University of Nairobi

MOLECULAR CHARACTERIZATION AND ANTIBIOTIC RESISTANCE PROFILES OF *SALMONELLA* ISOLATED FROM FECAL MATTER OF DOMESTIC ANIMALS AND ANIMAL PRODUCTS.

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DECLARATION

I declare that this thesis is my original work and has not been submitted for examination in any other institution. Any works cited herein have been clearly referenced.

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DEDICATION

This thesis is dedicated to my family for their constant support and encouragement.

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LIST OF ABBREVIATIONS ACRONYMS AND SYMBOLS

ADH	Arginine dihydrolase
AMY	Amygladin
API	Analytical Profile Index
ARA	Arabinose
AMP	Ampicilin
BLAST	Basic Local Alignment Search Tool
CDC	Centre for Disease Control
CFU	Colony Forming Unit
CHEF	Contour camped Homogenous Electrical Field
CIT	Citrate
CLSI	Clinical & Laboratory Standards Institute
CoT	Cotrimoxazole
DNA	Deoxyribonucleic Acid
DT`	Definitive phage Type
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme Linked Immunosorbent Assay
GEL	Gelatinase
GEN	Gentamycin

GLU	Glucose
IND	Indole
INO	Inositol
LDC	Lysine decarboxylase
MAbs	Monoclonal Antibodies
MAN	Mannose
MCMC	Markov Chain Monte Carlo
MDR	Multi Drug Resistance
MEL	Melibose
MLEE	Multilocus Enzyme Electrophoresis
MLST	Multilocus Sequence Typing
NAL	Nalidixic acid
NCBI	National Center for Biotechnology Information
NIT	Nitrofurantoin
NTS	Non Typhoidal Salmonella
ODC	Ornithine decarboxylase
ONPG	o-nitrophenyl-D-galactopyranoside
PFGE	Pulse Field Gel Electrophoresis
QRDR	Quinolone Resistance Determining Region

RHA	Rhamnose
RNA	Ribonucleic Acid
S	Streptomycin
SAC	Sucrose
SOR	Sorbitol
ST	Sequence Type
SPI	Salmonella Pathogenicity Island
SXT	Sulphamethoxazole
TAE	Tris-acetate-EDTA
TDA	Tryptophan deaminase
TET	Tetracycline
TSI	Triple Sugar Iron
URE	Urea
VP	Voges Proskauer Test
WHO	World Health Organization
XLD	Xylose lysine deoxycholate agar

ABSTRACT

Salmonella species are among the most genetically diverse and a common cause of food borne diseases worldwide. The species is associated with important losses in animal produce and it has significant public health implications due to its role as a food borne and zoonotic pathogen. The situation is aggravated by the ever increasing number of antimicrobial resistant strains due to the use of antimicrobials in agriculture at sub therapeutic doses for growth promotion *Salmonella* is transmitted through the fecal oral route with domestic animals such as chicken, pigs and cattle acting as major reservoirs of human transmission. Despite being of significant public concern limited data is available regarding the prevalence and antimicrobial susceptibility patterns of circulating serovars for non typhoidal *Salmonella* (NTS) isolated from domestic animals and animal products in Kenya. This project therefore was conducted to determine the prevalence of *Salmonella* in domestic animals, characterize the circulating *Salmonella* serovars and their evolutionary relationships as well as determine patterns of drug resistance.

A cross sectional study was conducted and a total of 740 samples fecal samples of cows (n=150), pigs (n=182), chicken(n=191) and chicken eggs (n=217) were collected from various markets and abattoirs in Nairobi, Mombasa, Machakos, Meru, Thika and Kakamega counties from November 2013-October 2014. The samples were pre enriched in buffered peptone water or nutrient broth followed by selective enrichment using selenite cysteine broth. Isolation was done by inoculating the selectively enriched sample on XLD agar, followed by confirmation of presumptive colonies biochemically using API 20E strips and PCR using 16s rRNA and finally phylogenetic analysis was carried out using the Bayesian method. The sensitivity to commonly used antimicrobial drugs was also carried out using the Kirby disk diffusion method.

Out of the samples collected, the prevalence of *Salmonella* by PCR was 3.8%, 3.6%, 5.9% and 2.6 % for pigs, chicken, eggs and cows respectively. Two serovars *S.* Typhimurium and *S.* Enteritidis which are responsible for most *Salmonella* associated outbreaks in humans were isolated with *S.* Typhimurium (18/21 =85%) being more common. The two serovars formed distinct clades on the phylogenetic tree. Antimicrobial resistance was detected was detected in 40% of the isolates with 20% of the isolates being resistant to more than one drug. The highest resistance was observed against sulphamethoxazole at 30% followed by ampicillin 20% while 10% of the isolates were resistant to cotrimoxazole and tetracycline.

These findings indicate that domestic animals act as reservoirs of *Salmonella* and since contamination can occur at multiple points in the food chain, it is important to conduct regular surveillance amongst domestic animals and animal products. This will help provide appropriate risk management options to manage transmission to humans as well as reduce the economic losses to the farmers. The resistance to sulphamethoxazole may be an indicator that the drug is commonly used in animal husbandry thus creating selection pressure for *Salmonella* to develop resistance. There also needs to be concerted efforts towards raising awareness among the communities and health care workers about the consequences of inappropriate use and emergence of antimicrobial resistance

CHAPTER ONE

1.0: INTRODUCTION

The bacteria in the genus *Salmonella* are gram-negative, facultative anaerobic rod-shaped bacilli in the family Enterobacteriaceae. The *Salmonella* genus is divided into two species: *Salmonella enterica* and *S. bongori. S. enterica* species are further divided into six subspecies, on the basis of biochemical and antigenic characteristics as well as genome phylogeny (Dougan *et al.,* 2011). *Salmonella* causes significant morbidity and mortality worldwide with an estimated 21.7 million cases of systemic typhoid fever and 93.8 million cases of non-typhoidal gastroenteritis in humans being reported of which 80.3 million of the NTS cases being foodborne (Chaudhuri *et al.,* 2013; Majowicz *et al.,* 2010). Salmonellosis is also a significant cause of economic loss in farm animals because of cost of clinical disease, diagnostic laboratory costs and cost of disinfection, control and prevention. Analysis of five outbreaks in North America gives a direct cost with range from $36.4-$62 million (Kemal, 2014).

There are various *Salmonella* subtypes in existence. These subtypes are differentiated from one another by serotyping analysis, a technique that exploits differences in the polysaccharide portion of lipopolysaccharide layer (O antigen) and the filamentous portion of the flagella (H antigen) present on the surface of *Salmonella* and groups strains into distinct serotypes (Foley & Lynne, 2007). This has resulted in over 2600 serovars with most of the humans and domestic animals pathogenic serovars belonging to the subspecies I (Stevens *et al.,* 2009). Based on the host range, *Salmonella* serotypes are normally divided into two groups; host restricted and the ubiquitous (unrestricted) serotypes. Host restricted serotypes are almost exclusively associated with one particular host species and typically cause systemic disease in a limited number of

related species. Ubiquitous or host generalist serotypes although capable of causing systemic disease in a wide range of host animals, usually induce a self-limiting gastroenteritis in a broad range of unrelated host species (Uzzau *et al.,* 2000). Non typhoid *Salmonella* serotypes can cause acute gastroenteritis, bacteremia, extraintestinal localized infections involving many organs as well as abortions in animals (Chiu *et al.,* 2004). In sub-Saharan Africa, NTS invasive infections are a public health concern for infants, young children, and adults suffering from malnutrition, malaria, and HIV infection (Kariuki & Dougan, 2014). A feature that all *Salmonella* infections share is colonization in the gastro-intestinal tract with associated fecal shedding that provides a source of infection for other animals and humans (Jin *et al.,* 2011).

Salmonella is a stealth pathogen with very effective mechanisms of evading detection by the host system during an infection (Liang *et al.,* 2013). The symptoms of the disease in humans usually appear 12-72 hours after ingestion of the bacteria and include fever, abdominal pain, diarrhea, nausea, malaise and sometimes vomiting. Complications occur in about 10-15% of the patients with gastrointestinal bleeding being the most common (Pui *et al.,* 2011).

Farm animals are the major reservoir for non typhoidal *Salmonella* and large outbreaks of host generalist *Salmonella* serovars have been associated with food-borne transmission including those from contaminated poultry and poultry products, meat, and milk and other dairy products (Kikuvi *et al.,* 2010; McClelland *et al.,* 2001). The prevalence of *Salmonella* in domestic animals in Eastern Africa ranges from 5% to 12% (Onyango et al., 2014; Endris *et al.,* 2013; Mdegela *et al.,* 2000; Kikuvi *et al.,* 2010).

The emergence of antibiotic-resistant strains, principally due to therapeutic use of antimicrobials in animals as growth promoters, is a further threat to human and animal health (Forshell *et al.,*

2006). Bacterial resistance to antibiotics results from the mutation of normal cellular genes, the acquisition of foreign resistance genes, or a combination of these two mechanisms (Rice, 1998). General mechanisms which are responsible for bacterial resistances to antimicrobial agents include: alteration of the sites where the drugs are targeted, enzymes that inactivate the antibiotics, decreased membrane permeability, and active efflux of antimicrobials (Kumar *et al.*, 2013). Bacteria may be intrinsically resistant to more than one class of antimicrobial agents, or may acquire resistance by de novo mutation or via the acquisition of resistance genes from other organisms (Tenover, 2006).

There are various methods for detecting *Salmonella* in many biological matrices. Bacteriological methods involve culturing of bacteria present in blood or stool followed by confirmation using biochemical tests. Serological tests based on an antigen antibody reaction are routinely carried out (Chiu *et al.,* 2004). Molecular methods such as the ribotyping (Hald *et al.,* 2007), pulse-field gel electrophoresis (Foley & Lynne, 2007), the Polymerase chain reaction with its various modifications such as the nested PCR, multiplex PCR have also been used to identify *Salmonella* but each with varying results. Molecular based methods have mainly been applied in research institutions to determine the source of outbreaks (Baker *et al.,* 2010; Liang *et al.,* 2013).

The classification of *Salmonella* based upon serotype and other physiological properties has provided limited information regarding the genetic relationship of the serovars and moreover is not sufficient for making disease association. DNA sequencing is the gold standard for detecting DNA changes (Ngoi & Thong, 2014). This project used molecular methods and DNA sequence analysis to identify and characterize isolated *Salmonella* from the fecal matter of key reservoirs of the bacteria: chicken, cows and pigs as well as chicken eggs. The sequence information will provide a valuable resource from which we can begin to dissect the features of *Salmonella* that

are both shared and distinct between serovars and to start exploring how and why differences arose (Chan *et al.*, 2003).

The ultimate objective was to determine the prevalence of *Salmonella* in cows, chicken, chicken eggs and pigs, as well as to determine their antimicrobial susceptibility patterns and finally to construct a genetic framework for the *Salmonella* serovars isolated from animals and animal products within which to study various problems relating to pathogenicity, host specificity and the evolutionary origins of the organisms causing salmonellosis.

1.1: PROBLEM STATEMENT

Salmonella is the leading foodborne pathogen worldwide and disease has most often been associated with consumption of contaminated foods of animal origin, such as poultry, swine, dairy products and eggs (Loongyai *et al.,* 2010).

There are limited studies of prevalence of *Salmonella* isolated from domestic animals and these studies have used culture and serology to determine serovars. These two methods are insufficient to make genetic relationship and extract evolutionary information. The study therefore characterized the serovars using molecular methods and phylogeny.

In addition no study in Kenya has been done to determine the prevalence of *Salmonella* in eggs which have been associated with numerous outbreaks in the world (Bäumler *et al.,* 1998). Moreover a previous study done in Kenya did not isolate *Salmonella* from cows and chicken which have been identified as reservoirs of *Salmonella* worldwide (Kikuvi *et al.,* 2007; Santos *et al.,* 2001).. Therefore the study sought to determine the presence and prevalence of *Salmonella* in cows, chicken, pigs and eggs as well as determine the genetic relationship of the various serovars isolated.

1.2: JUSTIFICATION

Salmonella spp. are zoonotic bacterial agents which are significantly associated with diverse animal and human infections. Domestic animals including chicken, pigs and cattle have been identified as key reservoirs and a major transmission vehicle to humans (Pui *et al.,* 2011). *Salmonella* is responsible for two types of disease in humans: gastroenteritis, a localized infection or enteric fever (typhoid), a severe systemic infection. In sub-Saharan Africa, cases of nontyphoidal salmonellosis (NTS), frequently complicated by bacteremia, are now more numerous than cases of enteric fever. The changing epidemiology of *Salmonella* infections around the world and the emergence of new *Salmonella* strains e.g. multidrug resistant *Salmonella* serotype Typhimurium DT 104 and invasive non typhoidal salmonella Typhimurium ST 313 unique to sub Saharan Africa calls for specific strain identification and characterization. This study therefore was carried out to determine the prevalence of *Salmonella* from these species as well as show genetic relationship of the isolated serovars via phylogeny.

