure 3: PCR amplification of 16S rRNA. M (1kb DNA ladder), 1 (Negative control-Taq treated distilled water), 2 (Positive control - *S*. Typhi (KEMRI), 3 - 6 (Isolates 7a, 16a, 30a, 40a).

Table 10: Percentage of isolates positive by culture and PCR methods

Method	**Percentage positive**
Culturing	96%
PCR analysis	90%

4.3.1 Multiple sequence alignment of 16S rRNA gene sequences

Alignment of 16S r RNA sequences using CLUSTAL W algorithim showing variation in S. Typhi isolates' sequences and S. Typhi plasmid sequences that confer resistance in Salmonella Typhi.

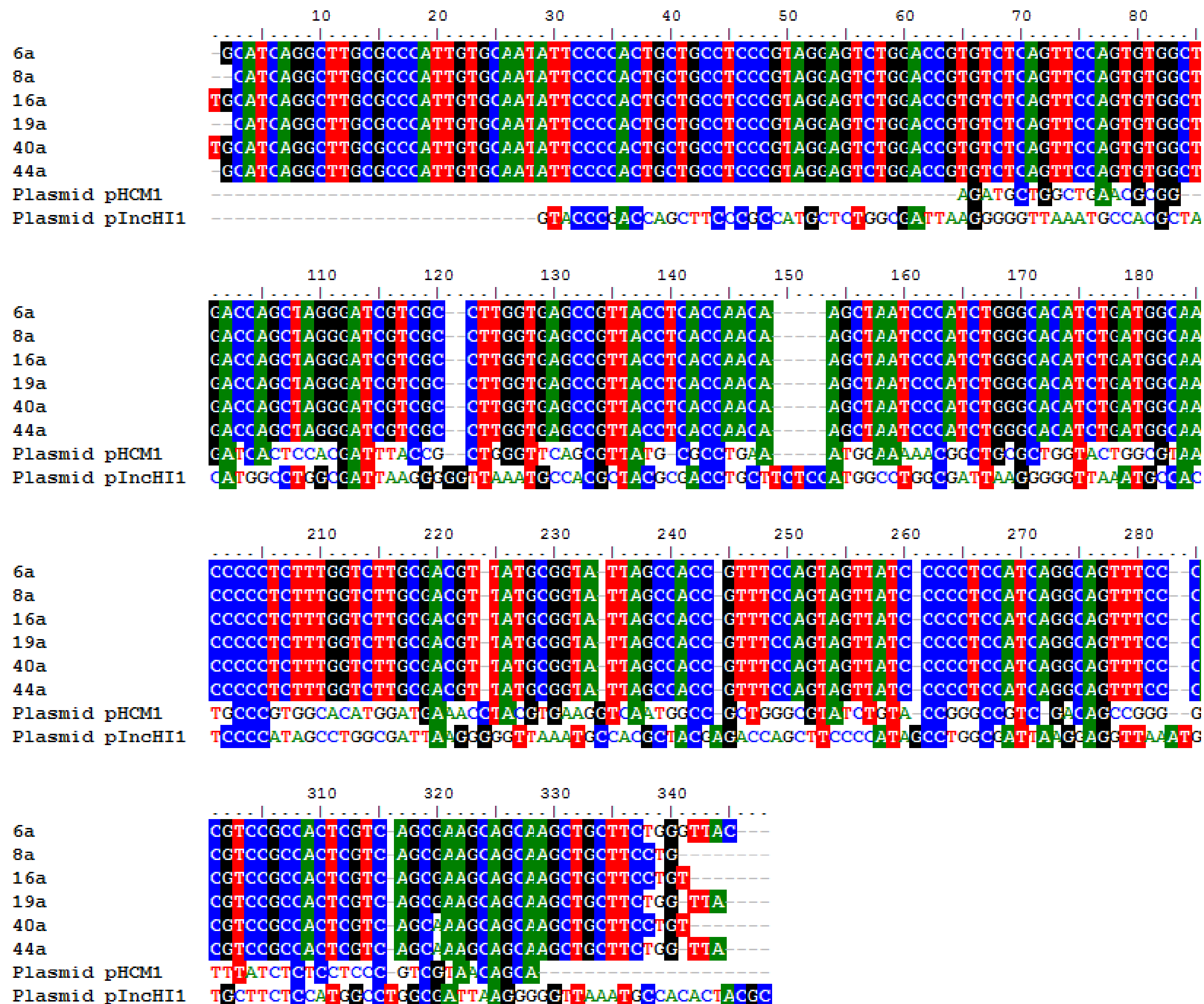

Figure 4: A multiple sequence alignment of 8 *S.* Typhi sequences. Isolates 6a, 8a, 16a, 19a, 40a, 44a are *S.* Typhi isolates; plasmid pHCM1 and plasmid IncHI1 are reference *S.* Typhi plasmid sequences. Shaded regions show similarity, unshaded regions show variation in nucleotidesequences. The differences in bases were seen in base positions 149, 153, 224, 244, 261, 263, 264, 316, 338 and 339.

