DIVERSITY OF *SALMONELLA* ISOLATES FROM WILDLIFE AND DOMESTIC ANIMALS IN SELECTED AREAS OF ZAMBIA

BY

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LUSAKA

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DECLARATION

I, CHARLES MIYANDA MUBITA, hereby declare that this study is a true reflection of my own research, and that this work, or part thereof has not been submitted for a degree in any other institution of higher education.

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APPROVAL

This thesis of Charles Miyanda Mubita has been approved as fulfilling the requirements for the award of Doctor of Philosophy in Microbiology by The University of Zambia.

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ABSTRACT

Domestic animals and wildlife are considered important reservoirs from which Non-Typhoid *Salmonella* serotypes have emerged to cause public health concerns worldwide. Identification of the zoonotic origin of such organisms is important to understand the risk factors associated with a diversity of host types. In this study, phenotypic and genetic properties of *Salmonella* strains isolated from domestic animals and wildlife sources were characterized to determine their potential for causing invasive salmonellosis. The isolates were characterized into serogroups, serotypes, fermentative groups, resistotypes, and beta-lactamase activity types. Further, *Salmonella* plasmid virulence genes responsible for extraintestinal manifestations were determined among *Salmonella* serotypes from domestic animals (bovine, horse, dogs, and chickens) and wildlife (Leopard, Sable and Impala). Sequencing of the invA gene of *Salmonella* strains from different hosts and locations was performed and compared with reference sequences on the Gene-Bank.

Thirty-four *Salmonella* strains belonging to four sero-groups; B, C1, D1, and a non- reactive group, were isolated and identified from the various animal hosts studied. The most prevalent sero-group was D1 (50.0%; 17/34) *Salmonella* strains, all from domestic animals followed by a non-reactive group (29.4%; 10/34) strains. Further, 12 serotypes, were detected among the strains. The most predominant serotype was *Salmonella enterica* serotype Enteritidis (47.1%; 16/34) isolated from horse and chicken, followed by *S*. Mbandaka (8.8%; 3/34) from dog and chicken. All *Salmonella* isolates were resistant to at-least four antimicrobial agents. Ampicillin, erythromycin, gentamicin, nitrofurantoin and penicillin G were observed as the most frequently resisted antimicrobial agents. *Salmonella* Enteritidis and *S*. Mbandaka isolated from the horse and dog respectively, each displayed multidrug resistance to 6 antimicrobial agents. All strains harboured *Salmonella* invasion A gene unique to the genus, while some strains possessed *Salmonella* plasmid virulence locus gene. The horse, leopard and chickens were found to be reservoirs of *Salmonella* isolates carrying *spv*A virulence genes and by consequence; these animals could serve as a reservoir for transmission of extra intestinal salmonellosis to humans and other animals through contamination of the environment. The homology percentage of nucleotide sequence (74.0 ~ 100.0) and amino acid sequence (60.0 ~ 100.0), showed genotypic differences between the strains isolated from domestic animals and wildlife, and the published sequences on the Gene-Bank.

In conclusion, the present study isolated and identified multi-drug resistant *Salmonella* serotype strains that possess virulence genes which are of potential public health concern.

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DEDICATION

This work is dedicated to Almighty God who ordained it before I was born.

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LIST OF ABBREVIATIONS AND ACRONYMS

^{O}C	Degree Celsius
µl	Microliter
AGDH	Australian Government, Department of Health
APHA	Animal and Plant Health Agency
AVMA	American Veterinary Medical Association
AVMF	American Veterinary Medical Foundation
bp	Base pair
CART	Combined Antiretroviral Therapy
CDFW	California Department of Fish and Wildlife
CDC	Centers for Disease Control and Prevention
CD_4	Cluster of Differentiation 4
CIDRAP	Center for Infectious Disease Research and Policy
CFIA	Canadian Food Inspection Agency
CFS	Cerebral spinal fluids
CFSPH	Center for Food Security and Public Health
CLSI	Clinical and Laboratory Standard Institute
DHHS-USFDA	Department of Health and Human Services, US Food and Drug Administration.
DNA	Deoxyribonucleic Acid
DT 160	Definitive Type 160
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
ESET	Euro surveillance Editorial Team
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
FBD	Foodborne diseases
FDA	Food and Drug Administration.
FT	Fowl typhoid
GMA	Game Management Area

HACCP	Hazard Analysis Critical Control Point
HCPH	Hamilton County Public Health
HIV	human immuno-deficiency virus
IFAH	International Federation for Animal Health
ISPAH	Intervet Schering-Plough Animal Health
invNTS	Invasive Non-Typhoid *Salmonella*
MAC	Ministry of Agriculture and Co-operatives
MAPKs	Mitogen-Activated Protein Kinases
MDR	Multi-Drug Resistance
mig-5	Segment polarity protein disheveled homolog
MLST	Multilocus sequence typing
NCCLSI	National Committee for Clinical Laboratory Standards Institute
NININ	National Nanotechnology Infrastructure Network
NHMIS	National Health Management Information System
NMCC	National Malaria Control Centre
NIV/WHO	National Veterinary Institute/World Health Organization
NSCFS	Norwegian Scientific Committee for Food Safety
NTS	Non-Typhoid *Salmonella*
OECD	Organization for Economic Cooperation and Development
OIE	World Organization for Animal Health
PAI	Pathogenicity Island
PCR	Polymerase Chain Reaction
PD	Pullorum Disease
PT	Phage Type
pef	Plasmid Encoded Fimbrae
RCK	Resistance to complement killing
rRNA	Ribosomal ribonucleic acid
SABC	The South African Broadcasting Corporation
SND	Sequence Nucleotide Differences
SPI	*Salmonella* Pathogenicity Island

spv	*Salmonella* virulence plasmid
srg	Thio:disulfide interchange protein
ssa	*Salmonella* system apparatus
ssc	*Salmonella* secretion effector chaperone
sse	*Salmonella* secreted effector protein
ssr	*Salmonella* secretion regulatory protein
SSNCISM	The *Salmonella* Subcommittee of the Nomenclature Committee of the International Society for Microbiology.
ST313	Sequence type 313
ST	*Salmonella* Typhi
ST14	Sequence type 14
TF	Typhoid Fever
TS	Typhoidal *Salmonella*
TTSS	Type III Secretory System
UNAIDS	The Joint United Nations organization for human immunodeficiency virus *(*HIV*)* and Acquired Immune deficiency Syndromes (AIDS)
UNICEF	United Nations Children Fund
USA	United States of America
USDA	United States Department of Agriculture
USPC	United States Pharmacopeial Convention
UV	Ultra-violet
VPs	Virulence Plasmids
VSCEAH	Veterinary Services Center for Epidemiology and Animal Health
WHO	World Health Organization
WTSI	Wellcome Trust Sanger Institute
ZAMPHIA	The Zambia Population-Based HIV Impact Assessment
ZNMCPPR	Zambia National Malaria Control Program Performance Review
ZAWA	Zambia Wildlife Authority

ZDM	Zambia Daily Mail
ZDA	Zambia Development Agency

CHAPTER ONE

INTRODUCTION

1.1 Background

Bacteria of the *Salmonella* genus remain a potential threat to human health, and are widely found in nature as common inhabitant of the intestinal tract of man, domestic animals and wildlife (Hendriksen *et al*., 2004; Lin-Hui *et al*., 2004; Chun-Yu *et al*., 2010; Gaffga *et al.*, 2012; WHO, 2017a). The genus *Salmonella* is currently divided into two validly published species called *Salmonella enterica* and *Salmonella bongori* (Reeves *et al*., 1989; Patrick and Grimont, 2007). Among *S. enterica* subspecies, non-typhoid *Salmonella* (NTS) is the cause of approximately 1.3 billion cases of human NTS salmonellosis yearly across the globe (Chimalizeni *et al*., 2010). A recent study revealed that *S. enterica* subspecies are one of the leading pathogens that cause substantial burden of food-borne diseases (FBD); almost 1 in 10 people fall ill and 33 million of healthy life years are lost through death annually (WHO, 2017a). Further, approximately 550 million adults, including 220 million children of less than five years old develop diarrhoeal diseases as a result of ingestion of *Salmonella* contaminated food (WHO, 2017a). *Salmonella* can also cause disease in domestic animals which then become the source of zoonotic infection to humans (Timme *et al*., 2013). A study elsewhere has shown that about 61 percent of animal diseases are zoonotic (IFAH, 2012). Transmission of non-typhoid *Salmonella* (NTS) pathogens to humans is mainly through consumption of animal-derived contaminated food products such as beef, pork, poultry meat, and eggs (Freitas *et al.*, 2010; Christou, 2011; Hendriksen *et al*., 2011; Bonnie and Stuart, 2011; Dallal *et al*., 2014; Kariuki and Onsare, 2015; Ford *et al*., 2018; Freidl *et al*., 2018).

However, within the NTS bacteria, a highly invasive form of non-typhoid *Salmonella* (invNTS) pathogen has emerged and is endemic in many countries of Africa region (Morpeth *et al.,* 2009; Crump *et al*., 2011; Crump and Heyderman, 2014) and other countries with low and middle incomes (Mogasale *et al*., 2014). Invasive NTS infections are less frequently reported in most developing nations, but pose a serious burden of infection in Asia and Africa (Gordon, 2011). These invNTS pathogens can cause a wide spectrum of life-threatening extra intestinal infections

in humans (Fierer and Guiney, 2001), including osteomyelitis and meningitis (Hung-Ming *et al*., 2013). Their impact was recognized as an important cause of bloodstream infection (BSI) (febrile illness) and mortality in Africa regions (Gibani *et al*., 2015; Crump and Heyderman, 2015). According to Reddy *et al*. (2010), invNTS is the second most common pathogenic bacteria to cause community-acquired BSI after *Streptococcus pneumoniae* in adults and in children across Africa. The case fatality rate of 20–25 percent was reported (Ao *et al*., 2015). The environmental reservoir from which these African *Salmonella* strains are transmitted to people is still unknown (WTSI, 2016). Lack of such information makes more difficult, controlling and preventing the spreading of the infections.

The most frequently reported NTS serotypes in human and animal salmonelloses across the world includes; *S.* Typhimurium and *S.* Enteritidis (Hendricksen *et al.,* 2011; Nagwa *et al*., 2012), but the numbers are gradually increasing and species such as; *S.* Infantis, *S.* Agona, *S.* Hadar, *S.* Heidelberg, and *S.* Virchow serotypes are also reported (Ruiz *et al*., 1995; Koch *et al.,* 2005; Freitas *et al*., 2010; Jackson *et al.,* 2013; Foley *et al*., 2014; Chironna *et al.,* 2014). In Zambia, *Salmonella enterica* serotype Senftenberg (*S.* Senftenberg) was among the clinical serotypes that caused severe human infections for which there were very few treatment options (Hendriksen *et al*., 2013).

On the wildlife front, Zambia is a home to a huge range of wild birds with more than 750 species (Zambia Advisor.Com, 2018). Reports indicate that wild birds represent the natural reservoir of some bacterial pathogens (Obukhovska, 2013), and have been associated with the transmission of human salmonellosis (Alley *et al*., 2002). Further, Zambia is naturally endowed with a rare privilege to be home of one of a spectacular Africa's great wildlife migrations which takes place across Liuwa Plain National Park, (Western Province). Therein wildebeests, zebra, tsessebe and buffalo converge in their thousands (African Parks, 2017). There are several other National Parks within the boundary of the country that host a diversity of wild animals and are open for game viewing by both national and international tourists. Wildlife has been implicated as reservoirs in the transmission and dissemination of *Salmonella* serotypes and can subsequently affect a large wildlife population, through shedding of pathogens in faeces of carrier animals (Sanchez *et al*., 2002). This was confirmed by a study that isolated *Salmonella* species from the intestines of

wildlife, indicating that these species could play a significant role in the epidemiology of zoonotic pathogens (Daszak *et al*., 2000). Wildlife species, particularly those that are at the point of extinction, are from time to time, trans-located to other National Parks or privately owned captive animal parks across the country. This is done in order to propagate or preserve the species and as such new pathogens are introduced in the new areas of habitation (Staji and Zandiar, 2017). In light of different wildlife species found in Zambia, due consideration needs to be put in place to monitor the environmental contamination caused by animal populations which are capable of disseminating pathogens such as *Salmonella* through faecal shedding.

In the period 2010 to 2015, statistics of livestock in Zambia revealed that the population of ruminant livestock sub-sector was estimated at 4.9 million (cattle), 4.8 million (goats), 149,420 (sheep) and 212.8 million (poultry) (Chapoto and Chisanga, 2016). However, plans are underway by the Government of Zambia, through the Ministry of Fisheries and Livestock, in conjunction with Central Statistics Office, to embark on updating the livestock Census as from November 2017 (Gideon, 2017). The disease problems at the wildlife/livestock interface are frequently bi-directional in cross infection of domestic and wildlife (Peighambari *et al*., 2011). Consequently, a long-standing conflict has existed between livestock owners and Animal Health Authorities on one hand and Wildlife Conservationists on the other, based on attitudes to controlling diseases of livestock which are associated with wildlife (Bengis *et al*., 2002). Anthropogenic activities such as animal husbandry have contributed to an increase in interspecies transmission of infectious agents (Iovine *et al*., 2015). For instance, shortages of grazing pastures are among the likely factors that can contribute towards the distribution of *Salmonella* serotypes into new environments (Chomel *et al*., 2007; Peighambari *et al*., 2011). Therefore, both wildlife and domestic animals play a pivotal function of being zoonotic reservoirs which are capable of spreading different *Salmonella* serotypes worldwide (Irfan *et al*., 2015). However, there is paucity of information regarding species diversity of *Salmonella* strains circulating in domestic animals and wildlife in Zambia, despite an increase in human population in Game Management Areas (GMAs) into what was wildlife habitats.

The human health risk factors of invasive salmonellosis are well elucidated and are associated with malaria and human immuno-deficiency virus (HIV) status (Bronzan *et al*, 2007; Morpeth *et*

al., 2009; Gordon, 2011; Feasay *et al*., 2012; Takem *et al*., 2014). Young children with an underlying condition such as malnutrition are also highly susceptible to invNTS infection (Gordon, 2011; Feasay *et al*., 2012). The highlighted human risk factors surrounding invasive NTS infections are not strange to the Zambian situation; considering that there are about 1.2 million people living with HIV infections, and about 20,000 patients with acquired immune deficiency syndrome (AIDS) related deaths (UNAIDS, 2015). Although, the introduction of combined antiretroviral therapy (cART) brought about the reduction of AIDS related deaths and occurrence of opportunistic infections worldwide. Human immuno-deficiency virus infected patients still remain a susceptible population to bloodstream infection (BSI) (Taramasso *et al.,* 2016). The reason being that the immune status of HIV infected patients is not fully competent until the CD_4 cell count increases to a value more than 750 cells/ml (Mocroft *et al.,* 2013). According to the report by WHO (2013a), Zambia has the highest burden of malaria in the world. The children below the age of five-year-old and pregnant women were the most vulnerable group (Chaponda *et al*., 2015), particularly those in remote and impoverished areas. A total of 3,783 deaths associated with malaria in children younger than five years were reported in 2007 in Zambia (WHO, 2010). A study by Munthali *et al*, (2015), reported that Kwashiorkor was the most common type of severe acute malnutrition (62 percent) among the children aged 0-60 months admitted to the University Teaching Hospital in Zambia from 2009 to 2013 (Munthali *et al*., 2015).

The ability of NTS pathogens to cause typical clinical manifestations of invasive NTS infections in humans and animals is facilitated by the presence of specific sets of virulence genes that are located on *Salmonella* pathogenecity Island 2 (SPI-2) and on large virulence plasmids (VPs) (Fierer and Guiney, 2001; Fluit, 2005; Biljana *et al*., 2010; Figueira and Holden, 2012). The type III secretion system (TTSS) protein effectors encoded by SPI-2 includes *Salmonella* secretion regulatory protein A and B (*ssr*A/*ssr*B), *Salmonella* secretion effector chaperones A (*ssc*A), *Salmonella* secreted effector protein C (*sse*C), *Salmonella* system apparatus protein H (*ssa*H) responsible for intracellular pathogenesis and plays a vital role for systemic infections (Yoon *et al*., 2009; Bhowmick, *et al.,* 2011; Fazl *et al.,* 2013; Fabrega and Vila, 2013), while *ssa*N, *ssa*P, *ssa*Q, *ssa*R, and *ssa*S are important for intracellular survival and bacteria replication (Dieye *et al*., 2009; Fazl *et al.,* 2013). Virulence plasmids are responsible for bacterial pathogenicity

during the systemic stage of infection (Feng *et al*., 2012). Within the virulence plasmid, exists a 7.8kb region of *Salmonella* virulence plasmid (*spv*) locus encoding *spv*R, *spv*A, *spv*B, *spv*C and *spv*D genes which confer the virulence phenotype and thereby facilitates bacterial multiplication in the reticulo-endothelial system (Rotger and Casadesus 1999; Chu *et al*. 1999). A few *spv* genes are also associated with antimicrobial resistance in addition to virulence function (Gothoh *et al*., 2003).

Most NTS diseases are uncomplicated and limited to gastroenteritis which rarely requires antimicrobial agents for treatment. On the other hand, human invasive salmonellosis requires use of antimicrobial agents for treatment (Hung-Ming *et al*., 2013; Mahon and Field, 2016), soon after diagnosis. According to Feasey *et al*. (2016), some strains of *S.* Enteritidis found in Africa have developed resistance to many antibiotics and behaved slightly differently to the strains commonly found in the West. Continued use of antimicrobial agents in farm animal husbandry and veterinary medicine is one of the factors that have contributed to the spread of antimicrobial resistance (Vangelis and Gousia, 2015). Interestingly, the approved antimicrobial agents by World Organization for Animal Health (OIE) for use in animal husbandry includes representatives of all major classes of antibiotics commonly used in human medicine (OIE, 2007). These include fluoroquinolones, trimethoprim-sulfamethoxazole, ampicillin, or third-generation cephalosporins (ceftriaxone or cefixime) (Liakopoulos *et al*., 2016a; Balasundaram *et al*., 2017).

Antimicrobial resistance among NTS pathogens was initially reported against the traditional first-line drugs; ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole (Morpeth *et al*., 2009; Crump *et al*., 2015). But now it has spread to fluoroquinolones and third-generation cephalosporins (Hung-Ming *et al*., 2013; Crump *et al*., 2015). This phenomenon brings about reduction in the choice among the traditional and newly developed antimicrobial agents (Lin-Hui *et al*., 2004). Simply, this explains why many health care facilities in African regions are failing to treat many invasive NTS diseases even when third-generation cephalosporins are used (Mahon and Field, 2016). Therefore, it is prudent to periodically monitor the prevalence of MDR *Salmonella* in both humans and animals for the purpose of controlling and preventing the spread

of the pathogen (Hung-Ming *et al*., 2013). Currently the prevalence of antimicrobial resistance of *Salmonella* pathogens in domestic animals and wildlife in Zambia remains unknown.

The present study sought to characterize *Salmonella* strains isolated from domestic animals and wildlife in order to understand the prevalence of serotypes, their virulence potential and multidrug resistance (MDR) phenotypes in Zambia. In order to achieve the objective of the study, we divided the present study into four aims. Firstly, was to isolate and confirm *Salmonella enterica* subspecies from domestic animals and wildlife by both conventional and molecular methods. Molecular methods involved the detection of *invasion* protein A (*inv*A) and universal bacteria (16S rRNA) genes specific to the genus *Salmonella* using PCR technique. The *inv*A gene was selected on the basis that it is found in all the *Salmonella* serotypes (Lostroh and Lee, 2001). Furthermore, the *inv*A gene is involved in the earliest stages of the invasion of intestinal epithelial cells by the facultative intracellular pathogenic *Salmonella* spp. (Galan *et al*., 1992; Boyd *et al*., 1997). On the other hand, 16S rRNA PCR amplification product of *Salmonella* strains under investigation were sequenced and compared with those in the GenBank database. The comparison of the 16S rRNA gene sequences facilitated the identification and confirmation of the bacterium both at the genus level and species or subspecies level (Clarridge, 2004).

The second objective was to determine the diversity among the *Salmonella* strains isolated from various wildlife and domestic animal sources from selected areas of Zambia. We used phenotypic (serogroups, serotypes, biotypes and resistotypes) and genotypic (virulence genes) determinants to characterize the strains. The accessory virulence genes called *Salmonella* plasmid virulence A, B and C (*spv*ABC) genes were selected on the basis that they are indicative of being potentials to facilitate extra-intestinal salmonellosis in humans and animals. Characterization of *inv*A gene has been reported for *Salmonella* strains isolated from beef and poultry meat (Hang'ombe *et al*., 2008), dogs and chickens (Ulaya *et al*., 2012) in Zambia. To our knowledge the occurrence of *spv* genes in *Salmonella* strains from domestic animals and wildlife have not been documented in Zambia.

Thirdly, we sequenced the *inv*A-284bp gene of selected serotypes to determine the level of sequence nucleotide differences (SND) by comparison with the same region of the published

sequences on GenBank, and lastly phylogenetic trees were constructed based on SND of *inv*A-284bp fragment gene of *Salmonella* serotypes from different sources to determine their genetic relatedness.

1.2 Study justification

In Zambia, a severe human invasive non-typhoidal *Salmonella* infection in HIV patients was reported for which there were very few treatment options (Hendriksen *et al.* 2013). This indicated a narrow treatment option for the diagnosis, which if not well handled can lead to a compromised antibiotic treatment; consequently increasing the chance of developing antibiotic resistance. Furthermore, sporadic outbreaks of diarrhoeal diseases were recently reported countrywide of which both the aetiological agent and their environmental reservoir was unknown. This makes it difficult to apply any control intervention. Therefore, it is important that such antimicrobial resistant pathogen is identified whether it is associated with environmental contamination, food consumption of animal origin and over use of antimicrobials in animal husbandry.