In addition to the high incidence rate of salmonellosis worldwide, increased levels of antibiotic resistance has been reported worldwide. This is due to an increased use of antimicrobial agents as both prophylactic measures and growth promoting agents in most farming systems. In most cases many of these antimicrobials have been frequently administered in sub therapeutic doses leading to an enormous selection pressure of antimicrobial resistance in zoonosis causing bacteria such as *Salmonella*.

Due to the effects of *Salmonella* both in animals and humans, there is therefore need to determine the various circulating *Salmonella* serovars, their prevalence among domestic animals and antibiotic resistant profiles in Kenya. This will help to assess the risk of hygiene failure and provide appropriate risk management options an effective way of managing the transmission of

Salmonella to the human populations as well as help in the understanding of the mechanism of *Salmonella* resistance to conventional drugs that is slowly and steadily appearing in human populations.

1.3: OBJECTIVES

1.3.1: Main objective

The main objective of this work was to isolate and carry out molecular characterization of the various *Salmonella* serovars found in domestic animals and animal products in Kenya.

1.3.2: Specific Objectives

1. To determine the prevalence of circulating *Salmonella* serovars from fecal samples of chicken, cattle and pigs as well as eggs in Kenya

2. To identify the evolutionary relationship of *Salmonella* within and between circulating serovars in different hosts.

3. To determine the patterns of drug resistance among the prevailing *Salmonella* serovars

CHAPTER TWO: LITERATURE REVIEW

2.1: Genus *Salmonella*

Salmonella is named after an American bacteriologist, D. E. Salmon, who first isolated *Salmonella* choleraesuis from porcine intestine in 1884 (Su & Chiu, 2006). *Salmonella* species are pathogenic bacteria that are members of the family *Enterobacteriaceae.* They are facultative anaerobes, non-spore forming, gram negative with a peritrichous flagella (graded in all directions) and exhibit an optimal growth at 37°C (Yan *et al.,* 2003).

2.1.1: Classification and nomenclature

Salmonella nomenclature is complex hence different systems have been used to classify members of this genus. The current nomenclatural systems divides the genus into species, sub species, sub genera (Brenner *et al.,* 2000). The antigenic classification system used by the CDC, WHO and the American Biological Society, is a result of extensive studies of antibody interactions with bacterial surface antigens by Kauffman and White. Three kinds of surface antigens, somatic O (somatic), H (flagella) and Vi (virulence) antigens, determine the reactions of the organisms to specific antisera resulting in over 2600 serovars (Chiu *et al.,* 2004; Pui *et al.,* 2011). These serovars differ greatly in their host range and their degree of host adaptation. Some of these serovars are host specific e.g. *S.* Typhi and Paratyphi that affects humans while some are host generalists or zoonotic e.g. *S.* Typhimurium and *S.* Enteritidis (Liang *et al.,* 2013).

Salmonella is made up of 2 species: *S. enterica* and *S. bongori* based on DNA- DNA hybridization. Current taxonomy is based on the Kauffman-Le Minor scheme of serotyping where S. *enterica* is further subdivided into six sub species (Waldner *et al.,* 2012). These sub

species are designated by taxonomic names or can be abbreviated using Roman numerals: *S. enterica* subsp. enterica (I), *S.enterica* subsp. salamae (II), *S.enterica* subsp. arizonae (IIIa), *S.enterica* subsp. diarizonae (IIIb), S.*enterica* subsp. Houtenae (IV) *S.enterica* subsp. Indica (VI). The Subgenus III has been further divided into IIIa and IIIb based on both DNA similarity and phenotypic characteristics (Lin-Hui Su *et al.,* 2007, Brenner *et al.,* 2010). In subspecies I, serovars are designated by a name usually indicative of the associated diseases, their geographic origins, or their usual habitats. On the other hand, antigenic formulae determined according to the Kauffmann-White scheme are used for the classification of the remaining subspecies as well as those of S. bongori. Serotype names are designated by the formulae: the sub species, O (somatic) antigens, followed by a colon then the H flagella antigens (phase 1) and H antigens phase 2 if present e.g. *Salmonella* enteric type II 39:z_{10}:$z_{6.}$ For *S*. bongori the V antigen is used for uniformity e.g. S. V 61:z_{35}-. To avoid confusion between serovars and species during citation, the serovar name is not italicized and starts with a capital letter (de Jong *et al.,* 2012; Su & Chiu, 2006).

Among the *Salmonella* species, the most pathogenic human and animal serotypes belong to *Salmonella enterica* subsp. *enterica*. Subspecies I is mainly isolated from humans and warm blooded animal whereas subspecies II, III, IV, VI and *S. bongori* are isolated from cold blooded animals and other environmental matrices (Stevens *et al.,* 2009). These serovars differ greatly in their host range and their degree of host adaptation. Some of these serovars are host specific e.g. *S.* Typhi and Paratyphi that affects humans while some are host generalists or zoonotic e.g. *S*. Typhimurium and *S*. Enteritidis (Liang *et al.,* 2013).

Table1: *Salmonella* nomenclature adapted from (Brenner *et al.*, 2000)

Taxonomic position	**Nomenclature**
Genus (italics)	*Salmonella*
Species (italics)	• *enterica*, which includes subspecies I, II, IIIa, IIIb, IV, and VI
	• *bongori* (formerly subspecies V)
Serotype (capitalized, not italicized)	• The first time a serotype is mentioned in the text; the name should be preceded by the word "serotype" or "ser."
	• Serotypes are named in subspecies I and designated by antigenic formulae in subspecies II to IV, and VI and *S. bongori*
	• Members of subspecies II, IV, and VI and *S. bongori* retain their names if named before 1966

2.1.2: Morphological and biochemical characteristics of *Salmonella*

Salmonella are facultative anaerobic, gram negative, non-spore forming and non-capsulated bacteria. They are generally 2-5µm long and 0.8-1.5 µm wide and most are motile and grow on nutrient agar. Most of them have type 1 mannose sensitive adhesive properties and have type 2 fimbriae. *S.* Paratyphi A do not have fimbriae, *S.* Gallinarum is non-motile and *S.* Typhi has no capsule (Grimont *et al.*, 2000). The biochemical characteristics of *Salmonella* are summarized in Table 2

Table 2: Biochemical reactions of *Salmonella*

#	Test or substrate	Result		*Salmonella* species reaction[a]
		Positive	**Negative**	
1.	Glucose (TSI)	yellow butt	red butt	+
2.	Lysine decarboxylase (LIA)	purple butt	yellow butt	+
3.	H_2S (TSI and LIA)	Blackening	no blackening	+
4.	Urease	purple-red color	no color change	−
5.	Lysine decarboxylase broth	purple color	yellow color	+
6.	Phenol red dulcitol broth	yellow color and/or gas	no gas; no color change	+[b]
7.	KCN broth	Growth	no growth	−
8.	Malonate broth	blue color	no color change	–[c]
9.	Indole test	violet color at surface	yellow color at surface	−
10.	Polyvalent flagellar test	Agglutination	no agglutination	+
11.	Polyvalent somatic test	Agglutination	no agglutination	+
12.	Phenol red lactose broth	yellow color and/or gas	no gas; no color change	–[c]
13.	Phenol red sucrose broth	yellow color and/or gas	no gas; no color change	−
14.	Voges-Proskauer test	pink-to-red color	no color change	−
15.	Methyl red test	diffuse red color	diffuse yellow color	+
16.	Simmons citrate	growth; blue color	no growth; no color change	V

[a] +: 90% or more positive in 1 or 2 days; −: 90% or more negative in 1 or 2 days; v: variable.

[b] Majority of *S. arizonae* cultures are negative. [c] Majority of *S. arizonae* cultures are positive.

Adapted from BAM : Salmonella. Manual, Bacteriological Analytical 2009

2.2: Transmission and clinical manifestations of *Salmonella*

The most common vehicles of transmission of *Salmonella* are meat, meat products, dairy products, eggs or egg products containing *Salmonella* serotypes either because animals are infected or because fecal contamination occurs during processing (Santos *et al.,* 2001). In humans *S. enterica* species are typically orally acquired pathogens that cause one of four major syndromes: enteric fever (typhoid), gastroenteritis, bacteremia and chronic asymptomatic carriage (Coburn *et al.,* 2007). Some serovars are highly adapted to the human hosts such as *S.* Typhi and Paratyphi collectively known as typhoidal serovars. *S.* Typhi causes enteric fever (typhoid) whereas *S.* Paratyphi A, B and C cause paratyphoid fever whose symptoms are milder and is associated with a lower mortality rate compared to *S.* Typhi. Infection typically occurs through ingestion of food or water contaminated with human waste. Non typhoidal salmonellosis is caused by at least 150 *Salmonella* serotypes with *S.* Typhimurium and *S.* Enteritidis being the most common serovars identified in many parts of the world. Infection occurs through ingestion of food or water contaminated with animal waste (Pui *et al.,* 2011). The most common manifestation of nontyphoidal salmonellosis in humans is mild to moderate gastroenteritis, consisting of diarrhea, abdominal cramps, vomiting and fever. Typically, symptoms of gastroenteritis develop within 6 to 72 h following the ingestion of the bacteria. The incubation period ranges from five hours to seven days, but clinical signs usually begin 12 h to 36 h after ingestion of the bacterial contaminated food. Shorter incubation periods are generally associated with either higher doses of the pathogen or highly susceptible people (Forshell *et al.,* 2006). Non-typhoid salmonellosis can later give rise to chronic diseases, including localized infections in specific tissues or organs and reactive arthritis, as well as neurological and neuromuscular

illnesses (Feasey *et al.,* 2012). Bacteremia (septicemia) is the least common clinical syndrome in man and it is associated with serotypes such as the porcine-adapted *S. enterica* serotype Choleraesuis and the bovine-adapted *S. enterica* serotype Dublin which may enter the food chain through undercooked pork products or unpasteurized milk, respectively. Bacteremia is often accompanied by a high spiking fever that distinguishes the syndrome from typhoid fever in which a more continuous fever is observed (Santos *et al.,* 2001).

2.3: Pathogenesis of *Salmonella*

In humans, upon ingestion both typhoidal and non typhoidal serovars initially adhere to and invade the intestinal epithelium of the small intestine (Gal-Mor *et al.,* 2014). The intestinal mucosa serves as the initial reversible or irreversible binding site for the bacteria. The Peyers patches which are aggregated lymphoid nodules of the terminal ileum play an important role in the transport of the pathogen into the underlying lymphoid tissue (Huang & DuPont, 2005).

The invasion step is believed to be a very important step related to virulence of *Salmonella* strains associated with infections. After invasion of the epithelial cells the bacteria reach the sub epithelial lymph tissue where the *Salmonella* meets host immune cells. The phagocytic process can be divided into two main parts: adherence and phagocytosis which involves the internalization of the adherent particle. During the bacteremic phase, the bacteria are widely disseminated throughout the body. Secondary infection can occur with liver, spleen, bone-marrow, gallbladder, and Peyer's patches as the most preferred sites (Bergeron *et al.,* 2009; Coburn *et al.,* 2007; Parry *et al.,* 2002). The virulence genes are located on pathogenicity islands of the chromosomes referred to as *Salmonella* pathogenicity island (SPI) which was acquired by plasmid or phage mediated horizontal transfer (Forshell *et al.,* 2006). The SPI genes are involved

directly in manipulating the host systems (Eswarappa *et al.,* 2009). SPI 1encodes virulence factors that mediate mechanisms used by *Salmonella* during the intestinal phase of infection including intestinal epithelial cells invasion, induction of neutrophil recruitment and secretion of intestinal fluid (Baumler *et al*.,1998). SPI-2 confers the ability to survive within the host cells especially macrophages while SP1-3 has a role in intramacrophage survival and virulence. SPI-4 is implicated in adhesion of *Salmonella* to host epithelial cells and SPI-5 is required for enteropathogenicity (Eswarappa *et al.,* 2009).

2.4: *Salmonella* incidence in animals

The incidence of *Salmonella* in farm animals is widespread. Common *Salmonella* occurring in animals include *S.* Choleraesuis, *S.* Typhimurium, *S.* Enteriditis, *S.* Gallinarium and *S*. Dublin.
S. Choleraesuis is a host-adapted pathogen that causes swine paratyphoid characterized by systemic disease that is often fatal (Boyen *et al.,* 2008). It is also highly pathogenic to humans, usually causing septicemic disease with little involvement of the intestinal tract. The resulting serotype Choleraesuis reservoir in swine is a concern, not only because of its disease-causing potential in young pigs but also because of its public health implications for humans (Chiu *et al.,* 2004, Santos *et al.,* 2001). The potential survival of *Salmonella* in manure and slurry poses a significant threat to public health because animal manure is often used as an organic fertilizer in agriculture. In particular, *S*. Typhimurium has been known to survive for considerable periods of time in pig slurries (Lim *et al.,* 2011). Gross pathology of the intestine commonly reveals enlarged Peyer's patches and a thickening of the ileal mucosa in *Salmonella* infected pigs (Uzzau *et al.,* 2000).