4.3.2 Phylogenetic analysis based on 16S rRNA gene sequences

All isolates appear to cluster close to plasmid pHCM1 (*Salmonella* Typhi strain CT18) and IncHI1 (*Salmonella* Typhi strain R27).

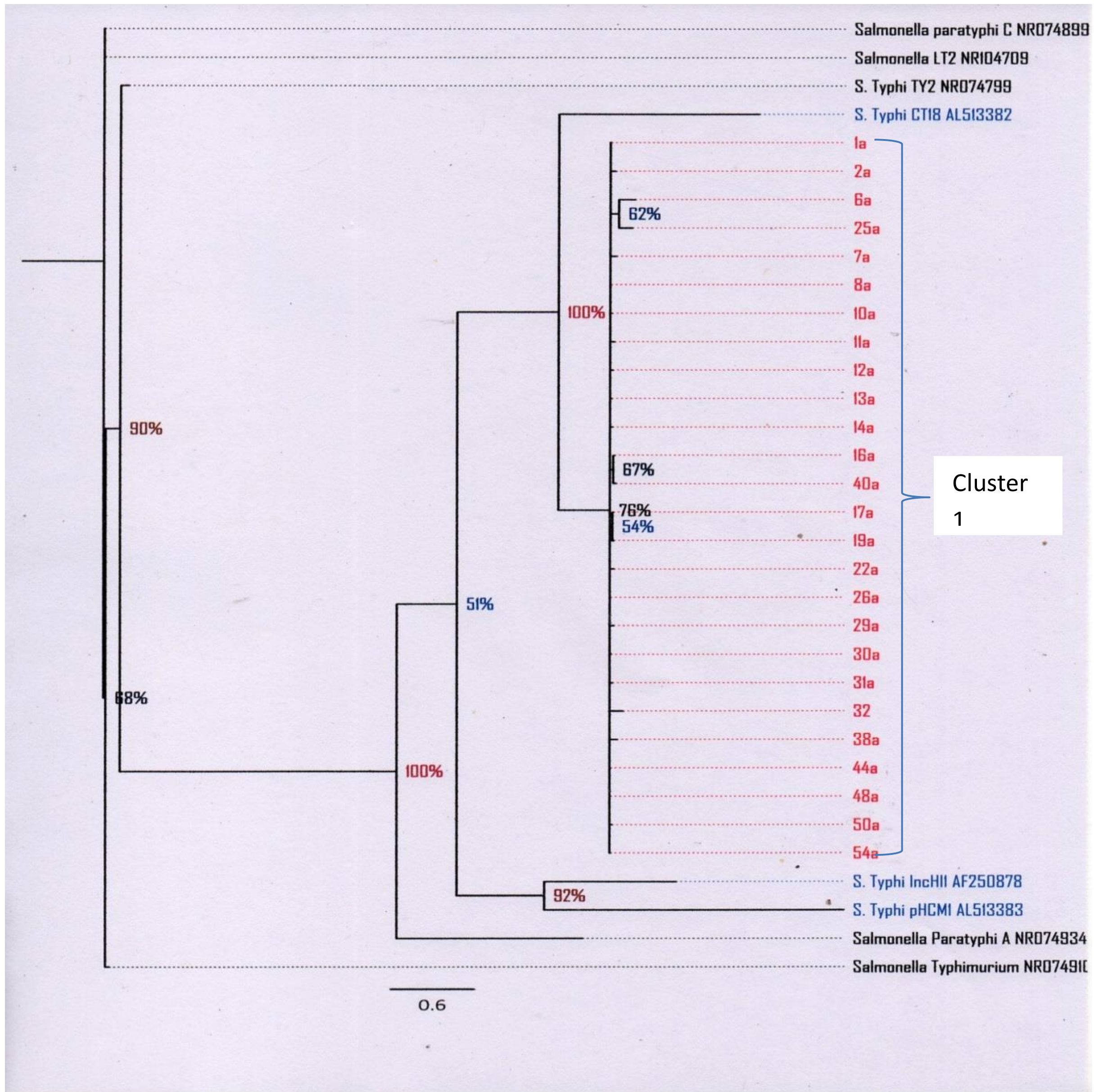

Figure 5: Phylogenetic tree of *Salmonella* Typhi isolates based on 16S rRNA gene sequences**.** Cluster 1 shows *Salmonella* Typhi isolates. Numbers at the nodes show posterior probabilities indicating topological robustness of the tree.

