PREVALENCE, GENETIC DIVERSITY AND ANTIMICROBIAL SUSCEPTIBILITY PATTERN OF CLINICAL *SALMONELLA* ISOLATES FROM KAPSABET AND KISUMU DISTRICT HOSPITALS IN KENYA

BY

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ABSTRACT

The re-emergence of invasive non typhoidal Salmonella (NTS) is exacerbated by antimicrobial resistance (AMR) to most commonly available antimicrobials. Extreme climatic changes, in addition to increase in susceptible host population risk factors has resulted in the emergence of successful clones of *Salmonella*. Prevalence of *S.Typhimurium* is documented at 4.2% while bacteria caused diarrhea at 8.5% in Kapsabet region. Prevalence of NTS in Kisumu is documented at 39% and MDR at 76%. This warranted the appraisal of antibiotic susceptibility patterns of clinical *Salmonella* isolates and determining correlation between antimicrobial susceptibility and genetic diversity. This study aimed to determine the prevalence, genetic diversity and antimicrobial susceptibility pattern of clinical *Salmonella* isolates from Kapsabet and Kisumu District hospitals. A cross sectional study design was adopted. Systematic random sampling technique was used and a total of 400 stool specimens were collected from each study site. *Salmonella* isolates were identified based on their morphological appearance, biochemical reaction on various bacteriological culture media in addition to indole, methyl red, voges proskauers, citrate (IMVIC) and *Salmonella* a nalytical profile index (API) 20E system and confirmed by *invA* gene sequencing. Antimicrobial susceptibility was done using disk diffusion method. Polymerase chain reaction was used to detect the presence of bla_{TEM} and *sul2* genes in addition to *spv* virulence genes. Single locus sequence typing was used to determine genetic diversity among *S. Typhimurium* isolates based on *fliC* gene. A total of 174 *Salmonella* species were isolated, 55.75% (97/174) and 44.25% (77/174) from patients treated at Kapsabet and Kisumu District hospitals respectively. The predominant *Salmonella* from Kapsabet was serovar *S. Typhimurium* 52.6% (51/97) followed by *S. Enteritidis* 26.8% (26/97). The highest *Salmonella* isolate from Kisumu was *S. Typhimurium* 57.1% (44/77), followed by *S. Enteritidis* 24.6% (19/77). The other serovars constituted a small proportion in both sites. Kapsabet had high distribution of serovars, $p=0.0288$. The difference in prevalence of NTS among patients was not significant, $p=0.0793$. Approximately 70% of *Salmonella* isolates were MDR, the resistant phenotype was; ampicillin, chloramphenicol, streptomycin, sulfamethoxazole, tetracycline (ACSSuT). Kisumu had high resistance to antimicrobials tested $p<0.0001$. bla_{TEM} and *sul2* genes were associated with antimicrobial susceptibility $p< 0.001$. A total of 4.54% (2/44) of *S. Typhimurium* from Kisumu lacked *spv* genes while 4.54% (2/45) of all *S. Enteritidis* lacked *spv* genes. Genetic diversity in *S. Typhimurium* strains correlated with AMR, $p= 0.0258$. Isolates exhibited geographic restriction. The overall interclade diversity was $p< 0.001$. Use of Multi locus sequence typing is recommended to mitigate phylogenetic discordance. Development of vaccines for human and vetenary use may be best alternative to antibiotics.

CHAPTER ONE

INTRODUCTION

1.1 Background

Salmonellosis, a water and foodborne infection is a major cause of high morbidity and mortality in sub Saharan Africa where it is ranked second to pneumococcal disease (Gordon, 2011). *Salmonella* infection can lead to severe invasive and focal infections (Feasey *et al.,* 2012). The invasiveness of *Salmonella* varies with respect to specific serovar that seems to display different invasive mechanisms (Gordon, 2011). Initial stage of invasion involves binding to specific receptors on epithelial cell surface of intestinal mucosa. This is followed by induction of enterocyte membrane to undergo 'ruffling' (Feasey *et al.,* 2012). Attachment and invasion of epithelial membrane by *Salmonella* involves multiple genes both chromosome and plasmid encoded (Wain *et al.,* 2012). Upon invasion of epithelium, *Salmonella* undergo intracellular multiplication and spreads to mesenteric lymph nodes and to the rest of the body via the systemic circulation. They are then taken up by reticuloendothelial cells. Reticuloendothelial system then confines and controls the spread of *Salmonella.* This invasive mechanism has been associated with the type of serovar, patient's age and geographical location (Gordon, 2011).

Worldwide examples of human *Salmonella* isolates are *S. Enteritidis*, *S. Typhimurium*, *S. Virchow, S. Newport, S. Hadar, S. Heidelberg, S. Agona* and *S. Indiana* (Threfall, 2000). The order of prevalence varies according to geographical location and over time (Threfall, 2000). The dominant strain throughout the world has been *S. Typhimurium*, however *S. Enteritidis* has also become the most prevalent serotypes in many western countries (Ducoffre, 2003).

Kisumu District hospital is located in Kisumu town, within Kisumu County in western part of Kenya. It lies on latitude, 00^0 60' 0"S, longitude, 34^0 45' 0"E. Kisumu is the third largest city in Kenya with a population of 968, 909 persons (Kenya National Bureau of Statistics, 2009). It is also the headquarter of Kisumu County. It serves as a communication and trading confluence for the Great Lakes region. Prevalence of NTS is reported at 568/100,000 persons per year observations (pyo) in the study region, while 76% of NTS isolates are multidrug resistant (Tabu *et al.,* (2012). A study by O'Reilly *et al.*, (2012) documents NTS to be associated with mortality in children aged below 1 year in the study region. Kisumu is characterized by high temperatures and frequent flooding due to its location on the shores of lake Victoria. Malaria, a co-factor for non typhoidal *Salmonella* infection is endemic in the area.

Kapsabet District hospital is located in Kapsabet town on latitude, 0^0 13' 07"N, longitude, 35^0 08' 35"E, along Kisumu- Eldoret road. Kapsabet has a population density of approximately 276 persons/km^2 (Kenya National Bureau of Statistics, 2009). The prevalence of bacterial diarrhea is documented at 8.5% (n=118) by Rono *et al.,* (2014) among HIV seropositive and seronegative children aged 5 years admitted at Moi Teaching and Referal hospital in western Kenya. The prevalence of *S.Typhimurium* and *S.Typhi* was 4.2% and 3.4% respectively in the same study. The prevalence is linked to socio economic status, housing, water, sanitation and seasonal variation in both study regions (Breiman *et al.,* 2012; Rono *et al.,* 2014).

Despite its robust drainage system, 75% of households in Kapsabet have limited access to safe and clean piped water for domestic use (FAO Kenya, 2007). Malaria is non endemic but does occur in Kapsabet due to its location in the highlands of Rift valley. Both sites (Kisumu and Kapsabet) have high adult prevalence of HIV at 15% and 13% respectively, a co- factor for non

typhoidal *Salmonella* infection (Tropical insititute of community health and development in Africa (TICH), 2006; FAO Kenya, 2007).

Prevalence of invasive NTS has been on the increase. Report from 22 studies in Africa between 1984 to 2006 indicated that *Salmonella enterica* serotypes are the second most prevalent organism recovered from children. Non typhoidal *Salmonella* were more commonly isolated than *S.Typhi*. *S. Typhimurium* and *S. Enteritidis* accounted for 65.2% and 33.1% of NTS isolates respectively (Oneko *et al.,* 2014)(unpublished data).

A cross sectional study in two hospitals in western Kenya by Onyango *et al.,* (2008), reported *S.Typhi* at 56% (n=33), *S.Typhimurium* at 34% (n=18) at St. Elizabeth Mukumu hospital. However, in the same study, only *S.Typhimurium* was isolated from Maseno Mission hospital. This implies that different geographical regions harbor different serovars and different serovars predominate at different periods of time (Galanis *et al.,* 2006). In this regard, circulating virulent strain of invasive non typhoidal *Salmonella* is evidence based that warrants establishment, hence this study in the respective study areas.

Non typhoidal *Salmonella* case fatality rate has been documented to be high and needs to be monitored through surveillance studies. This is supported by the fact that in Malawi, Gordon *et al.,* (2008) reported 21-24% case fatality rates in childhood NTS amidst availability of appropriate antibiotics. Its comorbidity with other infections e.g sickle cell disease is also documented by Williams *et al.*, (2009) in Kenya where 18% are known to suffer from NTS.

According to Talbert *et al.,* (2010) out of 748 children born out of hospitals, 11% suffer from invasive bacterial infection, 10% from bacteremia and 3% bacterial infection from meningitis, with case fatality of 33% in rural Kenya.

Similarly, the prevalence of NTS in rural western Kenya is reported at 65% of bacteremia in children of age group 0-4 years, with case fatality of between 12-24% in hospital based studies (Oneko *et al.,* 2014) (unpublished data). Without laboratory services in resource poor settings, the diagnosis and treatment of multidrug resistant infections is a challenge. This implies that there may be under reporting of NTS disease burden due to inadequate health facilities, inadequate diagnosis and lack of properly trained laboratory technologists to diagnose the disease burden in appropriate time, hence the total burden of invasive NTS in study areas has not been quantified.

The high prevalence rate of NTS is hypothesized to be as a result of antimicrobial resistance, climatic change as well as emergence of new strains of invasive *Salmonella*. This is supported by the fact that a new form of *Salmonella typhimurium* (ST313) that emerged in the Southeast of the continent in 1930s (Boor, 2006), followed by a second wave, which came from Central Africa in 1940s would be circulating geographically within the Asian and African continent (Boor, 2006). The second wave of invasive non typhoidal *Salmonella* is thought to have originated in the Congo Basin, and early in the event picked up a chloramphenicol resistant gene (Boor, 2006).

The variant is reported to be the cause of most invasive non typhoidal infections in sub Saharan Africa, as a result of a large proportion of susceptible population (HIV, malaria, malnutrition,

young age) (Okoro *et al.,* 2012). The variant (ST313) strain of *S.Typhimurium* in sub Saharan African countries between 1997-2010 (Okoro *et al.*, 2012) has adapted to humans. This is evidenced by the isolation of similar strain (ST313) in Malawi between 1997-2006 (Okoro *et al.,* 2012). ST313 is characterized by genome degradation a process associated with human host adapted *S. typhi* strain (De jong *et al.,* 2012) and it is able to efficiently spread around the human body.

As a basis of strain diversity, respective bacteria strains acquire antimicrobial resistance genes as a mode of survival in adverse environment, a condition refered to as phase variation. The aspect of phase variation has enabled the strains to survive in harsh geographical environment. To augment this fact, Tabu *et al.,* (2012) reports to have isolated multidrug resistant *S. Typhimurium* strain with pulse field gel electrophoresis (PFGE) pattern similar to that of antimicrobial resistance profile of ST313. However, without genetic sequencing, there was no conclusive evidence that the predominant MDR strain was ST313 clone. Limitations in such studies calls for conclusive data mining process and hence sequencing of isolates is expected to reveal additional information on the isolates. It is on this ground that sequencing and hence determination of genetic diversity of *S. Typhimurium* isolates from the study areas is warranted.

Antibiotic resistance is an increasing problem in *S. enterica* as in many other pathogens (Threfall, 2000). Many of the recent isolates obtained worldwide are resistant to a number of antibiotics (Threfall, 2000). Since antibiotics are the usual treatment for severe *S.enterica* associated disease, the emergence of MDR *S.enterica* displaying this phenotype is a major public health problem (Kariuki *et al.*, 2006).

Antibiotic resistance in bacteria can develop by accumulation of point mutation or by horizontal exchange of DNA between bacteria of the same or different species (Davis *et al.,* 2002). Increases in antibiotic resistance is generally attributed to the selective pressure exerted by the use of antibiotics both in treatment of disease in humans and at sub-therapeutic levels as additives to animal feed (Glynn *et al.,* 1998). In addition, increases in resistance in *S. enterica* may be at least partially due to clonal dissemination of resistant strains (Davis *et al.*, 2002).

Previously chloramphenicol, ampicillin and cotrimoxazole were first line drugs for treatment of typhoid disease. However, outbreaks of multidrug-resistant (MDR) *S. Typhi* (Phan *et al.,* 2009; Kariuki *et al.,* 2010) prompted the widespread use of fluoroquinolones. Fluoroquinolone usage was followed by the emergence of nalidixic acid-resistant *S. Typhi* exhibiting reduced susceptibility to fluoroquinolones in the early 1990s (Le *et al.*, 2007), which has since become widespread (Lewis *et al.*, 2005; Joshi and Amarnath, 2007; Kownhar *et al.*, 2007; Kariuki *et al.,* 2010). Thus, the spread of MDR and fluoroquinolone resistance in *S. Typhi* presents significant clinical challenges since resistance genes in *S.typhi* may be transferred to non typhoidal *Salmonella* strains.

Onyango *e t al.*, (2008) found *S.Typhimurium* isolates from two hospitals in western Kenya to be resistant to tetracycline, sulfamethoxazole, cotrimoxazole, ampicillin, chloramphenicol and streptomycin. In a related study in Siaya District hospital in western Kenya, *S.Typhimurium* isolates were resistant to most commonly used antimicrobials in addition to the development of ceftriaxone resistance (Oneko *et al.,* 2014)(unpublished data). This shows the fluidity in resistance patterns of *Salmonella* worldwide and regionally. Therefore characterization of

resistance phenotypes and genotypes in clinically recovered isolates is significant in order to respond to ever changing antimicrobial resistance surveillance data in these regions.

1.2 Problem Statement

The high prevalence of non typhoidal *Salmonella* is hypothesized to be linked to antimicrobial resistance, susceptible host population, climatic change as well as emergence of new strains of invasive *Salmonella*. Shifts in the prevalence of specific strains and serovars in human and animal population indicates a complexity of global epidemiology, as the frequency and occurrence of *Salmonella* change over time in countries and regions as has been observed in some countries (Malawi, Uganda, Democratic Republic of Congo, Mozambique) in addition to Kilifi and Nairobi in Kenya. Given the emergence of virulent invasive *Salmonella* strains, the predominant strains circulating in the study areas needs to be determined.