Another important justification to conduct such studies is to create evidence based scientific information. Such information may be key when generating a Zambian based pharmacopeia – for drug use and treatment options, which currently are based on studies conducted somewhere else, other than Zambia.

The *Salmonella* clones which have recently emerged in Zambia were a source of concern in the medical fraternity (Hendriksen *et al.*, (2013). The major risk factors for invasive NTS infection in the population are HIV and malaria infections which have become significant public health burden in Zambia (Masaninga *et al*., 2013; Banda *et al*., 2015; Chanda-Kapata *et al*., 2016; ZAMPHIA, 2016; Inambao *et al*., 2017). Annual incidence of HIV among adults aged 15 to 59 years in Zambia is estimated at 0.66 percent which corresponds to approximately 46, 000 cases of HIV annually among the adults between the ages 15 to 59 years (ZAMPHIA, 2016). By the end of 2016, there were about 1.2 million people living with HIV in Zambia (Lusakatimes.com, 2017). On the other hand, malaria disease accounted for more than 5.8 million reported cases in 2014 (Mukonka *et al*., 2015; Inambao *et al*., 2017). While the human risk factors to invasive

salmonellosis have been established, zoonotic origin of these serotypes is still unknown. Hence, the study on the diversity of *Salmonella* strains from wildlife and domestic animals included the prevalence of antimicrobial resistance profiles and detection of virulence (*inv*A, *spvA, spvB* and *spvC*) genes which together may contribute to the new emerging invasive strains.

1.3 Research Question(s)

a) Are there NTS serotypes circulating in wildlife or domestic animals in selected areas of Zambia?

b) Are there extended spectrum beta lactamase (ESBL) producing, and multiple drug resistant (MDR) *Salmonella* strains?

c) Do the NTS serotypes circulating in domestic animals and wildlife possess invasive (virulence) gene(s)?

d) Is the *inv*A gene of *Salmonella* strains isolated from domestic animals and wildlife related genotypically?

1.4 Study objectives

1.4.1 General objective

To characterize diversity of *Salmonella* strains from wildlife and domestic animals in selected areas of Zambia.

1.4.2 Specific objectives

a) To isolate and identify *Salmonella* circulating in wildlife and domestic animals.

b) To determine serogroups, serotypes, biotypes and resistotype profiles of *Salmonella* strains.

c) To assess the degree of extended spectrum beta lactamase (ESBL), MDR and multi-antibiotic resistance (MAR) Index among *Salmonella* strains from wildlife and domestic animals.

d) To detect the presence of *inv*A, *spv*A, *spv*B, and *spv*C using specific primers among *Salmonella* strains isolated from wildlife and domestic animal sources.

e) To detect nucleotide sequences differences (NSD) of *inv*A-284 bp fragment gene among the selected *Salmonella* strains from domestic animals and wildlife.

f) To determine phylogenetic relatedness of the selected *Salmonella* strains based on their *inv*A-284 bp gene.

CHAPTER TWO

LITERATURE REVIEW

2.1 General overview of the genus *Salmonella*

Salmonella is worldwide distributed and found in the intestinal tract of different domestic and wild animals, poultry and wild birds (Schukken *et al*., 2004; Coburn *et al*., 2007; Ojo and Adetosoye, 2009; Rajagopal and Mini, 2013; Gart *et al*., 2016; Holschbach and Peek, 2018). In addition to causing animal diseases, this pathogen has a growing food borne zoonotic impact (Stevens *et al.*, 2009; CFSPH, 2013; Ebani, 2017; Chlebicz and Slizewska, 2018). In the course of infection, these pathogens are capable of invading macrophages, dendritic and epithelial cells (Siriken, 2013). In human infection, *Salmonella* was first isolated from a sample in 1884 by bacteriologist called Georg Gaffky and the pathogen was later identified as *Salmonella enterica* subspecies *enterica* serotype Typhi (Gossner *et al*., 2016).

In animals, *Salmonella* pathogen was first isolated from porcine intestine which manifested classical swine fever, by Theobald Smith 1855 (Smith, 1894). The genus name *Salmonella* was derived from the last name of the veterinary scientist, Dr. Daniel Elmer Salmon, who was the administrator of the United States Department of Agriculture (USDA) research program, although a number of scientists were involved towards the research of *Salmonella* pathogen (Oldenkamp, 2004; Miller and Pegues, 2005). *Salmonella* was formerly called "*Bacillus choleraesuis*" and this name changed to *Salmonella choleraesuis* in 1900 by Lignnieres (SSNCISM, 1934). *Salmonella* species are relatively close to their counterparts *Enterobacteriaceae* (*Escherichia coli*, *Yersinia* and *Shigella*) in terms of the composition of guanine (G) and cytosine (C) content rated at about 52 percent (Salyers and Whitt, 2002; Thomson *et al*., 2008).

2.2 Classification of *Salmonella*

Salmonella is a facultative intracellular pathogen; non-sporing, oxidase negative, catalase positive, non capsulated; Gram-negative, straight rod shaped bacteria (Barlow and Hall, 2002; Jay *et al*., 2003) and are approximately 3 x 0.6 µm in size (Montville and Mathews, 2008).

Salmonella is flagellated and predominantly motile (Fabrega and Vila, 2013), with an exception of the non-motile mutants *S.* Gallinarum and *S.* Pullorum (Schofield, 1945; Lopes *et al*., 2016). The taxonomy of *Salmonella* is perceived to be complex and subject to evolving (Brenner *et al.*, 2000; Ryan *et al.*, 2017), such that it has taken several concepts to determine the taxonomy and nomenclature of the bacteria. Notable characteristics were based on clinical symptoms caused by the *Salmonella* strains (Grimont *et al*., 2000); the surface antigenic specificities (Kauffmann, 1960); biochemical tests including the ability to ferment substrates (Paccagnela, 2005); and last but not least, DNA relatedness (Crosa *et al*., 1973).

Hats off to the discovery of rRNA-DNA and DNA-DNA hybridization method, a "gold standard" method for identification of new species and through this method, definitive names are ascribed to the strain with ambiguous properties to the correct taxonomic unit (Le Minor, 1988; Janda and Abbott, 2007). Through DNA hybridization methods, all *Salmonella* strains were identified to belong to a single *Salmonella choleraesuis*, consisting of seven subspecies namely; *Salmonella choleraesuis* subsp. *arizonae, Salmonella choleraesuis* subsp. *bongori, Salmonella choleraesuis* subsp. *choleraesuis, Salmonella choleraesuis* subsp. *diarizonae, Salmonella choleraesuis* subsp. *houtane, Salmonella choleraesuis* subsp. *indica and Salmonella choleraesuis* subsp. *salamae* (Le Minor *et al.* 1982). In 1987, a proposal was made that the seven subgenera of *Salmonella* be referred to as subspecies I, II, IIa, IIIb, IV, V and VI (Le Minor and Popoff, 1987). Subsequently six of these subgenera (I, II, IIa, IIIb, IV and VI) were combined into one species called *Salmonella enterica* species (Brenner and McWhorter-Murlin, 1998; Popoff and Le Minor, 2001).

Therefore, genus *Salmonella* is currently divided into two validly published species called *Salmonella enterica* (six subspecies) and *Salmonella bongori* (one subspecies) (Reeves *et al*., 1989; Patrick and Grimont, 2007) based on their 16S rRNA nucleotide sequence analysis (Grimont *et al*., 2000; Popoffet *et al*., 2003). *Salmonella bongori* was previously known as *Salmonella* subspecies "V" (Reeves *et al*., 1989; Popoff and Le Minor, 1997). However, a new and third species "*Salmonella subterranean"* was proposed and subsequently approved by the judicial commission on March 18, 2005 (Shelobolina *et al*., 2004). *Salmonella subterranean* is

still written within parentheses, due to the reason that some researchers claim that the species should not belong to the *Salmonella* genus but rather to *Escherichia* genus.

Salmonella enterica is the type species and most diversified group represented by six phylogenetic subspecies namely, *enterica, salamae, arizonae, diarizonae, houtenae,* and *indica* (Patrick and Grimont, 2007). These subspecies can also be identified by Roman numerals in the same order; I, II, IIIa, IIIb, IV, and VI (Rotger and Casadesus, 1999; Patrick and Grimont, 2007). On the other hand, Lin-Hui and Cheng-Hsun (2007) attempted to name *S. enterica* subspecies I, as *S. enterica* subsp. *enterica* (subspecies I), *S.enterica* subsp. *salamae* (subspecies II), *S. enterica* subsp. *arizonae* (subspecies IIIa), *S. enterica* subsp.*diarizonae* (subspecies IIIb), *S. enterica* subsp *hountanae* (subspecies IV) and *S. enterica* subsp *indica* (subspecies VI. *Salmonella enterica* subspecies possesses the highest diversity of serotypes as shown in the table 2-1.

Table 2-1: Current classification of *Salmonella enterica* species (Achtman *et al*., 2012)

Serial No.	Sub species	Total No. of serotypes
1	*S. enterica* (I)	1587
2	*S. salamae* (II)	513
3	*S. arizonae* (IIIa)	100
4	*S. diarizonae* (IIIb)	341
5	*S. houtenea* (IV)	73
6	*S. indica* (VI)	13
	Total	2587

Salmonella enterica are considered the most important zoonotic pathogenic subspecies of the genus *Salmonella* in human and veterinary medicine worldwide and comprises more than 2,500 serotypes (Popoff and Le Minor, 2001; Tindall *et al*., 2005; Eswarappa *et al.,* 2008; Fookes *et al*., 2011; Feng *et al.,* 2012; Evangelopoulou *et al.,* 2013). *Salmonella* subspecies I, consists of the largest group responsible of causing disease in humans and animals, and of this group only about 150 serotypes are clinically significant as human or animal pathogens (Roer *et al*., 2016). These serotypes are responsible for 99 percent of all clinical isolates in humans (Crum-

Cianflone, 2008; CDC, 2008; Pui *et al*., 2011) and animals (Hernandez *et al*., 2012). Whereas, *Salmonella* serotypes belonging to subspecies II, IIIa, IIIb, IV and VI are associated with cold-blooded animals and the environment but occasionally cause disease in humans (Farmer *et al*., 1984).

Salmonella bongori was first isolated in 1966 from a lizard in the city of Bongor, Chad, upon which it derived its name bongori (Le Minor *et al*., 1969). *Salmonella bongori* is associated with infections with ectotherms (cold-blooded animals) such as reptiles (Tortora, 2008), but has also been isolated from endotherms such as humans and birds in Italy (Foti *et al.,* 2009). *S. bongori* and other subspecies belonging to *S. enterica* subspecies II, IIIa, IIIb, IV, and VI are commonly isolated from cold-blooded animals (Nataro *et al*., 2011).

2.3 Epidemiological determinants

Salmonella infections can be regarded as two types; the first is primarily of importance for public health which may cause illness in man (Meiying *et al*., 2016; Chong *et al.,* 2017) and animals (Schaer *et al*., 2010; Salisbury, 2011). The second type is caused by the serotypes *S.* Pullorum and *S.* Gallinarum, which cause severe disease in poultry and wildbirds but rare in humans (Government of Canada, 2012; Rajagopal and Min, 2013). Therefore, the principal agents of salmonellosis can be classified into two groups; Typhoid pathogens and non-typhoid *Salmonella* (Feasey *et al*., 2012). Although these two groups are genetically related, they are different in the way they evoke diseases in humans (Gal-Mor *et al*., 2014). Typhoid pathogens are responsible for enteric fever (typhoid), while gastroenteritis is caused by NTS pathogenes (Strugnell *et al*., 2014). Invasive non-typhoidal salmonellosis or extra-intestinal infection is characterized by bacteriemia (Dzierska *et al*., 2008; Guiney and Fierer, 2011).

2.3.1 Salmonellosis in wildlife

Although published reports on the outbreak of *Salmonella* in wildlife are few, the infection had been reported from some wild animals as described below;

2.3.1.1 Global burden of fowl typhoid (FT): Wildlife.

Fowl typhoid was first described more than one century ago (Klein, 1889). Although the disease affects mainly chickens; game birds, guinea fowl, ostriches, parrots, peafowl, ring doves, sparrows and turkeys can also be infected (Government of Canada. 2012; Rico, 2014). In the USA, an outbreak of fowl typhoid among guinea fowls was diagnosed on a farm near the City of Newark, Delaware in 1945 (Moore *et al*., 1946). Thereafter, several outbreaks have been reported in other countries; For instance in Japan, FT outbreak was reported in a flock of 400 Japanese quail (*Coturnix coturnix japonica*) at 91 days of age, of which 222 died suddenly (Casagrande *et al*., 2014). In Rumania, an outbreak of TF was reported on a flock of 7914 pheasants, of which 22 died and 49 slaughtered (Macovei *et al*., 2010)

2.3.1.2 Global burden of non-typhoidal salmonellosis: Wildlife

Non-typhoidal *Salmonella* has been isolated from various wildlife hosts species such as white tailored deer (Renter *et al*., 2006), wild birds (Afema *et al*., 2016; Staji and Zandiar, 2017), pet birds (Losson *et al*., 2013), snakes, lizards, and turtle (Chen *et al*., 2010; Marin *et al*., 2013; Tomastikova *et al*., 2017), red fox (Chiari *et al*., 2013), sea lion (Berardi *et al*., 2014), hedgehogs (Handland *et al.,* 2002), key deer (*odocoileus virginianus clavium*) (Nettles *et al*., 2002), and from various wildlife species that were admitted to two rehabilitation centers in Ohio, USA (Jijon *et al*., 2007). Distribution of NTS serotypes frequently isolated from wildlife in selected countries is shown in Table 2-2.

Table 2-2: World distribution of the predominant NTS serotypes isolated from different captive and wild animal sources in selected countries

Region	Period	Animal source	No. of strains	No. of serotypes	Predominant serotype	Rate (%)	Reference
Africa							
Burkano Faso	-	Hedgehog	24	8	*S.* Muenster	11 (45.8)	Kagambega *et al*., 2013
South America							
Chile	-	Sea gull	54	4	*S.* Selftenberg	19 (35.2)	Lopez *et al*., 2016
Argentina	2012	Chinchillas	5	1	*S.* Typhimurium	5 (100.0)	Churriaa *et al*., 2014
Asia							
Iran	2015-2016	Wild mammals	32	2	*S.* Enteritidis	19 (12.5)	Staji and Zandiar, 2017.
Taiwan	2005-2006	Snakes	59	11	*S.* newport *S.* Othanarschen	7 (11.9) 7 (11.9)	Chen *et al*., 2010
	2005-2006	Lizard	74	17	*S.* Cotham	10 (13.5)	Chen *et al*., 2010
Europe							
Italy	2007-2010	Wild boars	259	21	*S.* Coeln	71 (21.8)	Chiari *et al*., 2013
Italy	2009-2010	Red fox	29	16	*S.* Typhimurium	8 (27.6)	Chiari *et al.*, 2014
Italy	2009-2010	Badger	2	2	*S.*Typhimurium *S.* Infantis	1 (50.0) 1 (50.0)	Chiari *et al.*, 2014
Poland	2011-2013	Wild birds	52	-	*S.* Typhimurium	18 (28.1)	Krawiec *et al.*, 2015
North Amerca							
Nebraska	1998	White-Tailed Deer	5		*S.* Infantis	2 (40.0)	Renter *et al*., 2006
California		Sea lion	11	4	*S.* Enteritidis	7 (64.0)	Berardi *et al*., 2014

In Hokkaido, Japan, an outbreak of salmonellosis in wild passerines caused mass mortality of eurasian tree sparrows (*Passer montanus*) in the period 2005–2006 (Fukui *et al*., 2014). Elsewhere, an outbreak of salmonellosis was reported in a population of 45 lorikeets and lories in

an open-air zoologic exhibit, during August 2001 with 22 percent mortality rate (Ward *et al*., 2003). During 1998–2012, an extended outbreak of *Salmonella enterica* serovar Typhimurium definitive phage type 160 (DT160) killed wild birds in New Zealand (Bloomfield *et al*., 2017). In Italy, unusual mortality of wild boars occurred from 2012 to 2015 due to *Salmonella* Choleraesuis infection (Longo *et al*., 2019). Cases of sepsis due to *Salmonella* Choleraesuis have been on increase in Taiwan since 1995 (Shio *et al*., 2006). In another study, in Taiwan, a nationwide food-borne disease outbreak associated with sandwiches purchased from an online shop was reported in 2010 (Wei *et al*., 2014).

In La Plata City, Buenos Aires Province, Argentina, adult chinchillas (Chinchilla lanigera) were reported to have suddenly died in a commercial farm in July 2012 (Churria *et al*., 2014). In another study, two blue and gold macaw (*Ara ararauna*) chicks died of fatal Salmonellosis in Buenos Aires Province, Argentina, (Vigo *et al*., 2009).

2.3.1.3 Burden of Non-typhoid salmonellosis in selected countries of Africa: Wildlife

Salmonellosis in two captive African elephants and a black rhinoceros were reported in Kenya (Windsor and Ashford, 1972).

2.3.1.4 Burden of Non-typhoid salmonellosis in Zambia: Wildlife

Salmonellosis has been isolated from captive diseased wild animal (leopard and warthog) (Ngoma *et al.,* 1993). Apart from this there is no other information, hence the current study.

2.3.2 Salmonellosis in domestic animals

Salmonella pathogens can cause disease in all animals and poultry, characterized by septicaemia, acute enteritis (sub-acute) and chronic enteritis (Ayachi *et al*., 2012; Rajagopal and Min, 2013). Several outbreaks of classical animal salmonellosis have been reported worldwide as described below:

2.3.2.1 Global burden of fowl typhoid: Domestic animals

Fowl typhoid is an avian host specific salmonellosis caused by *Salmonella* Gallinarum (*S.* Gallinurum) (Kumar *et al*., 2010; Rajagopal and Mini, 2013) and is of worldwide concern

(Sannat *et al*., 2017). The disease was first noted in England in 1888 (Klein, 1889) affects birds of all ages (Kabir, 2010; Batista *et al*., 2015). Since then it has remained a serious economic problem to livestock in countries where control measures are not efficient and or where climatic conditions favour the environmental spread of the pathogen (Barrow and Neto, 2011). The disease was first described as "fatal septicemia" or "white diarrhoea" by Rettger (1909). Data on the outbreaks of FT is available for many countries; for instance in Korea, FT was reported in commercial broilers, baeksemi (a mixed breed of male meat-type breeder and female commercial layer), commercial layers, native chickens, and broiler breeders and the incidence rate was 47.7, 28.4, 17.2, 5.1, and 1.3 percent, respectively (Kwon *et al*., 2010).

In the year 2012, two FT outbreaks were reported in August and October out of which 34,000 and 4,000 were reported dead respectively, in Northern Ireland, UK (The Poultry Site, 2012a). Several FT outbreaks were previously reported in India (Kumar *et al*., 2010; Barrow and Neto, 2011; Rajagopal and Mini, 2013). For instance, during the period January 2011 to December 2013, 309 outbreaks of FT were recorded in chickens (Arora *et al*., 2015). In Raigarh, in the state of Chhattisgarh, India, an outbreak of FT was reported in adult layer birds of about 7-8 months at a Government Poultry Farm (Sannat *et al*., 2017). In Romania, 71 cases of FT outbreak in pheasants was reported, of which 22 died and 49 were slaughtered for laboratory investigation (Macovei *et al*., 2010). Recently an outbreak due FT was reported in Brazil (Celis-Estupinan *et al*., 2017).

2.3.2.2 Global burden of Non-typhoidal salmonellosis: Domestic animals

Non-typhoidal salmonellosis is a worldwide disease of animals which are the main reservoir (Baron, 1996). Non-typhoid *Salmonella* bacteria are responsible for a wide variety of pathological diseases in domestic and wild animals (Coburn *et al*., 2007). Some *Salmonella* strains have predilection for one kind of animal while others are found in many different animals and countries (Uzzau *et al*., 2000a). Several NTS outbreaks have been described involving domesticated animls; for instance, between 1996 and 1997, a high case fatality rate (44 percent) of horses in a veterinary hospital, led to the closure of the hospital to allow extensive cleaning and disinfection (Schott *et al*., 2001). Other reports include an outbreak of salmonellosis involving horses in large animal Veterinary Teaching Hospital (Tillotson *et al.,* 1997; Schaer *et*

al., 2010); and small veterinary clinics involving dogs (Prescott, 2005; Botha *et al*., 2018), and also involving pigs (Ha *et al*., 2005). In Brazil the first description of a nosocomial outbreak of multidrug resistant *S*. Typhimurium in a veterinary hospital was reported between 2016 to 2017; involving 65 calves with 1 to 3 days old (Ramos *et al.,* 2019).

Non-typhoidal *Salmonella* pathogens could have negative impact on the health of domestic animals (Cummings *et al*., 2010a; Cummings *et al*., 2010b; Kiflu *et al*., 2017).

2.3.2.3 Global burden of invasive non-typhoidal salmonellosis: Domestic animals

Invasive non-typhoid *Salmonella enterica* subspp. can cause a wide range of clinical signs in flocks of domestic animals such as joint infections, chronic pneumonia, abortion and sudden death. In Italy, 399 abortion samples were collected from 107 ovine and caprine farms in northern Sardinia, during 2003–2005 caused by *S.* Abortusovis (Masala *et al*., 2007). In Western part of Switzerland, abortion storms with up to 70 percent fetal losses were reported in several sheep flocks during the lambing seasons of 2003/2004 to 2007/2008 (Von Tavel *et al*., 2005; Belloy *et al*., 2009). An epidemic of abortions in sheep caused by *S.* Abortusovis, was reported in Dalmatia, south Croatia, in winter 2003-2004 (Habrun *et al*., 2006).