4.4 Evaluation of antimicrobial resistance

Only one of the *Salmonella* Typhi isolates was fully susceptible to all of the antimicrobial drugs used (Figure 7). Twenty three percent (23%) of the isolates were susceptible to most of the drugs used but also showed intermediate resistance to either Nalixidic acid, Amoxicillin-Clavulanate, Ciprofloxacin, Cefuroxime or a combination of two or three of these drugs. Seventy three percent (73%) of the isolates were multidrug resistant, showing resistance to four or more drugs (Figure 6). Nalixidic acid and Amino-Clavulanate exhibited the least resistance, seven percent (7%) of the isolates were resistant to these drugs, followed by ciprofloxacin and ceftazidime which exhibited fifteen percent (15%) resistance. Nineteen percent (19%) of the isolates were resistant to ceftriaxone, cefuroxime and Gentamicin. Tetracycline exhibited Fifty seven percent (57%) resistance in the isolates. The highest resistance (73%) was seen with Ampicillin, Trimethoprime-Sulfamethoxazole, Streptomycin and Chloramphenicol (Figure 8).

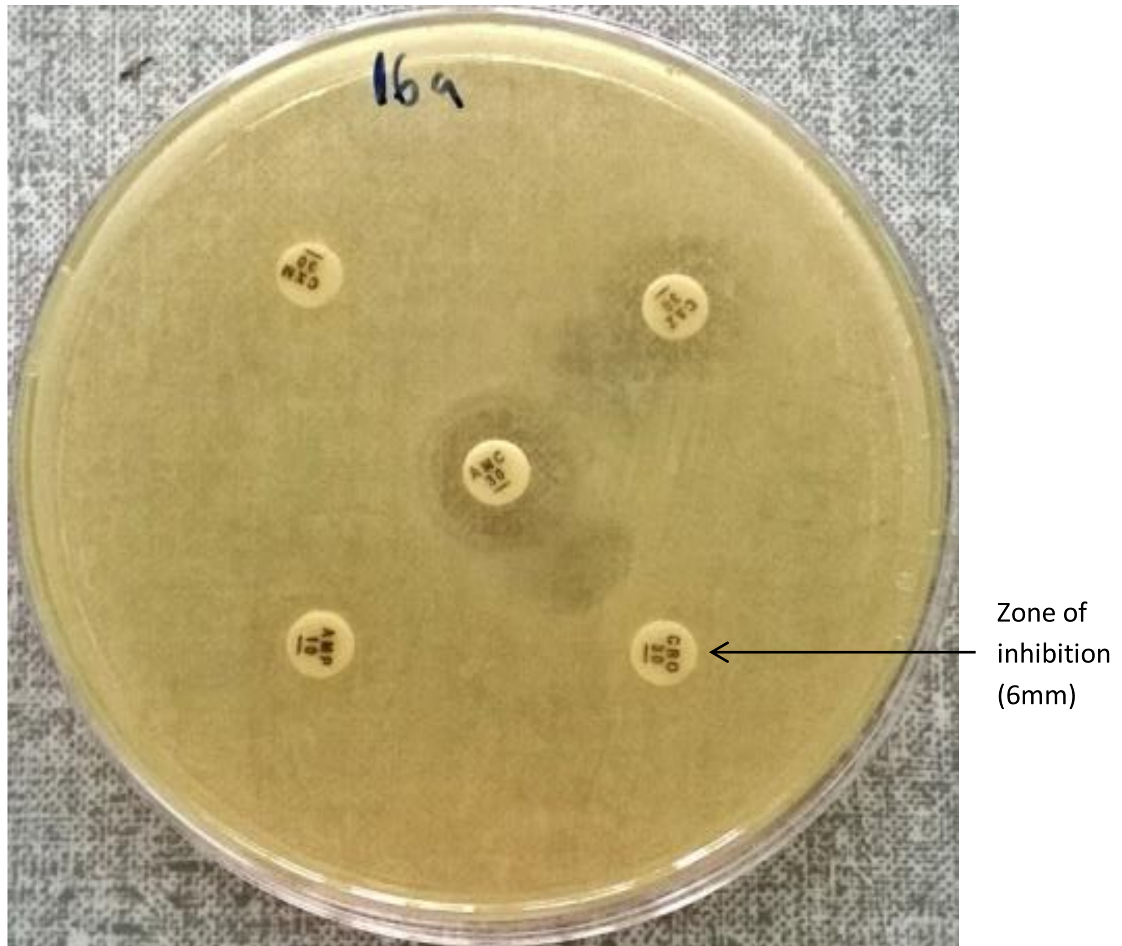

Figure 6: Multi-drug resistant isolate (16a)**,** Amino-Clavulinic acid (AMC), Ampicillin (AMP), Cefuroxime (CXM), Ceftazidime (CAZ), Ceftriaxone (CRO).