Poultry products have constantly been identified as important sources of *Salmonella* infection in humans because of the vertical transfer of infection from breeding hens to progeny (Bae *et al.,* 2013). The serovars associated with poultry reproductive tissues are *S.* Enteritidis, *S.* Typhimurium and *S.* Heidelberg. Among these, *S.* Enteritidis may have better invasive properties and therefore, found more frequently in reproductive tissues (Bayu *et al.*, 2013).

S. Gallinarium is the host specific pathogen found in chicken. *S.* Gallinarum is divided into two biotypes, gallinarum and pullorum, which can be differentiated both biochemically and genotypically. Biotypes gallinarum and pullorum are the causative agents of two different disease syndromes, fowl typhoid and pullorum disease respectively (Uzzau *et al.,* 2000). Although largely eradicated from the commercial poultry industry in many developed countries, outbreaks have occurred and the prevalence of the disease in poultry in areas such as Eastern Europe, Africa and South America, where the poultry industry is undergoing rapid expansion, remains high. Fowl typhoid generally presents as septicaemia, affecting birds mainly those over 3 months, whereas pullorum disease tends to be restricted to an enteric infection of birds under 6 weeks of age (Özbey *et al.,* 2008). The course of Gallinarum infection varies greatly depending on the age, breed, nutritional and immune status of the birds involved. Gallinarum is the only non-flgellated, and therefore non-motile serotype of *S. enterica*. Despite its phenotypic non-motility, *S.* Gallinarum contains the gene *fliC* which encodes the phase 1 structural flagella protein (Rabsch *et al.,* 2002). Pullorum disease and fowl typhoid frequently infect the reproductive organs of adult chickens establishing a chronic infection with direct passage of the organisms into the egg as formation takes place (Endris *et al.,* 2013).

Eggs can be infected by *Salmonella* via two major routes, vertical and horizontal. Vertical transmission (transovarian infection) occurs when the egg contents are contaminated with

Salmonella during their formation prior to shelling Horizontal transmission includes trans shell infection of the contents of the egg during transit through the cloaca or after oviposition and fecal contamination of the external surface of the shell (Martelli & Davies, 2012). The ability of *S.* Enteritidis to transmit by the transovarian route is an important factor for possible infection of shell eggs and the transmission of systemic infection to broiler chicks (Davies & Wray, 1994). *S.* Enteritidis frequently colonizes the alimentary tracts of chicken without causing disease. It can produce a systematic infection in young chicks which can lead further to infection of eggs (Betancor *et al.,* 2010). The prevalence of *Salmonella* has been reported to be higher in the yolks than the shell membranes (Munang'andu *et al.,* 2012).

S. Dublin is host-adapted to bovine and aff ects both young and adult cattle causing enteritis and or systemic disease. Acute disease is characterized by fever, anorexia and abruptly reduced milk yield (Uzzau *et al.,* 2000, Santos *et al.,* 2002). *S.* Dublin can cause systemic infections, and may cause abortion in pregnant cows (Rabsch *et al.,* 2002).

2.5: Treatment of Salmonellosis

The empiric treatment of choice in humans is a fluoroquinolone drug for treatment of salmonellosis caused by isolates that are not quinolone resistant. On the other hand, ceftriaxone and azithromycin are alternatives (Crum, 2003). Fluoroquinolones and third-generation cephalosporins are now commonly used in adults for treatment due to widespread resistance to chloramphenicol, ampicillin, and cotrimoxazole (Fashae *et al.,* 2010). Fluoroquinoles are also often used to treat severe enteric salmonellosis in different animal species (Boyen *et al.,* 2008). Antibiotic treatment in animals is however usually not advised except for rare cases because it can prolong the presence of bacteria in the stool (Pui 2011).

2.6: Antibiotic resistance

Antibiotic resistance is defined as the ability of a pathogenic microorganism to multiply beyond some critical mass in the face of invading antimicrobials (Zhang *et al.,* 2006). Antimicrobial resistance is now a global threat and the use of antimicrobial agents in any environment have been reported to create selection pressures that favor the survival of antibiotic-resistant pathogens (White *et al.,* 2001). It is of critical concern in African countries, where multidrug-resistant nontyphoidal salmonellosis is one of the most common causes of bacteremia in children (Boyle *et al.,* 2007). Furthermore, a distinct genotype of *Salmonella enterica* var Typhimurium, ST313, has emerged as a new pathogenic clade in sub-Saharan Africa, and might have adapted to cause invasive disease in human beings. Multidrug-resistant ST313 has caused epidemics in several African countries, and has driven the use of expensive antimicrobial drugs in the poorest health services in the world (Feasey *et al.,* 2012). Resistance of *S.* Cholerasuis to ampicillin, chloramphenicol, trimethoprim-sulfamethoxazole, tetracycline, gentamicin, and, notably, fluoroquinolones has been reported in Western Kenya (Onyango *et al.,* 2014). There are many causes of resistance but the application, misuse and prolonged antibiotic treatments in farm animals with therapeutic and prophylactic purposes creates selective pressure for antimicrobial resistant commensals and /or zoonotic foodborne bacterial pathogens (Morar *et al.,* 2015; Huttner *et al.,* 2013).

2.6.1: Mechanisms of action of antibiotics

Antibiotics are molecules that kill, or stop the growth of microorganisms, including both bacteria and fungi. Antibiotics that kill bacteria are called bactericidal. Antibiotics that stop the growth of bacteria are called bacteriostatic (Thenmozhi *et al.,* 2014). Antibiotics target essential bacterial

physiology and biochemistry, causing microbial cell death or the cessation of growth. There are five major antibiotic targets: the bacterial cell wall, the cell membrane, protein synthesis, DNA and RNA synthesis, and folic acid (vitamin B9) metabolism. These bacterial targets are different or nonexistent in eukaryotic cells (including those of humans), which means that antibiotics are relatively nontoxic drugs (Wright, 2010).

2.6.2: Antibiotic resistance mechanisms

Bacterial resistance to antibiotics can be intrinsic or acquired. Innate resistance is characteristic of a particular bacterium and depends on biology of a microorganism. For example, E. coli has innate resistance to vancomycin (Giedraitiene, 2011). There are two general strategies of acquired resistance. One comprises mechanisms that transfer resistance vertically from a bacterium to its progeny. Examples are mutations in chromosomal genes that give rise to drug-insensitive products, such as the point mutations in the genes encoding DNA gyrase or topoisomerase IV that result in resistance to fluoroquinolone antibiotics such as ciprofloxacin (Wright, 2010). Resistance to quinolones in *Salmonella* is associated with mutations in the QRDR (quinolone resistance determining region) of the *gyrA* and *parC* genes which encompasses amino acids 51 to 106 in *gyrA* and 23 to 176 in p*arC*. The most commonly described mutations in gyr A are codon 83 (serine to tyrosine, phenylalanine, or alanine) and codon 87(aspartic acid to asparagine, glycine or tyrosine). In par C the most common mutation is in codon 80 (serine to arginine or isoleucine) (Bae *et al.*, 2013, Kim & Hooper, 2014; Thenmozhi *et al.*, 2014). In some cases, alterations in a single gene can confer resistance, whereas in the majority of cases, a consortium of genes is involved in the development of resistance against a particular drug (Afzal *et al.*, 2013).

Although mutations in gyrB, parC, and parE do not play an important role in quinolone resistance, they contribute to the acquisition of high-level resistance (Jeong *et al.*, 2011).

Mutations are rare and commonly determine resistance to structurally related compounds. They occur as errors in replication or incorrectly repaired DNA fragment. Second strategy is acquired resistance which occurs from acquisition of exogenous genes by plasmids which are extrachromosomal, circular DNA molecules that typically contain genes that impart selective advantage to the host, such as virulence or antimicrobial resistance. When resistance determinants are on plasmids, they will spread quickly within the genus to even unrelated bacteria (Giedraitienė *et al.*, 2011). Resistance genes can be transferred by three main ways: transduction, transformation or conjugation. Transformation is the uptake of naked DNA from a lysed bacterium. Transduction is the transfer of genetic material using bacteriophages. Conjugation is the most efficient means of DNA transfer and it involves mating that requires cell to cell contact (Levy, 2002).

The development of resistance in *Salmonella* toward antimicrobial agents is attributable to one of multiple mechanisms, including production of enzymes that inactivate antimicrobial agents through degradation or structural modification, reduction of bacterial cell permeability to antibiotics, activation of antimicrobial efflux pumps, and modification of the cellular target for drug (Foley *et al.,* 2008).The most common resistance mechanism to beta-lactam antibiotics is mediated by beta-lactamase enzymes. Some βlactamases have affinities for the structures of a limited number of antimicrobial agents, whereas others are extended- or broad spectrum. Their coding genes are located on mobile genetic elements: plasmids that facilitate intra and inter species transfer (Wright, 2010; Carlet *et al.,* 2012). The most specific and evolved mechanism of antibiotic resistance are enzymes that recognize antibiotics and modify them in such a way as to

eliminate the functional characteristics and chemical transformation of these compounds. Modifications include acetylation, phosphorylation, adenylation, glycosylation, and hydroxylation (Tanwar *et al.,* 2014).

Efflux pumps transport drugs against their concentration gradient across the cell membrane. They are located in the cytoplasmic or plasma membrane and overexpression of one or more of these efflux pumps prevents the intracellular accumulation of the agent to thresholds necessary for its inhibitory activity. This efflux pump overproduction is generally accompanied by an increase in resistance to two or more structurally unrelated antibiotics [multidrug resistance (MDR)] and significantly contributes to the emergence and spread of MDR pathogens (Mahamoud *et al.,* 2007). Resistance to tetracycline and chloramphenicol is associated with the expression of these pumps (Kumar *et al.,* 2013).

Trimethoprim resistance is associated with *dhfr* genes which encode altered dihydrofolate reductases that reduced affinity for the antimicrobial agent, allowing folic acid biosynthesis to occur in the presence of trimethoprim. Resistance to sulphonamides is associated with acquisition of either sul I or sul II which encode altered dihydropteorate synthetase enzymes. (Foley *et al.,* 2008; Silva *et al.,* 1996).A survey conducted by Kariuki et al., (2002) on use of antibiotics by farmers discovered extensive use of tetracyclines in poultry rearing. It is added to commercial poultry feeds and in drinking water for birds of all ages. Tetracycline, penicillin and sulfonamides were also used extensively in dairy animals for prophylaxis (Kariuki et al., 2002).

2.7: Control strategies

Animals play a vital role in transmission of *Salmonella* and this has resulted in several outbreaks (Waldner *et al.,* 2012). The emergence of multi drug resistant *Salmonella* strains further calls for

strict measures to minimize transmission. *Salmonella* control measures can be implemented at three levels: the pre-harvest level (on farm), the harvest level (transport to and procedures in the slaughterhouse) and the post-harvest level (cutting, processing, retail and food preparation at home).

Pre-harvest control of *Salmonella* at the farm level has long been considered an important part of pathogen reduction schemes, not least because traditional meat inspection may not be able to detect *Salmonella*-contaminated carcasses (Forshell *et al.,* 2006). The main control method that has proved to work is raising livestock in separate groups, without mixing animals from different sources and ages, has proved to be an effective health measure. The 'all-in, all-out' system, with careful cleaning and disinfection between batches, has long been essential in broiler production, and is now also routine in *Salmonella* control programmes for beef and swine production. A live attenuated vaccine against *S.* Gallinarum in poultry is available and there is currently demand for a vaccine to control *Salmonella* infections associated with human food poisoning, in particular *S.* Enteritidis (Forshel *et al.,* 2006).

Implementation of monitoring programs and coordination of control measures at harvest and post-harvest, have been used to prevent non-typhoidal *Salmonella* infections in humans from pork in Denmark by monitoring the whole food chain from "feed to food." The program successfully reduced the level of *Salmonella* in pork from 3.5% in the year 1993 to 0.7% in the year 2000 (Malorny & Hoorfar, 2005).

2.8: Methods of *Salmonella* characterization

The presence of *Salmonella* pathogen in a biological sample matrix has been reported to be characterized using morphological (WHO, 2003), biochemical and antigenic characteristics (Chiu *et al.*, 2004) as well as by genome phylogeny (El Allaoui *et al.*, 2014).

2.8.1 Culture

Culture is the gold standard method for the detection of *Salmonella* in a biological matrix such as a blood or stool sample. It has been shown that the best culture results are obtained when the media is inoculated with freshly drawn blood (WHO, 2003). However, the sensitivity of blood culture tests are often highest during the first week of *Salmonella* infection and reduces with advancing illness, prior use of antibiotics. However, the sensitivity increases with the volume of blood cultured and the ratio of blood to broth (Zhou *et al.*, 2010).