In Croatia, an abortion outbreak occurred in a herd of 38 horses, 26 of which were pregnant mares and bacteriological examinations were carried out on 4 aborted foetuses, *S*. Abortusequi was the single causative agent in each case (Madic *et al.,* 1997). In Argentina, an outbreak of *S.* Abortusequi abortion occurred among recipient mares derived from five different embryo transfer centers (Llorente *et al*., 2016). In England and Wales, infection due to *S.* Dublin is the most frequently diagnosed cause of salmonellosis in cattle, and outbreaks occur most often in dairy herds, with enteritis or abortions being the most common clinical manifestations in adult cows, while enteritis is usually seen in calves (APHA, 2015).

2.3.2.4 Burden of fowl typhoid in Africa: Domestic animals

In South Eastern Nigeria, an outbreak of FT affected 11,000 laying birds in Undi region of which the total mortality was 25 percent (Ezema *et al*., 2009).

2.3.2.5 Burden of Non-typhoid salmonellosis in Africa: Domestic animals

In Lagos, Ogun and Oyo states, Nigeria, non-typhoid *Salmonella* serotypes causing high mortality (40 per cent to 80 per cent) were reported in poultry farms (Mshelbwala *et al*., 2017).

2.3.2.6 Burden of fowl typhoid in Zambia: Domestic animals

Studies by Sato *et al*. (1997), reported isolation of *S.* Gallinarum-Pullorum and characterized as fowl typhoid in five cases of 5-to-18-month-old layer chickens in Zambia. Apart from this, there are no other studies.

2.3.2.7 Burden of non-typhoid in Zambia: Domestic animals

Previous studies on *Salmonella* in Zambia have been limited and focused on aspects such as isolation of *Salmonella* from domestic animals. There is paucity of information on the disease outbreaks due to NTS salmonellosis. However, *Salmonella* has been isolated and identified from various sources in Zambia such as; beef at the point of retail (Hang'ombe *et al*., 1999b; Hang'ombe *et al.,* 2008), poultry carcasses from processing plants (Tuchili *et al*., 1996b; Hang'ombe *et al*., 1999a), diseased broiler and layer chicks at hatcheries (Sato *et al*., 1997; Munang'andu *et al*., 2012), chicken embryos and environmental samples (Tuchili *et al*., 1996a; Kabilika *et al*., 1999), slaughtered healthy cattle at abattoirs (Ngoma *et al.,* 1993).

2.3.3 Salmonellosis in humans

Human salmonellosis comprises three major disease forms: typhoid fever, non-typhoid gastroenteritis, and non-typhoid invasive (bacteremia) disease (Darby and Sheorey, 2008; Guiney and Fierer, 2011), in addition to a carrier state (Shu *et al.,* 2015).

2.3.3.1 Global burden of typhoid fever (TF) in humans

In the year 1907, about 3,000 New Yorkers in the United States (US) had been infected by *Salmonella* Typhi and Mary Mallon a "healthy carrier" and a cook at the time was probably the main source for the outbreak (Marineli *et al.,* 2013). During that period, immunization against *Salmonella* Typhi was not yet ensued not until about 1911, while on the other hand antibiotic treatment was not developed until 1948 (Fanning, 1997). The risk of further transmission of ST increases in the event that a chronic carrier is involved in food handling activities (ECDC,

2017a). In the mid-nineteenth-century in England, typhoid became a serious public health problem, until the introduction of piped and filtered water supply in most urban areas.

In the year 2000 the incidence rate of TF had risen to approximately 22 million cases resulting in 200,000 deaths worldwide especially those nations that are still under development (Crump and Mintz, 2004; ECDC, 2017b). The exact global burden of this disease may not have been captured in the year 2003, but WHO estimate was 16 million cases and 600,000 deaths of TF each year (Kalra *et al*., 2003). By 2010, a study by Buckle *et al*. (2012), showed a gradual global increase to 26.9 million cases of illness and up to 600,000 deaths to TF annually in the year. Although, TF is commonly found in many areas of the Asian and African continent, other countries in Europe, South and Central America and Middle East have also reported the incidences (Shu *et al.*, 2015). For instance, in 2017, the French authority reported to the European Centre for Disease Prevention and Control (ECDC), three cases of typhoid fever linked to the European Rainbow gathereing that took place in Tramonti di Friuli-Venezia Giulia region, Italy (ECDC, 2017a).

The incidence rate of TF in the USA and parts of European countries is low, at less than 10 percent per 100,000 persons annually (Shu *et al*., 2015), largely due to improved sanitation facilities and accessibility to clean and safe food. Cases of TF in USA are mostly reported in returning travellers from Asia where they could have gone to visit relations (Basnyat *et al*., 2005). A major outbreak of TF was reported in Guatemala during 2017, which affected more than 60 patients and caused two deaths (Alvarez, 2017). The pathogens originated from contaminated water sources.

Many parts of Asia (Dworkin *et al*., 2014; John *et al*., 2016; Meiying *et al*., 2016; Andrews *et al*., 2018) have high incidence of TF in particular South East Asia (274 per 100,000 people per year) (Kothari *et al*., 2008). The mortality rate due to TF infection varies from one geographical area to another, but could be as high as 5-7 percent despite the patients receiving antibiotic regimes (Pui *et al.,* 2011a). Asian countries, accounts for approximately 90 percent of global morbidity and mortality caused by TF, and this are largely due poor sanitation which affects the quality of drinking water (Gopinath *et al*., 2012). Among the Asian countries; India, China, Vietnam, Pakistan and Indonesia have had high incidence rates of TF, more than 100 cases per

100,000 populations annually (Shu *et al*., 2015). In India alone, the toll effect of TF due to *S.* Typhi accounts for more than 300,000 cases per year (Mandal *et al*., 2012). In 2014, an outbreak of typhoid fever occurred in Jorhat town, in Assam, India in which 46.15 percent of patients were found positive with *S.* Typhi (Jashbeer *et al*., 2016). In a district of Malatya-Turkey, an outbreak of *S.* Typhi occurred affecting 10 patients in two days due to consumption of contaminated water from the same well-water (Iseri *et al*., 2009).

In 2015, there were about 8850 TF due to *S.* Typhi cases reported in China from the surveillance system, with incidence rate as 0.65 per 100 000 (Liu *et al*., 2017). In the same period the number of paratyphoid fever cases reported in China were low at about 2,794, with incidence rate at 0.21 per 100 000. A study in rural Cambodia revealed that the majority of TF infections occurred during the rainy season, and the incidences was as high as 11.36 per 1,000 in children aged <15 years in the period 2007-2014 (Pham *et al*., 2016). The actual burden of *S.* Typhi in Fiji, a state in the Pacific Ocean is not certain, though the island had an increase of reported confirmed case notifications of enteric fever caused by *S.* Typhi (Watson *et al.,* 2017).

In other developing countries such as Iraq, thousands of people are infected with TF caused by *S.* Typhi annually. Typhoid salmonellosis continues to be an important cause of morbidity and mortality, particularly among children and adolescence in South-Central and South East Asia where the disease is associated with poor sanitation and unsafe food and water (Crump and Mintz, 2010). The diseases due to *S.* Typhi and *S.* Paratyphi are also more pronounced in crowded and impoverished populations with inadequate sanitation facilities (Whitaker *et al*., 2009; Dewan *et al*., 2013). Contrary to several efforts being made by the affected countries to curb the spread of typhoid cases, millions of new cases have risen to about 21 million cases per year of TF and paratyphoid especially in areas where sanitation facilities are still poor (Keestra *et al*., 2015; Franco *et al*., 2016**)**. Distribution of global TF is shown in Figure 2-1.

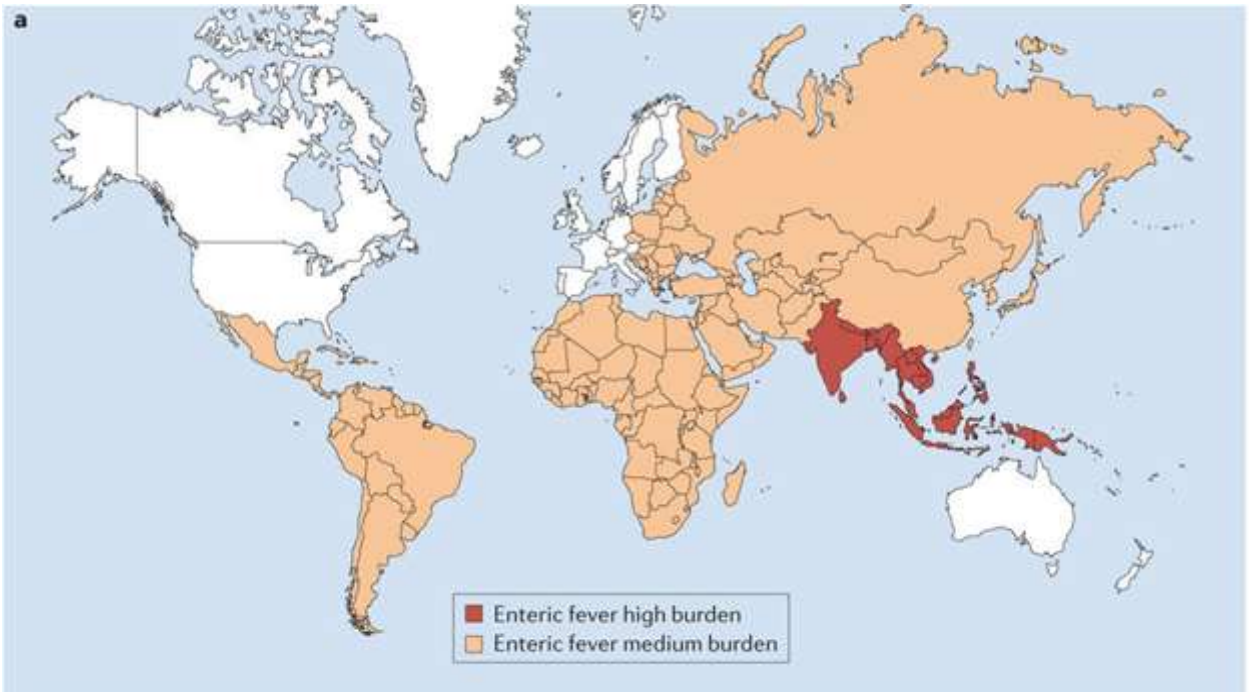


Figure 2-1: Global distribution of TF (Gilchrist *et al*., 2015)

2.3.3.2 Global burden of non-typhoidal salmonellosis in humans

The global burden of human gastroenteritis due to NTS remains high at 93.4 million cases, with 155,000 deaths and an average incidence of 1.14 episodes per 100 persons each year (Majowicz *et al*., 2010). The previous studies revealed slightly high estimate of 1.3 billion cases of gastroenteritis and 3 million deaths globally (Bhunia, 2008; Kemal, 2014). Overall, Asia accounted for the highest, with 83.4 million cases, 137,700 deaths, and an average incidence of 4.72 per 100 episodes in 100 persons each year (Majowicz *et al*., 2010). The incidence rate of NTS infections is higher than TF globally because NTS strains are widely distributed in domestic animals, wildlife and natural environment (Shu *et al*., 2015). A recent study by WHO, reported about 22 most common foodborne diseases worldwide caused by bacterial, protozoal and viral pathogens, and among these diarrhoeal and invasive infections due to NTS pathogens were the highest causing 4.07 million cases with African region accounting for the highest cases followed by the South East Asian region (Kirk *et al*., 2015). Therefore, NTS is equally most problematic disease which contributes to major public health concerns worldwide, because of its high endemicity, and the difficulty to control the disease (Antunes *et al*., 2003), coupled with significant morbidity and mortality rates (Cardoso *et al*., 2002).

In the USA, NTS was one of the major causes of acute bacterial gastroenteritis, and 2 to 4 million cases were reported with about 500 deaths per year (DHHS-USFDA, 2009; Pui *et al.*, 2011a). In 2007, there was a nationwide increase in *Salmonella* Tennessee infections; the illness was associated with consuming peanut butter (Sheth *et al*., 2011). Other reports have indicated that outbreaks of human *Salmonella* infections in the USA were increasingly associated with contact with live poultry; in particular mail-order hatchery was identified in 81 per cent of the trace-back investigations (The Poultry Site, 2012b). In 2017, a total of 220 people from 23 states, US, were reported to have been infected with the outbreak strains of *Salmonella* Thompson (144), *Salmonella* Kiambu (54), *Salmonella* Agona (12), *Salmonella* Gaminara (7), or *Salmonella* Senftenberg (3) which were linked to imported Maradol papayas from four different farms in Mexico (CDC, 2017).

On the other hand, human salmonellosis was until 2005, the most common food-borne disease in the European Union (EU) (EFSA, 2008; EFSA, 2012). A survey conducted to assess the country's burden of salmonellosis in Europe on the nationals returning to their respective countries from abroad revealed prevalence estimates at 2741 per 100,000 (Swedish), 2344 per 100,000 (Turkey) and 2141 per 100,000 (Malta), (Jong and Karl, 2006). In all cases, *S.* Enteritidis serotype was the most predominant strain in the three countries. However, there are still sporadic outbreaks, for instance there was a nationwide outbreak of *Salmonella* Enteritidis phase type (PT) 1B involving 53 cases in 2012, associated with consumption of chicken salad in Finland (Huusko *et al*., 2017). Further, a large multi-country outbreak of *Salmonella enterica* serotype Enteritidis in the EU and European Economic Area was investigated (EEA). The case-control study results showed that cases more often ate in food establishments (Pijnacker *et al*., 2019).

In Netherland, six related cases with gastroenteritis were reported in 2015; they suspected consumption of filet américain, a raw beef spread, to be the source of infection (Freidl *et al*., 2018). In Northern France, the regional health agency in Picardy was notified by a hospital laboratory of five cases of salmonellosis in young children in December 2014, of which two were confirmed *S.* Enteritidis and three were *Salmonella* spp. (Jones *et al*., 2016). In 2014, an outbreak of *Salmonella* enterica ssp. enterica serovar Bovismorbificans was detected in

Switzerland which was associated with consumption of contaminated salads as well as sprouts (Knoblauch *et al*., 2015). In 2018, an outbreak of monophasic *Salmonella* Typhimurium was detected across Denmark which was associated with consumption of raw Danish pork sausage (medister sausage), pork chops and ground veal/pork (Helmuth *et al*., 2019). Since the year 2017, there were 63 cases of *Salmonella* Kentucky infections reported by Swedish health authorities associated with food-borne (News Desk, 2018). All cases involved patients that had lived in homes for the elderly or had been hospitalized prior to becoming ill. On the other hand, an outbreak of salmonellosis among the students from one primary School was reported in Belgrade, in 2018, which was associated with consumption of contaminated food (Maris *et al*., 2019).

In Asia, in particular India, an outbreak of food poisoning was reported from a Military establishment in 2011, when 43 cases fell sick in a span of few hours (Kunwar *et al*., 2013). In another study, an outbreak of food poisoning due to *S.* Weltevreden, was reported in a tea garden of an upper Assam district, India, in 2014 (Saikia *et al.*, 2015). Cases were associated with drinking water contaminated with dead livestock and poor environmental sanitation. In Saudi Arabia, an outbreak of food borne *Salmonella* among guests of a wedding ceremony was reported; Meat and rice served at the wedding party were the food items incriminated (Aljoudi *et al*., 2010).

Elsewhere, an outbreak of foodborne gastroenteritis that infected 114 of 161 people who ate at a restaurant in December 2001, was reported in Aracaju, the State of Sergipe, Brazil (Carneiro *et al*., 2015). In Australia food-borne outbreaks due *Salmonella* were reported between 2001 and 2016. Of the 990 *Salmonella* outbreaks reported, 79% (778/990) were confirmed to have been transmitted through contaminated food (Ford *et al*., 2020). Other reports have indicated that in Australia, there have been more *Salmonella* outbreaks than any other country in the world, with the number of cases doubling over the last decade (ScienceDaily, 2019). In 2000, an outbreak of salmonellosis due to *S.* Typhimurium DT160 was reported in New Zealand which caused enteric disease in humans during the winter and spring months (Alley *et al*., 2002). However, the study conducted by Hendriksen *et al*. (2011), showed that *S.* Enteritidis serotype was the most common serotype isolated from human infections during 2007 from the selected regions worldwide.

Distribution of common *Salmonella* serotypes isolated from different human infection in selected countries is shown in Table 2-3.

Table 2-3: Global distribution of the common NTS serotypes isolated from human cases in the year 2007 (Hendricksen *et al.*, 2011)

Region	Country	No. of serotyped isolates/samples	Most frequently isolated serotype	No. of of dominant strains	%
Africa	Cameroon	99	*S.* Enteritidis	19	19.2
	Senegal	102	*S.* Typhimurium	11	10.8
	Tunisia	243	*S.* Enteritidis	119	49.0
Asia	Japan	963	*S.* Enteritidis	431	44.7
	Malaysia	859	*S.* Enteritidis	298	34.7
	Thailand	2720	*S.* Enteritidis	449	16.5
Europe	Bulgaria	500	*S.* Enteritidis	347	69.4
	Croatia	1113	*S.* Enteritidis	892	80.1
	Denmark	1554	*S.* Enteritidis	566	36.4
	Greece	977	*S.* Enteritidis	618	63.2
	Israel	2389	*S.* Enteritidis	511	21.4
	Serbia	3626	*S.* Enteritidis	3249	89.6
North America	Canada	6455	*S.* Enteritidis	1659	25.7
Oceania	New Zealand	1341	*S.* Typhimurium	595	44.4
Latin America	Argentina	520	*S.* Enteritidis	105	20.2
	Brazil	867	*S.* Enteritidis	602	69.4
	Chile	1990	*S.* Enteritidis	975	49.0
	Costa Rica	62	*S.* Typhimurium	18	29.0

2.3.3.3 Global burden of invasive non-typhoidal salmonellosis in humans

Globally, the disease due to invNTS in humans had annual incidence of 1.02 per 100,000 populations, as reported by an international bacteraemia surveillance study (Laupland *et al*., 2010). The bacterium has the capability to disseminate from the intestines to cause systemic disease (Baron, 1996). However, the recent studies on the global estimates of invNTS disease, was between 2.1 to 6.5 million cases with an overall incidence rate of 49 cases per 100,000 populations per annum (Trong *et al*., 2015). Another study indicated global invNTS to be approximately 3.4 million illnesses and about 681 deaths (Ao *et al*., 2015). The incidence of invasive salmonellosis in developed countries is less, although a number of outbreaks have been

reported in some countries such as Italy (Huedo *et al*., 2016) and New-Zealand (Bloomfield *et al*., 2017).

In the US, San Diego alone, five cases of salmonellosis were found positive with invasive *S.* Dublin, following drinking of raw cow milk, and of these one died (Fierer, 1983). On the other hand, the New York City Department of Health and the New York State Department of Health investigated the largest nosocomial outbreak of *S.* Enteritidis to have occurred in 1987, in which 404 of the 965 patients (42 percent) at one hospital were affected, and 9 patients died (Telzak *et al*., 1990). Further investigation revealed that *S.* Enteritidis with the same phage type were found in patients, epidemiologically implicated raw eggs, and the ovary of a hen from the farm corporation that supplied the implicated eggs (Telzak *et al*., 1990). In June 2015, an outbreak of salmonellosis occurred among people who had eaten at a restaurant in Darwin, Northern Territory of Australia (Draper *et al*., 2017). The distribution of invasive NTS serotypes isolated from human sources is shown in Figure 2-2 and Table 2-4.