As the epidemiology of associated risk factors change in Kenya, the epidemiology and the burden of invasive NTS may also change thus affecting the prevalence rate as well as the risk ration in disease free population. However, the total burden of invasive NTS in the proposed study areas has not been adequately quantified

As a basis of strain diversity, respective bacteria strains acquire antimicrobial resistance genes as a mode of survival in adverse environment, a condition refered to as phase variation, a situation which need routine appraisal of antibiotic susceptibility pattern of the clinical *Salmonella* isolates and determine whether there is any correlation between antimicrobial susceptibility and genetic diversity of *Salmonella* isolates in the study areas.

1.3 Justification and Significance of the study

Invasive non typhoidal *Salmonella* (NTS) infection is in a flux due to selective pressure in resource poor settings in Kenya. To minimize the burden of this pathogen, it is important to monitor *Salmonella* serovar distribution in study areas and implement control measures through out food production chain and monitor the effectiveness of these control measures.

Invasive non typhoidal *Salmonella* infection is currently thought to be enhanced by increase in susceptible host population, therefore, as the epidemiology of these associated risk factors evolve in Kenya, the epidemiology and burden of NTS may also change. Thus updated data on prevalence and severity of NTS in study population can serve to guide future policy decisions on treatment and prevention.

Salmonella Typhimurium has undergone tremendous genetic modification, involving genome degradation, a phenomenon observed in human host adapted *S. Typhi* strain, having evolved from ST19 strain previously associated with gastroenteritis to highly invasive ST 313 strain. This has been aggrevated by the existence of antimicrobial resistance strains. Hence data on genetic diversity of *S. Typhimurium* strains is epidemiologically important in order to monitor the disease prevalence.

Antimicrobial resistance patterns are regularly changing. A study by Onyango *et al.,* (2008) in western Kenya reported 100% of *S. Typhimurium* isolates were resistant to tetracycline, sulfamethoxazole, cotrimoxazole, ampicillin, chloramphenicol and streptomycin. In a related study in Western Kenya, Tabu *et al.,* (2012) reported 76% multidrug resistance to more than three antimicrobials tested (chloramphenicol, trimethoprim-sulfamethoxazole and ampicillin). Therefore knowledge of prevalent phenotypes and genotypes in study areas is important in

surveillance and determining the spread of antibiotic resistance, this is critical for clinicians to keep abreast on treatment options in the study areas.

1.4 Research questions

1. What strains of *Salmonella* are present in the study areas?
2. What is the prevalence of non typhoidal *Salmonella* in study population?
3. What is the genetic diversity of *S. Typhimurium* isolates in the study areas?
4. What are the antimicrobial susceptibility profiles of *Salmonella* isolates in study areas?

1.5 Main Objective

To determine the prevalence, genetic diversity and antimicrobial susceptibility patterns of clinical *Salmonella* isolates from Kapsabet and Kisumu District hospitals in Kenya.

1.5.1 Specific Objectives

1. To identify *Salmonella* strains in human stool samples from Kisumu and Kapsabet District hospitals
2. To determine prevalence of *Salmonella* infection among study population in Kisumu and Kapsabet District hospitals
3. To determine genetic diversity of *Salmonella Typhimurium* isolates from Kisumu and Kapsabet
4. To determine phenotypic and genotypic antimicrobial susceptibility patterns of *Salmonella* isolates in the study areas

CHAPTER TWO

LITERATURE REVIEW

2.1. General characteristics and distribution of *Salmonella enterica serovars*

The genus *Salmonella* consists of rod-shaped, gram-negative, flagellated facultative anaerobic bacteria and belongs to the family *Enterobacteriaceae* (Feasey *et al.*, 2012). *Salmonellae* are intestinal and intracellular pathogens in many mammalian hosts. They are found in many other hosts, including birds, reptiles, amphibians, and plants (McQuiston *et al.*, 2008).

The genus consists of two species, *S. enterica* and *S. bongori* (Lan and Octavia, 2009; Tajbakhsh *et al.*, 2010). Species *S. enterica* has been subdivided into over 2600 serotypes according to Kauffman-White scheme based on the serologic identification of 'O' antigen (somatic) and H (flagellar) antigens (Brenner *et al.*, 2000). These serovars were originally designated by Latin binomial species names, but due to close relatedness of the serovars, the species names were subsequently retained as the serovar names of the single *Salmonella* species known as *Salmonella enterica* (Le Minor and Popoff, 1987; Brenner *et al.,* 2000). For example, the name *Salmonella Typhi* refers to *S. enterica Typhi*, or simply *Typhi* (Octavia and Lan, 2010). This is based on phenotypic traits i.e according to markers (Nataro *et al.*, 2007).

Molecular wise, chromosomal DNA hybridization and multilocus enzyme electrophoresis analysis has been used to characterize *Salmonella* (Brenner, 2000). In this regard, *S. enterica* has been classified into six subspecies viz:- *S. enterica* subsp. *enterica* subspecies (I), *S. enterica* subsp. *salamae* (II), *S. enterica* subsp. *arizona* (IIIa), *S. enterica* subsp. *diarizona* (IIIb), *S. enterica* subsp. *indica* (VI), *S. enterica* subsp. *houtenae* (IV) (Brenner *et al.*, 2000; Prokaryotes, 2005; Tajbakhsh *et al.*, 2010). Subspecies VII was described by Boyd *et al.*, (1996)

while using multilocus enzyme electrophoresis (MLEE) data. However, this subspecies is not identifiable by unique biochemical properties listed above. The group was originally identified as subspecies V *Salmonella* subspecies *bongori*, but it is now recognized as a separate species; *Salmonella* subspecies *bongori* (McQuiston *et al.*, 2008). This shows the limitations of the previous methods used for the identification of *Salmonella*, hence the need for more advanced methods and techniques herein proposed (sequencing and use of bioinformatics) to study evolutionary patterns for precise isolate identification.

The most frequently encountered *Salmonella* subspecies is *Salmonella* subspecies I. This subspecies is the one most associated with human gastroenteritis (McQuiston *et al.*, 2008). The other six subspecies of *Salmonella* as well as *Salmonella bongori,* are found primarily in non human hosts and may occasionally be associated with human gastritis. Many (2,541) *Salmonella* subtypes have been identified and among this subtypes, 1504 belong to *Salmonella enterica* subsp. I according to Popoff *et al.*, (2004). However, according to the International *Salmonella* surveillance system done in 2005, a total of 36,183 isolates were isolated. Approximately 1% of these were those that caused gastritis and belonged to other subspecies of *Salmonella* other than subsp. I (McQuiston *et al.*, 2008).

Most subspecies of *Salmonella enterica* are not associated with salmonellosis and are known to be commensals in cold blooded animals (Baumler *et al.*, 1998). However, subspecies I strains cause gastroenteritis in warm-blooded animals, and are responsible for 99% of *Salmonella* related infections in human (Selander *et al.*, 1996; Popoff, 2001; Octavia and Lan, 2006).

Serovars *Typhimurium* and *Enteritidis* are broad-host-range serovars capable of causing systemic infection in a wide range of animals but are usually associated with gastroenteritis in a broad range of phylogenetically unrelated host species (Fossler *et al.*, 2005; Heithoff *et al.*, 2012). Serovars *Dublin* and *Choleraesuis* are host-adapted serovars that are often associated with systemic *Salmonella* infection in cattle and pigs respectively and can cause infection in other animals, including humans (Saeed *et al.*, 2003; Bergsbaken *et al.*, 2009). Serovars *Typhi, Gallinarum* and *Abortusovis* are host restricted serovars that are associated nearly exclusively with systemic infection in human (Nagy *et al.*, 2004), fowl (Brenner *et al.*, 2000), and ovine (Brenner *et al.*, 2000) hosts respectively.

Non typhoidal *Salmonella* infection in Africa is highly associated with other conditions particularly HIV, malnutrition and malaria (Mtove *et al.*, 2010). This implies that as the epidemiology of these associated diseases evolve in sub Saharan Africa and resource poor settings in Kenya, the epidemiology and the burden of non typhoidal *Salmonella* also changes (Breinman *et al.*, 2012). Recent reports indicate that multidrug resistant NTS clone causing invasive disease in Kenya may be in a flux due to selective pressure (increase in susceptible host, climatic changes, pathogenic adaptation of respective isolates) (Tabu *et al.*, 2012). With change in local conditions, increased trade of food, animal feed and livestock as well as increased travel and human migration, infectious disease is no longer confined to a single country or region. Thus prevalence of *Salmonella* in one region presents a potential problem to other regions and beyond. To minimize the burden of this pathogen it is important to monitor serovar distribution in respective study areas.

2.2. Prevalence of *Salmonella* infection

Salmonella enterica subspecies *enterica* is an important pathogen world wide. It is responsible for a large burden of foodborne gastroenteritis. Globally, non typhoidal *Salmonella* (NTS) gastroenteritis is estimated to cause 93.8 million illnesses and 155,000 deaths each year (Crump *et al.*, 2010; Majowicz *et al.*, 2010). Invasive NTS infection occurs when the organism spreads beyond the gastrointestinal mucosa to infect normal sterile sites, such as the bloodstream, the meninges, bone, and joint spaces (Hohmann, 2001) hence transforming to systemic salmonellosis.

Documented studies on invasive NTS in children in sub Saharan Africa indicate that in Tanzania, 5 out of 24 children with suspected meningitis and or septicemia was caused by *S. Enteritidis* all of whom died (Vaagland *et al.*, 2004; Blomberg *et al.*, 2005). In Uganda bacteraemia affects 1 out of 6 of severely malnourished children with high mortality especially among HIV positive individuals (Bachou *et al.*, 2006).

Case-fatality estimates for invasive NTS disease among hospitalized patients in Africa range from 4.4% to 27% for children (Brent *et al.*, 2006) and 22% to 47% for adults (Morpeth *et al.*, 2009). Meningitis case-fatality rates are higher for NTS than for any other common cause of bacterial meningitis. It is documented that in Malawi, 64% of neonates with NTS meningitis died, compared with 26% of neonates with group B *Streptococcus* meningitis (Morpeth *et al.*, 2009).

A study by Kariuki *et al.,* (2006) among children admitted in a hospital in Nairobi, Kenya reported an overall NTS infection in children at 51.2%, gastroenteritis and bacteremia at 8.4%, gastroenteritis alone at 40.4%.

In comparison, it is found that six years down the lane, non typhoidal *Salmonella* accounted for 39% of blood culture isolates in the rural western Kenya and 3% in urban (Kibera) sites. The adjusted incidence in the rural site was 568/100,000 person-years (Tabu *et al.,* 2012). This is an aspect of complete dynamism of NTS within a population.

Previous studies in various settings in sub Saharan Africa and Kenya have identified children ≤ 5 years to have co-morbidity between *Salmonella* and malnutrition, malaria prevalence, prolonged hospitalization and HIV infection in adults as risk factors for non typhoidal *Salmonella* diarrhoea infection (O' Reilly *et al.*, 2012). The above data acts as pointer to high burden of invasive disease.

In rural Kenya, the estimated minimum incidence of bacteremia is reported to be 505 cases per 100,000 person-years in the age group ≤ 5 years old, of which 88 cases per 100,000 person-years is NTS bacteremia (Berkley *et al.,* 2005). The correct incidence of bacteremia is likely to be 2 to 3 times higher than this figure, since the incidence of bacteremia among children who died before reaching the district hospital was unable to be ascertained in either study (Berkley *et al.,* 2005; Sigauque *et al.*, 2009).

A cross sectional study by Onyango *et al.,* (2008) isolated 23.3% and 60% of *S.Typhimurium* among immunocompromised and immunocompetent children in Kisumu District. In a related study by Oneko *et al.,* (2014) (unpublished), found invasive NTS to be responsible for 65% of bacteremia in children of age group ≤ 4 years in Siaya District in western Kenya, with the prevalence being higher in HIV positive children. However, in HIV negative children the high prevalence was closely related to recent or concurrent malaria (Oneko *et al.,* 2014) (unpublished data), an indication that HIV and malaria are risk factors for NTS in western Kenya. However, without microbiology services in resource poor settings, the diagnosis and treatment of multidrug resistant infections is difficult (Oneko *et al.,* 2014) (unpublished data) .Therefore, there may be under reporting of NTS disease burden due to inadequate health facilities, inadequate diagnostics and lack of properly trained laboratory technologists to diagnose the disease burden in appropriate time, hence the total burden of invasive NTS in study areas may have been missed out.

2.3 Mechanism of genetic diversity in *Salmonella enterica* species

Genetic diversity (polymorphism) within *Salmonella enterica* species is prominent in genes that encode surface structures e.g flagella, and specific virulence genes (*spv* genes). These genes encode factors that modify host cell physiology and protect bacteria from antimicrobial systems of host (Fierer, 2001; McQuiston *et al.*, 2004 and 2008). In addition, specific virulence determinants may be clustered together on polymorphic pathogenicity islands located on transposible genetic elements. Hence, *Salmonella* pathogenicity islands (SPIs), plasmids, functional prophages and phage remnants contribute significantly to the genetic diversity among *S. enterica* strains (Boyd and Brussow, 2002).

Since the divergence of *Escherichia coli* more than 100 million years ago, *Salmonella* has acquired, through lateral gene transfer (LGT) a repertoire of genes that confers a set of physiological features that define its niche from *E. coli* (K12) (Fierer, 2001). Some of these chromosomally encoded genes can be considered as part of "core genome" of *Salmonella* i.e present in all strains (Fierer, 2001). *Salmonella* pathogenicity island (SPIs) are large gene cassettes within *Salmonella* chromosome, they encode determinance for specific interactions with host and they are required for virulence in a given animal. Most SPIs have become part of core set of genes of *S. enterica* and encode species specific traits (Boyd and Brussow, 2002).

One of the genes that influence *Salmonella* virulence is *fliC* gene. The *fliC* gene encodes the major component of the flagellum which plays a key role for the Type III secretion system, the most widely used mechanism to secrete proteins from cytoplasm of bacterial cell (Yonekura *et al.*, 2003; McQuiston *et al.*, 2004; Phillip *et al.*, 2006). In case of *S. enterica Typhi*, the H antigen gene (*fliC-d*) i.e phage-1 flagellin gene for d-antigen (H:d) encodes for flagellin (Hirose *et al.*, 2002). The ability of *Salmonella* to swim is critical survival trait, flagellin activates eukaryotic defense pathways and thus *Salmonella* restrict the population of flagellin during systemic infection (Stewart *et al.*, 2011).

Salmonella modifies *fliC* gene expression during invasion, dissemination and colonization of intestinal mucosa (Yonekura *et al.*, 2003; Cummings *et al.*, 2006). This enhances virulence by generating molecular diversity, selected upon by environmental or host pressures, hence the best adapted phenotype survives during infection (Arunavas Das *et al.*, 2012).