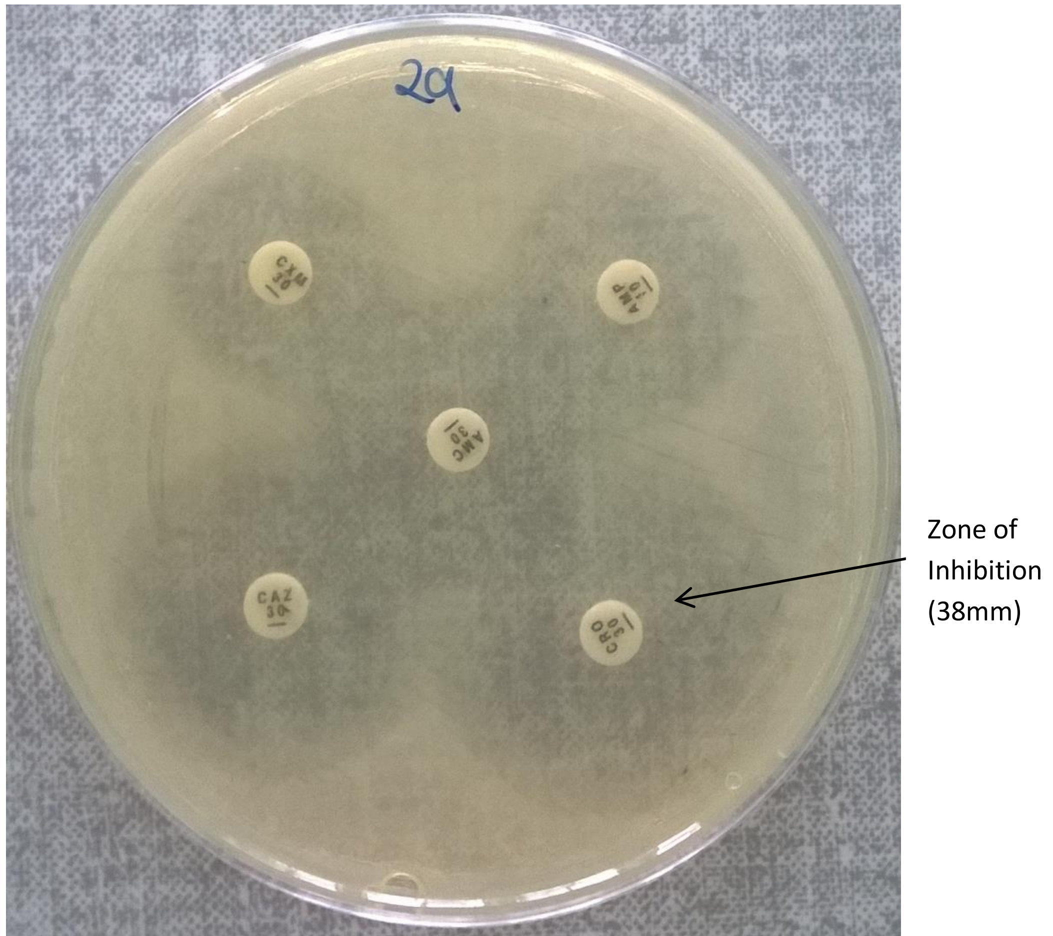

Figure 7: Susceptible isolate (2a), Amino-Clavulinic acid (AMC), Ampicillin (AMP), Cefuroxime (CXM), Ceftazidime (CAZ), Ceftriaxone (CRO).

The *Salmonella* Typhi isolates formed 7 distinct antibiotic resistant patterns (Table 10). Pattern AMP^R, TE^R, SXT^R, STR^R, CHL^R was the most common (34%).

Table 11: Antimicrobial resistant patterns of *Salmonella* Typhi isolates to 12 drugs used in the study. AMP (Ampicillin), SXT (Trimethoprim-Sulfamethoxazole), STR (Streptomycin), CHL (Choramphenicol), TE (Tetracycline), CAZ (Ceftazidime), CRO (Ceftriaxone), CXM (Cefuroxime), GEN (Gentamicin), NA (Nalixidic acid), CIP (Ciprofloxacin), AMC (Amino-Clavulinic acid)