The culture method is conducted with pre enrichment and selective medium plating. The selective agar includes an inhibitor substance and inhibitor system, that either changes the colour of colonies or the agar area under the colony. The samples are taken into a non-selective enriched medium and incubated for 24-48 hours then an aliquot is taken to the selective medium such as Selenite F and incubated for 24 hours. Most laboratories use one medium with low selectivity, such as Mac-Conkey agar, and one with higher selectivity, such as Hektoen enteric agar or XLD agar (Hohmann, 2001).The best agar is blood agar, if it is not available nutrient agar can be used. Mac Conkey agar is sometimes preferred because it allows the growth of only bile-tolerant bacteria and does not allow the growth of many Gram-positive

contaminants. Bile containing medium is used for isolation of enteric fever pathogens from blood. In addition to inhibitory activity against many of the common bacterial contaminants, bile has the additional advantages of a greater frequency of isolation of *Salmonella* and more rapid isolation (Kaye *et al.,* 1966, deJong *et al.,* 2012). In the case of stool samples, the selective agars used include: brilliant green agar, Mac Conkey agar, *Salmonella*-shigella agar (SS agar), bismuth sulphite agar and Xylose lysine deoxycholate agar (WHO, 2003; Park *et al.,* 2012). Colonies from solid media can be used for agglutination with specific antisera. Several *Salmonella*e have been shown to share the same antigenic structure consequently, thus it is necessary to confirm the presence of *Salmonella* by means of biochemical tests.

The limitation for the use of culture as a method of diagnosis is that many *Salmonella* endemic countries lack adequate microbiological diagnostic infrastructure especially in poor rural setting of developing countries (Parry *et al.,* 2011).

2.8.2: Serological typing

Currently, *Salmonella* isolates are identified using the White-Kauffman-Le Minor scheme which subtypes antibody interactions with three antigens the somatic O antigens, the flagellar H antigens and the Vi antigen. Although extensive serotyping of all surface antigens can be used for formal identification, most clinical microbiological laboratories perform a few simple agglutination reactions to define specific O antigens into serogroups, designated as the A, B, C1, C2, D, and E groups. This grouping system is useful in epidemiologic studies and can be used clinically to confirm genus identification. However, the method cannot quickly identify whether the organism is likely to cause enteric fever, because considerable cross-reactivity among serogroups occurs. For example, serotype Infantis, which typically causes gastroenteritis, and serotype Choleraesuis, a prominent cause of invasive infections, are both C1group members.

Similarly, serotype Enteritidis, another common cause of gastroenteritis and serotype Typhi, that cause enteric fever, are both group D members (Chiu *et al.*, 2004, Brenner *et al.,* 2000). Many of the genes for the biosynthesis of the O antigen are organized in the *rfb* cluster located between the *galF* and *gnd* genes. In this cluster, the sequences of the sugar transferases are relatively conserved and two genes coding for the O antigen flippase (*wzx*) and polymerase (wzy) are highly variable and are responsible for most of the genotypic and phenotypic differences of the 46 *Salmonella* O serogroups identified in the Kauffman White scheme.(Braun *et al.*, 2012).

The H antigens are heat labile proteins and are primarily encoded by two genes *fliC* and *fljB* which express phase 1 H antigen and phase 2 H antigens respectively. There are currently 114 H antigens as described by the Kauffman White scheme. Most of the *Salmonella* strains are biphasic and express two serologically distinct flagellar antigens. Some serovars such as *S.* Typhi and *S.* Enteriditis express only one flagellar antigen either phase 1 or 2 or are considered to be monophasic (Mc Quiston *et al.,* 2011). Phase I is known as the specific group and more than 80 have been found and are designated as small letters of the alphabet a to z and subsequently z1-z68. Phase II is known as group or nonspecific phase because many *Salmonella* show the same antigens when they are in phase II (Braun *et al.*, 2012).

The Vi antigen, the capsular polysaccharide is the major distinguishing feature of the serovars Typhi, Paratyphi C and Dublin (Wray *et al.*, 2004).

The current serotyping method only allows detection of a single antibody-antigen reaction at a time, requires well-experienced technologists to perform, consumes relatively high volumes of reagents and takes a minimum of 3 days to perform a minimum of three antibody-antigen

reactions to determine a serotype. The number of reactions and the time required can be many times greater if a less-common serovar is tested (Cai *et al.*, 2005).

2.8.3: ELISA

The assay is based on antigen and antibody reaction and a 'label' attached to the antibody allow the reaction to be visualized. Depending upon the substrates used, enzyme assay either can be colorimetric or fluorogenic. The technique most commonly used to detect the bacterial antigens in foods is a version of noncompetitive ELISA called the sandwich ELISA (Robison 1997). Several enzyme-linked immunosorbent assays (ELISAs) have been developed, using both polyclonal antibodies and monoclonal antibodies (MAbs) that will detect most *Salmonella* serotypes (Lee *et al.*, 1990). ELISA has been used to detect either the presence of the organism or the humoral response to the organism. The former has mainly been used in the detection of *Salmonella* in food and feedstuff (Fredoka-Cray *et al.*, 2002). It allows rapid analysis of multiple samples, thus can be used for sero-epidemiological studies of large population-based serum collections in order to estimate the population incidence of *Salmonella* infections and it has been used to detect S. Typhimurium in poultry (Brooks *et al.*, 2014) and pigs (Farzan *et al.*, 2007). Limitations of this method is that they require 10^4-10^5 CFU *Salmonella* ml^{-1} to detect the organism therefore it requires a pre enrichment step (Fredroka-Cray *et al.*, 2002).

2.8.4: Molecular characterization

Nucleic acid amplification is considered an improvement of the culture method for *Salmonella* identification. PCR is the most widely detected DNA technique that utilizes genus specific primers targeting specific genes. This method allows 10^7 -fold amplification of the target DNA from as little as one copy in 2 to 3 h. Several PCR methods for the detection of *Salmonella* for

instance, invA gene has been introduced for the effective, rapid and accurate detection of *Salmonella* in foods of animal origin (Nagappa *et al.,* 2007). In conventional PCR, the amplicons generated are separated by electrophoresis, DNA stained and the size of the DNA bands determined by comparison to a standard. The detection limit is 1-5 CFU per mixture (pure culture) (Kim *et al.,* 2006). This method therefore has limitations in terms of sensitivity and speed. The very low ratio of bacterial to human DNA means that the PCR template in clinical preparations is dominated by mammalian DNA and could cause false-positive PCR signals due to the non-specific binding of primers and false-negative results due to reduced sensitivity (Zhou *et al.,* 2010). Multiplex polymerase chain reaction (PCR) is a variant of PCR in which two or more loci are simultaneously amplified in the same reaction. This method has been successfully applied in many areas of DNA testing, including analyses of deletions, mutations and polymorphisms, or quantitative assays. This technique saves time and labor since more than one target DNA sequence can be detected in each reaction. On the other hand, nested PCR increases the sensitivity and specificity of the test through two independent rounds of amplification using two discrete primer sets. The second round of amplification delays results, increases the possibility of cross-contamination, and may complicate automation (Imen *et al.,* 2012).

Genetic diversity of *Salmonella* species can also be identified using pulse field gel electrophoresis (PFGE), plasmid fingerprinting, multilocus enzyme electrophoresis (MLEE), IS-200 profiling and random amplified polymorphic DNA analysis.(Grimont *et al.,* 2000). Pulse gel field electrophoresis (PFGE) can separate large DNA molecules upto 10Mb whereas the standard electrophoresis separates fragments upto 50 Kb. PFGE uses restriction endonucleases which have infrequently occurring restriction sites in bacterial DNA. Very large molecules tend to unravel and by introducing an alternating of an electric field in more than one direction the

fragments can be separated. The time required for the different fragments to re-orientate to a new electrical field is a factor of their molecular weight. PFGE has the capacity to fingerprint *Salmonella* isolates at the origin of an outbreak and has hence rapidly become very popular, to the point where it is considered the gold standard for *Salmonella* molecular subtyping. It has been used to examine the genotypic and phenotypic relationships from pigs and their farm environment (Kyung *et al.,* 2010). CHEF (contour camped homogenous electrical field) which uses an array of hexagonally arranged electrodes at angle of 120°C to each other ensuring that the DNA migrate through the electrical field in a straight line (Herschleb *et al.,* 2007, Kauffman, 1998, Vieira-Pinto *et al.,*2012). MLEE has been used to assess allelic variation in multiple genes in a collection of isolates (Grimont *et al.,* 2000). Comparing the sequence diversity at multiple conserved housekeeping genes, multilocus sequence typing (MLST) is a newly developed approach for determining the population structure of pathogenic bacteria than MLEE because it identifies all sequence changes, including synonymous changes that do not result in amino acid replacements (Kidgell *et al.,* 2012).

2.9: Phylogenetic analysis

Phylogenetic tree analysis is often used as a method to classify organisms (Fukushima *et al.,* 2002). The nucleic acid sequence particularly is of large potential value since it contains more evolutionary information than the traditionally used phenotypic traits and because it is precisely defined and relatively simple to determine (Hedegaard *et al.,* 1999). Molecular phylogeny supports the understanding of organismal relationships and provides the basis for the classification of microorganisms according to their natural affiliations. Comparative sequence analysis of ribosomal RNAs or the corresponding genes is currently the most widely used

approach for the reconstruction of microbial phylogeny (Ludwig *et al.,* 1994). Analysis of PCR sequences of various genes, including 16S/23S rRNA (Fukushima *et al.,* 2002), housekeeping genes (McQuiston *et al.,* 2008), and invasion genes (Boyd *et al.,* 1997) has been used to determine the evolutionary relationships of *Salmonella*..

Identification based on the 16S rRNA sequence is of interest because ribosomal small subunit exists universally among bacteria and includes regions with species-specific variability, which makes it possible to identify bacteria to the genus or species levels by comparison with databases in the public domain (Bakkali *et al.,* 2013). Public databases such as GenBank contain a vast number of bacterial 16S rRNA sequences, allowing for rapid analysis and providing phylogenetically meaningful information (Bosshard et al., 2006; Janda & Abbott, 2007).

It can be used as the gold standard for the speciation of bacteria including *Salmonella*. This method utilizes universal primers to amplify and sequence either a partial region approximately 500bp of the 16SrRNA or the full gene approximately 1500bp (El Allaoui *et al.,* 2014). rRNA based phylogenetic trees can be reconstructed and the significance of their topologies evaluated by applying distance, maximum parsimony and maximum likelihood methods of phylogeny (Ludwig *et al.,* 1994).

Application of sequencing and bioinformatics has succeeded in stratifying the *Salmonella* population into distinct phylogenetic lineages based on the differences in the nucleotides in the genetic makeup of the bacteria. Analysis of these differences in nucleotides provides an unequivocal test of relatedness which can be inferred from their relative positions on the phylogenetic tree (Kariuki *et al.,* 2010).

The genetic factors that influence each serovar's level of host adaptation, how they evolved or were acquired, their influence on the evolution of each serovar, and the phylogenic relationships

between the serovars are of great interest as they provide insight into the mechanisms behind these differences in host range and disease progression (Chan *et al.,* 2002).

CHAPTER THREE: METHODOLOGY

3.1: Study sites

The study site for this work was carried out in Nairobi and its environs. The fecal matter of cows was collected at Dagoretti slaughter house complex that has 3 different abattoirs and slaughters a large number of animals that come from different parts of the county. Sampling of pigs was done in Ndumbuini abattoir. Pigs are sent to this abattoir originate from Nairobi and Kiambu counties which are among the main pig farming counties (Kikuvi *et al.,* 2007).

Chicken samples were collected from Gikomba and Machakos markets as well as from slaughterhouses in Nairobi (Bama and Kariokor which handles exotic chicken and Maziwa which handles the indigenous breeds) in Nairobi and Machakos counties.

Eggs were collected from the same venues where the sampling of chicken was done.

3.2: Study design

This was a cross sectional study. This is a study that involves observing the incidence of a particular disease and the measurements are taken at a particular point in time (Pearce, 2012).

3.3: LABORATORY PROCEDURES

3.3.1: Sample collection

Samples for *Salmonella* analysis were obtained from the fecal material of chicken, cattle and pigs. Eggs were also collected for this study.

Sample collection was done between December 2013 and October 2014. The sample size was calculated according to the formula of Daniel *et al.,* 1999

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

Where n = sample size, Z = Z statistic for a level of confidence, P = expected prevalence or proportion, d = precision

Using this formula the sample sizes was obtained: Pigs= 182 (Kikuvi *et al.*, 2010), Chicken- 191 (Endris *et al.*, 2013), Eggs=217 (Bayu *et al.*, 2013), Cattle=148 (Addis *et al.*, 2011).