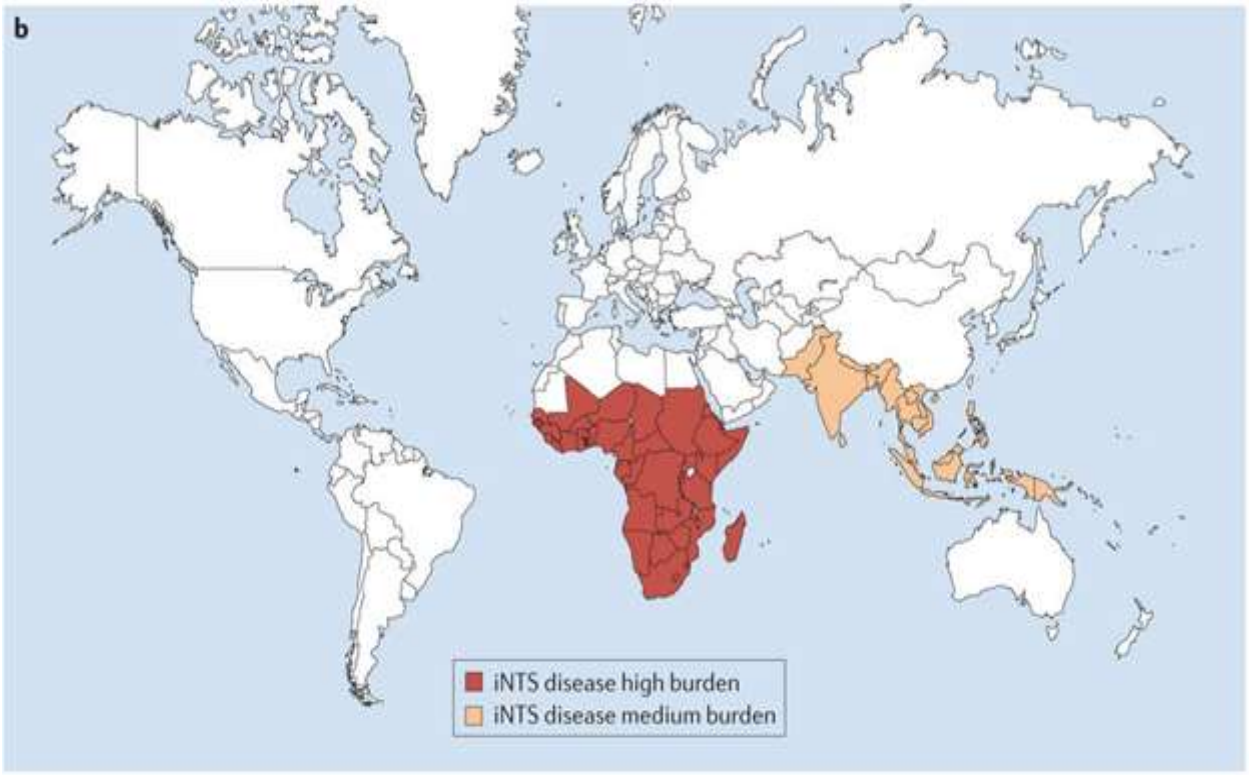


Figure 2-2: Global distribution of iNTS (Gilchrist *et al*., 2015)

Table 2-4: World distribution of the predominant invasive NTS serotype isolates from human sources in selected countries

Region	Country	Year of study	No. of strains (samples)	Dominant serotype	Prevalence rate (%)	Reference
Africa	Mozambique	2001-2006	401	*S.* Typhimurium	263 (66.0)	Mandomando *et al*., 2009
	Kenya	1998-2014	351	*S.* Enteritidis	160 (45.6)	Muthumbi *et al*., 2015
	Kenya	2005-2013	192	*S.* Typhimurium	114 (59.38)	Kariuki and Onsare, 2015
	Malawi	1998-2004	10,628	*S.* Typhimurium	4,956 (75.0)	Feasey *et al*., 2010
	South Africa	2003-2004	1,318	*S.* Typhimurium	883 (67.0)	Feasey *et al*., 2010
Asia	Vietnam	2008-2013	89	*S.* Enteritidis	43 (48.31%)	Nguyen *et al*., 2016
Europe	Italy	2014	25	*S.* Napoli	25 (100.0)	Huedo *et al*., 2016

2.3.3.4 Burden of typhoid fever in Africa: Humans

Estimates for the year 2000 suggested a moderate incidence of typhoid of 10–100 cases/100,000 person years in most African countries, with the incidence highest in childhood (Mweu and English, 2008). East Africa alone, the incidence was estimated at 39/100,000 person years (Mweu and English, 2008). Typhoid fever (TF) is predominantly in under developed countries (Hardy, 2004) in particular African countries (Mweu and English, 2008; Marks *et al*., 2010; WHO, 2011; Labi *et al*., 2014; Keddy *et al*., 2016; Africa, 2017). In early November 2011, there has been a surge of TF outbreaks in Central (Democratic Republic of Congo (DRC) and Southern Africa (Zambia and Zimbabwe), affecting children and adults alike (Nelson, 2012; Muti *et al*., 2014). In Zimbabwe's capital city, an outbreak in typhoid was reported in 2017, nine deaths resulted from the disease (Masiyiwa, 2017). Uncollected garbage, sewer bursts and contaminated water were cited as the major causes of typhoid.

In 2005, one person died and at least 50 were taken ill in an unprecedented outbreak of TF in the northern rain forests of Gabon, following repeated breakdowns of the local water supply system

(The New Humatarian, 2017). During the period, December 2007 to July 2009, a large outbreak of typhoid fever was reported in Kasese District, Uganda; involving 577 cases, 289 hospitalizations, 249 intestinal perforations and 47 deaths (Neil *et al*., 2012). In the same country, an outbreak of *Salmonella* typhoid was reported in 2015, which spread from Kampala City, to all divisions in the capital city and neighbouring districts to which a total of 1940 suspected cases were involved (WHO, 2015a). In 2011, a large outbreak of TF was reported in Kikwit, Democratic Republic of Congo, affected an estimated 1430 people (Brainard *et al*., 2018). The outbreak started in military camps in the city but then spread to the surrounding areas. An upsurge of typhoid fever cases were reported in Moyale Sub-County (MSC), Kenya, by reviewing records from five health facilities in MSC of patients admitted between 2014 to 2015 (Galgallo *et al*., 2018). In the year 2015, *S.* Typhi outbreak was reported in a Burundian refugee camp in Rwanda, despite increased hygiene promotion activities and hand-washing facilities instituted to prevent and control the outbreak (Nahimana *et al*., 2017).

In South Africa, an outbreak of TF was reported which claimed three human lives and left more than 300 people suffered within one week (Farooqui *et al*., 2009). In the same country, over 600 clinically diagnosed TF cases occurred in South Africa in 2005, where an outbreak had been previously described in 1993 (Keddy *et al*., 2010). In another study, an outbreak of TF in Limpopo, South Africa was reported; at least 68 children were admitted to hospitals in Sekhukhune (SABCNewsOnline, 2017). All of the children were suspected of drinking water from a contaminated furrow. In Mozambique, TF struck an area near the Malawian border in 2009; 111 people were affected and 17 of these died (Allafrica.com. 2009). To date TF continues to remain a major health problem in the developing world, and the emergence of multidrug-resistant (MDR) strains has further complicated management of the disease by reducing therapeutic options for treatment of the disease (Kumar *et al*., 2013).

2.3.3.5 Burden of non-typhoidal salmonellosis in Africa: Humans

In KwaZulu-Natal, South Africa, *Salmonella* Enteritidis outbreak was reported due to consumption of contaminated food served at the school function (Niehaus *et al*., 2011). In the same country a nosocomial outbreak of diarrheal disease caused by extended-spectrum β-lactamase-producing multidrug-resistant *Salmonella* enterica serovar Typhimurium, primarily

affecting a pediatric ward in South Africa was reported in 2012 (Smith *et al*., 2014a). Similarly, between 2013 and 2015, seven suspected outbreaks were reported to ORU, in which NTS was the implicated pathogen in South Africa (Muvhali *et al*., 2017). In Ibadan, Nigeria, an outbreak of food poisoning due to a new phage type of *Salmonella* Typhimurium claimed about 20 lives (Ojeniyi and Montefiore, 1986).

2.3.3.6 Burden of invasive non-typhoidal salmonellosis in Africa: Humans

Non-typhoidal *Salmonella* strains found in Africa are also capable of causing invasive disease such as bacteraemia, unlike those strains found in developed countries which are only responsible for diarrhoeal diseases (Crump *et al.,* 2015). These pathogens can cause a significant burden of life-threatening invasive infection (Feasey *et al*., 2012). The disease is characterized by mortality rate of about 20 percent, even when appropriate therapeutic antimicrobial agents are used (Mahon and Field, 2016). Infants, young children and young adults are the most affected with the incidence rate of 227 cases per 100,000 populations (Trong *et al*., 2015; Ao *et al*., 2015). The disease has preference for immune-compromised patients (MacLennan *et al*., 2010), in particular the HIV patients are highly susceptible to invasive NTS but tend to resist typhoid fever (Gordon, 2011).

Surprisingly, invNTS salmonellosis is less frequently reported in Asia than Africa (Khan *et al*., 2010), suggesting that certain areas of Africa are more predisposed to invNTS diseases than in other regions of the world (Deen *et al.,* 2012). In Africa, the disease is more endemic in the rural than urban areas of sub-Saharan Africa countries, where the effect of causing bloodstream infections is more pronounced (Morpeth *et al.,* 2009). In sub-Saharan Africa alone, an annual incidence rate of invNTS among young children of less than 5 years of age was in the range of 175–388 per 100, 000 cases (Sigauque *et al*., 2009). For instance in the rural Kenya alone, Morpeth *et al*. (2009) estimated 88/100,000 persons under the age group of 5 years suffered from blood stream infections, while Kariuki and Onsare (2015), reported an increase of the the incidence rate in children at 166 to 568 cases per 100, 000 persons per year. A similar study in the rural area of Mozambique, showed *inv*NTS infection rates were lower at 120/100,000 person in children below 15 years (Sigauque *et al*., 2009). Another study in Kenya, compared the incidence of invasive NTS disease between two locations; rural setting in western Kenya (Lwak)

and urban settlement in Nairobi (Kibera) from 2009 to 2014, whereby invasive NTS disease was reported to be higher in Lwak (839.4 per 100,000 persons), while the rate in Kibera was 202.5 per 100,000 persons (Verani *et al*., 2015).

Other notable countries in Africa with varying invNTS estimates include; Malawi (Gordon and Graham, 2008; MacLennan *et al.,* 2017), Tanzania (Mtove *et al*., 2010), and in Gambia, where community-acquired invasive NTS bacterial infections were reported in children aged 2-29 months (Enwere *et al.*. 2006). The most prevailing NTS serotype, responsible for bacteraemia in adults and children across sub-Saharan Africa were *S.* Typhimurium and *S.* Enteritidis (Kingsley *et al.,* 2009; Okoro *et al.,* 2012; Muthumbi *et al*., 2015), which accounted for 65.2 percent and 33.1 percent respectively of all the NTS isolates (Reddy *et al.,* 2010). In West Africa, three serotypes, *S.* Typhimurium, *S.* Enteritidis and *S.* Dublin, accounted for the majority of NTS strains isolated from patients in Mali (Tennant *et al*., 2010). Seemingly, the incidence rates are on the increase in Africa and this should be a source of public health concern. However, due to lack of comprehensive data on the disease epidemiology in sub-Saharan Africa region, it has been difficult to implement appropriate preventive measures (von Kalckreuth *et al*., 2016).

2.3.3.7 Burden of typhoid fever in Zambia: Humans

In the recent past, frequent typhoid like outbreaks due to unknown aetiological agents and mode of transmission have been reported in different parts of Zambia. In the year 2012, Zambia recorded a number of typhoid fever outbreaks. The most affected area being Mufulira district in the Copperbelt province (Syapiila, 2018). According to Mweetwa, (2014), the resurgence of typhoid started in 2008, through 2013. Following these suspected typhoidal outbreaks, human salmonellosis was confirmed in Zambia during the same period, hence *Salmonella enterica* serovar Senftenberg (*S*. Senftenberg) were among the clinical serovars that caused severe human infections for which there were very few treatment options (Hendriksen *et al*., 2013).

However, during 2010 to 2012 there was a massive outbreak of *S.* Typhi in Zambia which affected 2,040 patients with a fatality rate of 0.5 percent (Hendriksen *et al*., 2014**)**. In 2015, a large outbreak of typhoid fever was reported in the Kanyama constituency of the capital city of Lusaka, during which 845 patients were treated with typhoid fever in Lusaka local hospital

(Lusakavoice.com, 2015). In 2017, there was a suspected typhoid outbreak in North -western Province, including two deaths (Mwansa *et al*., 2017). Diarrheal outbreaks are still persistent in different parts of Zambia and require a concerted effort to establish the etiological agents. Yet, little is known about the environmental reservoirs and predominant modes of transmission of these pathogens.

2.3.3.8 Burden of non-typhoidal salmonellosis in Zambia: Humans

Previous studies on *Salmonella* in Zambia have been limited and focused on aspects such as isolation of *Salmonella* from humans. A study by Dube and Bhagwat, (1983), conducted at the University Teaching Hospital (UTH) in Zambia, showed 45 strains of various NTS species were isolated from stool, blood and cerebral spinal fluid (C.S.F) of patients. Of these isolates, 93 percent (41/45) strains were isolated from infants less than two years old. Among these strains *S.* Heidelberg was the most common serotype isolated from stools. Only *S.* Typhimurium and *S.* Bovismorbificans were isolated from blood and C.S.F (Dube and Bhagwat, 1983).

2.3.3.9 Burden of invasive non-typhoidal salmonellosis in Zambia: Humans

Salmonella enterica serovar Senftenberg (*S.* Senftenberg) were among the clinical serovars that caused severe human infections for which there were very few treatment options (Hendriksen *et al*., 2013).

2.4 Treatment of salmonellosis

The first antibiotic to be discovered was penicillin in 1928 by bacteriologist Alexander Fleming from a mold culture, and recognition of its therapeutic potential was done at St. Mary's Hospital, London in England (Siang and Tatsumura, 2015). The discovery of how to produce penicillin in mass was done in the US at the Peoria Laboratory (USDA, 2016), and thereafter a cascade of different antibiotics were made available. To that effect, penicillin was used successfully to control bacterial infections among World War II soldiers (Sengupta *et al*., 2013). On the other hand, ampicillin was the first broad-spectrum penicillin for the treatment of infectious caused by *Enterobactrecieae* (The Lancet, 2017). Out of this innovation, both pathogenic bacteria and the disease they cause were under control for almost 70 years, from 1930s to 1990s, during the period of discovery of many new other antibiotics (Waglechner and Wright, 2017). These

antimicrobial compounds have been successfully used since the middle of the twentieth century to treat various diseases in both humans and veterinary medicine (Frye and Jackson, 2013).

The use of antibiotics in animal husbandry and veterinary medicine has resulted in increase of health productive farm animals, which in turn contributes to the welfare and health of humans (Vangelis and Gousia, 2015). However, in the case of NTS infections, administration of antibiotics should be reserved for the septicemia, and focal infections syndromes (Giannella, 1996). The NTS infections are often self-limiting gastroenteritis not requiring treatment (Politi *et al*., 2005; Angulo *et al*., 2009). However, patients at risk of developing invasive salmonellosis such as immune-compromised patients or infants of less than 3 months of age may be recommended to be prescribed antibiotics even when the infection is gastroenteritis only (Christenson, 2013). By and large the clinical condition of a patient will usually determine the choice of the antibiotics to be prescribed. A range of traditional first line antibiotics includes; ampicillin, amoxicillin or trimethoprim/sulfamethoxazole; and chloramphenicol (Shu *et al*., 2015). Of these antibiotics, chloramphenicol, ampicillin and co-trimoxazole were considered drugs of choice to treat typhoid fever (Kalra *et al*., 2003). On the other hand, antibiotics may also be administered to patients manifesting complications of salmonellosis (Hanes, 2003).

The advent of antimicrobial resistant pathogens towards traditional antibiotics prompted a renewed effort to counter the effect by the introduction of new antibiotics such as extended-spectrum cephalosporins; ceftriaxone, cefotaxime, and fluoroquinolones to treat MDR *S.* Typhi strains (Sood *et al*., 1999). Among these antibiotic agents, fluoroquinolones and third-generation cephalosporins are the drugs of choice for invasive *Salmonella* infections in humans (Angulo *et al*., 2009; Kariuki *et al*., 2015b). Whereas, ciprofloxacin is used against NTS infections only in situations showing signs of severe gastroenteritis (Pui *et al.,* 2011a), but also can be a first-line drug of choice for treatment of typhoid fever (Jashbeer *et al.,* 2016). Azithromycin is effective in the management of uncomplicated typhoid fever (Harish and Menezes, 2011). Empirical treatment of typhoid fever can be backed by other measures such as oral or intravenous hydration, lukewarm bath, sponging, appropriate nutrition and blood transfusion (Kalra *et al*., 2003).

2.5 Antimicrobial resistance of *Salmonella* in humans and animals

Antimicrobial resistance is one of the most serious threats to public health globally and threatens the ability to treat human and animal infectious diseases (Lin *et al*., 2004; Davies and Davies. 2010; Anderson, 2013; Mshana *et al.*, 2013; Michael and Schwarz, 2016; Paulson and Zaoutis, 2015; Prestinaci *et al*., 2015; WHO, 2017b). The review of antimicrobial resistance worldwide by Department of Health, United Kingdom, revealed a gloomy and worrisome scenario which is likely to endanger the effectiveness of treating a number of diseases in humans. Further, the report showed that about 700,000 people die annually worldwide due to antimicrobial resistance pathogens (O'neil, 2016).

The factors that facilitate the occurrence and spread of antimicrobial resistance in bacteria population are complex; in animals, the overuse of antimicrobial agents in food animals for prevention and treatment of infections, or in animal feed as growth promoters is the most commonly cited factor (Kalra *et al*., 2003; Lin *et al*., 2004; Marshall and Levy, 2011; Economou and Panagiota, 2015; Vangelis and Gousia, 2015; Bremer, 2017; Naveed and Fogoros, 2018). By 1951, the use of antibiotics as animal additives without veterinary prescription was approved in the United States by Food and Drug Administration (FDA) (Jones and Ricke, 2003). Similarly, member countries of the European Union were also allowed to use antibiotics as growth promoters in animal feeds during the last 50 years (Castanon, 2007). However, it is interestingly to note that only antibiotics that are not absorbed in the digestive tract are permitted as growth promoters (Castanon, 2007).

A research conducted in 2015, calculated for the first time usage of antibiotics by farmers globally; approximately 63,000 tons of antibiotics is used in feeds of chickens, pigs and cattle every year as growth promoters and the figure is expected to rise by nearly 70 per cent to 106,000 by 2030 (News.com.au, 2016). It is also interesting to note the current use of antibiotics in food animals outstretches that of human consumption (Phys.org 2017) which has become a source of public health concern. Therefore, the high usage of antibiotics in food-producing animals contributes to the development of antimicrobial-resistant bacteria, particularly in sectors of intensive animal production (WHO, 2017b). On a global scale, antibiotic consumption is more pronounced with four countries; China, US, Brazil and India accounting for almost 50 percent of

global usage (OECD, 2016). In the US alone it is estimated that 80 percent of all antibiotics used are given to food animals, mainly for growth promotion (Phys.org, 2017). The global average annual consumption of antimicrobials per kilogram of animal produced was estimated at 45 $mg \cdot kg^{-1}$, 148 $mg \cdot kg^{-1}$, and 172 $mg \cdot kg^{-1}$ for cattle, chickens, and pigs, respectively (Van Boeckel *et al.*, 2015).

There is a trend that consumption of antibiotics has been on the increase worldwide; for instance in the period from 2000 to 2010, consumption of antibiotic drugs increased from $52,057,163,835$ to $70,440,786,553$ standard units (35 percent). Of the increase; Brazil, Russia, India, China, and South Africa accounted for 76 percent (Boeckel *et al*., 2014). Antibiotic consumption in livestock worldwide is likely to rise by 67 percent between 2010 and 2030 in particular the countries that have high demand for animal protein for human consumption such as Brazil, Russia, India, China, and South Africa, due to the growing number of animals raised for food production (Van Boeckel *et al.*, (2015). About 60 percent of antibiotics used in agriculture industry comprise tetracycline, penicillin and macrolides (OECD, 2016) which at the same time are recommended for human use. However, these major concerns about the steep rise in the use of antibiotics in animal farming around the world have recently been recognized by the United Nations (UN) Food and Agriculture Organization (FAO) and a great effort is being paid to control the situation (News.co.au, 2016).

In 2013, the distribution of antimicrobials used for animal husbandry management in the world is shown in Figure 2-3.

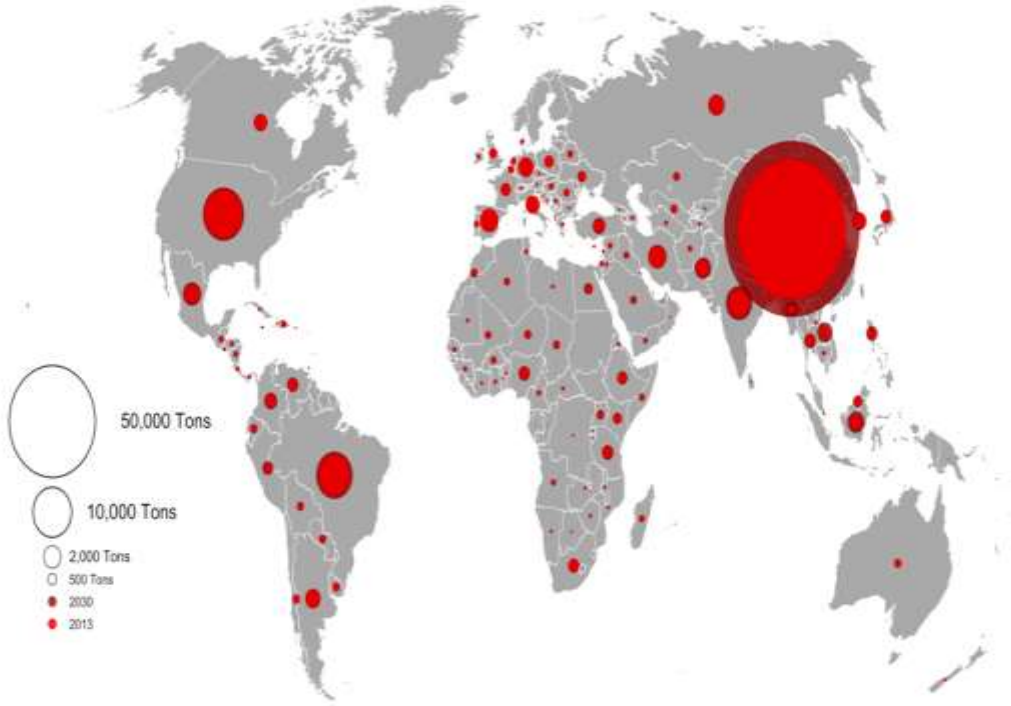


Figure: 2-3: Antimicrobial consumption for food animal production by country in 2013 (light red) and projected for 2030 (dark red). Credit: Center for Disease Dynamics, Economics and Policy (CDDEP) (Phys.org., 2017).