Salmonella Typhi isolates	Pattern of Antimicrobial Resistance	Proportion	Percentante (%)
6a, 11a, 13a, 14a, 38a	AMP^R, SXT^R, STR^R, CHL^R	5/26	19
7a, 12a, 19a, 22a, 26a, 29a, 32a, 40a, 50a	AMP^R, TE^R, SXT^R, STR^R, CHL^R	9/26	34
31a	AMP^R, CAZ^R, CRO^R, CXM^R, TE^R, SXT^R, GEN^R, STR^R, CHL^R	1/26	3
8a	AMP^R, CAZ^R, CRO^R, CXM^R, TE^R, CIP^R, SXT^R, GEN^R, STR^R, CHL^R	1/26	3
10a	AMP^R, CAZ^R, CRO^R, CXM^R, NA^R, TE^R, CIP^R, SXT^R, GEN^R, STR^R, CHL^R	1/26	3
16a	AMC^R, AMP^R, CRO^R, CXM^R, NA^R, TE^R, CIP^R, SXT^R, GEN^R, STR^R, CHL^R	1/26	3
30a	AMC^R, AMP^R, CRO^R, CXM^R, TE^R, CIP^R, SXT^R, CN^R, STR^R, CHL^R	1/26	3

R-Resistant

The response of *Salmonella* Typhi isolates to 12 antimicrobial drugs AMC(Amino-Clavulinic acid), AMP (Ampicillin), CAZ (Ceftazidime), CRO (Ceftriaxone), CXM (Cefuroxime), NA (Nalixidic acid), TE (Tetracycline), CIP (Ciprofloxacin), SXT (Trimethoprim-Sulfamethoxazole), GEN (Gentamicin), STR (Streptomycin), CHL (Chloramphenicol), was classified as Resistant, Intermediate resistant or Susceptible (Table 12) according to the zones of inhibition guidelines by CLSI (Table 8). Nalixidic acid (NA) and Amoxicillin-Clavulinic acid (AMC) were the most effective drugs exhibiting only seven percent (7%) resistance. However these drugs also exhibited an intermediate response between susceptibility and resistance whereby 19% of the isolates showed and intermediate resistance to Nalixidic acid (NA) while 38% of the isolate showed and intermediate resistance to Amoxicillin-Clavulinic acid (AMC). A relatively high intermediate resistance was also seen in Ciprofloxacin (CIP) and Cefuroxime (CXM), where 30% of the isolates were intermediately resistant to Ciprofloxacin (CIP) and 50% of the isolates were intermediately resistant to Cefuroxime (CXM) (Table 12)

Table 12: Response of *Salmonella* Typhi isolates to 12 antimicrobial drugs, shown in zones of inhibition (mm)