5 grams of fecal matter from 150 cows and 182 pigs was aseptically removed from the large bowel after evisceration at the slaughtering line and put in a sterile jar and transported to the lab for further processing.

In the case of chicken, clocal swabs were obtained from 191 chicken using a sterile cotton swab and put in a sterile container and transported to the lab.

3.3.2: Isolation and identification of *Salmonella* from the fecal material and eggs

In the laboratory, 1g of the fecal matter of cows and pigs as well as the clocal swabs of the chicken was inoculated in 10 ml of selenite broth (Oxoid, UK) and incubated at 37 °C for 24

hours. A loopful of the selenite broth was plated on XLD agar (Oxoid, UK) and incubated at 37 °C for 24-48 hours. The plates were then examined for the presence of *Salmonella* colonies characterized by pinkish colonies with black centers.

Isolation of eggs was done according to Bayu *et al.*, (2013) where the eggs were thoroughly cleaned first with soap and then surface cleaned by immersion in 70% ethanol, air dried and then cracked with a sterile knife. Each egg contents was then mixed thoroughly and 1ml of the mixed egg content was inoculated into 25 ml buffered peptone water (International Diagnostic Group, Lancashire, UK) and incubated at 37 °C for 24 hours for pre enrichment. The pre-enriched sample (1ml) was aliquoted and transferred to 10 ml selenite F broth and incubated for 24 hours. A loopful of the selenite broth was plated on XLD agar (Oxoid, UK) and incubated at 37 °C for 24-48 hours. The plates were then examined for the presence of *Salmonella* colonies.

3.3.3: Gram staining

Gram staining was carried out on all the presumptive *Salmonella* samples. A pure colony was obtained from the XLD plate and a smear was made and heat fixed on a microscope slide. The slide was first flooded with crystal violet and left to stand for 1 minute before the stain was washed off under running water. The slide was then flooded with iodine and allowed to stand for 1 minute and then the stain was washed off under a tap. Acetone was used to flood the slide and immediately washed off under running water. The slide was finally flooded with phenol red, allowed to stand for 1 minute and washed off with water. The slides were placed on a mesh to dry for 10 minutes. The slides were then viewed under a Leica DM 500 microscope (Leica microsystems, Wetzlar, Germany).

3.3.4: Biochemical tests

The positive samples that were distinguished morphologically and conformed to be gram negative rods via microscopy were subsequently subjected to the biochemical tests using the API (Analytical Profile Index) 20E strips (bioMérieux, Marcy-l'Etoile, France). The strips were first prepared by dispensing water from a dropper to create a humid environment for the bacteria. A pure colony was then picked from the XLD plate using a sterile cotton swab and placed into an ampule containing 2 ml of 0.85% saline solution suspension media. The suspension was then aspirated using a syringe and aliquoted into each of the 20 micro tubules about half way with the exception of GEL which was filled to capacity. Mineral oil was then added to the chambers labeled ADH, LDC, ODC, H_2S and UREA. The strips were then placed in the humidity chamber and incubated at 37 °C for 24 hours. After incubation the color reactions were read and recorded as either positive or negative. The results were then converted to a seven-digit profile number, and identifications were made with the API Profile Index software v40.

3.4: Extraction of genomic DNA

Upon confirmation of *Salmonella* by biochemical tests, a pure colony of the positive samples was inoculated in 5 ml of nutrient broth (Oxoid, UK) and incubated at 37 °C for 24 hours. *Salmonella* DNA was extracted using the QIA prep miniprep kit (Qiagen Valencia CA, USA). In this extraction protocol, the culture was centrifuged at 2500g for 10 minutes. The supernatant was discarded, 250 µl of suspension buffer P1 was added to each sample tube and vortexed till the pellet was dislodged. The suspension was then transferred to a 1.5 ml eppendorf tube where 250 µl of lysis buffer P2 was added to each tube and the contents mixed by gently inverting the tube 6 times till the solution became clear. Buffer N3 (350µl) was then added to each tube which

was immediately inverted 4-6 times till the solution became cloudy after which the mixture was centrifuged for 10 min at 13000 rpm. The supernatant was transferred to a spin column and spun at 13000 rpm for 1 minute. Binding buffer PB (500µl) was added to each column and centrifuged at 13000 rpm for 1 minute after which the flow through was discarded. Wash buffer PE (750 µl) was added to each column and centrifuged at 13000rpm for 1 minute. The flow through was discarded and a dry spin was done at 13000 rpm for 1 minute to remove any residual wash buffer. DNA was eluted using 50 µl of elution buffer EB by spinning at 13000 rpm for 2 minutes. The extracted DNA was stored at -20 °C for further processing. Presence of DNA was confirmed by electrophoresis on 0.8% agarose gel. Electrophoresis was done at 100V for 45 minutes and the DNA bands were visualized under UV transilluminator (Herolab, Wiesloch, Germany) before storage at -20 °C.

3.5: Polymerase Chain Reaction (PCR)

Forward primer 16SF1 (5'-TGTTGTGGTTAATAACCGCA-3') and reverse primer 16SIII (5'-CACAAATCCATCTCTGGA-3') primers (Inqaba Biotech, South Africa) of the16S rRNA gene (Lin & Tsen, 1996) targeting the 16sRNA gene were used in PCR amplification of the 572bp product. PCR was performed in a TProfessional thermocycler (Biometra, Gottingen, Germany).

Amplification was carried out in 50 µl reaction volumes containing 25 µl of Dream Taq Master mix (Thermoscientific, USA), 15 µl of nuclease free water, 2.5 µl of each primer and 2.5 µl of the extracted bacterial DNA. The amplifications were done in 35 cycles with an initial denaturation at 95 °C for 5 minutes, a denaturation step of 95 °C for 2 minutes, primer annealing at 55 °C for 30 seconds and primer extension 72° C for 1 minute. Finally an additional extension was done for 10 minutes at 72 °C. The PCR products were visualized on 1% agarose gel in 1X

TAE buffer stained with ethidium bromide for 1.5 hours at 100V. The PCR bands were visualized under UV transilluminator (Herolab, Wiesloch, Germany).

3.6: Gel extraction

The PCR products obtained were extracted using QIAquick gel extraction kit (Qiagen, Valencia CA, USA). Briefly, the amplified PCR product was excised from the agarose gel with a scalpel and placed in an eppendorf tube which was then weighed. The dissolving buffer QG (3 volumes) were added to1 volume of gel in each tube where 100 mg of gel ~100 µl of buffer. The eppendorf tube was then placed in a water bath at 56 °C until the gel dissolved and the tube was vortexed every 2 minutes to facilitate quick dissolution of the gel. After the gel dissolved, 1 gel volume if isopropanol was added to each tube and mixed thoroughly. The mixtures were then transferred to spin columns and centrifuged for 1 minute at 13000 rpm to enable the DNA to bind to the column. The flow through was discarded and 500 µl of buffer QG was added to each column and then centrifuged for 1 minute at 13000 rpm. The resulting flow through was discarded after which 750 µl of wash buffer was added to each column. The column was allowed to stand for 5 minutes after which it was centrifuged for 1 minute at13000 rpm. The flow through was discarded and a dry spin was done for an additional 2 minutes at 13000 rpm to remove any residual wash buffer. The DNA was eluted using 30 µl of the elution buffer and allowed to stand for 4 minutes before it was centrifuged at 13000 rpm for 2 minutes. The purified DNA was then analyzed on 1% agarose gel in 1X TAE buffer stained with ethidium bromide for 1.5 hours at 100V. The DNA bands were visualized under UV transilluminator (Herolab, Wiesloch, Germany).

3.7: Sequencing of *Salmonella* isolates

The purified DNA (30µl) of each of the samples after gel extraction were then sent to a commercial vendor (Macrogen, Netherlands) for DNA sequencing.

3.8: Phylogenetic analysis

A consensus sequence of 16srRNA for each of the isolates was generated using the Bioedit software. The sequences obtained were compared with known 16S rRNA sequences at National Center for Biotechnology Information (NCBI) database using BLASTn (Basic Local Alignment Search Tool) algorithm obtained from; http://www.ncbi.nlm.nih.gov/BLAST. Identification at both the genus and species level was defined as a 16S rRNA sequence similarity of ≥ 99% with that of the prototype strain sequence in GenBank. The sequences together with reference sequences derived from the Genbank were aligned using CLUSTAL W. The topology, distance and probability of the phylogenetic tree was determined using Mr. Bayes software. The topological robustness of the trees was evaluated by a bootstrap analysis involving 10000 replications. The tree was then visualized using fig tree software v. 13.1.

3.9: *Salmonella* antimicrobial susceptibility tests

Antibiotic susceptibility testing was tested Kirby-Bauer disk diffusion method using guidelines established by the Clinical and Laboratory Standards Institute (CLSI). Briefly, by taking pure isolated colonies, bacterial suspension in test tubes was adjusted and compared to 0.5McFarland turbidity standards. The diluted bacterial suspension was then transferred to Mueller-Hinton agar plate using a sterile cotton swab and the plate was seeded uniformly by rubbing the swab against the entire agar surface. Antibiotic impregnated discs were then applied to the surface of the

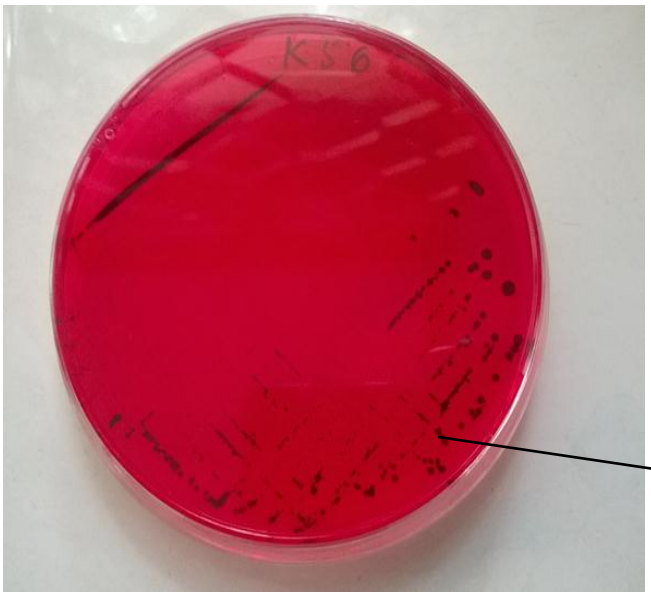
inoculated plates using sterile forceps. The plates were then incubated aerobically at 37 °C for 24 hours. *E. coli* (ATCC 25922), which was susceptible to all tested drugs, was used for quality control. A total of 8 selected antibiotic disks including tetracycline nitrofurantoin, nalidixic acid, streptomycin, sulphamethoxazole, cotrimaxazole, gentamycin and ampicillin were applied. Finally, the zone of inhibition was measured including the disk diameter and the susceptible intermediate and resistant categories were assigned on the basis of the critical points recommended by the CLSI.

CHAPTER 4: RESULTS

4.1: Morphological characterization

4.1.1: Culture and gram stain of *Salmonella* isolates

Following incubation of the samples in nutrient broth 49 out of the 740 samples showed the presence of characteristic *Salmonella* colonies i.e. pinkish colonies with black centers as a result of formation of hydrogen sulphide (H_2S) on XLD agar (Figure 1) and appearance of gram negative rods (Figure 2) after being subjected to gram staining. Of the samples 15/182 (8.2%) of pigs, 6/150 (4%) of cows, 8/191 (4.6%) of chicken and 20/217 (9.2%) of the eggs were positive as seen in figure 3. The positive samples are tabulated per species in table 3.



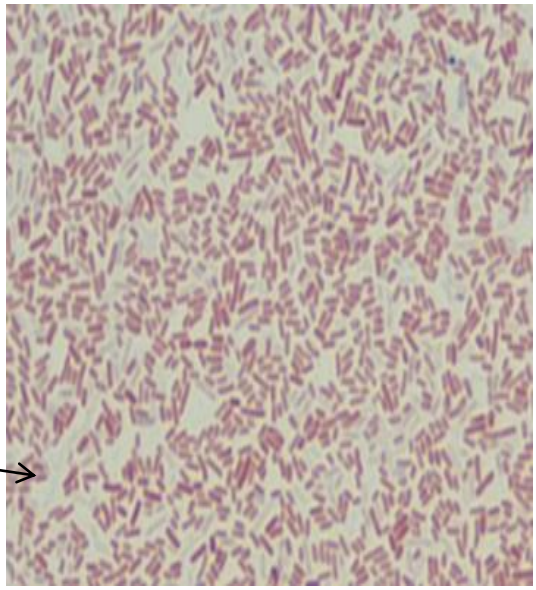


Figure 1 Figure 2

Figure 1: Photograph of *Salmonella* growth on XLD agar characterized by pinkish colonies with black centers.