With time, the reservoir of antimicrobial resistant strains in animals can be transmitted directly or indirectly to humans through food supply, direct or indirect contact with animal faeces and the environment (Angulo *et al*., 2009; Vangelis and Gousia, 2015). For instance, in 2002, data on salmonellosis outbreaks indicated that drug-resistant strains of *Salmonella enterica* serotype Newport were becoming increasingly common in dairy cattle and are causing a growing share of infections in humans (Roos, 2002). Foodborne pathogens that are resistant to multiple antibiotics are usually considered more virulent, and capable of causing an increase in the mortality rate of infected patients (Chiu *et al.,* 2002). A cascade of potential public health concerns involving the use of antibiotics as growth promoters in animal feed and for treatment or prevention of animal diseases in animal husbandry veterinary medicine is presented in Figure 2-4, respectively (McEwen and Fedorka, 2002), which subsequently result into the development of MDR (OECD, 2016).

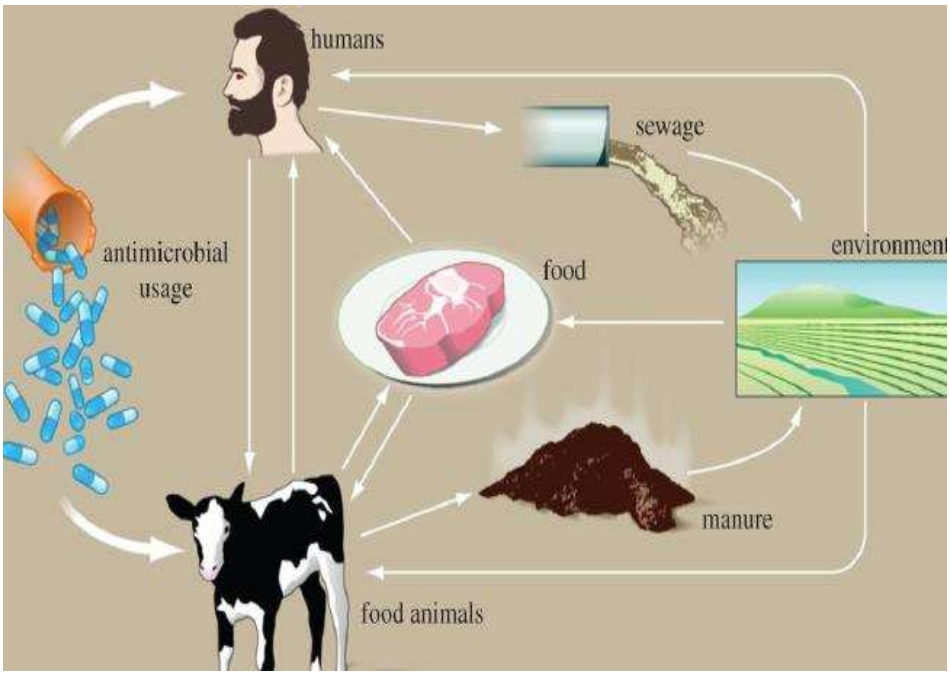


Figure 2-4: The problematic cycle of antibiotic use has excessively spread through global food supplies (Cited by News.com.au., 2016).

On the other hand, uses of antibiotics in humans to treat a variety of bacterial infections have been linked to the development of bacterial resistance (Giannella, 1996). Antimicrobial resistance can also be attributed to the serotype of *Salmonella* and the type of antibiotic used (Lin *et al*., 2004). Similarly, propagation of antibiotic resistance is also considered to be linked to environmental reservoirs that are connected to anthropogenic activities such as agronomic practices and wastewater treatment (Waglechner and Wright, 2017). Therefore, the problem of antibiotic resistance profiles in humans can be an indication of the most likely used antibiotics in a particular area (O'Brien *et al*., 1982).

In addition, there are several alternative ways through which *Salmonella* develops antimicrobial resistance such as the production of enzymes that degrade cell permeability to antibiotics, activation of antimicrobial efflux pumps and production of β-lactamase to degrade the chemical structure of antimicrobial agents (Foley and Lynne, 2008). In principle antimicrobial resistance evolves around the acquisition of genetic resistance elements via horizontal gene transfer and selection of resistant variants in the population (Giannella, 1996; McEwen *et al.,* 2002). These resistance genes in a bacterium are from time to time controlled by the discovery of new and

effective antibiotics (Mayer, 1988; Waglechner and Wright, 2017). It is worth mentioning that these mechanisms to resist antibacterial substances have continued to evolve in bacteria over a period of several billion years (Saliu *et al*., 2017).

The first emergence of multiple drug-resistant (MDR) *S.* Typhimurium D104 strains was reported in 1990, from 29 primarily industrialized countries that participated in the survey through WHO Global *Salmonella*-Survey (GSS) network (Helms *et al.*, 2005). In Scotland, England, Wales, and the Channel Islands, MDR *Salmonella enterica* serovar Paratyphi B variant Java (*S.* Java) has been isolated with increasing frequency from patients (Threlfall *et al*., 2005). Similarly, several other NTS strains have developed MDR phenotype and this phenomenon has been reported in many countries such as the United States of America (USA) (Lee *et al*., 1994; Mukherjee *et al*., 2019), Greece (Tassios *et al*., 1997), Thailand (Hoge *et al*., 1998; Srijan *et al*., 2018), Singapore (Phoon *et al*., 2015), Turkey (Yildirmak *et al*., 1998), Northern Nigeria (Al-Mustapha *et al*., 2018), Tanzania (Msemo *et al*., 2019), and Kenya (Kariuki *et al*., 2019). Another study in Kenya, reported MDR resistant strains of NTS were more frequently isolated from patients presenting bacteremea due to NTS than diarrheae (Akullian *et al*., 2018). In Zambia, NTS serotypes were isolated from stool, blood and cerebral spinal fluid (CSF) from children less than two years and revealed MDR profiles (Dube and Bhagwat 1983). A recent investigation of the epidemiology of the salmonellosis disease in Zambia yielded 94 *S.* Typhi isolates which revealed a high rate of antimicrobial resistance. Most of the isolates (83 percent) exhibited resistance to ampicillin, chloramphenicol, streptomycin and sulfamethoxazole (Hendriksen *et al*., 2014**)**. Other studies by Kalonda *et al.*, (2015), at University Teaching Hospital (UTH) in Zambia, demonstrated that all the 52 *S.* Typhi isolates under investigation were resistant to ampicillin, sulphamethoxazole, trimethoprim and cotrimoxazole, while 84 percent were resistant to chloramphenicol and 4 percent to both ciprofloxaxin and amoxicillin. Similarly *S.* Paratyphi B isolates were resistant to ampicillin, cotrimoxazole, sulphamethoxazole, trimethroprim and streptomycin, while 11.1 percent were resistant to amoxicillin/clavulanic acid and 7.4 percent to both ciprofloxacin and tetracycline.

In recent years, MDR *Salmonella enterica* serovars have been associated with numerous human foodborne illness outbreaks due to consumption of poultry meat and products (CDC, 2014c;

Bearson *et al*., 2017; CDC. 2018d) or beef and products (Plumb *et al*., 2019; Physicians Weekly, 2019). In other studies, human MDR *Salmonella* infection outbreaks have been linked to contact with dairy calves and other cattle (CDC, 2018c), or dogs and pigs (Fleming, 2019). Distribution of *Salmonella* antimicrobial resistance profiles from different animal sources in selected countries is shown in Table 2-5 and Table 2-6.

Table 2-5: Distribution of *Salmonella* antimicrobial resistance profiles from different animal sources and the environment in selected countries in Asia

Region	Period	Animal source	No. of strains	Most prevalent antimicrobial resistance profile (%)	Reference
Asia					
China	2008-2009	Domestic animals	163	carbenicillin (65.4), nalidixic acid (48.8), tetracycline (46.9), sulfafurazole (45.7), ampicillin (43.2), streptomycin (38.3) trimethoprim/sulfamethoxazole (33.3).	Pan *et al*., 2010
China	2010-2014	Domestic animals	92	trimethoprim–sulfamethoxazole (94.6), tetracycline (55.4), carbenicillin (41.3), amoxicillin/A.clav (32.6), and piperacillin (26.1).	Zeng *et al*., 2019
China	2018	Poultry	63	nalidixic acid (100.0), streptomycin (100.0), ampicillin (98.4), erythromycin (93.7)	Yang *et al*., 2019
China	2016	Commercial broilers	21	ampicillin (95.2), nalidixic acid (85.7), cefotaxime (81.0), and tetracycline (61.9);	Li *et al*., 2017
China	2016	Spent hens	19	nalidixic acid (100.0), tetracycline (100.0), ampicillin (94.7), ciprofloxacin (68.4)	Li *et al*., 2017
Nothern India	-	Chicken meat	70	tetracycline (100.0), erythromycin (100.0), nalidixic acid (98.57), ampicillin (95.71) ciprofloxacin (82.9).	Sharma *et al*., 2019

Table 2-6: Distribution of *Salmonella* antimicrobial resistance profiles from different animal sources and the environment in other countries

Region	Period	Animal source	No. of strains	Most prevalent antimicrobial resistance profile (%)	Reference
South America					
Brazil	-	Pigs	20	ciprofloxacin and tetracycline (90.0 each), nalidixic acid (80.0)	Filho *et al.*, 2016
Europe					
Serbia	-	Meat products	25	ampicillin and nalidixic acid (80.0), tetracycline (72.0), cefotaxime/clavulanic acid (48.0), gentamicin (8.0) trimethoprim/sulfamethoxazole (0).	Raseta *et al*., 2017
Latvia	2015	Read to eat meat	20	sulfamethoxazole (40.0), nalidixic acid (25.0), ciprofloxacin (25.0), ampicillin (25.0), tetracycline (20.0).	Terentjeva *et al*., 2017
Africa					
Ghana		Poultry farm	200	nalidixic acid (89·5), tetracycline (80·7), ciprofloxacin (64·9), sulfamethazole (42·1), trimethoprim (29·8) ampicillin (26·3).	Andoh *et al*., 2016
Nigeria	2014 - 2015	Poultry farm	31	tetracycline (89.0), ampicillin (86.0), nalidixic acid (86.0) Sulfamethoxazole (71.0).	Al-Mustapha *et al.*, 2018
Ethiopia	2014 - 2015	Food of animal origin	21	tetracycline (42.6), sulfamethoxazole-trimethoprim (28.6), ampicillin (14.3)	Ejo *et al*., 2016
South Africa	-	Swine	48	tetracycline (100.0), oxytetracycline (100.0), ampicillin (75.0), sulphamethoxazole-trimethoprim (75.0), nalidixic acid (75.0), and streptomycin (75.0).	Iwu *et al*., 2016

2.6 Risk factors of salmonellosis

According to Gras *et al*. (2014), most risk factors for human infections have been attributed to different food and animal reservoirs. Their study identified that about 90 percent of human cases were linked to layers production and pig husbandry. For instance in urban areas, human adults' salmonellosis has been linked to layers and broilers as the reservoirs of infection, and most likely in spring/summer (Gras *et al*., 2014). On the other hand, pigs and cattle were the most likely reservoirs of salmonellosis in children, in rural areas, and in autumn/winter (Gras *et al*., 2014).

Host risk factors may include humans and animals that have been exposed to high consumption of antimicrobial agents (Ruddat *et al*., 2014), immunocompromised patients (Gordon, 2008), malaria and anemia patients (MacLennan and Levine, 2013), malnutrition (Rosanova *et al*., 2002), HIV infection (Levine and Farag, 2011), gastric acid suppression (Dial *et al*., 2005; Tennant *et al*., 2008), sickle cell disease (Hand *et al*., 1978), and schistosomiasis (Hsiao *et al*., 2016). Children under the age of 3 years are particularly at risk for invasive invasive non-typhoid salmonellosis (Morpeth *et al*., 2009; Gilchrist *et al*., 2018). Children are vulnerable to gastroenteritis due to under developed immune system of their intestinal barrier to pathogens (Cremon *et al*., 2014). At household levels, parameters that influence risk for human salmonellosis were identified as poor pork handling practice, use of a chopping board for raw meat and consuming raw/undercooked meat were risk factors for infection with salmonellas originating from pigs, cattle and broilers (Sadeleer *et al*., 2009; Abdunaser *et al*., 2009; Gras *et al*., 2014).

In the central markets, traders of pork meat play a major role towards contamination of several surfaces due to poor hygiene practice (Xuan *et al*., 2017). Similarly, ownership of backyard flocks can be a source of poultry associated *Salmonella* infections and outbreaks, in particular the urban areas where zoonotic diseases from farm animals are considered rare (Shah, 2015).

In animal feed production, the temperatures used to produce feed are sufficient to eliminate *Salmonella,* but often high concentrations of *Salmonella* in other feed ingredients, may result in the production of *Salmonella*-contaminated feed (NSCFS, 2006). Wild birds or animals having access to animal feed storage facilities may contaminate feed (McGuirk and Peek, 2003).

Similarly, practicing of mixed farming (dairy herds, beef herds and calf herds) sourcing of animals such as cattle from unclean herds, confinement of animals in relatively small area, and having a high population of feral cats around the farm area can also be a risk factor (McGuirk and Peek, 2003).

Human salmonellosis may be acquired by direct animal contact with faecal matter of domestic animals such as pigs and cattle (Gras *et al*., 2014; Hoelzer *et al*., 2011; Cummings *et al*., 2012; Stipetic *et al.,* 2016), and also wildlife such as visits to the zoo in particular for the children (Mermin *et al*., 2004; Morpeth *et al*., 2009). Other potential areas that humans may contract infectious diseases in particular for children include; Health Centers, such as the hospitals, or clinics or any other place designated for patients to receive medical services, or diagnostic laboratories (Morpeth *et al*., 2009). On the other hand, floods during rainy seasons are risk factors due to their potential to contaminate the environment with faecal organisms, which include the sources of drinking water (Morpeth *et al*., 2009). Occupational exposure to animals may contribute to NTS colonization in particular among the chicken farmers (Trung *et al*., 2015). Further, risks factors can also be associated with highly dynamic population of animals with limited space as well as movements of food animals as reservoirs of pathogens across boundaries (Stipetic *et al*., 2016).

2.7 Prevention and control of salmonellosis in humans and animals

Most human salmonellosis cases are foodborne, but infections are also acquired through direct or indirect animal contact in homes, veterinary clinics, zoological gardens, farm environments or other public, professional or private settings (Hoelzer *et al*., 2011). Therefore, prevention of infection or contamination with *Salmonella* requires control measures at all stages of the food chain, from agricultural production, to processing, manufacturing and preparation of foods in both commercial establishments and at home (Arguello *et al*., 2013; WHO, 2018).

2.7.1 Control of *Salmonella* on animal husbandry farms:

Prevention is preferable to treatment. There are several methods that can be used to prevent *Salmonella* infection from spreading onto a farm. Disease prevention promotes the health and wellbeing of both farm and companion animals and prevents suffering. In the case of poultry

farming, monitoring is one such effective method which involves checking that upon the arrival at the site chicks should be free from *Salmonella* pathogens by testing samples such as chick box liners, swabs from bases of boxes and chicks found dead-on-arrival or cull chicks. On the other hand, flocks should be monitored frequently for possible *Salmonella* infection. Samples for culture can be taken from litter, faecal samples, boot or drag swabs nd dust samples.

In pig production, the farm-to-fork approach is currently used, in which specific measures are employed in each respective sector of the production chain, in order to reduce *Salmonella* contamination (Callegari *et al*., 2020). Fundamentally, thorough sanitation between groups is most desirable. Pigs of different ages or sources should not be commingled.

2.7.1.1 Hygiene and biosecurity:

Good control measures at the farm level are likely to produce lower occurrence of *Salmonella* infection; for instance, use of biosecurity measures in pig farms can help to control important pig diseases as well as reducing the within-herd prevalence of *Salmonella* (Andreas and Davies, 2015). It is important that organic farmers should consider the control of rodents, wild birds and flies, as well as disinfection of boots/clothes and equipment for farm workers. Further control of the influx of visitors is another integral part of hygiene measures to prevent transfer of food-borne pathogens (Meerburg and Kijlstra, 2007).

2.7.1.2 Good Management practices:

Prevention of diseases depends largely on good management and husbandry practices. According to Linden (2013), critical *Salmonella* control points include breeder houses, feed in various form, water, the farm environment, vermin control and prohibiting cross-contamination. Preventive measures such as vaccination and biosecurity, also makes for good farm management as healthy animals make food production more efficient and sustainable and improve food security. Strict management of waste such as effluent is important; to reduce the organisms that are discharged into the environment such as waste effluent is another important measure that can be employed to curb the spread of *Salmonella* (Murray, 1991).

2.7.1.3 Vaccinations:

Vaccines protect livestock against infectious diseases that could potentially be passed to people through food chain such as eggs, milk and meat products (Neuman, 2010). A WHO report recommended that poultry farmers should consider vaccination of birds as an additional measure to reduce *Salmonella* infection within the flock (WHO Consultation, 1994). Therefore, vaccination program against any *Salmonella* serotype will only be effective if it is part of a sound and comprehensive biosecurity program designed for *Salmonella* control in poultry farms (Paiva *et al*., 2009). A previous study, demonstrated that vaccinated birds produced antibodies earlier than non-vaccinated birds (Hesse *et al*., 2018). Consequently, high titers of circulating antibodies have been associated with protection against systemic infections (Woodward *et al*., 2002).

Further, production systems also need to be developed that will produce *Salmonella*-free stock, grandparent, and great grandparent's flocks (WHO Consultation, 1994). For this reason, fowl typhoid vaccinations with a special *S.* Gallinarum (9R strain) has been practiced in poultry industries worldwide and have conferred protection (Mitra *et al*., 2015). Further, poultry industries are urged to observe stringent measures to control the spread of *Salmonella* through key areas such as trade in live parent stock and grandparent flocks within and between countries (Forshell and Wierup, 2006). Although, live attenuated *Salmonella* vaccines have in rare cases been used successfully to evoke cell-mediated immune response in pigs, cattle, and chickens, it was on the hand proven to have protected such animals against both systemic disease and intestinal colonization (Gruenberg, 2014). Diarrhea due to *Salmonella* infection is a cause of neonatal calf diarrhea. Studies have shown that stimulation of passive immunity in the calf by vaccinating the dam against *Salmonella* has shown some success; although, there are no data on the use of currently licensed vaccines in the United States (Smith *et al*., 2014).

Vaccination of pregnant cows with an autogenous *Salmonella* bacterin had no effect on faecal shedding of *Salmonellae,* whereas vaccination with a modified live *S.* Choleraesuis vaccine reduced the frequency of fecal shedding of serogroup C1 *Salmonellae* during the peripartum period (House *et al*., 2001). In another study, the use of a commercially available *Salmonella* Dublin vaccine was shown to stimulate antibodies that are passed on to the calf via colostral transfer (Smith *et al*., 2015). However, a recent study, developed a two novel *Salmonella*

bivalent vaccines, SLT26 (pCZ11) and SLT27 (pCZ11), suggesting that the delivery of a heterologous O-antigen in attenuated *Salmonella* strains is a prospective approach for developing *Salmonella* vaccines with broad serovar coverage (Zhao *et al*., 2017).

2.7.2 Control of *Salmonella* in slaughter houses

Food from animal origin such as meat and meat products are important in regardsare among the main sources of *Salmonella* infection in humans, as such several countries have implemented *Salmonella* surveillance and control programs (Arguello *et al*., 2013; Wambui *et al*., 2017). Studies have shown that the risk of contamination by *Salmonella* increases along the food chain, reaching its summit in the slaughter houses (Botteldoorn *et al*., 2003). Therefore, a slaughter house should be considered as one of the critical points in which effective processes can be applied to reduce contamination.

It is worthynoting that in the slaughter houses, *Salmonella* is contionusly being introduced along the processing plant. For instance, in pig meat processing plants, attention should be paid to avoiding contact of the faeces and tonsilles of contaminated pigs with the carcass (Sadeleer *et al*., 2009). Therefore, it is imperative that good health practice is essential to ensure the prevention or minimization of carcass contamination with *Salmonella* in the slaughter houses (FAO, 2016b; Andreas and Davies, 2015). According to Skaarup (1985), a well-executed and controlled cleaning and sanitation programme for rooms, machines and equipment is very important to achieve a hygienic standard. Further, it is important that personal hygiene is equally considered as a part of hygiene practices. For instance, hand washing, protective clothing, training needs, medical examination and equipment handling to avoid prohibited practices (Wambui *et al*., 2017).

2.7.3 Control of *Salmonella* in food and feed processing industries

Salmonella is found everywhere and can survive, even at low moisture levels, for a long time even in the feed (Li *et al*., 2011). Therefore, the control of *Salmonella* in animal feedstuffs is critical, to prevent the human food chain from contamination by *Salmonella* derived from infected animals (Tomičić *et al*., 2019). According to Tomicic *et al*., (2019), there are multiple ways in which *Salmonella* can reach the animal feed during production stages. The common

routes of contamination include ingredients, co-products, dust, rodents and contaminated equipment. All stages of the food chain, from agricultural production, to processing, manufacturing and preparation of foods in both commercial establishments and at home requires strict prevention and control measures against salmonellosis (WHO, 2015b). In this case, animal feed is widely considered to be an important critical point to control *Salmonella*. Therefore, application of Hazard Analysis Critical Control Point (HACCP) in commercial establishments provides the most effective use of control sources by emphasizing monitoring of Critical Control Points (CCP) rather than concentrating on the analysis of end product.