The *spv* operon is common to all *Salmonella* virulence plasmids. *spv* virulence locus located on large plasmids in *Salmonella* subsp I serovars is associated with severe infection (Guiney and

Fierer, 2011). The *spv* locus consists of five genes: the positive regulatory gene *spvR*, and four structural genes; *spvA, spvB, spvC*, and *spvD* (Boyle *et al.,* 2007). *spv* gene expression is co-ordinately regulated by *spvR* through activation of the *spvA* promoter. Deletion of *spvA* promoter abolishes expression of *spvB, spvC, spvD* (Lesnick *et al.*, 2001). *spv* genes promote transmission of organism through systemic spread and localization in key target tissues (Libby, 2000). Transcription of *spv* genes is regulated by *rpoS* genes, the *spv* genes in turn encodes stress response proteins which act to increase resistance to environmental stress conditions including nutrient deprivation, heat oxidative and osmotic shock (Libby , 2000).

invA gene located on SPI-1, is essential for full virulence in *Salmonella* (Malorny *et al.*, 2003). It is believed to trigger the internalization required for invasion of deeper tissues (Malorny *et al.*, 2003). *invA* gene is a structural gene that forms invasion gene operon *invA, B, C, D* . *invA, B, C, D* structural genes are mediated through transcription from *invA* promoter. Amplification of *invA* is significant in the detection of *Salmonella* (Malorny *et al.,* 2003). It is documented that evolutionary diversification of invasion and housekeeping genes among subspecies of *S.enterica* has been similar in pattern and average rate (Li *et al.*, 1995; Prescott *et al.*, 2008).

A bacterial clone is an isolate of bacterial species that are genotypically indistinguishable hence are believed to have descended from same recent ancestor (Whithead *et al.,* 2004). However, recombinational replacements and the fact that bacteria are not truly asexual have enhanced diversity of the genotypes of a clone and this has led to clonal complexes (Whithead *et al.*, 2004).

2.4 Genetic Diversity of *S. Typhimurium*

The full genome sequence of numerous *S. enterica* serovars have been determined. The *S. Typhi genome*, compared to that of *S. Typhimurium*, harbors many inactivated or disrupted genes

(De jong *et al.*, 2012). Analysis of *S. Typhimurium* isolates from the unusually invasive infections isolated in sub-Saharan Africa have shown a predominant multi locus sequence (MLST) type , ST313, being distinct from the usual *S. Typhimurium* sequence type, ST19, associated in other *S. Typhimurum* isolates from Malawi (Kingsley *et al.*, 2009). It is characterized by loss of gene function, including genes previously implicated in the virulence of *S. Typhimurium* in the murine model of infection, such as *sseI* (encoding a type III-secreted effector protein) and *ratB* (encoding a secreted protein associated with intestinal persistence), and of the 44 novel pseudogenes or deletions in the strain relative to LT2, 26 are pseudogenes or deletions in *S. Typhi* or *S. Paratyphi A* (Kingsley *et al.*, 2009).

These observations indicate that a similar process of adaptation to the human host may be happening in Africa's *S. Typhimurium* as has been observed in *S. Typhi.* Comparative genetic analysis indicated that about 90% of the genes in *S. Typhi* and *S. Typhimurium* serovars are identical (McClelland *et al.*, 2001; de jong *et al.*, 2012). This is an indication of a single ancestry but diversity due to survival requirements between the two strains. The 10% of genes that differ include virulence factors, which determine their pathogenic potential (Sabbagh *et al.*, 2010). The microevolution of virulence gene expression has been associated with environmental stress (Boor, 2006). Due to climatic changes, countries in sub Saharan Africa were prone to extreme draught followed by rain storms, hot days are then followed by cool nights, these conditions could lead to the emergence of successful clone of *S. Typhimurium* (Boor, 2006).

Multi locus sequence typing (MLST) analysis of invasive *S. enterica Typhimurium* from Malawi and Kenya identified a dominant type designated as ST 313 rarely reported outside Africa

(Kingsley *et al.*, 2009). A comparison of a collection of *S. Typhimurium* isolates from invasive disease in urban and rural areas of Blantyre region in Malawi and from urban Nairobi and rural Kilifi in Kenya indicated that they were of single MLST type ST313. All Malawi isolates (31) and some Kenyan isolates (13) were ST313. The remaining (6) of Kenyan isolates were of ST19, the most common ST for *S. Typhimurium* in database (Kingsley *et al.,* 2009). The most previously common *S.enterica Typhimurium* ST19 isolates includes LT2, SI 1344, DT 104, and NCTC 13348 (Kingsley *et al.*, 2009). Most of ST313 isolates described are phage types DT 56var or untypeable (Wain *et al.,* 2012). The above data indicates that there is evolution of invasive non typhoidal *Salmonella* in sub Saharan Africa and Kenya in particular. This is thought to be enhanced by increase in susceptible host population, climatic changes during which seasonal peaks occur with the rainy season among adults and children (Morpeth *et al.,* 2009).

Pathogenic adaptation of respective isolates significantly enhances *Salmonella* evolutionary adaptation (Rogers *et al.*, 2011). Most bacterial survival mechanisms are geared towards adaptation in changing environments. Some of these include; development of mutator phenotypes, adaptive mutation, phase variation and bacterial persistence phenotypes (persister cells) (Rogers *et al.,* 2011). They protect *Salmonella* from various stress conditions including antibiotics (Kussel *et al.,* 2005). *S. Typhimurium* has evolved various adaptations for survival including tolerance to acidic conditions encountered both in environments and the infected host (Kussel *et al.*, 2005).

Two lineages are responsible for invasive *S. Typhimurium* disease in sub Saharan Africa (Okoro *et al.,* 2012). The spread of lineage I clones occurred from Malawi to Kenya. This was followed

by spread from both Kenya and Malawi to Uganda and from Malawi to Democratic Republic of Congo and Mozambique (Wain *et al.*, 2012). The spread of lineage II clones occurred across the continent to Mali and Nigeria (Kingsley *et al.*, 2009; Msefula *et al.,* 2012 and Okoro *et al.*, 2012). The successful transmission and clonal expansion of this strain is thought to be linked to acquisition of multidrug resistance genes.

Acid adaptation in *S. Typhimurium* induces cross-protection against stresses including salt, heat, and organic acids (Kussel *et al.,* 2005). Acid Tolerance Response (ATR) is triggered in *Salmonella* at pH values of between 6.0 and 5.5 but protects cells against stronger acids (pH 3.0 to 4.0). Adaptation to a number of stress conditions and starvation enhances thermal tolerance in *Salmonella* by triggering synthesis of heat shock proteins, a genetic response to environmental stress. *S. Typhimurium* also expresses outer membrane proteins (OmpC and OmpF) to respond to osmotic stress (Kussel *et al.,* 2005). Hence studies relating to emergence and re-emergence of these infective strains are imperative during this period of climatic change that lead to environmental degradation. Sequence analysis of the isolated *S.Typhimurium* strains in Kisumu and Kapsabet will therefore provide additional data on genetic diversity of local strains of non typhoidal *Salmonella* responsible for significant proportion of reported invasive NTS in study areas.

2.5 Antimicrobial resistance in *Salmonella enterica* species

Two million people suffer from bacterial infections each year with 90,000 resultant fatalities (CDC, 2006). 70% of these 90,000 resultant fatalities are due to organisms that are resistant to at least one antibiotic (CDC, 2006). The occurrence of isolated *Salmonella* strains showing resistance to one or more antimicrobial agents have been on the rise probably due to continuous antibiotic pressure (Lawson *et al.,* 2000; Kariuki *et al.*, 2006b). Among antimicrobial drugs used

for the management of *Salmonella* are; ampicillin, sulfamethoxazole-trimethoprim, and chloramphenicol which have been used as first line drugs for the treatment of severe *Salmonella* infections (Fonseca *et al.*, 2006). However, increasing rates of resistance to these agents have significantly reduced their efficacies (Olsen *et al.,* 2001; Fonseca *et al.,* 2006) and hence their current potencies on circulating *S.Typhimurium* need to be determined.

Exposure of bacterial pathogen to antibiotics enhances resistance by selecting for those cells that are able to tolerate them (Rogers *et al.*, 2011). This creates positive correlation between level of antibiotic use and prevalence of antibiotic resistance in bacteria in the same human population both at national and regional levels (Hogber *et al.*, 2010). The rate of emergence of resistance is also related to total antibiotic use, including widespread prescription of antibiotics for respiratory viral infection (Ben-David and Rubinstein, 2002: Rogers *et al.*, 2011) in addition to use of antibiotics in low doses as growth promoters in livestock farming (FDA, 2009). The above data implies that there is need for strategies to maintain spectrum of activity of existing antibiotics especially in resource poor settings in Kenya.

The emergence of antimicrobial resistance, especially multidrug resistance to ampicillin, chloramphenicol, and cotrimoxazole, has further complicated the treatment and management of enteric fever (Kariuki *et al.,* 2010). In Kenya, ampicillin, chloramphenicol, sulfamethoxazole, tetracycline, cotrimoxazole are used as first line therapy for enteric fever and other non typhoidal *Salmonella* infections (Kariuki *et al.*, 2010). The emergence of resistance to β-lactam antimicrobial agents is a great challenge in management of adult and pediatric infections (Kariuki *et al.,* 2006).

This indicates that there has been change in resistance patterns of *Salmonella.* Given the clinical importance of the above antibiotics, it was in order to test susceptibility of *Salmonella* isolates to ampicillin, chloramphenicol, tetracycline, sulfamethoxazole, kanamycin and gentamicin.Characterization of resistance genes in *Salmonella* isolates is clinically and epidemiologically important for clinicians to keep abreast on treatment options in the study areas.

2.6 Molecular detection and phylogenetics of *Salmonella*

In *S. enterica* genetic diversity is based on serovar. Serovar is determined by the lipopolysaccharide 'O' antigen and the two flagellar 'H' antigens, and has been widely used historically to differentiate *S. enterica* strain (Guiney, 2005). Although serotyping has been an epidemiologically useful resource for classifying isolates, it provides relatively limited information regarding bacterial diversity, evolutionary relatedness, and pathogenicity (Guiney, 2005; Ruzante *et al.*, 2010; Heithoff *et al.,* 2012).

A variety of methods have been used to examine phylogenetic history of *Salmonella.* Crosa *et al.*, (1973) used DNA disassociation by DNA-DNA hybridization to define the species and subspecies of *Salmonella* and differentiated them from other members of *Enterobacteriaceae.* In a related study, Boyd *et al.*, (1996) defined the relationship of *Salmonella* based on MLEE and DNA sequence analysis of housekeeping and invasion genes. Multilocus enzyme electrophoresis (MLEE) looks at similarity of the enzymes produced by an organism and would therefore seem to be well suited to evolutionary analysis, however, it relies upon protein electrophoresis which is difficult to perform reproducibly, making global comparisons a challenge (Verghese *et al.,* 2012). In a related study, Porwollik *et al.,* (2002) used microarray analysis of gene presence or absence to compare *Salmonella* subspecies and serotypes.

These studies resulted in similar conclusions but with notable exceptions. The phylogeny of MLEE data by Boyd *et al.*, (1996) conflicted at many points with DNA sequence based phylogeny and microarray data.

Other methods that have been used to estimate genetic relatedness in groups of *S.enterica* isolates include pulse field gel electrophoresis (PFGE)(Peters *et al.,* 2007), ribosomal RNA analysis, phage typing, plasmid analysis, multilocus sequence typing (MLST) and single locus sequence typing (SLST) (Peters *et al.*, 2007). PFGE uses an electric field of changing orientation to move large DNA fragments, usually obtained by restriction endonuclease digestion through a gel (Kraulandm *et al.,* 2008).

PFGE is highly discriminatory, since it is based solely on the electrophoretic mobility of restriction endonuclease fragments, it measures only a small subset of the mutations that occur in an organism's DNA (Lim *et al.,* 2005). A single point mutation in a restriction site may lead to large changes in the PFGE band pattern, while more numerous changes in other locations may not be identified (Kraulandm *et al.,* 2008). Bands which appear identical by PFGE may be composed of entirely different nucleotide sequences (Lim *et al.,* 2005). I n addition, PFGE results do not contain any phylogenetic signal and hence cannot be used for phylogenetic analysis (Kraulandm *et al.*, 2008). Ribosomal RNA is highly conserved in *S.enterica* and is therefore not useful for determining genetic lineage (Lim *et al.,* 2005).

Phage typing has been used for many years to differentiate *S.enterica* strains. In this method, *S.enterica* isolates are grown on solid media and treated with stock solutions of phages. The resulting pattern of lysis determines the phage type (Kraulandm *et al.*, 2008). Since this method relies on genetic material that is external to the *S.enterica* genome and may be lost without

significantly impacting the viability of the organism, the usefulness of phage typing for estimating relatedness is limited (Kariuki *et al.,* 1999).

Other studies of *Enterobacteriaceae* have used DNA sequence based approaches to dissect the natural history of these organisms. Dauga (2002) and Fukushima *et al.*, (2002) each compared the DNA gyrase subunit gene *grB* to 16S rRNA and found that the congruence of phylogenetic relationships is not always clear with *Enterobacteriaceae*. Similar study by Roggenkamp (2007) compared *oriC* to 16S rRNA and found that *oriC* gave robust phylogenies for species within *Enterobacteriaceae.*

Paradis *et al.,* (2005) compared *tuf* and *atpD* genes to 16S rRNA phylogenies within *Enterobacteriaceae* and found that these phylogenies were comparable and gave better discrimination than 16S rRNA alone. McQuiston *et al.*, (2008) analyzed four house keeping genes, *gapA, phoP, mdh* and *recA* to characterize phylogenetic relationship of species and subspecies of *Salmonella.*

Multilocus sequence typing measures the DNA sequence variations in a set of house keeping genes (McQuiston *et al.*, 2008). Ahuge set of data is produced during sequencing and identification hence bioinformatics techniques are used (Sabat *et al.,* 2013). MLST data can be used to investigate evolutionary relationships among bacteria (Tankou-Sandjong *et al.,* 2007). Single locus sequence typing (SLST) is a variant of MLST. It is used to determine the relationships among bacterial isolates based on the comparison of sequence variations in a single target gene (Sabat *et al.,* 2013). SLST is automated, combines advances in high throughput sequencing and bioinformatics with established population genetics techniques. However no single method is optimal for all forms of investigation.