Isolate No.	AMC 20µg	AMP 10µg	CAZ 30µg	CRO 30µg	CXM 30µg	NA 30µg	TE 30µg	CIP 5µg	SXT 23.75µg	CN 10µg	S 10µg	C 30µg
1a	>18^{S}	26^{S}	30^{S}	32^{S}	23^{S}	21^{S}	22^{S}	28^{I}	27^{S}	26^{S}	14^{I}	29^{S}
2a	18^{S}	25^{S}	31^{S}	38^{S}	23^{S}	22^{S}	23^{S}	31^{S}	28^{S}	26^{S}	16^{S}	31^{S}
6a	14^{I}	6^{R}	27^{S}	29^{S}	23^{S}	20^{S}	19^{S}	32^{S}	6^{R}	23^{S}	6^{R}	6^{R}
7a	22^{S}	6^{R}	30^{S}	31^{S}	22^{S}	25^{S}	6^{R}	31^{S}	6^{R}	22^{S}	6^{R}	6^{R}
8a	18^{S}	6^{R}	10^{R}	6^{R}	6^{R}	15^{I}	7^{R}	19^{R}	6^{R}	12^{R}	6^{R}	6^{R}
10a	15^{I}	6^{R}	14^{R}	6^{R}	6^{R}	12^{R}	8^{R}	20^{R}	6^{R}	11^{R}	6^{R}	6^{R}
11a	17^{I}	6^{R}	23^{S}	27^{S}	22^{I}	22^{S}	17^{S}	31^{S}	6^{R}	20^{S}	6^{R}	6^{R}
12a	>18^{S}	6^{R}	31^{S}	35^{S}	22^{I}	26^{S}	6^{R}	34^{S}	6^{R}	23^{S}	6^{R}	6^{R}
13a	15^{I}	6^{R}	27^{S}	29^{S}	22^{I}	21^{S}	17^{S}	32^{S}	6^{R}	21^{S}	6^{R}	6^{R}
14a	16^{I}	6^{R}	29^{S}	30^{S}	23^{S}	25^{S}	6^{R}	32^{S}	6^{R}	21^{S}	6^{R}	6^{R}
16a	13^{R}	6^{R}	18^{I}	6^{R}	6^{R}	6^{R}	10^{R}	17^{R}	6^{R}	10^{R}	6^{R}	6^{R}
17a	>18^{S}	24^{S}	24^{S}	28^{S}	22^{I}	20^{S}	16^{S}	32^{S}	22^{S}	23^{S}	14^{I}	24^{S}
19a	14^{I}	6^{R}	21^{I}	27^{S}	22^{I}	21^{I}	6^{R}	37^{S}	6^{R}	22^{I}	6^{R}	6^{R}
22a	>18^{S}	6^{R}	39^{S}	40^{S}	31^{S}	36^{S}	7^{R}	41^{S}	6^{R}	31^{S}	6^{R}	6^{R}
25a	>18^{S}	28^{S}	29^{S}	29^{S}	20^{I}	24^{S}	20^{S}	30^{I}	25^{S}	20^{S}	15^{S}	30^{S}
26a	20^{S}	6^{R}	30^{S}	31^{S}	22^{I}	24^{S}	6^{R}	31^{S}	6^{R}	26^{S}	6^{R}	6^{R}
29a	22^{S}	6^{R}	29^{S}	28^{S}	20^{I}	21^{S}	6^{R}	33^{S}	6^{R}	22^{S}	6^{R}	6^{R}
30a	10^{R}	6^{R}	10^{R}	6^{R}	6^{R}	15^{I}	9^{R}	19^{R}	6^{R}	9^{R}	6^{R}	6^{R}
31a	17^{I}	6^{R}	13^{R}	6^{R}	6^{R}	15^{I}	10^{R}	22^{I}	6^{R}	10^{R}	6^{R}	6^{R}
32a	>18^{S}	6^{R}	27^{S}	29^{S}	26^{S}	24^{S}	6^{R}	27^{I}	6^{R}	24^{S}	6^{R}	6^{R}
38a	17^{I}	6^{R}	28^{S}	30^{S}	22^{I}	20^{S}	16^{S}	33^{S}	6^{R}	22^{S}	6^{R}	6^{R}
40a	22^{S}	6^{R}	30^{S}	30^{S}	23^{S}	22^{S}	6^{R}	30^{I}	6^{R}	25^{S}	6^{R}	6^{R}
44a	>18^{S}	22^{S}	27^{S}	30^{S}	21^{I}	24^{S}	24^{S}	35^{S}	23^{S}	24^{S}	17^{S}	24^{S}
48a	>18^{S}	20S	26^{S}	29^{S}	20^{I}	20^{S}	19^{S}	30^{I}	24^{S}	22^{S}	14^{I}	25^{S}
50	15^{I}	6^{R}	26^{S}	26^{S}	21^{I}	16^{I}	6^{R}	29^{I}	6^{R}	18^{S}	6^{R}	6^{R}
54a	>18^{S}	22^{S}	25^{S}	29^{S}	21^{I}	19^{S}	18^{S}	30^{I}	22^{S}	22^{S}	19^{S}	25^{S}
E. coli ATCC 25922	22^{S}	19^{S}	28^{S}	28^{S}	22^{S}	23^{S}	22^{S}	31S	28^{S}	22^{S}	15^{S}	28^{S}