Figure 2: Photograph of gram stain of a Salmonella colony showing a gram negative rod

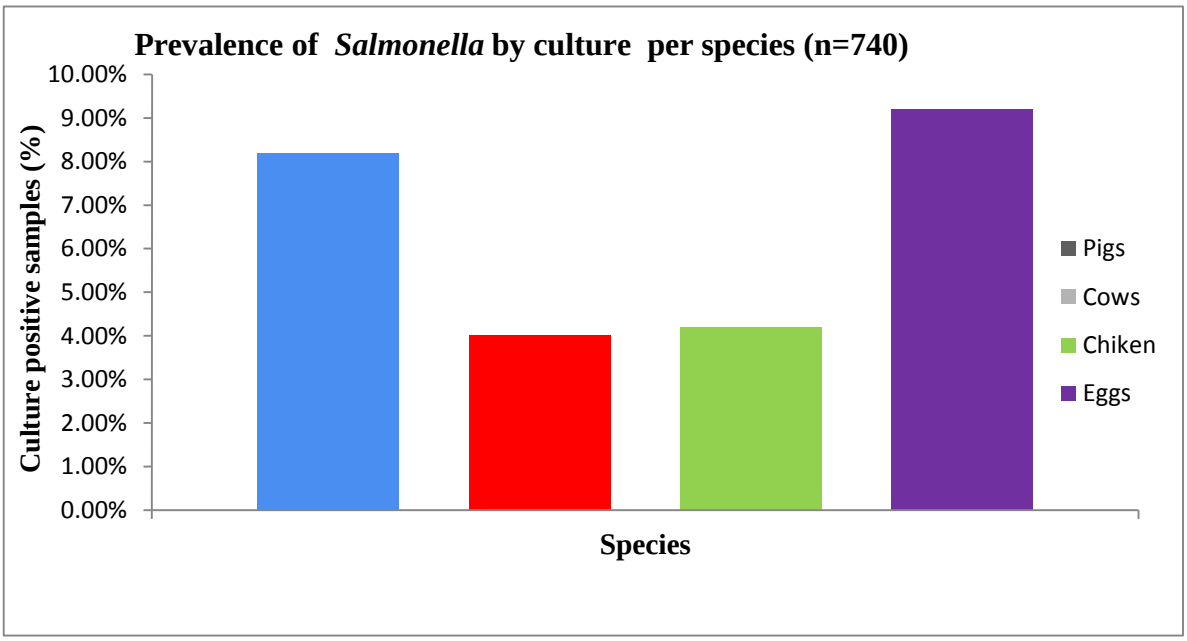


Figure 3: Prevalence of *Salmonella* from samples of different species (cows, pigs, chicken and eggs)

Table 3: Prevalence of *Salmonella* by culture from different species (cows, chicken, pigs and eggs)

Species	**Pigs**	**Cows**	**Chicken**	**Eggs**
Positive samples	15	6	8	20
Total number of samples tested	182	150	191	217
Percentage	8.2%	4%	4.2%	9.2%

4.1.2: Biochemical tests

Positive colonies by culture (n=49) were subjected to biochemical tests using API 20E strip and 31 out of 49 samples turned positive for *Salmonella* (Table 4, Table 5 & Figure 4). The negative samples were identified as Citrobacter, Serratia or unidentified profiles using the API software. Figure 4 shows the difference in colour changes between the negative sample (Citrobacter) and a

positive *Salmonella* sample and table shows utilization of each of the 20 substrates and table 4 denotes the utilization of each of the substrates.

Figure 4: Biochemical test using API 20 E strips showing a negative control Citrobacter no.21 and a positive *Salmonella* no. 2. The microtubules are labeled as follows ONPG: o-nitrophenyl-D- galactopyranoside; ADH: arginine dihydrolase, LDC: lysine decarboxylase, ODC: ornithine decarboxylase, CIT: citrate, H_2S: hydrogen sulphide production, URE: urease, TDA: tryptophan deaminase, IND: indole, VP: Voges Proskauer test, GEL=gelatinase, GLU: glucose, Man: mannose, INO: inositol, SOR: sorbitol, RHA: rhamnose, SAC: sucrose, MEL: melibiose, AMY: amygdalin, ARA: arabinose.

Table 4: Biochemical test results showing positive and negative reactions to different substrates for *Salmonella* (sample number 2) and a negative control *Citrobacter* (sample number 21).

Sample	**ONPG**	**ADH**	**LDC**	**ODC**	**CIT**	**H_2S**	**URE**	**TDA**	**IND**	**VP**	**GEL**	**GLU**	**MAN**	**INO**	**SOR**	**RHA**	**SAC**	**MEL**	**AMY**	**ARA**	**Identification**
2	-	+	+	+	+	+	-	-	-	+	-	+	+	-	+	+	-	+	-	+	*Salmonella*
21	+	+	+	+	+	+	+	+	+	+	+	+	+	-	+	+	+	+	+	+	Citrobacter

The results of the differences in utilization of various substrates that help distinguish between *Salmonella* and other Enterobacteriaceae. + indicates ability of the bacteria to utilize the substrate while – indicates inability to utilize the substrate.

Table 5: Identification of *Salmonella* by biochemical tests of different species (n=49)

Species	**Pigs**	**Cows**	**Chicken**	**Eggs**
Positive	7	4	7	13
Total samples	182	150	191	217
Total samples tested	**3.8%**	**2.7%**	**3.7%**	**6%**

4.2: Molecular characterization

4.2.1: PCR analysis

The positive samples by biochemical tests were subjected to genomic DNA extraction (sec 3.3.1) followed by PCR analysis of 16S rRNA. A 572bp band was observed in each of the wells as seen in figure 7. All 31 samples were positive for *Salmonella.*

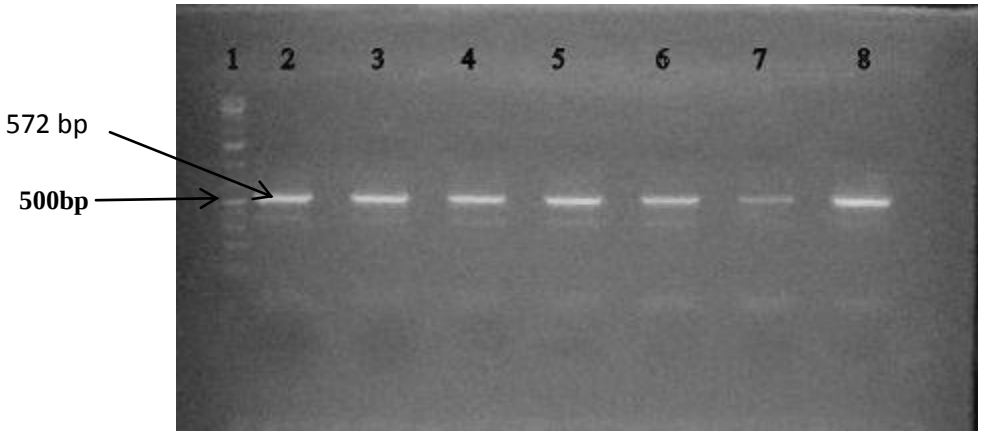


Figure 5: Agarose gel analysis of PCR (572bp) of Salmonella isolates. Lane 1: 100bp ladder; Lane 2: Positive control; Lanes 3, 4, 5, 8: S. Typhimurium; Lane 7: S. Enteritidis; Lane 9: Negative control.

4.3: Prevalence of *Salmonella* in cows, pigs chicken and eggs

The overall prevalence was determined by PCR to be 3.8% for pigs, 2.7% in cows, 3.7% in chicken and 6% in eggs (Figure 6; table 6).

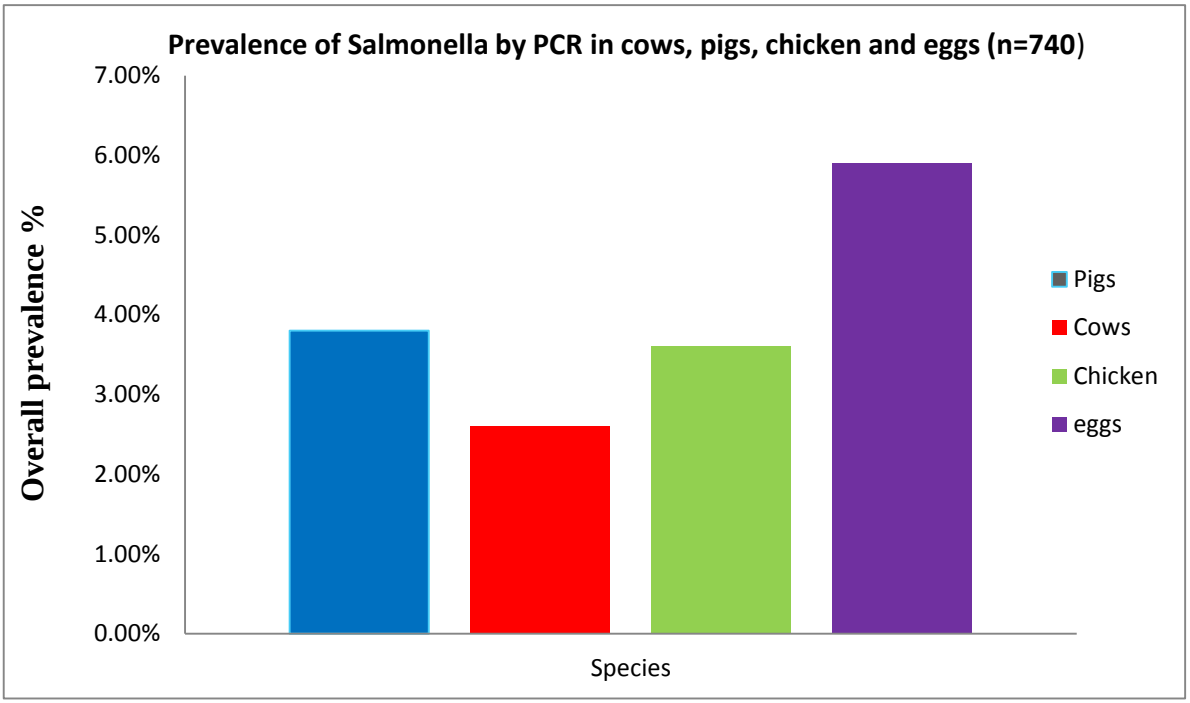


Figure 6: Prevalence of *Salmonella* by PCR analysis of samples from different species

Table 6: Prevalence of *Salmonella* PCR analysis of cows, pigs chicken and eggs following PCR analysis.

Species	**Pigs**	**Cows**	**Chicken**	**Eggs**
Positive for *Salmonella*	7	4	7	13
Total number of samples collected	182	150	191	217
Overall Prevalence by PCR	**3.8%**	**2.6%**	**3.6%**	**5.9%**

4.4: Sequencing of *Salmonella* PCR Products

A representative sequence of a *Salmonella* isolate results are shown in the figure 7 below.

Fig 9: A chromatogram one of the sequenced *Salmonella* isolates. The peaks represent called bases and different coloured peaks denote different nucleotides.