In most isolates of *Salmonella* two genes encode flagellar antigens *fliC* encode phase 1 antigens and *fljB* encodes the phase 2 antigens (Macnab, 1992). These genes are coordinately expressed by a phase variation mechanism (McQuiston *et al.*, 2004). *fliC* is located in one of the flagellar biosynthesis operons, is present in all *Salmonella* (Macnab, 1992). Genes that encode bacterial flagellin are typically highly conserved at their 5' and 3' ends while the middle region is quite variable. The conserved regions encode the flagellar filament backbone and are critical for the assembly of the filament. The central region corresponding approximately to amino acids 181-390 encode the surface exposed and antigenically variable portion of the filament (McQuiston *et al.*, 2004).

Comparative DNA sequence analysis of flagellin alleles have been done to determine genetic diversity (McQuiston *et al.*, 2008). This is due to the fact that genetic diversity of bacteria depends on the rate of recombination and recombinational exchange mobilizes small genome segments among lineages and species (Whitehead *et al.*, 2004). Therefore one of the objectives of this study was to analyse phylogeny of *S.Typhimurium* isolates from Kapsabet and Kisumu using single locus sequence typing based on DNA sequence analysis of *fliC* gene.

2.7 Antimicrobial susceptibility testing for non typhoidal *Salmonella*

In treatment and control of infectious diseases caused by pathogens that are often drug resistant, sensitivity testing is used to select effective antimicrobial drugs (Cheesbrough, 2000). This is essential for the guidance of clinical management. Sensitivity tests measure antimicrobial activity against bacteria under laboratory conditions and not in patient (Madigan *et al.*, 2000).

The antimicrobials currently in use for treatment, determination of MDR strains and patient medical history determine the choice of antimicrobials for the test. Using the interpretive chart,

zone sizes are interpretated reporting organism as, 'resistant', 'intermediate' or 'sensitive' (WHO, 2000).

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

Some of the antimicrobial testing methods include; Agar disc diffusion (ADD), Minimum inhibition concentration (MIC), Epsilometer strip test (E test).

2.7.1 Agar disc diffusion (ADD) / Kirby Bauer

These are paper discs impregnated with the antimicrobial drug of choice at varied concentration appropriate for either elimination or limit activity of bacteria in *vitro* or in *vivo*. The discs are placed on agar plate and the drug diffuses into the agar thus extending the bacteriocidal /bacteriostatic phenomena. In this method, activity of the drug against bacteria correlates with the zone of bacteria inhibition around the disk (Prescot *et al.*, 2008).

2.7.2 Molecular testing methods

To combat the rise in antibiotic resistance, it is necessary to understand how resistance spreads through the bacterial population. This requires the study of genes that determine resistance (Scott *et al.*, 2011). The location of antibiotic resistance genes on mobile genetic elements such as plasmids, transposons and integrons facilitates the mobilization of antibiotic resistance from one organism to another (Scott *et al.*, 2011). In addition resistance may be chromosomally encoded or conferred through chromosome mutation.

This involves the use of molecular testing methods, some of the methods includes; detection of antimicrobial resistance genes by Polymerase chain reaction (PCR), plasmid extraction and characterization, integron detection and characterization. Polymerase chain reaction (PCR) method uses specific antimicrobial resistance gene primers to validate the existence of resistance genes in the suspected microorganism (Scott *et al.,* 2011).

Plasmid extraction and analysis involves plasmid DNA extraction from isolates resistant to one or more antimicrobials. Plasmid DNA is then separated by electrophoresis on 1% horizontal agarose gel stained with ethidium bromide and photographed with ultraviolet transilluminator. Plasmid molecular sizes are then determined by coelectrophoresis with *E. coli* strains 39R861(NCTC 50192)(147,63,43.5, and 6.9kb) an V517(NCTC 50193)(53.7, 7.2, 5.6, 3.9, 3.0,2.7 and 2.1kb) on 1% horizontal agarose gels (Krauland *et al.*, 2008).

Integron detection is achieved when genomic DNA of bacterial isolates is prepared according to respective kits. Integron carriage is determined by PCR using primers specific to the *Intl* region of the integrase gene. Isolates positive for integrase are further characterized by PCR using primers specific for the 5' and 3' conserved segments (CS) of the integron structure. These methods have increased speed and reliability of resistance testing (Kraulandm *et al.,* 2008).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Areas

3.1.1 Kapsabet District Hospital

Sample collection was done in Kapsabet District hospital in Nandi County of Rift Valley Province. Nandi County lies on the western side of the Rift Valley and receives mean annual rainfall of 1200-2000mm p.a. Rainfall is bimodal with dry season experienced between December and March. It has a robust drainage system with a number of permanent rivers. The prevalence of bacteria caused diarrhea is 8.5% (Rono *et al.,* 2014). However, 75% of households have limited access to safe and clean sources of water for domestic use (FAO Kenya, 2007), this is an environmental risk factor for non typhoidal *Salmonella* infection. Approximately 50% of the population live below poverty level, with rural and urban areas registering counts of 50% and 27% respectively. Malaria non-endemic but does occur while HIV/AIDS pandemic, a co-factor for non typhoidal *Salmonella* infection is documented at 13% (FAO Kenya, 2007). Kapsabet District hospital is located in Kapsabet town on latitude 0^0 13' 07"N, longitude 35^0 08' 35"E, along Kisumu- Eldoret road. Kapsabet is the County headquarter and has a population density of approximately 276 persons/km^2 (Kenya National Bureau of Statistics, 2009). It is the main government hospital in Nandi Central of Nandi County as well as a referral hospital for the five administrative divisions (Fig. 1).

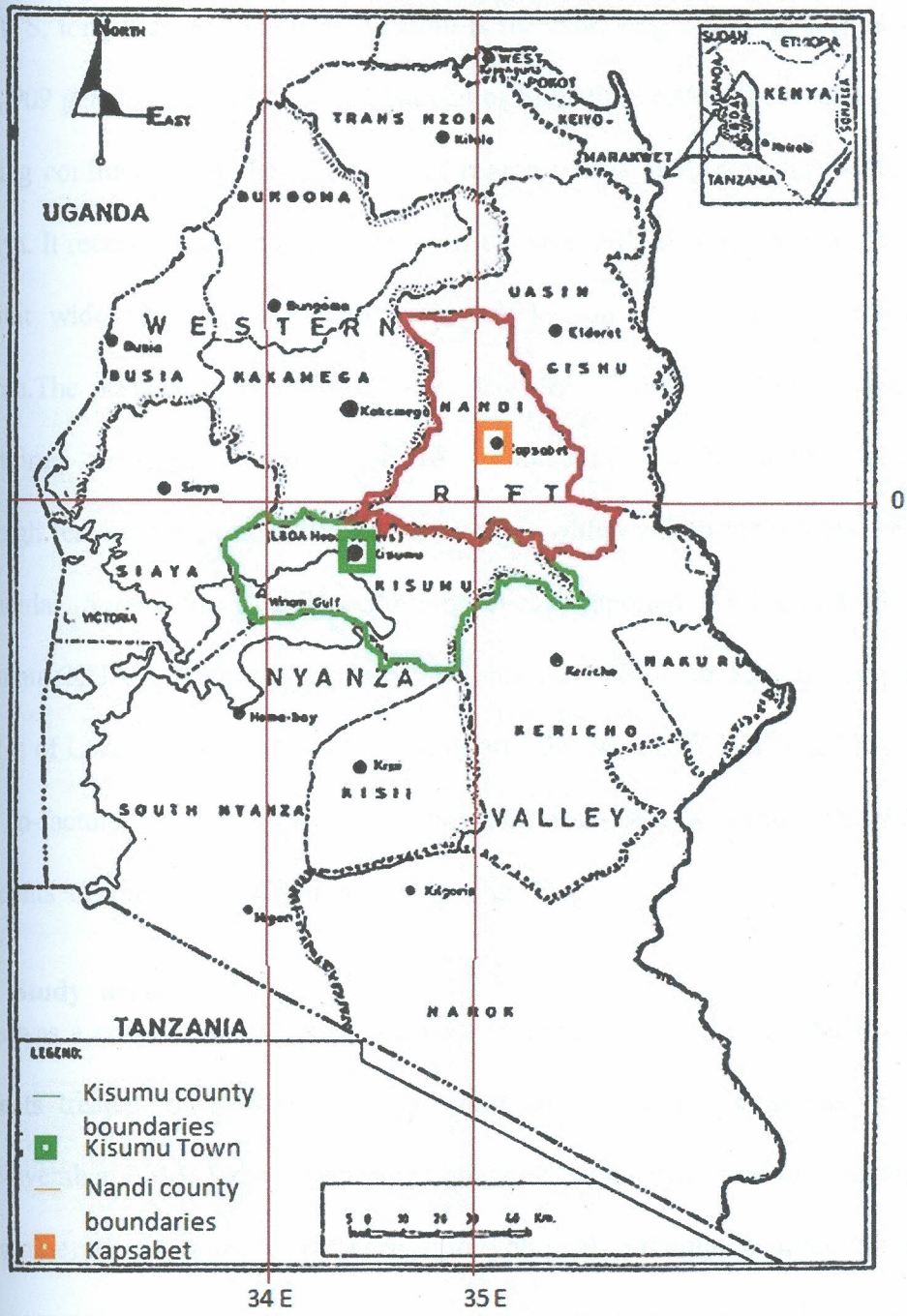

Figure 1. Map of Kenya showing location of study areas: Kapsabet and Kisumu towns (Marked in box) (Adapted from google map 2014).

3.1.2 Kisumu District Hospital

Kisumu District hospital is located in Kisumu town within Kisumu County and lies on latitude 0^0 60' 0"S, longitude 34^0 45' 0"E. Kisumu is the third largest city in Kenya with a population of 968, 909 persons (Kenya National Bureau of Statistics, 2009). It serves as a communication and trading confluence for the Great Lakes region. Kisumu County is located in western part of Kenya. It receives mean annual rainfall of between 600 to 1630mm p.a. Rainfall is bimodal and exhibit wide variation in distribution, dry season is experienced between December and March.The prevalence of NTS accounts for 39% of blood cultures in the study region while multidrug resistance is reported at 76% (Tabu *et al.*, 2012). Kisumu county is characterized by high temperatures and frequent flooding which are environmental risk factors for non typhoidal *Salmonella* infection. Poverty level is reported at 61% and 68% in rural and urban Kisumu (KUAP, 2008) respectively. Malaria is endemic in Kisumu given its location on the shores of Lake Victoria. It has high adult prevalence of HIV/AIDS at 15% (TICH, 2006), these are co-factors for non typhoidal *Salmonella* infection. Kisumu District hospital serves residents of the larger Kisumu county (Fig. 1).

3.2 Study design

This was a cross sectional study involving recovery of clinical *Salmonella* isolates from patients treated at Kapsabet and Kisumu District hospitals in Kenya between June 2011 to November 2013. Cross sectional study design was chosen because it attempts to determine the relationship between non typhoidal *Salmonella* infection, the study population and geographic location. Systematic random sampling was used, in which the first three (3) patients aged ≤ 5 years and the first three (3) patients aged > 5 years who met inclusion criteria were enrolled for the study.

3.3 Sample size determination

Sample size was estimated using formula by Glynn *et al.,* (1998) for estimating sample size from catchment population, of previous estimate of available proportion in target population with non typhoidal infection present in Kapsabet and Kisumu

$N = [Z^2 P (1-P)]/D^2$

$= [1.96^2 X 0.08(0.92)]/0.05^2$

$= 400$

Where:

N = Minimum sample size

Z = 1.96 standard error

P = Expected prevalence of condition of interest

D= 0.05(inverse of 95% allowable error)

Therefore a minimum sample of 400 stool specimen was randomly sampled from a catchment population of 483 of previous estimate available of the proportion of people in target population that have non typhoidal *Salmonella* infection in each study areas.

3.4 Study participants inclusion criteria

In order to be included in this study, all participants were expected to either be permanent residents or must have stayed in the respective area for a period not less than three weeks at the time of the study. Inclusion criteria for participants was that a patient had; a presentation with fever defined as $\geq 38.0^0$C, without acute respiratory illness, irrespective of malaria blood smear results and regardless of bloody diarrhoea (defined as presence of visible blood in stool). Systematic random sampling procedure was used in which; only the first three (3) patients ≤ 5 years and first three (3) patients > 5years who met this criterion per day were enrolled for the study. Laboratory personnel were provided with data collection forms to fill in secondary data (record) on anthropometric indices like age, sex, health status of patients. For children less

than 15 years old, parents or guardian gave consent to permit their participation. Consent to carryout study in respective hospital was sort from hospital administrative authority (Appendix I).

3.4.1 Study population and sampling procedure

In this study, patients aged ≤ 5year and patients aged > 5years who visited Kapsabet and Kisumu District hospitals presenting with symptoms of fever ≥ 38.0^{0}C and diarrhoea (≥ 3 bowel movements within 24hr period during the preceding 5 days) were included in the study. Both swabs of whole stool and rectal from patients enrolled for the study were obtained and immediately placed in Cary Blaire transport medium and transported within 6 to 12hr in iced cool box at 8^{0}C to the laboratory for analysis.

3.5 Culture and Isolation of *Salmonella*

Stool samples were cultured at 37^{0}C in Selenite F broth (Himedia laboratories Pvt Ltd Mumbai India) for 18 - 24hrs for enrichment after which the cultures were streaked onto plates of *Salmonella Shigella* agar from Himedia , a selective medium for *shigella* and *Salmonella* and cultured for 18-24hrs at 37^{0}C. Colony colour and morphology was determined by visualizing through hand lens. The red colonies with black centers were carefully selected, picked using dry heat sterile inoculating loop and streaked onto plates of Xylose lysine Deoxycholate (XLD) agar from Himedia, a selective medium for *Salmonella* then cultured for 18-24 hrs at 37^{0}C. Colonies from XLD agar were used to inoculate Tryptic Soy Broth (TSB in 15% glycerol) from Himedia, incubated for 18-24hr then frozen in small eppendorff tubes for later genetic analysis.