2.7.4 Control and prevention of salmonellosis in humans

Majority of cases of salmonellosis in humans are associated with the consumption of contaminated food products such as beef, pork, poultry meat, eggs, vegetables, juices and other kind of foods (Freitas *et al*., 2010). Other studies have associated the infection with the contact between humans and infected pet animals (Hoelzer *et al*., 2011). Therefore, it is important that appropriate steps are taken to prevent getting *Salmonella* infection which can include the following;

2.7.4.1 Good hygiene practices:

According Giacometti *et al*. (2015), the proposed quantitative risk assessment (RA) models revealed that boiling milk before drinking is a simple yet effective tool to protect consumers against the risk of illness inherent in the consumption of raw milk. Other measure includes; cross-contamination of foods should be avoided; uncooked meats should be kept separate from produce, cooked foods, and ready-to-eat foods. Hands, cutting boards, counters, knives, and other utensils should be washed thoroughly after touching uncooked foods. Hand should be washed before handling food, and between handling different food items. Similarly hands should be washed after contact with farm animals, because pets, animal feces, and animal environments have been associated with salmonellosis (Hoelzer *et al*., 2011). Further, raw fruits and vegetables can be washed before eating as they have been linked to human salmonellosis (CDC, 2019).

2.8 Public health importance of salmonellosis

Salmonellosis due NTS pathogens has brought about significant morbidity and mortality rate worldwide (Cardoso *et al*., 2002). These pathogens are associated with foodborne and waterborne diseases through faecal contamination and are the most problematic diseases in terms of control worldwide coupled with high endemicity (Antunes *et al*., 2003; Marshall *et al*., 2006). Food handlers play an important role in the transmission of food-borne diseases (Ogah *et al*., 2015). In 2010, Hamilton County Public Health (HCPH), Ohio, US, confirmed the existence of food-borne outbreak following the consumption of pork prepared in a private home and sold at the church festival (CDC, 2014b). Therefore, most salmonellosis infections are acquired by consuming contaminated food and food products, especially food from animal origins (Buzby and Tonya, 2009). There are several other ways of acquiring human salmonellosis such as through contact with faeces of companion and/or domestic animals (Finley *et al*., 2006; Gaffga *et al*., 2012), or exposure to faeces of zoological animals or wildlife (Jang *et al*., 2008; Hale *et al*., 2012). Frequently, infection associated with profession occupancy is reported as in fish industry (Musto *et al*., 2006) and veterinary practice (Hoelzer *et al*., 2011).

2.9 Socio-economic importance of salmonellosis

Health is an important part of welfare and whenever an animal is diseased, welfare is poorer than when there was no disease (Broom and Corke, 2002). Salmonellosis causes considerable morbidity and mortality in both humans and animals, and has a significant socio-economic impact worldwide (Coburn *et al*., 2007; Macovei *et al.*, 2010; Smith *et al*., 2016). The impact includes the costs that are associated with prevention and treatment of the diseases (Crump *et al*., 2004; Boyle, *et al*., 2007).

2.9.1 Socio-economic impact of Salmonellosis in humans

Salmonellosis associated with foodborne and waterborne constitutes a major economic problem which has the potential for loss of trust in public utilities such as water reticulation systems world wide following outbreaks (Ailes *et al*., 2013). Therefore, management of human salmonellosis outbreaks can be expensive in terms of medical costs, productivity losses and premature death, as well as considerable costs associated with control efforts (IFAH, 2012). Human cases of salmonellosis contributes significant burden on the health care system (Boyle *et*

al., 2007). For instance, in 1994, treatment of human salmonellosis due to *S.* Enteritidis was estimated to costs of approximately 1 billion US dollar per year on the USA economy (Roberts and Sockett, 1994). By 1999, the economic burden had swelled to to $2.8 billion (95 percent CI: $1.6 to $5.3 billion) annually, which translated to approximately $2,472 per case of *Salmonella* infection (Frenzen *et al*., 1999). This cost estimate was largely driven by the number of premature deaths followed by average cost of hospitalization (Frenzen *et al*., 1999). In 2008, a large *Salmonella* outbreak caused contamination of the municipal drinking water supply in Alamosa, Colorado, USA, and took the residents and businesses about 1.5 to 2.0 million dollars to contain the outbreak (Ailes *et al*., 2013).

In the European countries, majority (95 percent) of salmonellosis infections is food-borne, and the annual cost to control the infection is between 560 million and 2.8 billion euros (Galanis *et al*., 2006). In the year 2005, the overall economic burden of human salmonellosis for the Europan Union countries was estimated as high as €3 billion a year (EFSA, 2012). However, by the year 2008, about 131, 468 cases of *Salmonella* were reported in 27 countries of the European Union alone, which translated into an estimated cost of 132, 612, 837 British pounds to manage the situation (1,009 pounds per case) (IFAH, 2012). In England and Wales, a total cost of 996,339 pounds were spent as costs imposed upon public health authorities, the health sector, individuals and families in terms of wider economy resulting from loss of production due to absence from work and costs that were related to health care and local investigation of the cases (Sockett *et al*., 1991).

In humans, *Salmonella* contamination of food products of animal origin can bring about loss of consumer confidence which could result into substantial economic loss to the animal husbandry industry including poultry (Wisner *et al*., 2012). The cost of treatment has since increased to about 365 million dollars in direct medical costs and beyond 2 billion dollars in total societal costs by the year 2011 (IFAH, 2012). Majority of these cases were due to consumption of contaminated food products of which *S.* Enteritidis is the second most commonly isolated serovar after *S.* Typhimurium in North America (Callaway *et al*., 2008).

2.9.2 Socio-economic impact of Salmonellosis in animals

Animal diseases affect livestock production through direct costs such as treatment of cases, laboratory investigation and sometimes loss of livestock due death (Kemal, 2014), and reduction in productivity, such as lower milk yield in the case of cattle, weight loss, abortions and death (McGuirk and Peek, 2003; IFAH, 2012). In the case of sheep, *S.* Abortusovis causes abortion as the main symptom, and this is economically important in light of losses during ovine production systems particularly in the regions where sheep are reared extensively (Pardon *et al*., 1988). Sometimes loss of animals is brought about by contamination of animal feed (Davies and Wales, 2010; Jones, 2011; Davies and Wales, 2013). Animal feeds become contaminated with foodborne *Salmonella* pathogens either during harvesting, processing at the mill or during storage (Maciorowski *et al*., 2006).

There are also indirect or hidden costs that are associated with control and prevention of the spread of the disease (Kemal *et al.*, 2014), from subclinically infected animals which could pose a health risk to consumers (Evangelopoulou *et al*., 2015). Therefore, it would be economically profitable to vaccinate animals as a preventive measure as opposed to managing acute salmonellosis (Bearson *et al.,* 2016). *Salmonella* vaccinations of animals in particular poultry does not only reduce *Salmonella* colonization of the chickens, but also minimizes public health risks by decreasing the levels of environmental contamination through faecal shedding (Revolledo and Ferreira, 2012). In Swedish dairy herds, the average costs per herd for on-farm *Salmonella* control was 4.60 million SEK with a median of 1.06 million SEK corresponding to approximately 490 000 and 110 000 EUR (Agren *et al*., 2015).

However, in industrialized economies, the economic and social burden of this infectious disease is considered very important (Tabo *et al*., 2015). For instance, in North America alone, a cost benefit analysis to control five *Salmonella* outbreaks due to manufactured food was estimated in the range of 36,000 to 62 million US dollars (Wray, 1994). Whereas, in Denmark, the total annual *Salmonella* control costs incurred exclusively by the industry were U.S.A $14.1 million of pork and broiler or eggs (Wegener *et al*., 2003). Between 2009 and 2010, representatives of the major feed producers in the Swedish market, independently estimated the annual total cost was 1.8-2.3 Euro pound (€) per tonne of feed, for preventing *Salmonella* contamination (Wierup

and Widell, 2014). In contrast, economic analysis for animal diseases in developing countries are not always available and rarely conducted (FAO, 2016a). However, livestock diseases in developing countries have a bigger impact on the market value of the livestock, and subsequently contribute to poverty levels to livestock owners (Karl and Perry, 2011).

Humans derive their much needed dietary protein from food of animal origin and their products (Pasiakos *et al*., 2015; Elmadfa and Meyer, 2017). Therefore animal food products that are contaminated from subclinically infected animals are a risk for consumers (Evangelopoulou *et al*., 2015). *Salmonella* Pullorum and *S.* Gallinarum can be responsible for severe mortality among chickens resulting in huge economic loss, but rarely cause disease to humans (Rajagopal and Min, 2013).

In developing countries, animals that are apparently healthy are source of energy, and as such they are used for agriculture purposes to cultivate areas as well as being involved in transportation of goods (Ramaswamy, 1994). On the contrary, diseased animals may not provide these services. Against this background all forms of animal infection including salmonellosis should be a source of economical and political concern (Evangelopoulou *et al*., 2015).

2.10 Biology of *Salmonella*

The genus *Salmonella* constitutes many pathaogenic serotypes that are highly adaptive to vertebrates and are host specific (Evangelopoulou *et al.*, 2013; Muthumbi *et al*., 2013). Most serotypes are capable of causing diseases in multiple hosts but some are highly adapted to a single-host species (Baumler and Fang, 2013). This phenomenon greatly applies to *Salmonella enterica* subspecies by virtue of their broad host range and their degree of host adaptation (Eswarappa *et al*., 2008). *Salmonella enterica* subspecies I has the largest diverse group of serotypes that have evolved to survive in a wide range of environments and across several hosts (Foley *et al*., 2013) and 99 percent of human and animal infections are caused by this group (Achtman *et al*., 2012).

The diversity of *S. enterica* subspecies I is attributed to mutational evolution, as well as intra and inter species recombination that is potentially influenced by restriction-modification (RM)

systems (Roer *et al*., 2016). *Salmonella enterica* subspecies can conveniently be divided into three groups namely; host-restricted serotypes, host-adapted serotypes and broad host-range (Non-host adapted) (Rotger *et al*., 1999).

2.10.1 ` Host restricted *Salmonella* serotypes in humans

This group of *Salmonella* contains few serotypes which are exclusively limited to human for transmission of the disease (Baumler and Fang, 2013). *Salmonella* Typhi (ST) is a highly human adapted pathogen which causes enteric fever commonly known as typhoid fever, a serious global health infection which is responsible for life threatening infections in humans only (Jashbeer *et al*., 2016; Chong *et al.,* 2017). Other human restricted *Salmonella* serovars *S.* Paratyphi A, B and C which cause paratyphoid fever and collectively including ST are known as typhoidal *Salmonella* (TS) (Raffatellu, *et al.*, 2008; Crump and Mintz, 2010). *Salmonella* Paratyphi A exhibits symptoms similar to TF, but often less severe (WHO, 2011).

These serotypes manifest a systemic disease syndrome, which results into dissemination of pathogens beyond the lymph nodes in immune competent hosts, and they display a decreased intestinal involvement to a point where they are no longer associated with gastroenteritis (Kingsley *et al*., 2013). However, host restricted serotypes tend to cause higher mortality rates than those with a broad host range. The infection due to host restricted serotypes tends to persist in the tissue long after the clinical symptoms (Barrow *et al.,* 1994). Typhoid fever is a typical example of the disease caused by *S.* Typhi a highly adapted serotype in humans (Crump *et al.*, 2004) and higher primates (Evangelopoulou *et al*., 2013). Other serotypes include; *S.* Paratyphi A, B and C (Crump *et al.*, 2004; Gal-Mor *et al*., 2014), and *S.* Sendai (Baumler and Fang, 2013; Gal-Mor *et al*., 2014) which are are capable of causing acute infections called paratyphoid fever in humans and have animal reservoirs (ECDC, 2017b). *Salmonella* Paratyphi B can also cause sporadic gastroenteritis and less frequently paratyphoid fever in humans (Caumes *et al.,* 2001; Chart, 2003).

2.10.2 Host-adapted *Salmonella* serotypes in animal species

In birds the infection is called fowl typhoid caused by *S.* Gallinurum-Pullorum (Rajagopal and Min, 2013). Both *S.* Gallinurum and *S.* Pullorum biotypes primarily cause systemic infections in

chickens and turkeys (Kabir, 2010; Barrow and Neto, 2011). Several outbreaks due to *S.* Pullorum have been reported worldwide (Haider *et al*., 2013; Casagrande *et al*., 2014). However, both diseases are common in some countries of Central and South America, Africa and Asia (CFSPH, 2009). Although, these biotypes are bird adapted, they have been isolated from other sources such as humans and crocodiles (Menghistu *et al*., 2011), and also from few other animals after experimental inoculation or natural exposure such as pigs, cattle, cats, dogs, foxes, mink, rabbits, guinea pigs and laboratory and wild rats, and chimpanzee (CFSPH, 2009). In Zambia, ten outbreaks due to *S.* Gallinarum and *S.* Pullorum were reported on poultry farms involving day old broiler chickens and 5 to 18 months old layers chickens (Sato *et al*., 1997).

Other serotypes in this group includes; *S.* Abortusequi (horses) and *S.* Abortussuis (porcine) (Uzzau *et al*., 2000a; Crump *et al*., 2004). *Salmonella* Abortusequi (horses and donkeys) has been associated with abortion in pregnant mares (Madic *et al*., 1997; Buiques *et al*., 2012). On the other hand, *S.* Dublin serotype is predominantly adapted in cattle and rarely causes infections in sheep, swine, horses and zoological animals (Ferris *et al*., 1992). Both S. Abortusequi and *S.* Dublin serotypes are known to be invasive, and are a source of a major veterinary problem worldwide (Libby *et al*., 1997). But consumption of raw milk and undercooked beef, can lead to invasive bloodstream infection in humans (Roudier *et al*., 1990; Fang and Fierer, 1991).

Strains adapted to pigs include *S.* Choleraesuis (Methner *et al*., 2018), which have also been implicated in other animal host species such as humans (Roudier *et al*., 1990), where it is capable of causing severe systemic disease (Su *et al*., 2014; Ferstl *et al*., 2017). Last but not least, *S.* Abortusovis is adapted to sheep, and causes abortion as the main symptom (Pardon *et al*., 1988; Giannati *et al*., 2018). *Salmonella* Choleraesuis and *S.* Abortusovis serotypes are capable of causing disease in humans, which is often invasive and life-threatening (Jones *et al*., 2008; Eke *et al*., 2014), owing to their possession of *Salmonella* plasmid virulence (*spv*) (Uzzau *et al*., 2000b; Derakhshandeh *et al*., 2013).

2.10.3 Non-host adapted (Generalists) *Salmonella* serotypes

Non-typhoidal salmonellosis infections are caused by all serotypes of *Salmonella* except *S.* Typhi and *S.* Paratyphi A, B and C (Taramasso *et al*., 2016). This group of serotypes exhibits a broad host range and generally tends to colonize the intestinal tract and invade enterocytes of the intestinal mucosa. They manifest gastroenteritis, but in contrast fail to disseminate beyond the lymph nodes, unless in the event that the host has an underlying immune defect (Kingsley *et al.*, 2013). The group consists of serotypes such as *S.* Ttyphimurium and *S.* Enteritidis (Rotger *et al*., 1999), and is perceived as a major cause of gastroenteritis and septicaemia syndromes in humans (Roudier *et al*., 1990). *Salmonella* Typhimurium and *S.* Enteritidis serotypes can also cause disease in broad range of highly susceptible animal hosts such as poultry, cattle, rodents, pet hedgehogs (Costa *et al*, 2012; Gaffga *et al*., 2012; Loharikar *et al*., 2013; Basler *et al*., 2016; Bastin *et al*., 2016; Anderson *et al*., 2017; WHO, 2018). Further, these serotypes have been implicated in food-borne salmonellosis worldwide, and have been detected in most species of domestic and wild animals used as food animals for human consumption (Pande *et al*., 2016).

Other non-host adapted *Salmonella* serotypes that have been associated in food-borne diseases (FBD) include; *Salmonella* Infantis, *S.* Heidelberg, *S.* Bovis-morbificans and *S.* Agona (Lax *et al*., 1995). However, reported death due to zoonotic *Salmonella* serotypes are few less than 0.5 percent of cases of human illness (Miller *et al.*, 1995). However, a variable number of common broad-host-range serotypes carry virulence plasmids which encode *spv* genes (Libby *et al*., 1997). These are potential serotypes capable of causing systemic disease in a wide range of animals including humans and can also manifest diverse clinical symptoms, from asymptomatic infection to serious typhoid-like syndromes (Uzzau *et al*., 2000b).

2.11 Pathogenesis of *Salmonella*

A simplified sequence of events after ingestion of contaminated food or water can be summarized as shown in Figure 2-5. Pathogenic *Salmonella* in food survives the gastric acid barrier to colonize the mucosa of the ileum and the colon and attach to the intestinal epithelium (Cooke *et al*., 2007). The intestinal surfaces comprise non-specific frontline barrier such as mucus, intestinal peristalsis, lactoferrin and lysozyme (Baron, 1996), that limits the invasion of both commensal and pathogenic bacteria (Ribet and Cossart, 2015). However, adhesion of

pathogens to the host mucosa facilitates host colonization, and minimizes the chances of the pathogens being eliminated from the intestines by mechanical action of peristalsis (Ribet and Cossart, 2015). In mammals, *Salmonella* serotypes exhibit a predilection for intestinal lymphoid tissues (Reis *et al*., 2003; Santos and Baumler, 2004), but the specialized epithelia microfold cells overlying the lymphoid follicles of Peyers patches are important in the mucosal immune system (Vazquez-Torres *et al.,* 1999). Despite, the presence of host defence mechanisms at this level, the pathogens invade and proliferate within the epithelium and lymphoid follicles (Giannella, 1996; McGuckin *et al*., 2011).

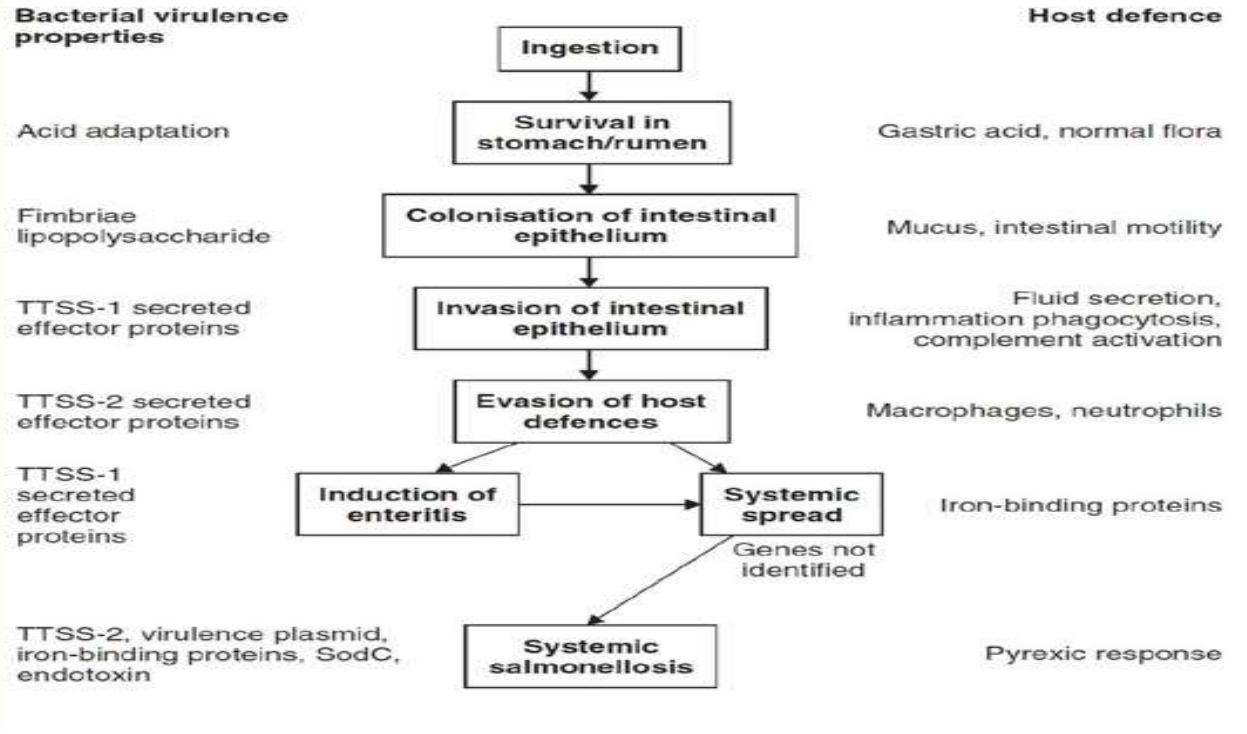


Figure 2-5: The attachment, colonization, invasion, virulence property and host defense mechanism of *Salmonella* once ingested from food and pathogenicity island function (Kemal, 2014).

An experiment by Martinez-Argudo and Jepson (2008) showed *Salmonella enterica* is capable of invading cultured cells by deploying a type III secretion system (T3SS) encoded within *Salmonella* Pathogenicity Island 1 (SPI-1) to translocate effector proteins into the host cell. The effector proteins stimulates cytoskeletal rearrangements of the host cell that facilitates membrane agitation usually referred to as "ruffling" followed by bacterial internalization through

micropinonocytosis (Knodler *et al*., 2010). Thereafter, the pathogens spread to mesenteric lymph nodes and throughout the body via the systemic circulation, upon being taken up by the reticuloendothelial cells which controls the spread of the pathogens (Baron, 1996). The invasion of the host cells are determined by genetic control and involves several genes (Baron, 1996), such as the involvement effector proteins encoded within SPI-2, virulence plasmids, iron binding proteins, *sod* C, and endotoxin encoded by *Salmonella*.

Salmonellosis infections in humans, tend to vary from gastroenteritis to typhoid fever and the degree of infection will largely depend on other key factors such as; virulence properties possessed by the bacterial strain (Fierer *et al*., 2001; van Asten and van Dijk, 2005), and immune status of the host (Giacomodonato *et al*., 2007), and the ability to colonize certain internal organs such as the liver, spleen, gallbladder, bones, meninges (Giannella, 1996). Other factors include; amount of the inoculum, genetic background of the host (Langridge *et al*., 2012) and virulence of the strains in non-adapted hosts (Baumler and Fang, 2013). *Salmonella* virulence is not fully understood but both chromosomal and plasmid genes are involved (Forshell and Wierup, 2006).