4.4.1: Alignment of 16s rRNA sequences

A multiple sequence alignment of the 16S rRNA sequences for selected isolates using Bioedit showed differences in nucleotides at particular positions in the alignment indicated by gaps and unshaded sections as seen in positions 10-18, 68, 131,135, 191,201, 237, 286, 302, 369 among others (Figure 8)

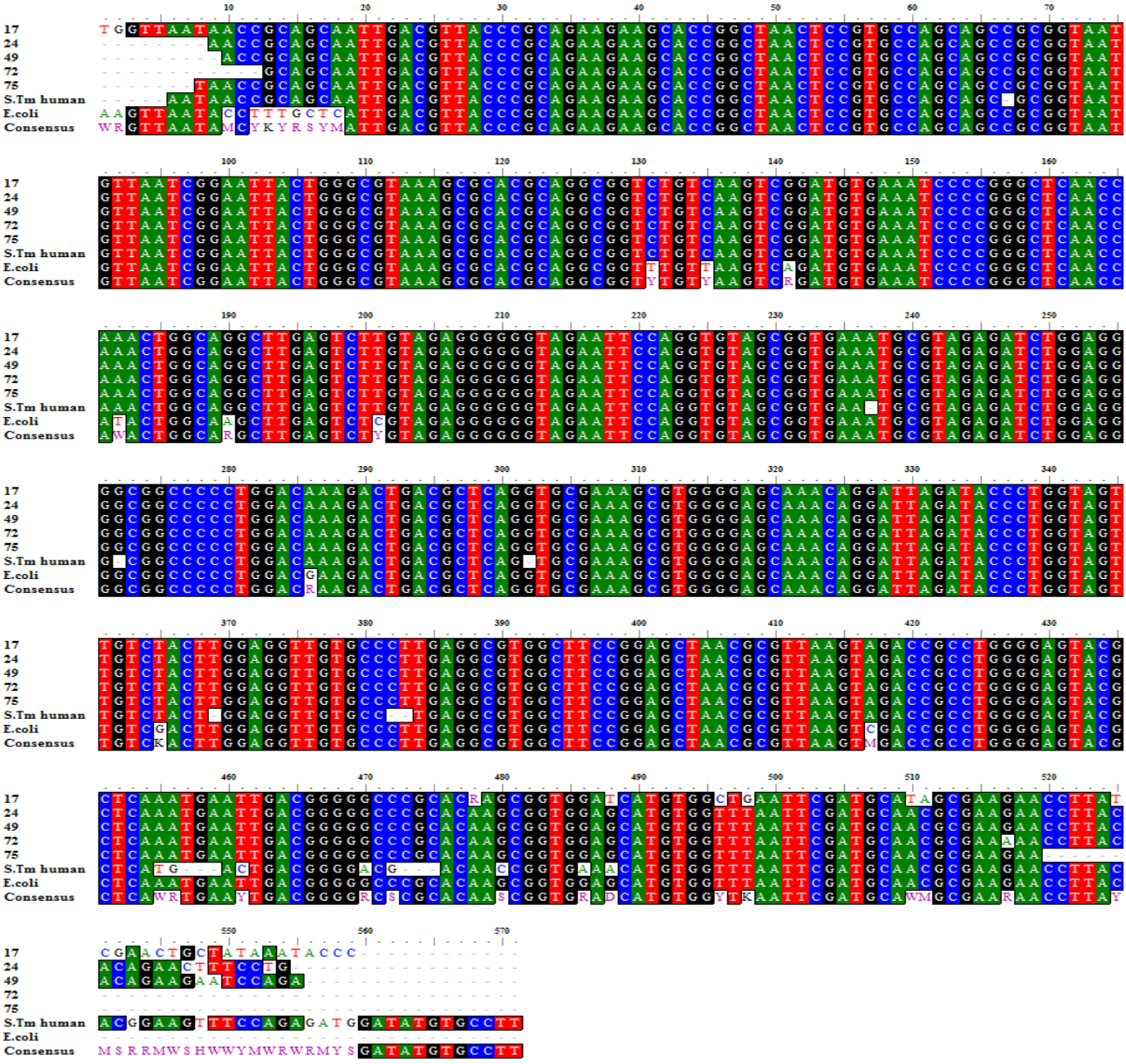


Figure 8: A multiple sequence alignment of 16S rRNA of field samples(17,24,49,72,75) contol (STm) and the root of the tree (*E. coli*) showing the differences in the bases of selected isolates as well as a consensus sequence. Shaded areas of a colour indicate similarity while gaps/unshaded areas indicate differences between the selected sequences.

4.4.2: Phylogenetic tree

A phylogenetic tree was constructed and the topological robustness of the tree was evaluated using posterior probabilities (Figure 9). From the NCBI blast results 2 serovars: *S.* Typhimurium and *S.* Enteritidis were identified and they formed two clades in the phylogenetic tree. *Escherichia coli* was used to root the tree. *S.* Enteritidis clade shows a probability of 68% from the majority *S.* Typhimurium clade in this tree. There is a 91% probability between the *S.* Choleraesuis and *S.* Paratyphi that were used as reference sequences in this analysis. From the branch length it appears that more variation has occurred in *S.* Typhimurium human isolate (*S.* Tm NR074800.1) than the *S.* Typhimurium field samples used in this study.

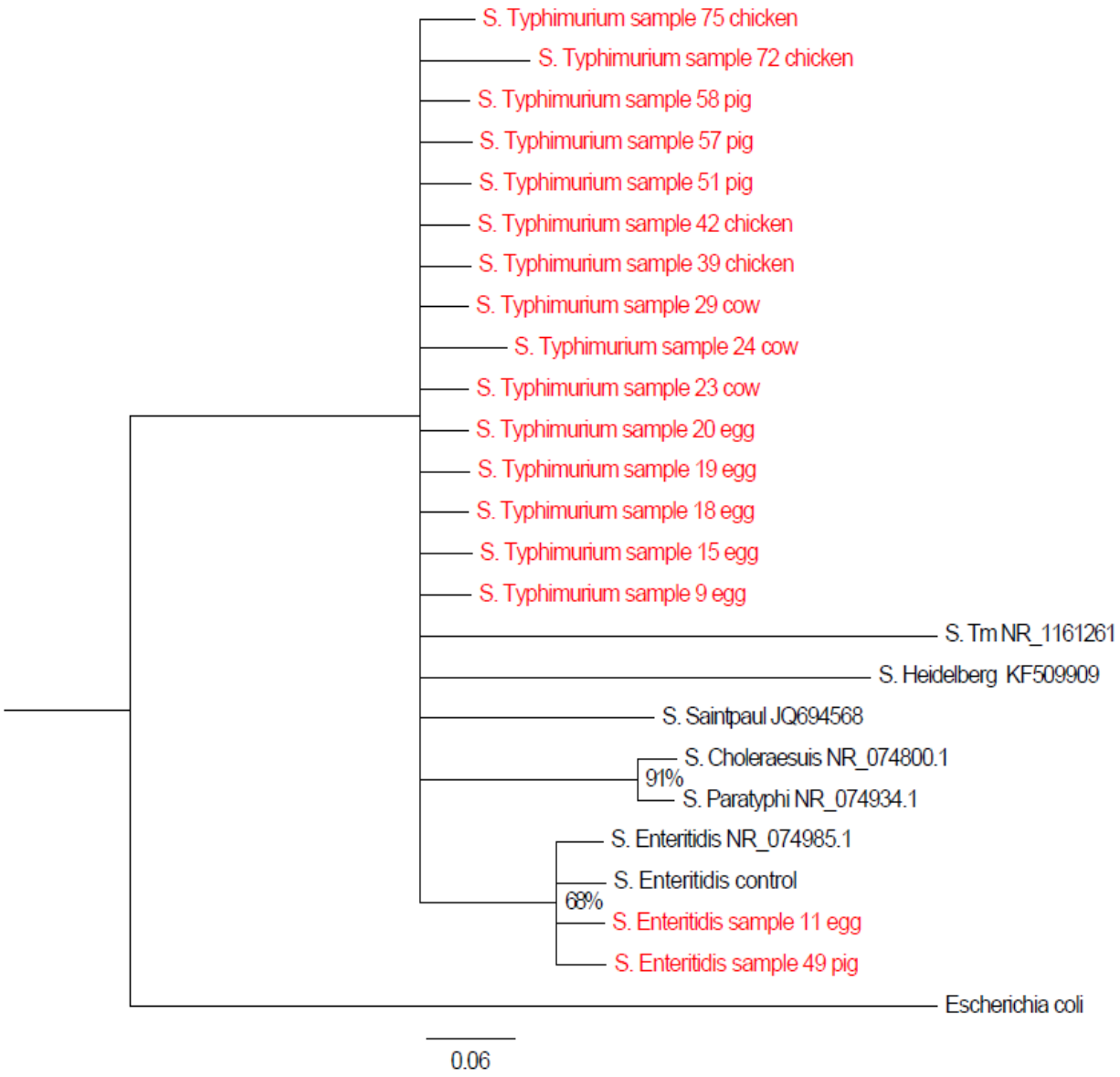


Figure 9: A phylogenetic tree based on 16s rRNA sequences of *Salmonella* isolates. The phylogeny was inferred by Bayesian method using the Markov Chain Monte Carlo (MCMC) method from an alignment performed using Bioedit. The Phylogenetic tree was visualized using Fig Tree v. 13.0. Numbers at the nodes show percentage of posterior probabilities indicating topological robustness.

4.5: Antibiotic resistance profiles of *Salmonella* isolates

Antimicrobial resistance was detected in 40% of the *Salmonella* isolates and 20% of the isolates resistant to two drugs: SXT COT (n= 3), streptomycin sulphamethoxazole (n=1), tetracycline sulphamaethoxazole (n=1). Intermediate resistance of the *Salmonella* isolates was observed against nitrofurantoin (84%), ampicillin (76%), sulphamethoxazole (52%), streptomycin (40%), tetracycline (36%), gentamycin (28%), cotrimoxazole (20%) and nalidixic acid (12%) as shown in Figure 10, 11 and table 7.

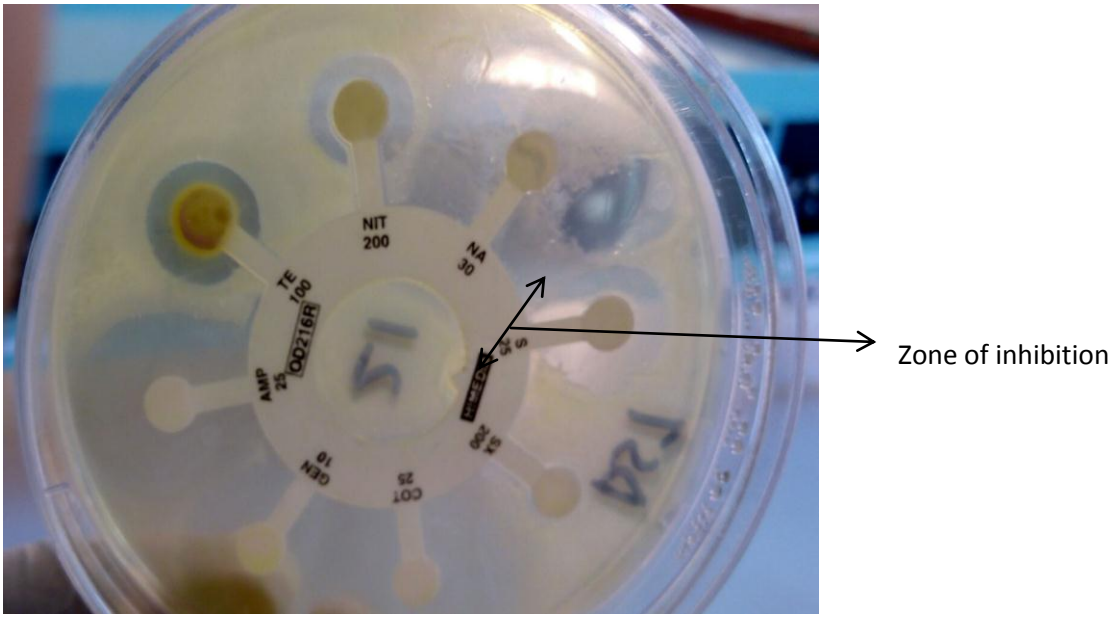


Figure 10: Antimicrobial resistance tests using the Kirby disk diffusion method using the following drugs: tetracycline100µg (TET), nitrofurantoin 200µg (NIT), nalidixic acid 30µg (NAL), streptomycin 25µg (S), sulphamethoxazole 200µg (SXT), cotrimoxazole 25µg (CoT), gentamycin 15µg (GEN) and ampicillin 25µg(AMP).

Table 7: A table showing the antibiotic resistant profiles of *Salmonella* isolates (n=25).

Sample no	**TET 100µg**	**NIT 200µg**	**NAL 30µg**	**S 25µg**	**SXT 200µg**	**CoT 25µg**	**GEN 15µg**	**AMP 25µg**
1	+++	+	+++	++	+	+++	+++	++
2	+++	+	+++	++	**R**	**R**	+++	+++
3	+++	+	+++	+++	++	+++	+++	++
4	++	+	+	+++	+++	+	+++	+
5	++	+	+++	+++	+	+++	+++	+
6	++	+++	++	++	+	+++	++	**R**
7	++	++	+++	++	+++	+	+++	**R**
8	+++	++	+++	+++	+	+++	++	+
9	+++	++	+++	++	**R**	++	++	+
10	**R**	++	+++	++	**R**	+++	++	+
12	+++	+++	+++	+++	++	+++	+++	++
13	+++	+++	+++	+++	**R**	**R**	+++	++
14	+++	++	+++	+++	**R**	**R**	++	+
17	+	++	+++	+++	++	+	+++	+
20	+++	++	++	+++	+	++	+++	+++
21	++	++	+++	++	**R**	+++	+++	+++
22	+++	++	+++	+++	+	++	++	++
25	+	++	+++	+++	+	+++	++	+
26	+++	+++	+++	+++	++	+++	+++	++
27	++	+	+++	++	+	+++	+++	++
30	++	++	+++	+++	+++	+++	+++	++
31	++	++	+++	++	**R**	+++	+++	+
32	+	+	+++	**R**	**R**	+++	+++	++
33	+++	++	+++	+++	+++	+++	+++	++
34	++	++	+++	++	+	+++	+++	++

The table shows the resistance profiles of the *Salmonella* strains isolated where: +++ represents susceptible strains; ++ and+ represents intermediate susceptibility while R represents resistant *Salmonella* strains.