The non lactose fermenting colonies were subjected to biochemical tests comprising of Indole, Methyl Red, Voges, Proskauers, Citrate (IMVIC) to identify specific bacteria genus based on

their biochemical activities in different culture media. Isolates from Triple Sugar Iron (TSI) (Himedia pvt Ltd Mumbai, India) which showed characteristics of *Salmonella* were subcultured in methyl red medium and incubated at 35^0C for 48± 2hr. Five to six drops of methyl red indicator was then added to 5ml of 48hr glucose phosphate broth culture (Himedia pvt Ltd Mumbai, India). Voges proskauer (VP) test was performed by transferring 1ml of 48hr culture isolates to a culture test tube and broth incubated for additional 48hr at 35^0C. 0.6 ml α-naphthol was added to the mixture and vortexed. 0.2ml of 40% KOH solution was later added to the mixture and vortexed. The results were read after 4hrs. In addition, API 20E system (Biomerieux, Marcy L'etoli, France) was done to confirm identity of *Salmonella.*

3.5.1 *Salmonella* antimicrobial susceptibility testing by Kirby-Bauer disks diffusion method (ADD)

Salmonella isolates from Kapsabet and Kisumu were tested for susceptibility to antimicrobials chosen on the basis of the most commonly prescribed in respective health facilities. And those easily bought over the counter, these antibiotics are also classified according to WHO in category 1 and 2 as critically important (ampicillin, tetracycline) and highly important (kanamycin, sulfamethoxazole, chloramphenicol) to human medicine (WHO, 2013). Break points for antimicrobial drugs were based on guidelines provided by clinical laboratory standard institute (CLSI) (2011). The following antimicrobial agents were considered in this study: ampicillin (AMP) (25mcg), gentamicin (GEN) (10mcg), kanamycin (K) (30mcg), tetracycline (TET) (25mcg), co-trimoxazole (COT) (25mcg), streptomycin (ST) (10mcg), sulfamethoxazole (SX) (200mcg) and chloramphenicol (C) (30mcg). The antimicrobial susceptibility was done following Kirby-Bauer disks diffusion method (ADD) 1996 (Himedia pvt Ltd Mumbai, India), according to manufacturer's instructions. Fresh *Salmonella* colonies were inoculated in 0.85% NaCl suspension to turbidity equivalent to 0.5 MacFarland standards which is equivalent to

$1.0x10^8$ colonies. The culture was swabbed onto a Muller-Hinton agar from Himedia. Antibiotic discs were applied using sterile forceps onto the culture plates after drying the plates for 5 min on working bench, the plates were incubated at 37^0C for 24hrs. *E. coli* ATCC 25922 was used as a positive control for potency of antibiotic discs.

Diameters of zone of inhibition around the disc were measured to the nearest millimeter using a ruler (0-30cm) and isolates classified as sensitive, intermediate or resistant according to guidelines provided by clinical laboratory standard institute (CLSI) (2011) (Appendix II).

3.6.0 Genetic characterization of *Salmonella* species

3.6.1 *Salmonella* DNA extraction by boiling (according to protocol by Amini *et al.*, 2010).

Pure *Salmonella* isolates obtained from a series of sub cultures in XLD medium (Himedia pvt Ltd Mumbai, India) and stored in Tryptic Soy Broth from Himedia were allowed to thaw at room temperature and then reconstituted in 200ml of 0.9% NaCl solution (normal physiological saline).

Salmonella colonies were freshly grown in nutrient agar (Himedia pvt Ltd Mumbai, India). The isolates were then picked using dry heat sterile inoculating wire loop and suspended in 150µl of sterile distilled water in eppendorf tube (2ml) (Sarstedt Ltd, Germany). This was gently vortexed using a vortexer and homogenate was then heated at 100^0C for 10min in a water bath. Homogenate was then centrifuged at 10000rpm (Spectrafuge 16M, Labnut International USA) for 5min at 4^0C. The obtained supernatant was carefully aliquoted by micro pipette, stored at -20^0C and later used as a source of DNA template (Amini *et al.*, 2010).

3.6.2 Primer sequences used for amplification reactions

Published primers used for amplification reactions are listed (Table 1) below.

Table 1. Primer sequences for PCR amplification

Primers	Sequence (5' – 3')	References
InvA	*F* – ACAGTGCTCGTTTACGACCTGAAT	Chui *et al.*, (2006)
	R- AGACGACTGGTACTGATCTAT	
fliC fli15	*F*- CGGTGTTGCCCAGGTTGGTAAT	Kilger and Grimont (1993)
fliC Tym	*R*- ACTCTTGCTGGCGGTGCGACTT	
spvA	*F*- GTCAGACCCGTAAACAGT	Del Cerro *et al.*,(2003)
	R- GCACGCAGAGTACCCGCA	
spvB	*F*- ACGCCTCAGCGATCCGCA	Del Cerro *et al.*, (2003)
	R- GTACAACATCTCCGAGTA	
spvC	*F*- GTCCTTGCTCGTTTACGACCTGAAT	Chui *et al.*,(2006)
	R –TCTCTTCTGCATTTCGTCA	
bla_{TEM}	*F* – ATGAGTATTCAACATTTCCG	Aerestrup *et al.*,(2003)
	R- ACCAATGCTTAATCAGTGAG	
sul2	*F*- CACTGCCACAAGCCGTAA	Aerestrup *et al.*,(2003)
	R - GTCCGCCTCAGCAATATC	

F – Forward primer

R – Reverse primer

3.6.2 PCR amplification of invA gene sequences in Salmonella species, according to protocol by Chui et al., (2006), with modifications

PCR amplification of *invA* gene was performed using Ready-To-Go PCR beads (GE Healthcare UK Ltd Chalfont Buckingamshire UK) as summarized (Table 2) below:

Table 2. PCR master mix for DNA amplification reaction according to manufacturer's instructions

Component	Quantity for 1 reaction	Concentration
PCR H_2O	8.5µl	
Ready- to- Go PCR beads		
dNTPs		200Um
$MgCl_2$		1.5mM
Taq pol		2.5U
Forward Primer	6.25 µl	12.5pmol
Reverse Primer	6.25 µl	12.5pmol
DNA template	4 µl	5ng
Total Volume	**25** µl	

Amplification reactions were carried out using published primers listed in (Table 1). Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific Finland). The prepared mixtures were amplified according to conditions (Table 3) below:

Table 3. *invA* DNA amplification conditions

Reaction	Temperature	Time	cycles
Initial denaturation	94^0C	5min	
Denaturation	94^0C	1min	30cycles
Annealing	57^0C	1min	
Extension	72^0C	30sec	
Final extension	72^0C	7min	
Hold	4^0C	∞	

3.6.3 Agarose Gel Electrophoresis

PCR amplification products were loaded onto 1.5% agarose gel containing ethidium bromide alongside 0.5µg/ul; Fermentas: GeneRuler$_{TM}$ 1kbp DNA ladder and electrophoresed at 100 volts for 35min, visualized under Ultra violet light. Presence of bands on the gel indicated successful amplification of the DNA.

3.7.0 PCR amplification of spvA , spvB and spvC gene sequence in Salmonella species according to protocol by Del Cerro et al., (2003) with modifications

Amplification of each of *spv* genes: *spvA, spvB,* and *spvC* genes were performed in a final reaction volume of 25 µl (Table 2). Amplification reactions were carried out using published primers listed in Table 1. Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific Finland). The prepared mixtures were amplified according to conditions (Table 4) below:

Table 4. *spvA, spvB, spv C* DNA amplification conditions

Reaction	Temperature	Time	cycles
Initial denaturation	94^{0}C	5min	
Denaturation	94^{0}C	30sec	30cycles
Annealing	60^{0}C	30sec	
Extension	72^{0}C	1min	
Final extension	72^{0}C	7min	
Hold	4^{0}C	∞	

3.7.1 PCR amplification of blaTEM gene sequence in Salmonella species according to protocol by Aarestrup et al., (2003) with modifications

Amplification of *bla*$_{TEM}$ gene was performed in a final volume of 25 µl (Table 2). Amplification reactions were carried out using published primers listed in Table 1. Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific Finland). The prepared mixtures were amplified according to conditions (Table 5) below:

Table 5. *bla*$_{TEM}$ DNA amplification conditions

Reaction	Temperature	Time	cycles
Initial denaturation	95^{0}C	10min	
Denaturation	95^{0}C	30sec	30cycles
Annealing	55^{0}C	1min	
Extension	72^{0}C	1min	
Final extension	72^{0}C	7min	
Hold	4^{0}C	∞	

3.7.2 PCR amplification of sul2 gene sequence in Salmonella species according to protocol by Aarestrup et al., (2003) with modifications

Amplification of *sul2* gene was performed in a reaction volume of 25 µl (Table 2). Amplification reactions were carried out using published primers listed in Table 1. Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific Finland). The prepared mixtures were amplified according to conditions (Table 6) below:

Table 6. *sul2* DNA amplification conditions

Reaction	Temperature	Time	cycles
Initial denaturation	95^0C	8min	
Denaturation	95^0C	30sec	30cycles
Annealing	56^0C	1min	
Extension	72^0C	1min	
Final extension	72^0C	7min	
Hold	4^0C	∞	

3.7.3 PCR amplification of fliC gene sequence in Salmonella species according to protocol by Kilger and Grimont, (1993) ,with minor modifications

Amplification of *fliC* gene was performed in a reaction volume of 25 µl (Table 2). Amplification reactions were carried out using published primers listed in Table 1. Amplification was carried out in ARKTIK thermocycler (Thermofisher Scientific Finland). The prepared mixtures were amplified according to conditions (Table 7) below:

Table 7. *fliC* DNA amplification conditions

Reaction	Temperature	Time	cycles
Initial denaturation	95^0C	5min	
Denaturation	95^0C	1min	30cycles
Annealing	57^0C	1min	
Extension	72^0C	2min	
Final extension	72^0C	7min	
Hold	4^0C	∞	

3.7.4 Purification of PCR products

Prior to sequencing, *invA, spvC* and *fliC* PCR products were purified using Gene-Jet PCR purification kit (Inqaba biotechnology, South Africa) to retrieve DNA and to remove undesirable remnants of PCR, following manufacturer's protocol (Appendix III).

3.7.5 Salmonella invA, spvC DNA sequencing

Purified gene fragments of each of *invA* and *spv*C were subjected to direct sequencing in the forward and reverse directions using the same forward and reverse primers as in the PCR (Table 1) using the Big Dye Terminator 3.1 cycle sequencing Ready Reaction kit (Applied Biosystems). The automated sequence data were analyzed using the SEQUENCHER v.3.0 software (Gene Codes Corporation, Inc.). Chromatographs (Appendix III) were visually inspected and consensus sequences were aligned manually prior to further analysis. *Salmonella invA, spvC* gene sequencing was performed according to protocol by the ABI PRISM Big Dye Terminator Cycle Sequencing Ready Reaction Kit Protocol (Appendix IV).

3.7.5.1 Single locus sequence typing (SLST) of S.typhimurium using fliC gene

Purified gene fragments of *fliC* gene were subjected to SLST using forward and reverse primers as in PCR (Table 1) using the Big Dye Terminator 3.1 cycle sequencing Ready Reaction kit (Applied Biosystems). Sequencing was performed in a reaction volume of 20 µl which included , 8.5 µl H2O, 3 µl 5 x reaction buffer, 3 µl PCR product (20-80 ng), 3 µl Primer (= 6 pmol), 0.5 µl Pellet Paint, 2 µl Big Dye (includes 2.5 x reaction buffer). The following sequencing conditions were used: 2 min 95^0C initial denaturation, 1 min 94^0C denaturation, 30 sec 50^0C annealing, 4 min 72^0C extension, then 1 min 94^0C denaturation and repeat 25 cycles followed by 4^0C indefinite to stop the reaction. The extended extension time allows the AmpliTaq DNA polymerase to incorporate "gigantic" dye terminators. The automated sequence data were analyzed using the SEQUENCHER v.3.0 software (Gene Codes Corporation, Inc.). Chromatographs (Appendix IV) were visually inspected and consensus sequences were aligned manually prior to further analysis.

3.8 Data management

3.8.1 Genetic phylogenetic analysis of *S.Typhimurium* strains:

i. Contiguous sequences of the *fliC* gene were created from forward and reverse chromatograms and edited using BioEdit software. Multiple alignments of the contigs was conducted using Muscle 3.8.3.1 (http://update.musclesoftware.net.download.php) multiple alignment software, in which the most similar pair were aligned first and then progressively more distant pairs were added. Nucleotide sequence data of *fliC* gene sequences from all clusters were submitted to Basic Local Alignment Search Tool (BLAST) www.ncbi.nlm.nih.gov/blast) for

similarity searches in Gen Bank. Phylogenetic trees for *S. Typhimurium* using *fliC* gene were constructed based on mrBayes software (http://mrbayes.net), a program for the Bayesian inference of phylogeny that is based on the Markov Chain Monte Carlos (MCMC), bootstrap (MCMC) clades, distance and rooting

ii. The distribution of different serovars and prevalence of NTS among patients was analyzed using chi- square statistical method. Difference in antimicrobial susceptibility was analyzed using chi-square.

iii. Gel images were analyzed using the bionumerics software package version 3.5 (Applied-maths sint-martens-laterm, Belgium).

iv. Agar disk diffusion (ADD) - Kirby Bauer test was interpreted according to the guidelines provided by the clinical and laboratory standards institute (CLSI, 2011).

CHAPTER FOUR

RESULTS

4.1. Distribution of *Salmonella enterica serovars* in Kapsabet and Kisumu District hospitals

The distribution of NTS serovars obtained from patients in Kapsabet and Kisumu District hospitals is shown (Table 8).

Table 8: Distribution of NTS serovars from Kapsabet and Kisumu patients

NTS Serovars recovered	No. of NTS isolates from respective hospitals:	
	Kapsabet (n=97)	**Kisumu (n=77)**
S. Typhimurium	51 (52.6%)	44(57.1%)
S. Enteritidis	26 (26.8%)	19(24.6%)
S. Paratyphi A	4 (4.1%)	0(0%)
S. Paratyphi C	6 (6.2%)	6(7.8%)
S. Gallinarum	2 (2.0%)	0(0%)
S. Dublin	2 (2.0%)	0(0%)
S. Thompson	6 (6.2%)	3(3.9%)
S. Bareilly	0(0%)	5 (6.5%)

The predominant *Salmonella* isolate from both study sites was *S. Typhimurium,* followed by *S. Enteritidis*. The other serovars constituted a small proportion. Kapsabet had significantly high distribution of different serovars of *Salmonella* at p-value 0.0288 ($\alpha < 0.05$) using one tailed spearman correlation coefficient .