S=Susceptible, I=Intermediate and R=resistant

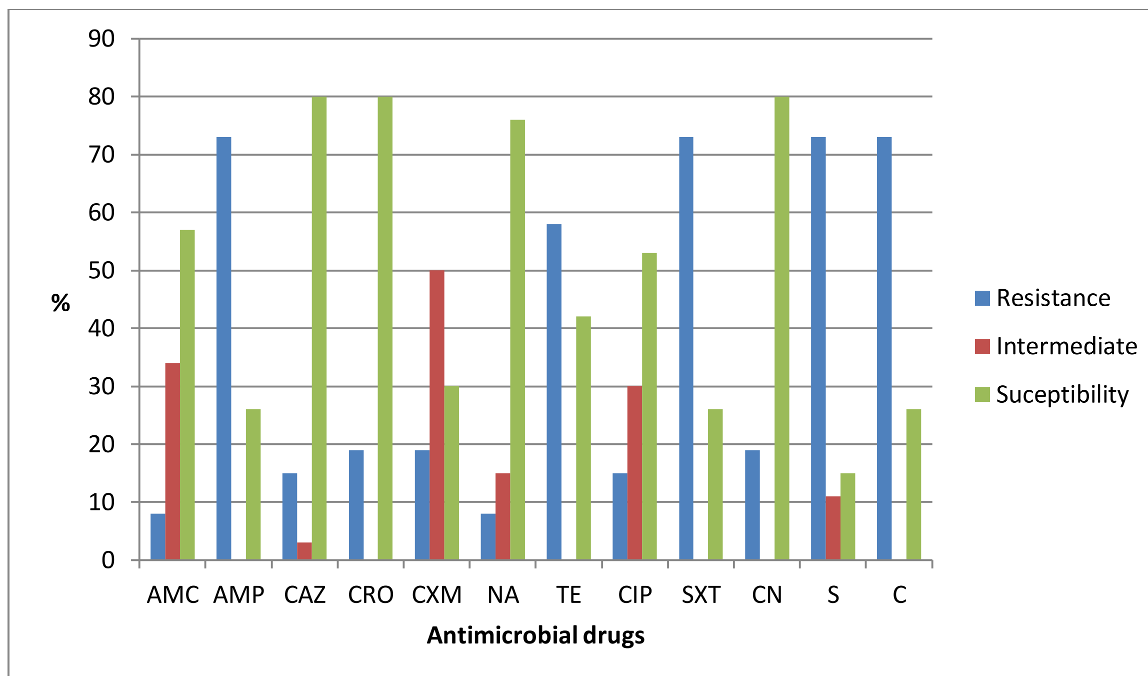

Figure 8: Percentage resistance, intermediate response and susceptibility of *Salmonella* isolates against 12 antimicrobial drugs. AMC (Amino-Clavulinic acid), AMP (Ampicillin), CAZ (Ceftazidime), CRO (Ceftriaxone), CXM (Cefuroxime), NA (Nalixidic acid), TE (Tetracycline), CIP (Ciprofloxacin), SXT (Trimethoprim-Sulfamethoxazole), CN (Gentamicin), S (Streptomycin), C (Chloramphenicol). High susceptibility (>50%) was observed with AMC (Amino-Clavulinic acid), CAZ (Ceftazidime), CRO (Ceftriaxone), NA (Nalixidic acid), CIP (Ciprofloxacin) and GEN (Gentamicin) groups. High resistance (>50%) was observed with TE (Tetracycline), AMP (Ampicillin), SXT (Trimethoprime-Sulfamethoxazole), STR (Streptomycin) and CHL (Chloramphenicol)

CHAPTER 5

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1 DISCUSSION

Antimicrobials have been used for more than 50 years for the treatment of infections, including typhoid fever leading to recovery (Aminov, 2010). However, the past few decades have seen the emergence and rise of antimicrobial resistance to the most commonly used drugs (Omulo *et al.,* 2015) leading to the use of more potent second and third generation drugs. There is a major public health concern that drugs of greater potency such as ciprofloxacin have begun to exhibit patterns of resistance in *Salmonella* Typhi (Gaind *et al.,* 2006).

In the current study, molecular techniques combined with phylogenetic analysis confirmed that the isolates from Kenyatta National Hospital and Agha Khan University Hospital were *Salmonella* Typhi strains. The isolates were 96% and 90% positive for *Salmonella* Typhi by culture and PCR respectively. There was no significant difference between these two identification methods used in this study since the samples used were *Salmonella* Typhi isolates. However, the study showed that culture method alone may not be enough to distinctly confirm the presence of *Salmonella* Typhi isolates rather a molecular technique especially the use PCR technique may be necessary as a confirmatory test. Previous studies have indicated that culture methods alone may show the presence of *Salmonella* sp but may not be able to distinguish *Salmonella* Typhi from the rest of the species since they show similar morphological and gram staining characteristics (Woo *et al.,* 2000).

Phylogenetic analysis showed that the isolates from infected patients from Kenyatta National Hospital and Agha Khan University Hospital were related to *Salmonella* Typhi strain CT18 and

Salmonella Typhi strain R27 because of their relativity with these reference strains which have been associated with multi-drug resistance (Kariuki *et al.,* 2010; Sherburne *et al.,* 2000).

Antimicrobial susceptibility tests showed that there was an overall high resistance (73%) to penicillins, sulfonamides, aminoglycosides and phenicols that are among the most commonly used drugs. After the development and rapid rise of resistance to Chloramphenicol, its use was withdrawn and replaced with second and third generation cephalosporins (Bhan *et al.,* 2005). The 2nd and 3rd generation cephalosporins used in this study (Cefuroxime, Ceftriaxone and Ceftazidime) exhibited a resistance of 15-19%. This indicates a development and rise in resistance to even the most potent antimicrobial drugs. The overall Multidrug resistance was 73% an increase from 60.4% in 2010 (Kariuki *et al.,* 2010) an indication of misuse of drugs while targeting multidrug resistant phenotypes with ineffective combinations of antibiotics. Some of the *Salmonella* Typhi isolates showed an intermediate resistance to Amino-Clavulinic acid, Cefuroxime, Nalixidic acid and Ciprofloxacin, an indication that these phenotypes may gain full resistance in the near future.