The virulence determinants of *Salmonella* serotypes evokes two distinct types of diseases namely; gastroenteritis due to the bacteria that remain localized in the intestinal tract (self-limiting gastroenteritis) and the disease arising from the bacteria that are disseminated beyond the gut lumen (associated with systemic infection) (Guiney and Fierer, 2011; Mastroeni *et al*., 2011; Abbott *et al*., 2012). In either of the infections, *S. enterica* subspecies will require multiple virulence genes for invasiveness of the pathogen into host cell and subsequently expression of different virulence phenotypes (Hensel, 2004). Several of these genes are located in pathogenicity islands (PAI) in the chromosome (D' Aoust and Maurer, 2007; Van Asten *et al*., 2005; Sirken, 2013), or mediated by mobile genetic elements such as; bacteriophages, plasmids, and transposons (Schmidt and Hensel, 2004; Fluit 2005). Collectively these virulence cassettes constitute packages for *Salmonella* pathogenesis. Virulence factors identified as major genes responsible for virulence in *Salmonella enterica* includes; *Salmonella* virulence plasmids (*spv*), toxins, fimbriae, flagella (van Asten and van Dijk, 2005) and *Salmonella* invasion genes (Swati *et al*., 2015).

Pathogenicity islands are present in both gram positive and gram negative bacteria and are linked to human, animal and plant pathogens, though they have been detected in non-pathogenic bacteria as well (Lloyd *et al*., 2007). A typical PAI is a unique DNA region found in the genome of pathogenic bacteria but absent from non-pathogenic bacterium of the same species (Schmidt and Hensel, 2004). Pathogenecity Island is usually large and unstable components of the chromosome (Lee, 1996; Ritter *et al*., 1995) and contains about 10-200 kb clusters of genes associated with virulence, survival, extra-intestinal and fitness of bacteria (Schmidt and Hensel, 2004; Siriken, 2013).

2.11.1 The role of chromosomal (*inv*A) virulence genes in *Salmonella* pathogenesis

Currently, there are at least 23 defined genomic islands on *Salmonella* pan-genome (Fookes *et al*., 2011; Hayward *et al*., 2013) referred to as SPIs (Elder *et al*., 2016) and range from SPI-1 to SPI-23 for *Salmonella.* In the chromosome of *Salmonella enterica* only five SPI-1 to SPI-5 are considered major SPIs, while *Salmonella bongori* harbours only 4 of them (SPI-1, SPI-3 to SPI-5) without SPI-2 (Porwollik *et al*., 2002) (Figure 2-6).

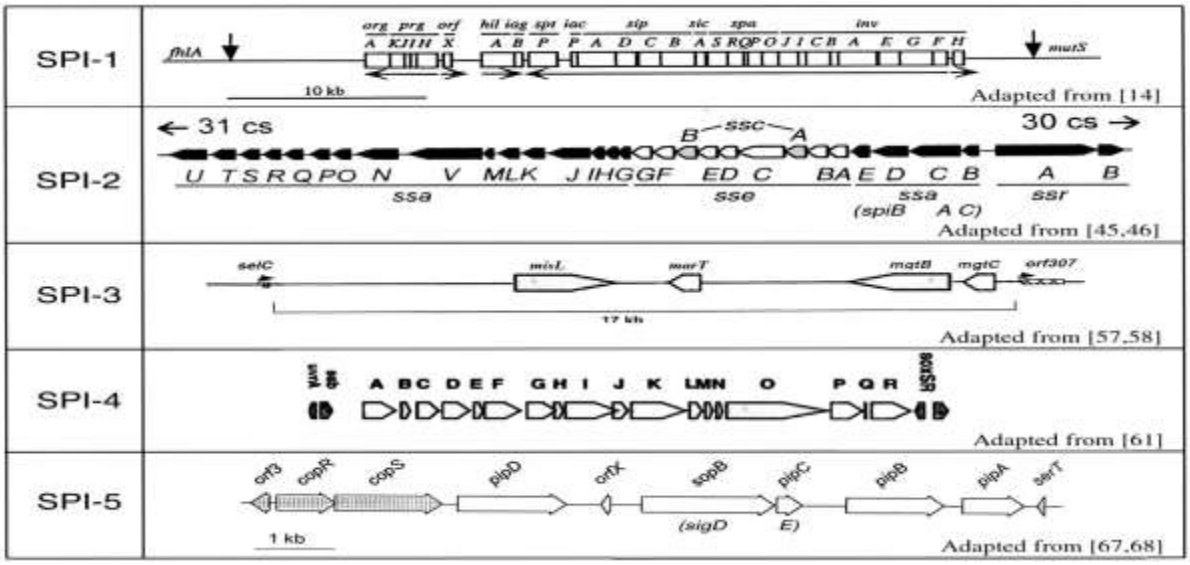


Figure 2-6: Schematic representation of SPI 1-5. (Marcus *et al.*, 2000).

However, most attention has been paid to SPI-1 and SPI-2 (Elder *et al*., 2016), which are critical for invasion of nonphagocytic cells in the intestinal tract, and for survival in macrophages and systemic spread respectively (Hansen-Wester and Hensel, 2001; Giacomodonato *et al*., 2007).

The SPI-1 and SPI-2 each encode a T3SS for virulence proteins that have a defined role during the intestinal and systemic phases of salmonellosis (Giacomodonato *et al*., 2007). These virulence proteins manipulate host signal transduction pathways and cellular processes to the advantage of the pathogens (Ramos-Morales, 2012), and their expression is activated by a transcription factor referred to as *hil*A, also encoded on SPI-1 (Murray and Lee, 2000).

The T3SS is complex assembly that consists of more than 20 genes for its activity (Schmidt and Hensel, 2004). Several Gram-negative bacterial pathogens causing diseases in animals as well as in plants utilize the T3SS to translocate bacterial proteins across three membranes into the cytoplasm of the host organism (Lilic *et al*., 2010). The SPI is located within a 40 to 50-kb segment of chromosomal DNA (Amavisit *et al.,* 2003; D' Aoust and Maurer, 2007) and are responsible for confering specific virulence traits (Marcus *et al*., 2000). Some SPIs are conserved across the *Salmonella* genus, while others are specific only to certain serotypes (Siriken, 2013). For instance SPI-1 has been detected in all *Salmonella* species (*S. enterica* subspecies and *S. bongori*) (Ochman and Groisman, 1996), and could have been passed on to all lineage ancestral to *Salmonella* serotypes (Li *et al.,* 1995).

Pathogenicity of NTS in human and animal infection is propelled by several factors which are beyond the scope of the present study. The virulence genes associated with the intestinal infection are located on SPI-1 and SPI-2 (Siriken, 2013). The SPI-1 encodes a first T3SS (Murray and Lee, 2000) which expresses several invasion genes such as *inv*A, *inv*H, *inv*E, *spa*M and *spa*N (Boyd *et al*., 1997). Other notable protein effectors of TTSS encoded by SPI-1 virulence factor includes surface presentation of antigens protein P (*spa*P), *spa*Q, *spa*R, cell invasion protein C (*sip*C), *sip*D, chaperone protein (*sic*A), transcriptional regulator A (*hil*A), *hil*C, *hil*D, invasion protein B (*inv*B), *inv*C, *inv*F and *inv*J genes responsible for invasion into epithelium and non-phagocytic cells (Li et *al*., 1995; Boyd *et al*., 1997; Wisner *et al*., 2012; Dietsche *et al.,* 2016). Majority of these genes are found within a 40 kb region of the chromosome (Mills *et al*., 1995).

A study by Fierer and Guiney, (2001), revealed that mutation in the region of SPI-1 secretory apparatus could bring about a reduded virulence if oral models of infections are used but retains

the virulence properties when administered systemically. However, the release of invasion protein A gene could be regulated by environmental stress condition within the intestinal tract (Galan *et al.*, 1989), such as pH, osmolarity, oxygen tension, bile, Mg^{2+} and short chain fatty acids (Bajaj *et al.*, 1996; Altier, 2005). *Salmonella* invasion *inv*A gene is found in all the *Salmonella* serotypes (Lostroh and Lee, 2001), and located on the *Salmonella* pathogenicity island (SPI-1) within the *inv* locus (Galan *et al*., 1992). It is an important virulence factor that plays a major role in invasion of the cells (Qiumei *et al*., 2012). The *inv*A gene is the first gene of an operon consisting of at least two additional invasion genes, involved in the earliest steps of the invasion of intestinal epithelium cells by the facultative intracellular pathogen *Salmonella* spp (Galan *et al*., 1992).

The specific functions of the rest of genes are beyond the scope of this study. These genes are involved in the early stages of the pathogenic cycle of the facultative *S. enterica* pathogen to adherence and which facilitates *Salmonella* pathogens to invade the cells of the intestinal epithelium (Galan *et al*., 1992; Collazo and Galan, 1997; Papezova *et al*., 2007).

On the other hand, SPI-2 encodes a second type T3SS in *S. enterica* subspecies which enables the bacterium to survive and proliferate in different cells but in particular macrophages (Murray and Lee, 2000; Hanisch *et al*., 2010). The discovery of SPIs genetic elements, underscores the distinction between serotypes of *S. enterica* sub species and *S. bongori* in the sense that the former was found to possess SPI-2 which is ancestral to the former and could have facilated the separation from *S. bongori* prior to diversification into seven groups namely; I, II, IIIa, IIIb, IV, VI and VII (Ochman and Groisman, 1996). The gene loss and gene gain in the genomes of bacteria are the propelling forces for genome evolution (Pallen and Wren, 2007). *Salmonella* pathogenicity island-2 is a major contributor of virulence factor which leads to the systemic spread of the bacteria and diseases in hosts (Ochman *et al*., 1996; Hensel, 2000; Imami *et al.*, 2013). Some described SPI-2 encoded effector *sse*F/G protein which are translocated by intracellular *Salmonella* and are required for the aggregation of endosomal compartments along microtubule and also to induce the formation of massive bundles of microtubules (Kuhle *et al.*, 2004), while *ssc*B also known to assist the subsequent secretion and translocation of their substrates (Shipan and Daoguo, 2004).

The acquisition of both SPI-1 and SPI-2 by *Salmonella enterica* species plays a pivotal role in the invasiveness of *Salmonella* as an intracellular pathogen (Ochman and Groisman, 1996). SPI-3 harbours *mgtC,* a *Salmonella*–specific gene that is required for intra-cellular survival (Blanc-Potard *et al*., 1999; Fierer and Guiney, 2001). *Salmonella* pathogenicity island-4 (SPI-4), encodes a type 1 secretion system (T1SS) and a giant nonfimbrial adhesin (protein called *Sii*E), which is involved in the adhesion of *Salmonella* Typhimurium serotype to polarized epithelial cells (Gerlach *et al*., 2007) and also the protein contributes to *Salmonella* colonization of the bovine intestines (Kiss *et al*., 2007). While SPI-5 is important in the virulence of pathogenic *Salmonella* (Cao *et al*., 2014), these SPI's are acquired from other bacteria within a population during evolution (Van Asten *et al*., 2005). The remaining SPIs are poorly defined and their contribution to *Salmonella* pathogenesis is still not clear (Elder *et al.,* 2016), and their contribution in the pathogenesis of *Salmonella* infections is beyond the scope of our study.

2.11.2 The role of *Salmonella* plasmid virulence genes in pathogenicity

A few *Salmonella* serotypes belonging to subspecies *enterica* carry specific large VPs (Rotger and Casadesus, 1999) and are homologous in nature (Montenegro *et al*., 1991). Virulence plasmids are mobile genetic materials which are transferred to new hosts across species boundaries (Skippington and Ragan, 2011). Virulence plasmids are the most described group of plasmids which contain *spv* genes associated with severe systemic infection by non-NTS serotypes of subspecies I (Guiney *et al*., 1995). Therefore, *spv* genes are indicative of being virulence determinants that are capable of spreading infection from small intestine into mesenterial lymph nodes, liver and spleen (Biljana *et al.,* 2010). It is understood, that the role of mobile virulence determinants is significant in the sense that they are the major elements that contribute to the evolution of bacterial pathogens, because their incorporation can, in a single step, transform normally benign organism into a pathogen (Blanc-Potard *et al*., 1999). For instance bacteraemia infection due NTS serotypes is associated with strains that harbour *spv* genes (Guiney and Fierer, 2011). In perspective, it is indicative that all *Salmonella enterica* subspecies serotypes are capable of causing bacteraemia if only they could have the possession of *spv* genes.

Virulence plasmids within the *Salmonella* genus are confined to few serotypes which can be grouped into two classes based on their epidemiology; one group is composed of serotypes such as *S.* Dublin (cattle), *S.* Gallinarum-Pullorum (poultry), *S.* Cholerasuis (pig) and *S.* Abortusovis (sheep) (D' Aoust and Maurer, 2007) which are considered host-adapted in domestic animals (Baumler *et al*., 1997; Rotger and Casadesus, 1999; Forshell and Wierup, 2006). These host adapted serotypes are highly associated with bacteremia condition (Woods *et al.*, 2008; Tennant *et al*., 2010). The second group consists of broad-host range or non-host-adapted serotypes (*S.* Typhimurium and *S.* Enteritidis) (Guiney *et al*., 1995). Non host adapted or usually referred to as generalists serotypes includes the invNTS strains that possess different invasive virulence properties (Jones *et al*., 2008a; Jones *et al*., 2008b) such as *S.* Typhimurium and *S.* Enteritidis which have been associated with invasive salmonellosis in human (Morpeth *et al*., 2009; Tennant *et al*., 2010; Reddy *et al*., 2010; Muthumbi *et al*., 2015; Mahon and Field, 2016). Interestingly VPs are not found in typhoid strains (Guiney and Fierer, 2011). The VPs are also carriers of other genes such as those responsible for plasmid replication and conjugation (Feng *et al*., 2012).

Depending on the variant of the *Salmonella* serotype, VPs are able to code for several key virulence-associated genes such as *rck* (resistance to complement killing), *pef* (plasmid encoded fimbriae), *srg*A (SdiA-regulated gene, putative disulphide bond oxidoreductase) or *mig*-5 (macrophage-inducible gene coding for putative carbonic anhydrase) (Rychlik *et al*., 2006). Others may include the conjugal transfer gene *tra*T (a surface exclusion effector for plasmid transfer as well as responsible for serum resistance), and the enigmatic *rsk* loci, required for serum resistance or could play a role in other stages of the infection process (Chu, *et al.*, 2001). Virulence plasmids are in the range of about 50-100 Kb in size and are commonly found in NTS serotypes such as *S.* Choleraesuis (50 kb), *S.* Enteritidis (60 kb), *S.* Dublin (80 kb), *S.* Gallinarum/Pullorum (90 kb) and *S.* Typhimurium (94 kb) (Haneda *et al*., 2001). All VPs contain a highly conserved 7.8 kb region called *spv* across the serotypes required for bacterial multiplication in the reticuloendothelial system (Guiney *et al*., 1995; Rotger and Casadesus, 1999).

The NTS serotypes in possession of *spv* genes are associated with severe systemic infection (Guiney *et al*., 1995; Heithoff *et al.*, 2008; Nhidza *et al.*, 2012; Feng *et al*., 2012). Dowman and

Meynell, (1970) were first to recognize the *spv* locus in bacterium, but it had taken 10 years before its role in virulence was established (Jones *et al.*, 1982). However, plasmid-encoded *spv* genes can also be found in the common broad-host-range serotypes such as *S.* Enteritidis, but only a variable proportion of isolates are considered to be carries of these virulence plasmids (Libby *et al*., 1997). Other serotypes reported to harbour *spv* genes include; *S.* Paratyphi C, *S.* Rostock, *S.* Newport and *S.* Moscow (Chu *et al*., 1999). *Salmonella* virulence plasmid genes have been detected in other *Enterobacterceae* such as; *Escherichia coli*, *Yersinia* spp., *Shigella* spp suggesting they are capable of conferring virulence phenotype (Rotger and Casadesus, 1999).

The *spv* locus encodes five genes operon called *spv*RABCD genes involved in intra-macrophage survival of *Salmonella* (Rychlik *et al.*, 2006) and are responsible for triggering systemic disease (Guiney *et al*., 1994). The three genes required for the virulence, are the positive transcriptional regulator spvR and two structural genes *spvB* and *spvC* (Guiney and Fierer, 2011; Derakhshandeh *et al*., 2013). The operon is positively regulated by the transcriptional regulatory protein *spv*R which also requires to be triggered by an alternative sigma factor RpoS for optimized expression (Fang *et al*., 1992). Conversely, lack of the *spv*R gene in *Salmonella* serotypes is an indication that the bacteria could be avirulent strains (Guiney *et al*., 1995). The *spv*A gene has been detected in *Salmonella* strains belonging to serogroup 4 (serogroups B), a group that includes the most common serovars of clinical significance, such as serovar *S.* Typhimurium and *S.* Heidelberg, which also often exhibits multi-drug resistance phenotype (MDR) (Gebreyes *et al*., 2009).

The role of *spv*A is to promote the macrophage phase avoiding destruction by neutrophils (Mrtchyan *et al*., 2016), and but also the carriage of *spv*A together with the MDR phenotype enables the bacterium to be of clinical importance when compared with MDR strains without the spv*A* gene or part of the *spv* operon (Wondwossen *et al*., 2009). The function of *spv*B encodes an enzyme ADP-ribosylates which modifies actin cytotoxin required for systemic survival (Heithoff *et al*., 2008), and it is responsible for the *spv* virulence phenotype (Lesnick *et al*., 2001), while *spv*C has phosphothreonine lyase activity which inhibits mitogen-activated protein kinases signaling (MAPKs) (Guiney and Fierer, 2011). In the same manner lack of *spv*C gene may be

indicative of partial virulence of *Salmonella* strains (Guiney *et al*., 1995). The function of *spv*D is not well defined. *Salmonella* serotypes in possession of *spv* genes are reported to have the propensity of causing serious *Salmonella* infections in patients receiving immunosuppressive therapy such as HIV patients with low levels of CD4 T cells, a strong indication that they cannot fight the disease (Guiney *et al.*, 1995; Guiney and Fierer, 2011).

Salmonella infection is usually initiated in the intestinal tract, there after it is disseminated through the lympatic system and blood streams where it is localized within the macrophages of the spleen and liver to produce cytotoxicity (Richter *et al*., 1997). *Salmonella* infection is initiated in the intestinal tract, there after it is disseminated through the lympatic system and blood streams where it is localized within the macrophages of the spleen and liver to produce cytotoxicity (Richter *et al*., 1997).

2.12 Transmission of *Salmonella*

In nature, the dissemination of the *Salmonella* pathogens is interrelated with various factors, such as hosts reservoirs, food, environment, vectors, utensils and equipment, production and processing procedures (Tessari *et al*., 2012) and will also depend on the serotype of *Salmonella*.

2.12.1 Transmission of salmonellosis in animals

Animals are the primary source of this pathogen, and animals of food origin are the main transmission route to humans (Ferrari *et al*., 2019). In the natural, both carnivorism and shedding of pathogens through faecal contamination of the environment are common routes of spreading *Salmonella* pathogens among wild birds (Tizard, 2004). Various transmission routes are shown in Figure 2-2. Since, *Salmonella* pathogens frequently colonize the intestinal tract of food producing animals such as chicken; this could result in the contamination of the avian reproductive tract and subsequently the egg during the laying (Humphrey *et al*., 1989). Animal feeds have been linked to source of salmonellosis (Jones *et al*., 1982). Therefore, understanding the global epidemiology of *Salmonella* serotypes is important to controlling and monitoring this bacterium (Ferrari *et al*., 2019).

2.12.1.1 Poultry typhoid salmonellosis

Fowl typhoid and Pullorum disease (PD) are caused by *S.* Gallinarum and *S.* Pullorum serotypes respectively and are avian host specific serotypes which in this case are rarely associated with zoonotic potential like *S.* Typhimurium and *S.* Enteritidis (Rajagopal and Mini, 2013). Both horizontal and vertical transmission is important in the epidemiology of FT and Pullorum diseases (CFSPH, 2009). Typhoid pathogens may be spread among animals by several routes which could include; mechanical transmission through contaminated material on bodies of animals such as rats, insects and other wild birds, dogs, and cats. Rats are known to be the worst culprits in the spread of diseases from one house to another house (Umali *et al*., 2012). Equally, birds are thought to be an important factor in spreading the disease from farm to farm as wild birds feed on carrion, and sometimes drink from range fountains (Andres-Barranco *et al*., 2014). Flies have been linked to the spread of the disease (Wang *et al*., 2011). Thus it is evident that improper disposal of dead chickens may be one of the greatest factors in spreading and perpetuating fowl typhoid in a community. If dead birds, are thrown out without burial or are not picked up from the range they become accessible to buzzards, rats, dogs, cats, raccoons, skunks, and other free-moving carnivorous animals which may carry the disease to disease-free areas.