4.2 Prevalence of non typhoidal *Salmonella* (NTS) among various age groups from Kapsabet and Kisumu District hospitals.

The prevalence of NTS among age groups from Kapsabet and Kisumu is shown (Table 9).

Table 9: Prevalence of NTS isolates among different age groups in Kapsabet and Kisumu District hospital patients

Age group (years)	Percentage of NTS recovered from different age groups	
	Kapsabet (n=97)	Kisumu (n=77)
0- 5	42.27	46.75
6-10	4.12	6.49
11-15	3.09	7.79
16-20	4.12	6.49
21-25	6.18	7.79
26-30	9.27	9.09
31-35	5.15	2.59
36-40	6.18	3.89
41-45	9.27	5.19
46-50	10.30	3.89

In Kapsabet, 42.27% (n=41) of patients aged ≤ 5 years and 57.73% (n=56) of patients aged > 5years presented with non typhoidal *Salmonella* infection. In Kisumu 46.75% (n=36) of patients aged ≤ 5 years and 53.25% (n=41) of patients aged > 5years presented with non typhoidal infection . The difference in prevalence of NTS among patients in the two study areas was not significant at p value = 0.0793, using paired t-test.

4.3 Antibiotic susceptibility of NTS from Kapsabet and Kisumu District hospitals

A total of 174 NTS isolates were tested for susceptibility to 8 antimicrobials in both study areas. In Kapsabet (Table 10), all the isolates 100% (n=97) were susceptible to three antimicrobials, viz;- gentamicin (MIC_{90}= 0.02µg/ml), kanamycin (MIC_{90}= 0.01µg/ml) and chloramphenicol (MIC_{90}= 0.12µg/ml). Resistance to ampicillin was 99% (MIC_{90}= 250µg/ml), followed by sulfamethoxazole at 19% (MIC_{90}= 32µg/ml), resistance to tetracycline (MIC_{90}= 0.200µg/ml), cotrimoxazole (MIC_{90}= 32µg/ml) and streptomycin (MIC_{90}= 252µg/ml) was less often at 6%.

In Kisumu (Table 11), all the isolates were resistant to ampicillin (MIC_{90} = 250 µg/ml) at 100% (n=77). Resistance to chloramphenicol (MIC_{90}= 256µg/ml) and tetracycline (MIC_{90}= 200µg/ml) was 96% , this was followed by resistance to streptomycin (MIC_{90}= 252µg/ml) at 92%, sulfamethoxazole (MIC_{90}= 32µg/ml) at 91%, gentamicin (MIC_{90}= 215µg/ml) at 84%, kanamycin (MIC_{90}= 214µg/ml) at 80% and cotrimoxazole (MIC_{90}= 32µg/ml) displayed the least resistance at 76%. A total of 70% of *Salmonella* isolates from Kisumu and 50% of isolates from Kapsabet were multidrug resistant (MDR) phenotype i.e resistant to ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT). Most common antibiotic treatment choices used for NTS infections in both health facilities was available (Appendix V).

Table 10: Antimicrobial susceptibility of *Salmonella* by disc diffusion of (n= 97) non typhoidal *Salmonella* isolates from Kapsabet

Antimicrobial Agent tested	Minimum inhibition concentration (MIC_{90})	Zone of diameter nearest whole mm			% Resistance	% Intermediate	% Sensitive
		R	I	S			
Ampicillin	0.25 - >256	≤13	14-16	≥17	99	1	0
Tetracycline	0.064 - >256	≤14	15-18	≥19	6	3	91
Cotrimoxazole	0.064 - >32	≤10	10-15	≥16	6	3	91
Streptomycin	0.38 - >256	≤12	13-16	≥16	6	6	88
Kanamycin	0.06 - >256	≤12	13-14	≥15	0	0	100
Gentamicin	0.06 - >256	≤12	13-14	≥15	0	0	100
Sulfamethoxazole	0.032-32	≤10	11-15	≥16	19	3	78
Chloramphenicol	0.19 - >256	≤12	13-17	≥18	0	0	100

Table 11: Antimicrobial susceptibility of *Salmonella* by disc diffusion of (n = 77) non typhoidal *Salmonella* isolates from Kisumu

Antimicrobial Agent tested	Minimum inhibition concentration (MIC_{90})	Zone of diameter nearest whole mm			% Resistance	% Intermediate	% Sensitive
		R	I	S			
Ampicillin	0.25 - >256	≤13	14-16	≥17	100	0	0
Tetracycline	0.064 - >256	≤14	15-18	≥19	96	0	4
Cotrimoxazole	0.064 - >32	≤10	10-15	≥16	76	4	20
Streptomycin	0.38 - >256	≤12	13-16	≥16	92	0	8
Kanamycin	0.06 - >256	≤12	13-14	≥15	80	0	20
Gentamicin	0.06 - >256	≤12	13-14	≥15	84	4	12
Sulfamethoxazole	0.032-32	≤10	11-15	≥16	91	1	8
Chloramphenicol	0.19 - >256	≤12	13-17	≥18	96	0	4

Comparison of antimicrobial susceptibility data between Kapsabet and Kisumu revealed significantly high antimicrobial resistance in Kisumu than Kapsabet at p value < 0.001 using chi square.

4.4 *Salmonella* antimicrobial resistance genes bla_{TEM} and *sul2*

Genetic analysis of *Salmonella* antimicrobial resistance genes by Polymerase Chain Reaction (PCR) were found to correlate to those of phenotypic antimicrobial susceptibility (Tables 10 and 11). A 643bp was obtained for all the isolates positive for bla_{TEM} gene (Fig. 2). Analysis of *sul2* genes displayed a band of 331bp responsible for sulfamethoxazole resistance in isolates (Fig. 3).

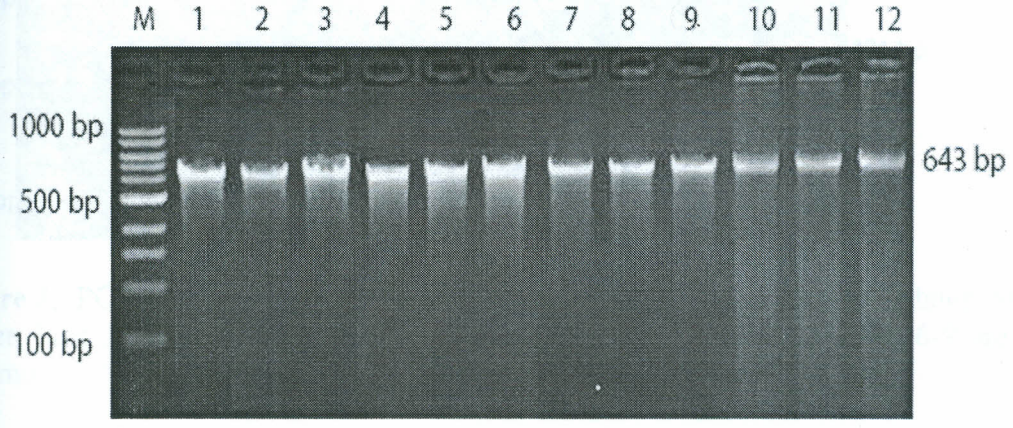

Figure 2. PCR gel showing positive bla_{TEM} gene products for *Salmonella* isolates. M: 100bp DNA ladder, lane 1, positive control, lanes 2-6 are isolates from Kapsabet, lanes 7-12 are isolates from Kisumu.

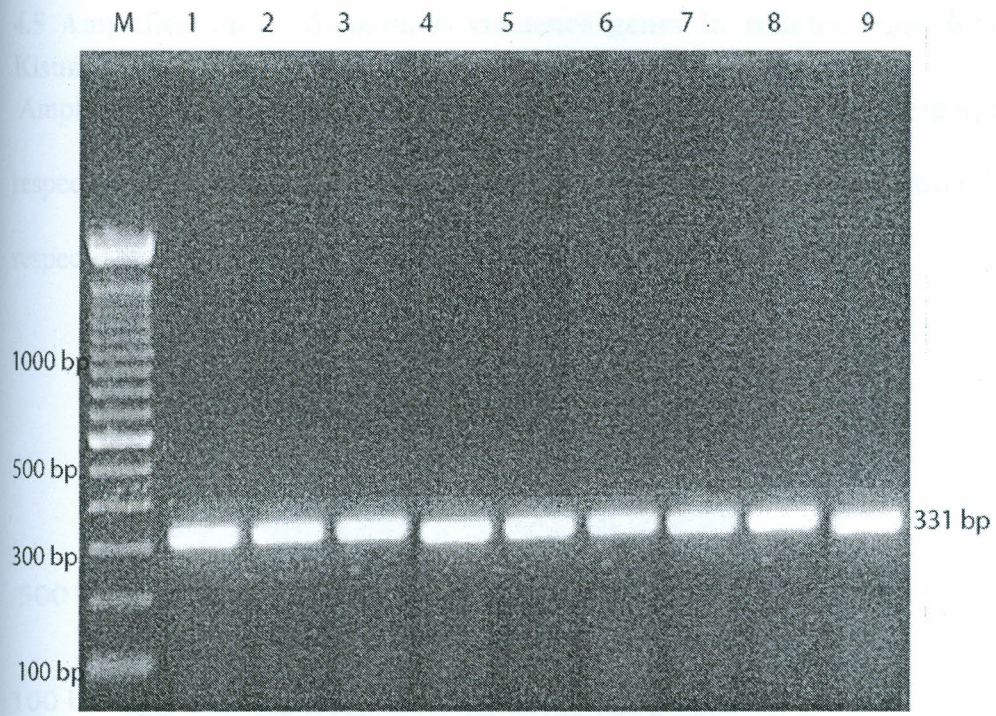

Figure 3. PCR gel showing positive *sul2* gene products for *Salmonella* isolates. M: 100bp DNA ladder, lane 1, positive control, lanes 2-5 isolates from Kapsabet, lanes 6-9 are isolates from Kisumu.

Table 12: Comparison between genotypes and antibiotic susceptibility profile

Genotype	**% Resistance**		**% intermediate**		**% sensitive**	
	KAP	KIS	KAP	KIS	KAP	KIS
bla_{TEM}	99	100	1	0	0	0
sul2	19	91	3	1	78	8

Comparison of genotype and susceptibility pattern between Kapsabet and Kisumu using chi-square revealed significant difference ($p < 0.001$).

4.5 Amplification of *Salmonella* virulence genes in isolates from Kapsabet and Kisumu District hospitals

Amplification of *spvA* gene and *spvB* gene gave bands of 604bp (Fig.4) and 1063bp (Fig.5) respectively in *Salmonella* isolates from Kapsabet and Kisumu District hospitals respectively.

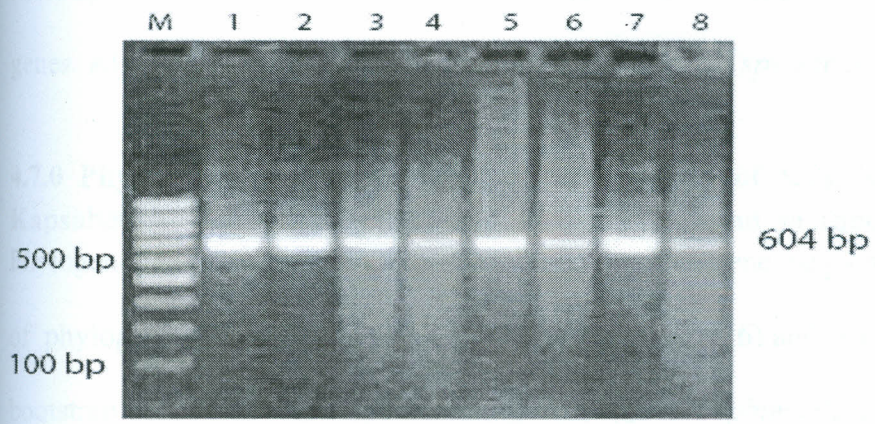

Figure 4: PCR gel showing positive *spvA* gene products for *Salmonella* isolates. M: 100bp DNA ladder, lane 1, positive control, lanes 2-5 are isolates from Kapsabet, lanes 6-8 isolates from Kisumu.

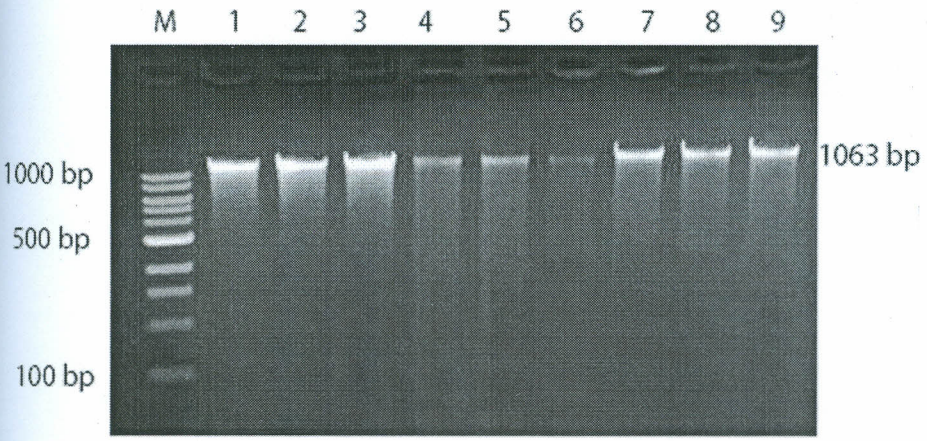

Figure 5: PCR gel showing positive *spvB* gene products for *Salmonella* isolates.M: 100bp DNA ladder, lane 1, positive control, lanes 2-5 are isolates from Kapsabet , lanes 6-9 isolates from Kisumu .

4.6 Genetic characterization of *Salmonella* isolates for *fliC* and *spvC* genes

A total of 54.6% (n=95) *Salmonella Typhimurium* isolates were found to posses *fliC* gene (559bp). A total of 11.8% (6/51) of *S. Typhimurium* isolates from Kapsabet did not display *spvC* (571bp) genes (Table 13; Appendix). 4.54% (2/44) of *S. Typhimurium* isolates from Kisumu did not display *spvC* gene , while 4.54% (2/45) of all *S. Enteritidis* isolates did not display *spv* genes. All the other isolates did not display both *fliC* and *spv* genes.