In a previous study done by Mengo *et al.* (2010) using isolates from the same Hospitals (Kenyatta National Hospital and Agha khan), resistance to Ceftriaxone was 6% compared to a 19% resistance in this study. In the same study, only 1% of the isolates were resistant to Gentamicin, compared to a 19% resistance to Gentamicin in this study. The rise in resistance to these drugs may indicate a misuse of the drugs in the time period between the two studies and that their use needs to be monitored and regulated to curb the rise in resistance.

In this study the 2 isolates (7%) that were resistant to Nalixidic acid showed resistance to 11 out of the 12 drugs including Ciprofloxacin. The study done by Mengo *et al.,* (2010) showed the

reistance to Nalixidic acid at 35%, an indication that of the use of fluoroquinolones may have reduced in the past few years and that of 2nd and 3rd generation cephalosporins may have increased owing to their higher resistance rate in this study. In a study done in two hospitals in Western Kenya by Onyango *et al.,* (2008) there was 90% resistance to Ampicillin and Streptomycin. There was no resistance to Tetracycline, Trimethoprim-Sulfamethoxazole and Ciprofloxacin. In another study in Nandi Central, Rift Valley by Wandili *et al.,* (2013) all *S.* Typhi isolates were resistant to Ampicillin and 19% were resistant to Trimethoprim-Sulfamethoxazole. Tetracycline, Streptomycin and Chloramphenicol exhibited 6% resistance. There was no resistance to Gentamicin, while the overall multidrug resistance was 25%. Resistance patterns in these studies show a much lower resistance compared that in the current study. This may be attributed to restricted antimicrobial use or under exposure to antimicrobials in these rural areas compared to the urban areas such as Nairobi where antimicrobials are readily available over the counter with little or no restriction.

This study has therefore provided insight to antimicrobial resistant patterns of *Salmonella* Typhi isolates which phylogenetically associate with *Salmonella* Typhi strain CT18 (pHCM1) and *Salmonella* Typhi strain R27 (IncHI1) that confer multi-drug resistance in *Salmonella* Typhi. The antimicrobial resistance profiles in this study point to a rise in antibiotic resistance to even the most potent antimicrobials if policies on drug use and administration are not put in place and implemented.

5.2 CONCLUSION

In this study, the *Salmonella* Typhi isolates from two hospitals in Nairobi, Kenyatta National Hospital and Agha khan Hospital were genetically characterized targeting 16S rRNA gene Circulating *Salmonella* Typhi isolates were clustered close to *Salmonella* Typhi plasmids (pHCM1 and IncHI1) that are linked to antibiotic resistance in *Salmonella* Typhi. This study showed the presence of antibiotic resistant *Salmonella* Typhi strains as well as a possibility for the development of new resistance.

5.3 RECOMMENDATIONS

There is need for continual monitoring of the circulating *S*. Typhi strains and their drug resistance patterns to enable appropriate anti-microbial therapy against typhoid fever. Proper policies should also be put in place to regulate the use of antimicrobials in order to control resistance that develops due to indiscriminate antibiotic use. Since this study was done only in Nairobi, it is also recommended that similar studies be carried out in other regions of Kenya to determine the circulating *Salmonella* spp. and evaluate their antimicrobial resistance patterns. It is important for future studies to evaluate resistant gene structures to establish whether the same resistant genes are being transferred between animals and humans, contaminated food and humans and between *Salmonella* serotypes.

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APPENDIX

APPENDIX I: Media/buffers and their composition

Media/Buffers	**Composition**
Xylose Lysine Deoxycholate Agar (XLD)	Yeast extract 3.0g/l; L-lysine Hcl 5.0g/l; Xylose 3.75g/l; Lactose 7.5g/l; Sucrose 7.5g/l; Sodium deoxycholate 1.0g/l; Sodium chloride 5.0g/l; Sodium thiosulphate 6.8g/l
Nutrient broth	Lab-Lemco' powder 1.0g/l; Yeast extract 2.0g/l; Peptone 5.0g/l; Sodium chloride 5.0g/l
Mueller Hinton Agar	Beef extract 2.0g/l; Acid hydrolyasate of casein 17.50g/l; Starch 1.50g/l ; Agar 17g/l
Resuspension Buffer (P1)	50 mM Tris-HCl pH 8.0; 10 mM EDTA; 100 µg/ml RNase A
Lysis Buffer (P2)	200 mM NaOH; 1% SDS
Neutralization Buffer (N3)	4.2 M Gu-HCl; 0.9 M potassium acetate, pH 4.8
Binding Buffer (PB)	5M Gu-HCl; 30% isopropanol
Wash Buffer (PE)	10mM Tris-HCl, pH 7.5; 80% ethanol
Elution Buffer (EB)	10 mM Tris-Cl, pH 8.5
Solubilization Buffer (QG)	5.5 M guanidine thiocyanate (GuSCN); 20mM Tris-HCl, pH 6.6