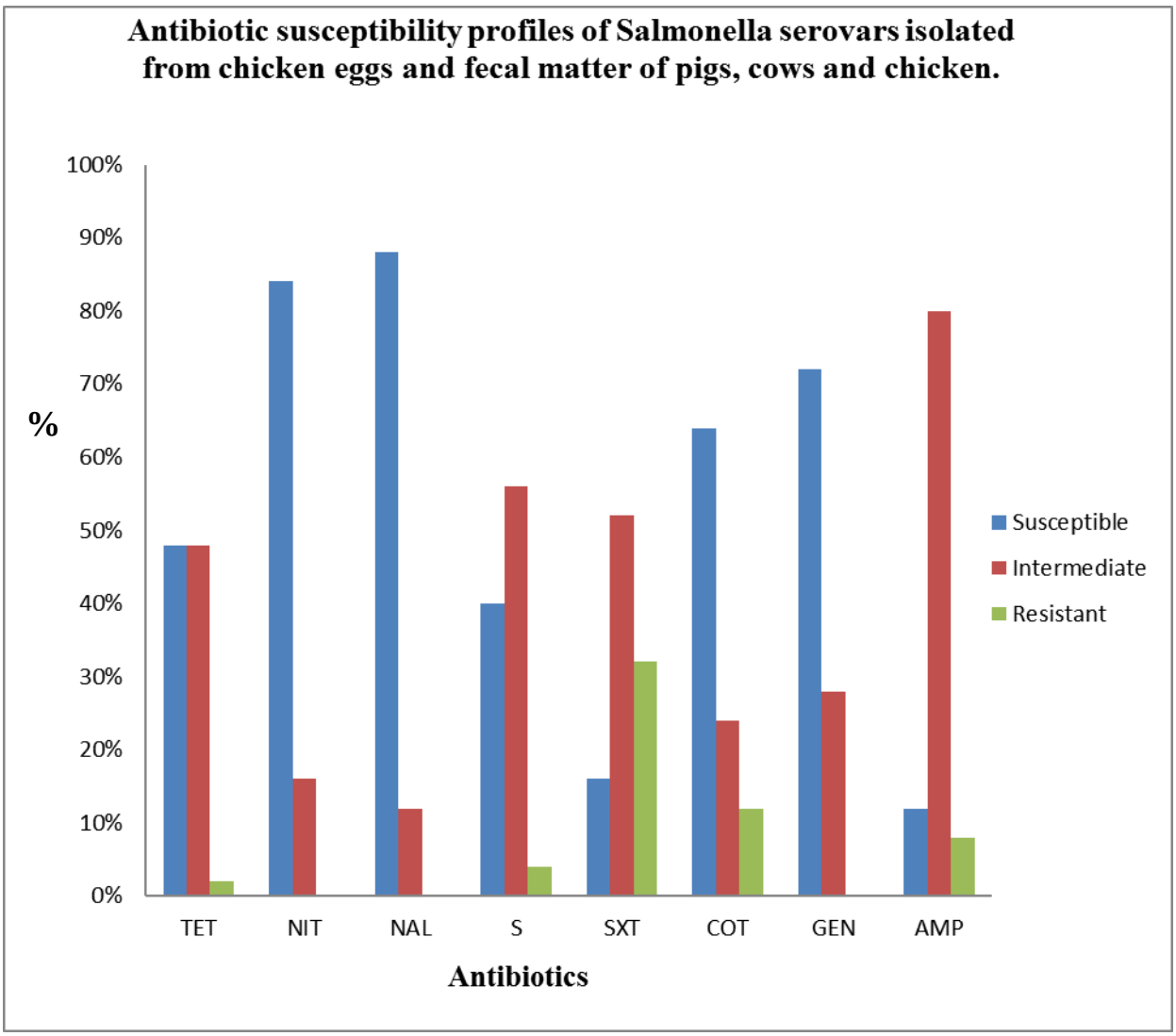


Figure 11: Percentage activity of *Salmonella* isolated from eggs and fecal matter of cows, pigs and chicken to various antibiotics. The activity was grouped as susceptible (blue), intermediate (red) or resistant (green) to the following drugs: tetracycline (TET), nitrofurantoin (NIT), nalidixic acid (NAL), streptomycin(S), sulphamethoxazole (SXT), cotrimoxazole (CoT), gentamycin (GEN) and ampicillin (AMP).

CHAPTER 5: DISCUSSION, CONCLUSION AND RECOMMENDATIONS

5.1: DISCUSSION

Salmonella generates serious health and economic consequences (Ammari *et al.,* 2009). Sub clinical *Salmonella* infections in animals are an important food safety problem because of the transmission route of *Salmonella* through the food chain to humans (Malorny & Hoorfar, 2005).

This is the first study in Kenya that sought to determine the prevalence of *Salmonella* in animals and animal products using a combination of culture, biochemical and molecular methods (phylogeny). Overall in determination of prevalence of *Salmonella* in the study there was a reduction in the number of positive samples after culturing method and after biochemical tests. A decrease in positive samples was observed ranging from as low as 12.5% in chicken to as high as 50% in pigs. This is because although XLD has high specificity for *Salmonella*, Proteus and Citrobacter can produce colonies indistinguishable from those of *Salmonella* (Park *et al.,* 2012).

In this study the prevalence of *Salmonella* species isolated from pigs was 3.8%. This is comparable to a study conducted in Korea (Lim *et al.,* 2011), but is lower than a study conducted in Kenya (Kikuvi *et al., 200* that reported a prevalence of 13.8% in Kenya as well as a study that reported 16% prevalence in Burkina Faso (Kagambèga *et al.,* 2013). The difference in the prevalence for the Kenyan study could be due to the difference in period of sampling, husbandry practices or the origin of the pigs since the pigs come from different parts of the country to the abattoir. Factors such as intermittent shedding and clustering have also been acknowledged to reduce the diagnostic sensitivity of fecal culture methods (Sanchez *et al.,* 2007).

The prevalence of *Salmonella* in eggs (5.9%) was higher in this study than in the Ethiopia study which established a prevalence of 4.69% (Bayu *et al.,* 2013). The presence of *Salmonella* in eggs in Kenya therefore is a concern because several outbreaks have been attributed to consumption of contaminated eggs especially in the United States (Andino & Hanning, 2015). Most food-borne *S.* Enteritidis infections are associated with the consumption of raw eggs and foods containing raw eggs such as homemade ice cream, mayonnaise and others egg products (Bayu *et al.,* 2013, Kariuki *et al.,* 2002). The detection of *Salmonella* in eggs demonstrates that improvements need to be made in controlling *Salmonella* transmission in farms. There are various sources of contamination on farms, the main sources being hens' droppings and contaminated litter. Therefore, effective egg surface disinfection is critical to reduce pathogens on eggs and potentially control egg-borne disease outbreaks. To the best of my knowledge this is the first study to investigate the prevalence of *Salmonella* in eggs in Kenya.

The prevalence of *Salmonella* in chicken in this study was 3.6%**.** *Salmonella* contamination rates for chicken reported in literature vary from 0.8% to 11% in Ethiopia (Aragaw *et al.,* 2010, Endris *et al.,* 2013, Menghistu *et al.,* 2011) and Nigeria (Fashae *et al.,* 2010 Raufu *et al.,* 2009). The results of this study are comparable to results obtained from a study conducted in in Tanzania (Mdegela *et al.,* 2000). The lower prevalence of 0.8% in Aragaw *et al.,* 2010 could be due to the fact that pre enrichment was not done. Pre enrichment helps to proliferate or regenerate cells thus increasing their viability when cultured on a solid medium (Zadernowska & Chajecka, 2012). The differences in prevalence could also be due to the geographical region, the type of chicken screened whether local indigenous or the exotic breeds. This study corroborates the work done by Endris *et al.,* 2013 where there was a higher prevalence of *Salmonella* in the indigenous chicken 71.4% compared to the grade chicken 28.6%. The levels of *Salmonella* in poultry can

vary depending on the country, the nature of the production system and the specific control measures in place (Kagambèga *et al.,* 2013).

The prevalence of *Salmonella* in cows was lower in this study as compared to studies done in Ethiopia (Addis *et al.,* 2011, Alemayehu *et al.,* 2003). This could be due to differences in environment, management and geographical distribution as well as husbandry practices. In the above studies a higher prevalence has been observed amongst dairy cattle compared to beef cattle (Addis *et al.,* 2011).

In this current study, two (2) serovars were identified: *S.* Typhimurium and *S.* Enteritidis. These two serovars are most commonly associated with food products and are the major causes of Salmonellosis in humans worldwide (Whiley & Ross, 2015; De Medici *et al.,* 2003). The serovars identified in this study are contrary to the study done in Kenya where *S.* Heidelberg, *S.* Agona and *S.* Saintpaul were the most common isolated serovars in pigs (Kikuvi et al., 2010). In cows *S.* Typhimurium and Newport were the most isolated in Ethiopia (Alemu *et al.,* 2011) while in another Ethiopian study *S.* Anatum and *S.* Newport were the most commonly isolated (Sibhat *et al.,* 2011). In eggs *S.* Enteritidis was the isolated serovar in Ethiopia whereas in Australia *S.* Typhimurium is the most isolated serovar (Whiley *et al.,* 2015). These results highlight the complexity of the global epidemiology of *Salmonella* as frequency and occurrence changes over time in countries and regions. Shifts in prevalence may follow introduction of the strain through animal feed and livestock trade (Hendriksen *et al.,* 2011).

Genotypic identification methods are emerging as an alternative or complement to established phenotypic identification procedures. For bacteria, 16S rRNA gene sequence analysis is a widely accepted tool for molecular identification (Bosshard *et al.,* 2006). From the multiple alignment data, there exists some differences in various positions in the sequences. These differences are

then exploited by the software that uses Bayesian inference method to show evolutionary relationship using a phylogenetic tree. The phylogenetic tree showed the two serovars: *S.* Typhimurium and *S.* Enteritidis resulting in two distinct clades.

Antibiotic resistance is the evolutionary response by bacteria to the strong selective pressure that results from exposure to these compounds (Wright *et al.,* 2010). The *Salmonella* isolates in this study were susceptible to most of the easily accessible and cheaper drugs such as tetracycline while resistance was observed against sulphamethoxazole and cotrimoxazole. This could be an indicator of the acquisition of the resistance genes for those drugs due to the indiscriminate use of these 2 drugs at recommended doses or at sub therapeutic doses in feed additives to promote growth creating on farm selection of antimicrobial resistant strains (Kariuki *et al.,* 2005). Two of the isolates are resistant to sulphamethoxazole and not to cotrimoxzole which is a combination of sulphamethoxazole and trimethoprim (a folic acid analogue). Cotrimoxazole works by inhibiting 2 steps in the enzymatic pathway for bacterial folate synthesis. The isolates therefore have not acquired the trimethoprim resistance, *dhfr* genes that encode altered dihydrofolate reductases that reduced affinity for the antimicrobial agent, allowing folic acid biosynthesis to occur in the presence of trimethoprim (Foley *et al.,* 2008). There was a high percentage of isolates that were intermediately resistant to the panel of antibiotics tested. This could be indicative of increasing resistance towards the commonly used drugs and a cause of concern in the treatment of non typhoidal *Salmonella*. In a previous study done by Kariuki *et al.,* (2002) all the isolates from animals were susceptible to the commonly used drugs. The detection of resistance in the samples in this study shows that there could be an indicator of the increased use of the antibiotics at sub-therapeutic levels or prophylactic doses which may promote on-farm selection of antimicrobial resistant strains.

5.2: CONCLUSION

The study isolated *Salmonella* Typhimurium and *Salmonella* Enteritidis from seemingly healthy animals and eggs poses a significant public health threat because it is indicative that there is presence of zoonotic organisms that have the potential of entering the food-chain. These two serovars have been associated with several outbreaks worldwide. It is therefore imperative that disease control strategies should not only focus on reducing the occurrence of bacterial infections in animals, but should include the need to reduce the threat of zoonotic pathogens from infecting humans. It also calls for surveillance and monitoring programs in the country. The chain of transmission should be viewed to ascertain sources of contamination.

The emergence of resistance is a problem and prudent use of antibiotics in animal husbandry and human therapy should be encouraged to help conserve the limited options of antibiotics available. Resistance limits the therapeutic options available to vets and physicians in treatment of salmonellosis. In view of this, the genes associated with the resistance of *Salmonella* to co trimoxazole, gentamycin, tetracycline, streptomycin and sulphamethoxazole should therefore be studied to assist in clinical management of non typhoidal salmonellosis.

5.3: RECOMMENDATIONS

The study should be extended and sample other parts of the country to give a better understanding of the prevalence rates countrywide. This will properly inform policy makers on the state of *Salmonella* infections in the country. Different types of samples such as blood or carcasses should be used to determine the level of cross contamination associated with slaughterhouses or markets. Further studies should also be carried out to determine the phage types of the two serovars to determine if they are comparable to those phage types that have been

associated with outbreaks in the global north and the invasive salmonellosis found in sub Saharan Africa.

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