4.7.0 Phylogenetic analyses of *fliC* gene sequences of *S.Typhimurium* isolates from Kapsabet and Kisumu District hospitals using Bayesian method of phylogeny

Phylogenetic analyses were conducted based on *fliC* gene sequences using Bayesian method of phylogeny.The resultant trees from Kapsabet (Fig. 6) and Kisumu (Fig.7) presented varied bootstrap consensus values, confirming that the *S.Typhimurium* isolates from the two study areas are genetically diverse, but belong to *Salmonella enterica* subsp. serovar *Typhimurium* str. DT2 and *Salmonella enterica* serovar *Typhimurium* str. UK-1 ancestral lineage.

Figure 6. Phylogenetic tree for *S. Typhimurium* isolates from Kapsabet District hospital based on *fliC* gene sequences. The tree was constructed in mrBayes, a program for inference of phylogeny that is based on the Markov Chain Monte Carlos (MCMC) method. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.

Bayesian analyses of *fliC* gene sequences from Kapsabet (Fig.6) revealed that strains KPH 017,001 are clustered in a clade which is a sister clade to KPH 013, 062. KPH 011, 022 and KPH 013, 062 are paraphyletic group. KPH 007,036 are clustered in a clade which is a sister clade to KPH 020, 051. KPH 030,075 and KPH 007, 036 are paraphyletic group. KPH 005, 054 and KPH 020,051 are paraphyletic group. The mean interclade diversity ranged between 2.1% and 6.6%, with overall interclade diversity of 4.4%. All the clades are rooted on *S. Typhimurium* strain UK-1.

4.7.2 Phylogenetic tree for S.Typhimurium strains from Kisumu District hospital

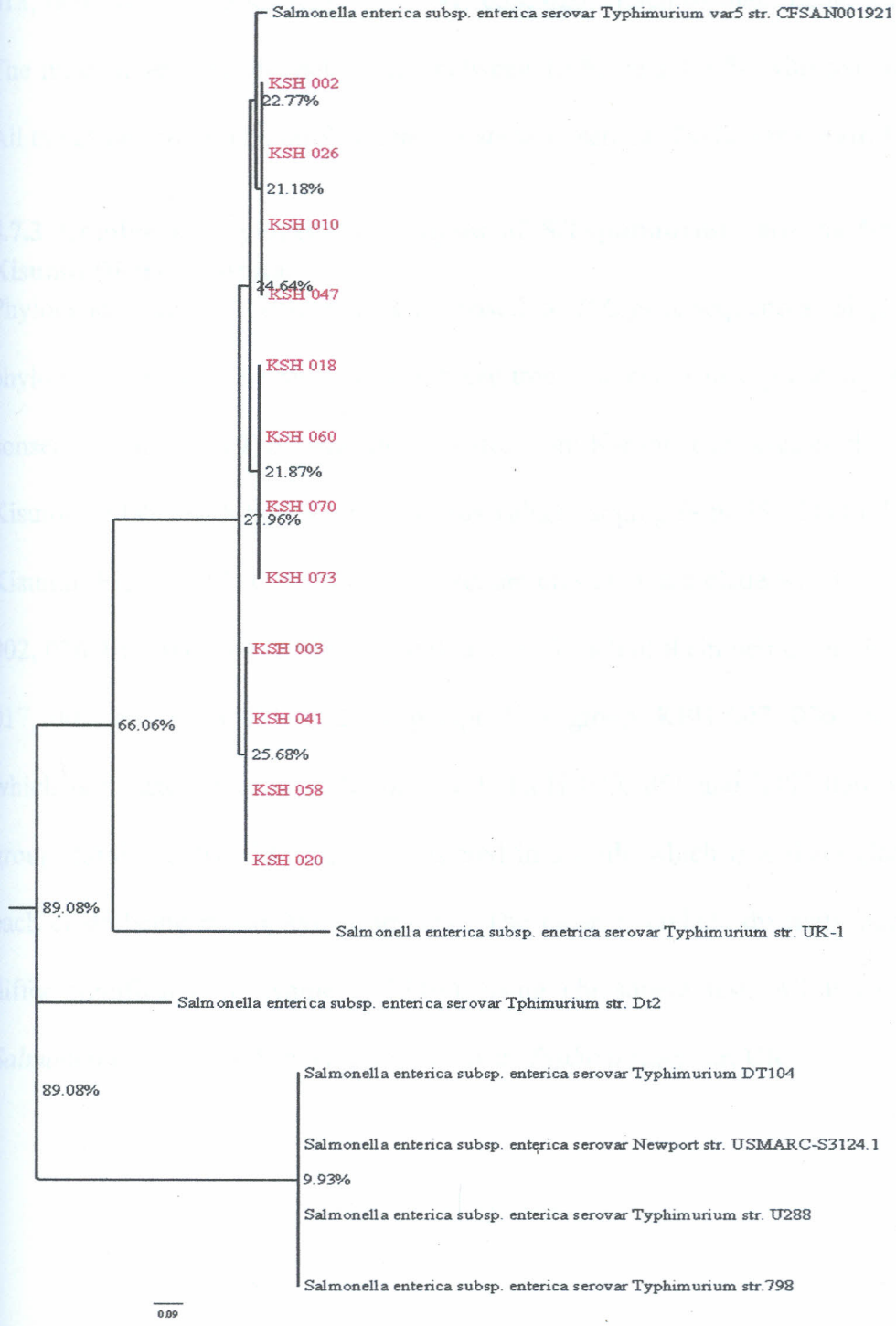

Figure 7. Phylogenetic tree for *S. Typhimurium* isolates from Kisumu District hospital based on *fliC* gene sequences. The tree was constructed in mrBayes, a program for inference of phylogeny that is based on the Markov Chain Monte Carlos (MCMC) method. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.

Strains KSH 002, 026, 010, 047 (Fig.7) are clustered in a clade which is a sister clade to KSH 018, 060, 070, 073 and KSH 003, 041, 058, 020. Each of them being a monophyletic group. The mean interclade diversity range between 1.6% and 4.3 % with overall diversity of 3.1%. All the clades are rooted on *Salmonella enterica* serovar *Typhimurium* str. UK-1.

4.7.3 Combined Phylogenetic analyses of S.Typhimurium strains from Kapsabet and Kisumu District hospital

Phylogenetic analyses were conducted based on *fliC* gene sequences using Bayesian method of phylogeny (Fig.8). The resultant combined tree is shown below, presented with varied bootstrap consensus values. *S.Typhimurium* isolates from Kapsabet are clustered in separate clade from Kisumu isolates with bootstrap consensus values ranging from 55.92% to 72.47%. Strains from Kisumu (Fig.8) KSH 003, 041, 058, 020, are clustered in a clade which is a sister clade to KSH 002, 026, 010, 047 and KSH 018, 060, 070, 073 each of them being a monophyletic group. KPH 017, 001, and KPH 011, 022 are paraphyletic group. KPH 007, 036 are clustered in a clade which is a sister clade to KPH 020, 051. KPH 020, 051 and KPH 030, 075 are paraphyletic group. Strains KPH 013, 062 are clustered in a clade which is a sister clade to KPH 005, 054 each clade being monophyletic groups. The mean interclade diversity between distinct clades differ significantly (p value < 0.001), using chi square test. All the clades are rooted on *Salmonella enterica* subsp. *enterica* serovar *Typhimurium* str. UK-1.

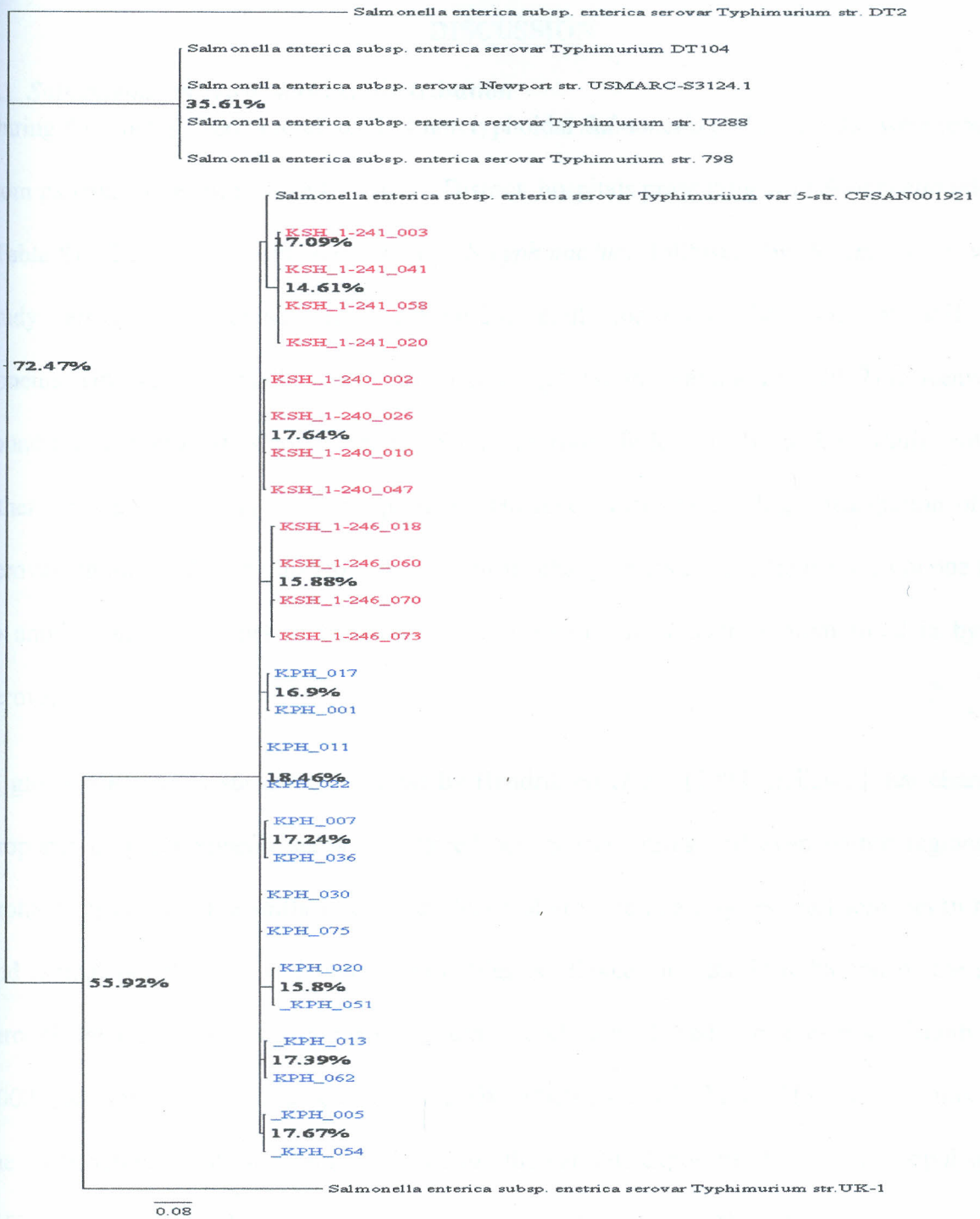

Figure 8. Combined phylogenetic tree for *S. Typhimurium* isolates from Kapsabet and Kisumu District hospitals based on *fliC* gene sequences. The tree was constructed in mrBayes, a program for inference of phylogeny. Numbers at the nodes indicate percentage of occurrence in 1000 bootstrapped trees.

CHAPTER FIVE

DISCUSSION

5.1 *Salmonella enterica* serovar distribution

During the study period, a total of 174 non typhoidal *Salmonella* (NTS) isolates were recovered from patients in Kapsabet and Kisumu District hospitals presenting with *Salmonella* infection (Table 8). The predominant serovar was *S.Typhimurium* followed by *S. Enteritidis* in both study areas. The other serovars constituted a small proportion. The results of this study concurs with related studies by Kariuki *et al.,* (2006) and Tabu *et al.,* (2012) in Kenya who found the commonest serovar to be *S.Typhimurium* followed by *S. Enteritidis* with the other serovars being in small proportion. However, there was a high distribution of other serovars in this study. The distribution of serovars change from time to time and from one region to another and the niches created by the decrease of one serovar is often filled in by other serovars.

A global *Salmonella* surveillance report by Hendriksen *et al.,* (2008) indicated that changes in proportions of *Salmonella* serovars differed between countries and even within regions. The probable reason for the shifts in the prevalence of specific strain types and serovars in human and animal populations in developed countries is linked to the introduction of the strains through international travel, human migration, food, animal feed and livestock (Crump *et al.*, 2002; Aerestrup 2007; Hendriksen *et al.*, 2008; Ethelberg *et al.,* 2010). However, in this study the distribution of the serovars is linked to the variable exposure of the study population to different animal and plant sources, use of unsafe drinking water, the different food processing procedures used for various animal products as reported by Wandili *et al.*, (2011) and difference in susceptibility of hosts to certain serovars.

In Kenya, Kisumu is characterized by high temperatures, heavy rainfall and frequent flooding while Kapsabet is characterized by low temperatures, heavy rainfall that ends up in open water sources. These are environmental stresses that lead to micro-evolution of virulence gene expression in *Salmonella*. Such mechanisms are geared towards adaptation in changing environment they protect *Salmonella* from various stress conditions as well as antibiotics (Kussel *et al.*, 2005). Some of the conditions attributed to this as documented by Rogers *et al.*, (2011) include; development of mutator phenotypes, adaptive mutation, bacterial persistence phenotypes, phase variation. In addition, *Salmonella* triggers synthesis of heat shock proteins to enhance thermal tolerance and express outer membrane proteins (OmpC and OmpF) to respond to osmotic stress.

Kapsabet had significantly high distribution of different serovars of *Salmonella* (p value = 0.0288). Among them is *S. Thompson* which is transmitted through plant sources and it is more prevalent in Europe and North America as reported by Hendriksen *et al.*, (2011), and hence could have been transmitted through human mobility. *Salmonella Dublin* causes systemic infection in cattle while *S. Gallinarum* is associated with salmonellosis in fowl. Thus due to agricultural nature of the area, these strains could have been disseminated through food animals.

The predominant isolate in this study belonged to *S.Typhimurium* strain DT2 lineage in both study areas. *Salmonella enterica serovar Typhimurium* DT2 is highly associated with systemic disease in pigeons. Previous studies by Helms *et al.*, (2004) indicate that pigeon associated strains share many qualities with host adapted strains. Therefore the presence of these strains is associated with interaction with the primary host directly or indirectly through contamination of food sources. This implies that failure to control *Salmonella* in this studyareas presents a potential problem for the other regions in Kenya and beyond.

5.2 Prevalence of NTS infection among study population

The highest prevalence of NTS was recorded in patients of age group ≤ 0-5 years in both study areas. The total prevalence of NTS in both areas was 44.25% (77/174) in patients of age group ≤ 5 years and the overall prevalence in patients of age group > 5 years was 57.47% (100/174). This implies that patients of age group ≤ 5 years were at risk of NTS infection in both study areas. These results are in line with previous studies in sub Saharan Africa by Feasey *et al.*, (2012), Okoro *et al.*, (2012) who reported high prevalence of NTS among children of the same age group. Rono *et al.*, (2014) also reported high prevalence of bacterial diarrhea in children aged <5 years admitted at Moi Teaching and referral hospital. In Kisumu fish has been implicated in spread of salmonellosis as documented by Wandili *et al.,* (2011). However, there was relatively high prevalence of NTS infection in patients of age group ≤ 5years in Kisumu than Kapsabet. This could be attributed to difference in climatic conditions in the two areas. Non typhoidal *Salmonella* infection in western Kenya has been associated with malaria prevalence since 2008 as documented by Tabu *et al.,* (2012). Oneko *et al.*, (2014) (unpublished data) in western Kenya also found that NTS in HIV negative children was closely related to recent or concurrent malaria. Therefore comorbidity with other infections e.g current or recent malaria and malarial anemia, could be responsible for the difference in prevalence in the two areas. Thus measures directed at malaria control may reduce invasive NTS in study areas.

In this study, two main *Salmonella* serovars were recovered from patients, *S.Typhimurium* and *S. Enteritidis.* The results concur with findings by Oneko *et al.,* (2014) (unpublished data) who found almost similar results from patients treated at Siaya District hospital. However, this results contrasts findings by Onyango *et al.,* (2008) in western Kenya who found all isolates from patients treated at Maseno Mission hospital to be only *S. Typhimurium.*

However, a major limitation of the study was that it was hospital based and hence patients who do not seek treatment in health facilities were not captured. Therefore the health seeking behaviour of the population affects the outcome of the study. In addition, the study concentrated on individuals with diarrhoeal NTS while NTS clinical features are diverse including bacteremia.

5.3 Genetic diversity of *S. Typhimurium* from study areas

The principal findings of this study indicated that *S.Typhimurium* isolates from the two study areas are genetically diverse with significant difference in interclade diversity (p value < 0.001). The results based on *fliC* gene sequences using Bayesian method of phylogeny indicated that there was intra and inter genetic diversity (Figs. 6,7 and 8).

Phylogenetic analysis of *S.Typhimurium* strains from Kapsabet (Fig.6) comprised of both monophyletic and paraphyletic groups. This is was due to genetic diversity within the isolates resulting to interclade diversity. The low bootstrap values could be due low number of characters as suggested by Harrison and Langdale, (2006). All the clades are rooted on *S.Typhimurium* strain UK-1 with bootstrap consensus value of 64.49% this implies that they belong to the same ancestral lineage.

Phylogenetic tree (Fig.7) depicts *S. Typhimurium* strains from Kisumu. The strains comprises of monophyletic group, implying that tree includes most recent common ancestor with all its descendants. All the clades are rooted on *S. enterica* serovar *Typhimurium* str. UK-1 with bootstrap consensus value of 66.06% implying that they belong to the same ancestral lineage but are new members of the lineage.

Gain and loss of genes during evolution of *Salmonella* is one of the mechanisms that drives diversification within the bacteria as documented by Retchless and Lawrence (2007). The low bootstrap values indicate high level of incongruence between individual trees. In this study phylogenetic tree was based on *fliC* gene, a gene that encode surface structure and hence under significant recombination pressure that contributes to genetic diversity. This is in agreement with findings by Prerak *et al.,* (2013) who noted that recently acquired AT- rich regions which encode virulence functions are undergoing selection to maintain high AT content. This could account for obscured phylogeny in this study. This results concurs with findings by Brown *et al.,* (2003) who found that a phylogeny derived from DNA that has been acquired laterally would display incongruence (phylogenetic discordance) when compared with evolutionary trees constructed from stable house keeping genes or whole chromosome. The low consensus bootstrap values indicates that recombination has occurred at a frequency sufficiently high to liminated phylogenetic signal as documented by Octavia and Lan (2006).

This could probably be linked to recombination mechanism that produce genomic diversity as documented by Baker *et al.*, (2010). *Salmonella* subspecies I strains share a common niche, one restricted to warm blooded animals, and endowed with compatible restriction modification systems that allow incorporation of larger gene segments of DNA among closely related *Salmonella* pathogens. The results of this study also concurs with findings by Brown *et al.,* (2003), Soyer *et al.,* (2009) and Didelot *et al.,* (2011) who found that *Salmonella* genomes undergo frequent intergenome recombinations and thereby disrupt the clonal inheritance pattern resulting to genetic diversity.

Comparative phylogenetic analysis of *S.Typhimurium* isolates from Kapsabet and Kisumu indicated that isolates from Kapsabet (Fig.8) indicated a significant interclade diversity (p

value = 0.001) using chi square. Genetic diversity correlated with antimicrobial resistance (Pearson correlation, P = 0.0258). The clades comprised of both monophyletic and paraphyletic groups, this accounted for significant interclade diversity. All the clades are rooted on *S. enterica* serovar *Typhimurium* str. UK-1 with bootstrap consensus value of 55.92% (<97%), this implies that they are highly diverse but have evolved from common ancestral lineage.

The above phylogenetic tree also indicates that *S. Typhimurium* strains from the two study areas are clustered in separate clades and hence *S. Typhimurium* isolates from the two study areas exhibited a geographic restriction. Geographical clustering may be a reflection of the clustering of local environmental factors and thus adaptation to prevailing environmental conditions. A study by Lewin *et al.,* (2010) found that the clustering of specific strains of *Salmonella* in Sweden correlated with the clustering of susceptible herds. Previous study by Brown *et al.,* (2003) reported that the distribution of some bacteria may be related to geographical patterns such as climatic zones and movement of human population. The two study areas are geographically variable.

5.4 Antimicrobial susceptibility pattern

In this study, a total of 174 *Salmonella* isolates were tested for their antimicrobial susceptibility profile. A total of 97 (100%) of the isolates from Kapsabet were fully susceptible to three antimicrobials; gentamicin, kanamycin and chloramphenicol. Susceptibility to the same antimicrobials was relatively low in Kisumu at 12%, 20% and 4% for gentamicin, kanamycin and chloramphenicol respectively. This indicates a decline in NTS susceptibility to these commonly used antimicrobials in the study areas. Previous study by Velge *et al.,* (2004) reported that resistance to antibiotics within a population is linked to widespread prescription of antibiotics for other infections.

In developing countries, multidrug resistance (MDR), particularly to commonly available antimicrobials remains a major challenge for the healthcare system (Bonfiglio *et al.,* 2002; Kariuki *et al.,* 2005; Kariuki *et al.,* 2010). In this study, 99% of the isolates in Kapsabet were resistant to ampicillin , followed by sulfamethoxazole at 19%, resistance to tetracycline, cotrimoxazole and streptomycin was low at 6%.

In Kisumu, all the isolates 100% (n=77) were resistant to ampicillin, followed by resistance to chloramphenicol and tetracycline at 96%, streptomycin at 92%, sulfamethoxazole at 91%, cotrimoxazole was least resistant at 76% . This is contrary to study by Kariuki *et al.,* (2010) who reported a total of 23.4% (n=45) of NTS were fully susceptible to all antimicrobials tested including ;ampicillin, tetracycline, cotrimoxazole, chloramphenicol. In addition, only 5% (n=45) of NTS were resistant to ampicillin. The results implies that there is increase in resistance to commonly available antimicrobials, and the highest resistance was recorded in ampicillin.

Ampicillin is one of the most available and commonly prescribed antibiotic in both hospitals as indicated by data from the two hospitals. It is available in combined form as ampiclox, and prescribed for respiratory infections. Ampicillin in combined form, ampiclox and uncombined form is easily sold over the counter without prescription. Therefore, widespread use and availability could have accounted for high resistance in study areas.

In this study, the resistance phenotype was ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT). Hence a total of 68.96% (120/174) of the isolates exhibited MDR phenotype. These results are consistent with findings by NARMS, (2010), which found 4.3% (107/2474) of non typhoidal *Salmonella* isolates were ACSSuT- resistant, including

18.6% (68/366) of *Salmonella* serovar *Typhimurium*. In addition, study by Kariuki *et al.*, (2010) reported that 34.2% (n=66) of NTS isolates from human subjects, were resistant to three or more antibiotics in Kenya. The most common resistant phenotypes were ampicillin, tetracycline and cotrimoxazole in 75% of the isolates. Resistance to tetracycline, gentamicin, chloramphenicol or ampicillin was low at 15%. This is contrary to this study in which the highest resistance was observed in ampicillin at 100% and 99% in Kisumu and Kapsabet respectively. These shows increase in resistance compared to previous study. Comparatively, antimicrobial resistance was significantly high in Kisumu than Kapsabet at $p< 0.001$.

This implies that there is a wide spread use of these antimicrobials in Kisumu. The results from Kisumu are consistent with previous studies by Brooks *et al.*, (2003; 2006) in western Kenya who recorded high resistance to most commonly used antimicrobials. Tabu *et al.*, (2012) in Lwak area of western Kenya also recorded high resistance by *S. Typhimurium* to most antimicrobials tested. A study by Geissler *et al.*, (2000) in western Kenya established prevalence of self- treatment of common illnesses.

Genetic characterization of antimicrobials resistance genes, their location and diversity is important in identifying factors involved in resistance (Menggen *et al.*, 2007). In this study, bla_{TEM} (643bp) gene was expressed in ampicillin resistant isolates. bla_{TEM} belong to the TEM family of β –lactamase genes. bla_{TEM} genes have a tendency to mutate and code for β -lactamase with extended spectrum of activity. The emergence of resistance to β -lactam antimicrobial agent (ampicillin) is a challenge to management of adult and paediatric infections in resource poor settings in Kenya.

Sulfonamide resistance was mediated by *sul2* gene (331bp) encoding forms of dihydropteroate synthase that are not inhibited by sulfamethoxazole (Antunes *et al.*, 2005). Both bla_{TEM} and *sul2* genes are associated with class 1 integrons that can capture mobile gene cassette and plasmids, bla_{TEM} gene is located on transposon *Tn3* (Schwarz, 2000). *sul2* genes are located on small non conjugative plasmids or on large transmissible multiresistant plasmids (Aerestrup *et al.*, 2003). Hence resistance to sulfamethoxazole is hypothesized to be plasmid mediated as supported by the fact that ; 50.6% (88/174) of all *Salmonella* isolates harboured *sul2* gene. The association of genotypes and susceptibility pattern between *Salmonella* isolates from Kapsabet and Kisumu was significant at $p< 0.001$.

spv virulence locus is found on large plasmids in *Salmonella* subspecies I serovars, associated with severe infection. *spv* genes encode factors that modify host cell physiology and protect *Salmonella* from antimicrobial systems of the host (Fierer 2001; McQuiston *et al.*, 2004 and 2008). In this study, 80.46% (140/174) of all the isolates expressed *spv* genes. This could have accounted for high resistance to antibiotics tested. The location of *spv* genes on pathogenicity islands on transmissible genetic elements enhances their role in antimicrobial resistance.

A total of 4.54% (2/45) of all the *S.Enteritidis* isolates from Kapsabet did not display *spv* genes while 11.76% (6/51) of *S.Typhimurium* strains from Kapsabet did not express *spv* genes. *spv* genes encode factors that protect *Salmonella* from antimicrobial systems and this could have accounted for the low resistance to antimicrobials in Kapsabet.

Data set and Limitations

The main limitation in this study is that the internal bootstrap values were lower than recommended threshold for the isolates to be considered as closely related as documented by

Harrison and Langdale (2006). Bootstrapping may overestimate or underestimate phylogenetic accuracy depending on the condition under which data was generated. Low bootstrap value indicates low confidence or phylogenetic discordance. Thus the use of multiple genes or use of amino acids sequences instead of nucleotide sequences would improve the data as suggested by (Harrison and Langdale, 2006). Bootstrap values may also be affected by alignment errors. In this study alignment was done in musle 3.8.3.1 in addition to manually checking alignment to remove less confident parts.

In addition, two mechanisms that can also introduce phylogenetic discordance between gene trees are; Intergenomic recombination, this can reintroduce previously lost allelic changes into recombining population . Another mechanism could be incomplete lineage sorting, given the short branch lengths within the trees (Galtiers and Daubin, 2008). However, it is not possible to distinguish between these two mechanisms using data from contemporary *Salmonella* strains.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

1. The predominant serovar in both study areas was *S. Typhimurium* (54.59%) followed by *S. Enteritidis* (29.88%) the other serovars constituted a small proportion (15.53%).
2. The prevalence in NTS is linked to host factors as well as climatic factors in the study areas. The difference in prevalence of NTS among patients in both areas was statistically insignificant ($p= 0.0793$) using paired t-test.
3. *S. Typhimurium* in the study areas are genetically diverse. The dominant *S. Typhimurium* isolate in both areas belonged to *S. typhimurium* str. DT2 lineage.
4. There is generally low susceptibility to antimicrobials tested in Kisumu compared to Kapsabet $p<0.0001$, using chi square. The resistance phenotypes were ampicillin, chloramphenicol, streptomycin, sulfamethoxazole and tetracycline (ACSSuT).

6.2.1 Recommendations

1. The frequency of variation in distribution of serovars in study areas and the wider western Kenya needs to be investigated
2. *Salmonella Typhimurium* in both areas exhibited geographic restriction. To improve on the investigation, phylogenetics can be combined with epidemiological informative data to study temporal and spatial distribution of bacterial pathogens aided by GPS devices.
3. *Salmonella* isolates exhibited ACSSuT resistance phenotype, therefore there is need to regulate the use and prescription preference of these antimicrobials to reduce their circulation within the population.

6.2.2 Recommendations for future studies

1. There is need to keep an inventory of the possible circulating *Salmonella* strains in western Kenya for the purpose of future reference and strain comparison.
2. Continuous surveillance to detect resistant isolates to various antimicrobials will be useful in treatment and management of salmonellosis in Kenya.
3. Multi locus sequence typing (MLST) method should be used since it involves a huge set of data, this will solve the problem of phylogenetic discordance.
4. Development of vaccines for human and veterinary use may be the most promising alternative to antibiotics.

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