2.12.1.2 Animal non-typhoid salmonellosis

Non-typhoid *Salmonella* strains are predominantly carried in animal reservoirs (Layton and Galyov 2007; Shu *et al*., 2015). However, *Salmonella* can be transmitted among animals; often through ingestion of *Salmonella* contaminated water or feed (Pender, 2003). Animals such as rodents carry *Salmonella* pathogens asymptomatically in their intestines or gall bladder and may shed continuously or intermittently the microorganism in the faeces from where it can spread and contaminate the soil, water and/ or feed (Pui *et al*., 2011a) for weeks or even months (Pui *et al*., 2011a). The resultant effect is environmental contamination which has become another potential source of *Salmonella* infection (CFSPH, 2013) because *Salmonella* can survive in the environment for a long time (Pui *et al*., 2011a). A vector such as rats, flies, and birds pick up the pathogens from the contaminated environment and can shed them in their faeces for weeks and longer periods (Pui *et al*., 2011a). In sheep, transmission of *S.* Abortusovis within a flock is through dissemination of bacteria from infected to susceptible sheep through the oral,

conjunctival, or respiratory routes, while venereal infections appear to be of minor importance (Uzzau *et al*., 2001).

Transmission in birds occurs through horizontal and vertical routes (Gantois *et al*., 2009) such as the contamination of the vitelline membrane, albumen and possibly the yolk of eggs resulting into infection of the chicks and via the respiratory and oral routes respectively (CFSPH, 2009). According to the National Veterinary Institute (NVI) of (Sweden)/World Health Organization (WHO), *Salmonella* in poultry production could be spread by the trade of live parent stock and grandparent flocks within and between countries (NVI/WHO, 1993). Similarly, trading in contaminated feed products has also greatly contributed to the spread of *Salmonella* (Forshell *et al*., 2006; Wales *et al*., 2009).

2.12.2 Transmission of salmonellosis in humans

Salmonella pathogens are maintained in animal reservoirs, and infection in humans follows ingestion of contaminated animal products or ingestion of farm produce contaminated with infected animal faeces, or in close contact with infected animals (Hendriksen *et al*., 2004; Soltan *et al*., 2014). The different transmission routes of *Salmonella* infections to man are elaborated in figure 2-7.

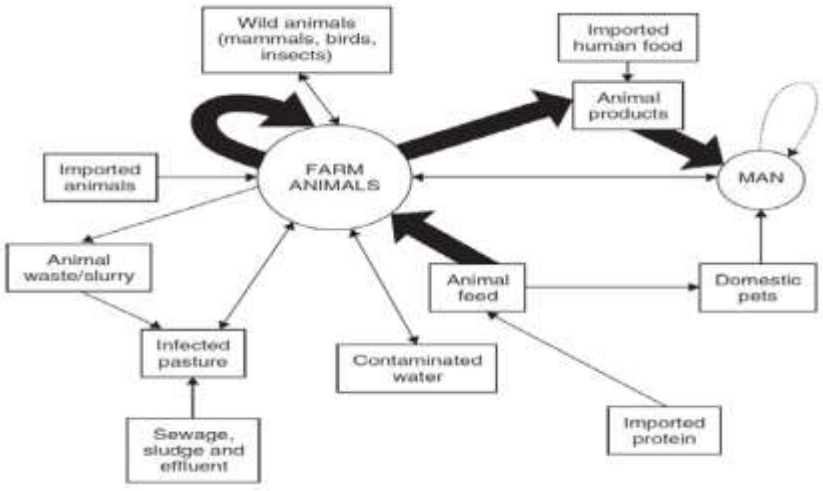


Figure 2-7: Transmission and maintenance of *Salmonella* spp to man through food, water, pasture, animals, and man making it a Public Health Significant microorganism (Kemal, 2014).

A variety of foods especially of animal origin such as poultry, eggs and dairy products are the most common vehicles of salmonellosis (Pui *et al*., 2011b; De knegt *et al*., 2014) and able to dessiminate *Salmonella* pathogens through shedding in faeces. Chickens are the main culprits, since they do not often show signs of the disease, as a result the entire flock can become colonized unnoticed and tend to shed the pathogens through their faeces over a period of time (Clavijo *et al*., 2006; Penha *et al*., 2009), and consequently the environment become contaminated with *Salmonella* pathogens. Although, human foodborne salmonellosis is largely attibibuted to animal reservoirs (De knegt *et al*., 2014), transmission of the disease will largely depend on the animal host susceptible to the type of *Salmonella* serotype (Baumler and Fang, 2013).

2.12.2.1 Human typhoid salmonellosis

The infection is spread mainly from person-to-person via the fecal-oral route and has no significant animal reservoirs (Baron, 1996; Giannella, 1996; CDC, 2010b; WHO, 2011). Human typhoid infection is usually spread through contaminated food or water (Kabwama *et al*., 2017). Other potential vehicles include; sea food (Heinitz *et al*., 2000; Bujjamma *et al*., 2015) and houseflies (Cirillo, 2006; Wang *et al*., 2011). Transmision of typhoid infection is dependent on several factors such as infective dose and immune-status of the host (WHO, 2011).

2.12.2.2 Human non-typhoid salmonellosis

Non-typhoid *Salmonella* subspecies *enterica*, are the most important causative pathogens of FBD, followed by other pathogens that cause localized infections such as parasitic helminthes (Havelaar *et al.*, 2015; Ferrari *et al*., 2019). Currently, the predominant route of infection is through foodborne transmission from animals to humans and is common among the highly-industrialized countries (Morpeth *et al*., 2009). The disease can also be spread from person to person through faecal contamination (Baron, 1996). A large number of food-producing animals are principal reservoirs of many pathogenic *Salmonella* serotypes in regard to human infections (Humphrey, 2000; Magwedere *et al*., 2015). Cattle, swine and poultry are the prime sources of *Salmonella* infections (Olsen *et al*., 2001; Mitchell *et al*., 2003; Shu-Kee *et al*., 2015). Other animals such as song birds (Hernandez, *et al*., 2012), and pet hedgehog (Anderson *et al*., 2017), have been linked to zoonotic transmission.

Human NTS infection is common among the highly-industrialized countries (Layton and Galyov, 2007; Morpeth *et al*., 2009). In 1968, foodborne outbreak of gastroenteritis was reported in New Jersey, USA, involving a family dinner Thanksgiving Day at which turkey was served to 18 persons with a 95 percent attack rate of illness and 14 percent case-fatality rate (Janeway *et al*., 1971). On the other hand, eggs have been prominently implicated as source of *S.* Enteritidis, a cause of human illness for several decades (Gantois *et al*., 2009). However, professional practice can also be a source of *Salmonella* infection, for instance, an outbreak of *S.* Typhimurium associated with a veterinary clinic was reported involving one cat, two veterinary technicians, four persons associated with clinic patients, and a nurse not linked to the clinic (Cherry *et al*., 2004). In 2012, an outbreak of *S.* Infantis was reported involving a total of 49 individuals, 47 from 20 states in USA and 2 from Canada, and the pathogen was traced to a Foods production facility (AVMF, 2012).

Several other studies also have indicated that ingestion of farm plant produce such as raw vegetables (lettuce) contaminated with animal waste can bring about salmonellosis infection (Freitas *et al*., 2010; Abakpa *et al*., 2015). Other potential routes for transmission of NTS pathogens includes the shedding of bacteria in animal excreta which can be transmitted directly to humans through contact (Zambrano *et al*., 2014), or by indirect route through contamination of the environment (Rabinowitz *et al*., 2009; Freitas *et al.*, 2010; Tadesse *et al.*, 2016).

Transmissions of *Salmonella* to humans through contaminated food processing plants are of great importance (Jones, 2011). Equally, fomites and mechanical vectors (insects and flies) are also known to spread *Salmonella* (Pui *et al*., 2011a). In the period 2006 to 2011, foods of animal origin were linked to most of the foodborne diseases in USA (CDC, 2013) (Table 2-7).

Table 2-7: Number of *Salmonella* foodborne outbreaks in the US linked to animals from 2006 to 2011 (CDC, 2013)

Food animal	**No. of illnesses**
Poultry	2580
Eggs	2938
Pork	1043
Beef	1138
Dairy	682
Wild game	48
Total	**8429**

2.12.2.3 Human invasive non-typhoid salmonellosis

At the time of emergence of invNTS diseases, it was considered not to have been through zoonotic route, but perceived to have been primarily due to environmental contamination related to human to human transmission as well as foodborne diseases (Kariuki *et al*., 2006). Other studies have indicated that invNTS strains were non-human serotypes but have adapted as human pathogens and can be transmitted from person to person (Giannella, 1996; Okoro *et al*., 2012). *Salmonella* Typhimurium ST313 serotype is one such strain that has become human adapted (Okoro *et al*., 2012; Feasey *et al*., 2012) and there is also evidence that suggests other serotypes such as *S.* Enteritidis and *S.* Isangi strains have become human adapted nosocomial infections (Raghavendra *et al*., 2009; Suleyman and Tibbe, 2016; Suleyman *et al.,* 2016). In Kenya, *Streptococcus pneumoniae,* nontyphoidal salmonella species, *Haemophilus influenzae,* and *E. coli* accounted for more than 70 percent of isolates cultured from blood collected from children admitted to a rural District Hospital (Berkley *et al*., 2005).

Salmonella serotypes; Typhimurium and Enteritidis are the most common serotypes within the food chain and therefore the large number of invasive salmonellosis linked to these serotypes is most probable due to exposure rather than increased virulence of the pathogens (Langridge *et al*., 2012). Another invasive NTS serotypes includes, *S.* Choleraesuis that occasionally infects humans causing extra-intestinal focal (sepsis) infections (Chiu *et al*., 1999). In Michigan, USA, of 6797 cases of salmonellosis, 347 (5.1 percent) were characterized as invasive, and among the dominant serotypes associated with invasive salmonellosis were *S.* Heidelberg, *S.* Typhimurium, and *S.* Enteritidis (Arshad *et al*., 2008).

However, anthroponotic route is strongly suggested to be responsible for transmission of invNTS organisms from person to person (Kariuki *et al.,* 2006) within or outside the healthcare set ups but associated with food-borne diseases in sub-Saharan Africa (Morpeth *et al.,* 2009). That being said, a closely related group of strains of serotype Typhimurium have been associated with HIV infections in sub-Saharan Africa, and may have become adapted to humans, therefore expelling the suggestion that not all isolates identified as "Typhimurium" should be regarded as a single group of strain (Langridge *et al*., 2012). In this particular case, a distinct genotype *var* Typhimurium, ST313, has emerged as a new pathogenic strain in sub-Saharan Africa which is adapted to cause invasive infections in humans (Feasey *et al*., 2012). On the other hand, only about 5 percent of NTS become invasive to cause extra-intestinal disease or bacteraemia or focal systemic infections in humans in developed countries (Suez *et al*., 2013).

2.13 Diagnosis of *Salmonella*

Salmonella serovars is a normal microbiota of the gastro intestinal tract (GIT) of animals, birds, humans and occasionally insects. These organisms are excreted in faeces from which they may be transmitted by insects and other animals to contaminate the environment and/ or water sources. Humans and animals that consume contaminated water may shed the bacteria through faecal matter continuing of the cycle of contamination (Andino and Hanning, 2015). As such, its detection in faeces does not always entail disease. However, in the event of *Salmonella* infection in humans and animals, pathogens can be diagnosed through laboratory testing of a stool, blood, urine or tissue samples for the presence of *Salmonella*. Currently there are three principal methods in use for identification and detection of *Salmonella* from clinical or environmental samples namely; bacteriological, serological and molecular.

2.13.1 Bacteriological method

This is a traditional method of cultivation and identification of *Salmonella.* It takes 3-5 days to obtain the results. This requires the isolation of *Salmonella* from routine samples such as blood from humans and animals with septicaemia, or faecal samples from humans or animals with enteritis and diarrhoea. Other samples could include suspected food ingredients, food products of animal animal origin. Similarly environmental samples such as water, and faeces from wild rodents and birds may also be tested.

2.13.1.1 Culture of *Salmonella*

There are several methods for isolation of *Salmonella* from different samples and for different serovars (OIE, 2012). However, isolation of *Salmonella* pathogens from samples such as; gastrointestinal flora, foods and environment requires use of buffered peptone water (BPW) or Lactose Broth (LB) incubated at 37^{o}C for 18 hours to resuscitate the stressed *Salmonella* pathogens before subculture into enrichment broths (Liao and Fett, 2005). The pre-enrichment culture in BPW is then subcultured into enrichment broth such as tetrathionate broth (Bohls and Mattman, 1950), Rappaport-Vassiliadis Enrichment (RVE) Broth (Peterz *et al.*, 1989; USPC, 2007), or Rappaport-Vassiliadis Soy Peptone (RVSP) Broth (Peterz *et al*., 1989) incubated for further 24 hours at 41.5^{o}C. This is followed by subculture on at least two highly selective media which contains selective agents to inhibit other bacteria. These media include xylose lysine desoxycholate (XLD) agar (MacFaddin, 1985), and desoxycholate citrate (DC) agar (Nye *et al*., 2002) which contains sodium deoxycholate as an inhibiting agent and incubated at 37^{o}C for 24 hours. Similarly, intermediate-selective media such as *Salmonella-Shigella* (SS) agar (CLSI, 2004), MacConkey agar, Brilliant green agar (MacFaddin, 1985), Rambach agar (Ruiz *et al*., 1995), Brilliant agar modified, or Mannitol lysine crystal violet brilliant green (MLCB) agar (Kodaka *et al*., 2000) may be used for the isolation of *Salmonella* pathogens. Suspect colonies showing typical characteristics reaction on selective agar are cultured onto non-selective media for use in confirmatory tests.

2.13.1.2 Biochemical identification of *Salmonella* – Gold Standard

Suspect *Salmonella* colonies on highly selective medium are confirmed by testing for the enzyme activities on the following mediums: triple sugar iron (TSI) for hydrogen sulphide (H_2S) production, decarboxylation of lysine and ornithithine; and non-hydrolysis of urea (D'Aoust *et al*., 2007; Abulreesh, 2012). Other tests specific for identification of *Salmonella* genus are Simmons citrate as a source carbon required for growth, and non-utilization of sucrose and salicin (Barrow and Feltham, 1993; Baron *et al*., 1995). The summary of additional typical biochemical tests required for *Salmonella* are indicated in Table 2-8.

Table 2-8: Additional typical biochemical reaction of *Salmonella enterica* subspecies I

Biochemical activity		Enzyme activity (VetBact, 2017)	
Test	Reaction	Enzyme	Reaction
Methyl red test	+	Oxidase	-
Voges-Proskauer	-	Hydrogen sulphide (H_2S)	+
		Ornithithine decarboxylase	+
		Lysine decarboxylase	+
		Tryptophanase Indole	-
		Urease production	_-

Further, biochemical confirmation of *Salmonella* can be achieved by using commercial identification systems such as API 20E test kit used to identify *Salmonella* isolates to the genus level (Yang *et al*., 2013), Micro-ID System (Edberg *et al*., 1979) and also automated systems such as Vitek (bioMerieux Vitek, Hazelwood, Mo.) and MicroScan (Beckman Coulter) (Odumeru *et al*., 1999). In addition, other tests such as Microbact-12E/24E bacterial identification systems (Ling *et al*., 1988; Keelan *et al*., 1988) and MALDI-TOF (Murray, 2012) have been developed that can identify members of the *Enterobacteriaceae* including *Salmonella* pathogens.

2.13.2 Serological and serotyping confirmation of *Salmonella* genus

Salmonella can also be confirmed by antigenic analysis of somatic (O) and flagella (H) antigens using polyvalent and specific antisera (Baron, 1996). There are more than 150 different somatic O and H antigens (Perch *et al*., 2003). The O surface antigen determines the group of the *Salmonella* isolate to which it belongs and the H antigen characterizes the serotype (Agasan *et al*., 2002). Polyvalent "O" antiserum is used for preliminary screening of *Salmonella* subspecies. *Salmonella* can be grouped into over 50 serogroups based on the lipopolysaccharides (LPS) structure often referred to as somatic antigen "O" present which forms the most outer layer around the NTS bacterium (Sabbagh *et al*., 2010). The test is simple, and requires to use polyvalent somatic O" antiserum on a slide agglutination test (Yang *et al*., 2013).

The somatic antigen is highly variable among the Gram-negative bacteria and this is the basis for partially distinguishing *Salmonella* strains into "O" antigen serogroups (Liu *et al*., 2010;

Kalynych *et al*., 2014). The most common "O" antigen serogroups are A, B, C1, C2, D and E (Brenner *et al.,* 2000; Fierer and Guiney, 2001; Shu *et al*., 2015). *Salmonella* strains under these serogroups are responsible for about 99 percent of *Salmonella* infections in humans and warm-blooded animals (Fierer and Guiney, 2001).

In contrast, a small number of serotypes such as *S.* Typhi, *S.* Paratyphi C and *S*. Dublin have a capsuler outer layer around them for the purpose of protection against unfavourable environment. The capsular antigens are heat-sensitive and are carbohydrate in nature (Hu and Kopecko, 2003; Yousef and Carlstrom, 2003). Bacterium in possession of capsular antigens cannot be sero-grouped (Fierer and Guiney, 2001) because the LPS are over masked by the layer of "Vi" antigen. On the other hand, *Salmonella* mutants that lack this protective layer are referred to as "Rough mutants" (Fierer and Guiney, 2001). The LOUIS test is another serological test that uses rapid enzymes to screen for lactose non-fermenting colonies such as *Salmonella* and *Shigella* (Wilson, 2004).

Most of the common "O" groups were originally designated by letters and are still commonly used. However, each "O" has now been designated by a number e.g *Salmonella* Enteritidis belongs to group D1 or referred to as O: 9. Majority of the NTS human infections are caused by *Salmonella* strains belonging to serogroups; A, B, C, D and E (Giannella, 1996; Fierer and Guiney, 2001). To carry out the serogrouping testing requires numerous O-grouping antisera along with the control antigens. Rarely does the cross-reaction between somatic O antigens of *Salmonella* and other genera of *Enterobacteriaceae* occur (Pui *et al*., 2011a).

Salmonella has a unique status among its counterpart's enteric bacteria in that it can express two different H antigens namely Phase 1 and Phase 2 (CDC, 2011). In reality, only one H antigen is expressed at any given time by a single bacterial cell and this is referred to as "Monophasic" isolates. According to Pui *et al*. (2011a), H antigens are heat-labile proteins associated with the peritrichous flagella. Either the tube agglutination or slide agglutination tests can be performed to determine the H antigen (Ryan *et al.,* 2017).

Differentiation of *Salmonella* strains into serotypes is based on its antigenic mosaic, a combination of "O", "H", and "Vi" antigens (Giannella, 1996; Popoff *et al.*, 2004; Grimont and Weill, 2007). Serotyping of *Salmonella* can be achieved according to the Kauffman-White-Le Minor scheme, developed as reference method for discrimination of *Salmonella* varieties (Dera-Tomaszewska, 2012). Serotyping of *Salmonella* subspecies is in itself important in the understanding of the natural history of bacterial diseases in particular those emanating from foodborne salmonellosis outbreaks (Furukawa *et al*., 2017). However, there is no doubt that serotyping is expensive because some notable challenges include the difficulty to standardize as well as to arrange a large number of known positive control strains (Mayer, 1988). On the other hand, virulence capsular has limited application because only a few serotypes (*S.* Typhi, *S.* Paratyphi C, and *S.* Dublin) have the ability to express Vi antigen in relation to virulence (Grimont *et al*., 2000).

Naming of *Salmonella* serotypes can be based on place of isolation, name of the patient or the tribe of the patient. About 60 percent of all described 2600 *Salmonella* serotypes are represented by *S. enterica* sub spp. I (Guibourdenche *et al*., 2010). On comparison serological tests are much sensitive and specific than the commercial biochemical identification systems and also reduce turn-around time. According to the recent report on the classification, *Salmonella* comprises 2610 serotypes under two species; of these 2587 (99.1 percent) belong to *S. enterica* species, while 23 (0.9 percent) serotypes are grouped under *S. bongori* (Achtman *et al*., 2012).

Other tests for detection and confirmation of *Salmonella* strains include several rapid serological assays. For instance an enzyme immunoassay (EIA) has been developed to detect and measure serum antibodies to *Salmonella* using commercialy available lipopolysaccharides (LPSs) with a combination *S.* Typhimurium and *S.* Enteritidis as antigens (Isomaki *et al*., 1989). This method is only valuable for the detection of *Salmonella* antibodies such as IgM, IgG and IgA during post-infectious complications, in particular when it is no longer possible to isolate the pathogens.

On the other, enzyme-linked immunosorbent assay (ELISA) asssays have also been widely applied based on lipopolysaccharides and Vi antigens to detect *Salmonella* infections in human (Appassakij *et al*., 1987; Araj and Daschugh, 1987), and poultry (Barrow, 1994; Brigmon *et al*.,

1995; Oliveira *et al*., 2006). In poultry, the method is primarily used to detect specific IgG produced in infections (Hassan *et al*., 1990).

Use of bacteriophages, in the ELISA based assays has been an alternative method to specific antibodies (Galikowska *et al*., 2011). The method by Thomason, (1971), detected *Samonella* from environmental, food, and feed samples using a microcolony fluorescent antibody (FA) procedure. Another method is the use of counterimmunoelectrophoresis serological for diagnosis of typhoid fever (Tsang and Chau, 1981). *Salmonella* Typhi infection can be diagnosed from a patient by using Widal agglutination assay which relies on patients' antibodies level against *Salmonella* O: 9, lipopolysaccharides (LPS), flagella (H) and capsular (Vi) antigens (Somily *et al*., 2011). Whereas, the work by Lanata *et al*., (1990), used an indirect hemagglutination test to screen a high-risk population for chronic *S.* Typhi carriers using a highly purified Vi antigen. The study by Krysinski and Heimsch, (1977), used an indirect enzyme-labelled antibody technique (ELAT), to detect *Salmonella* Typhimurium in food samples. A suspension array was used for *Salmonella* serodiagnosis in pigs (van der Wal *et al*., 2018). On the other, a pre-concentrated immunochromatographic assay has been developed to detect *S. enteritidis* based on the unique optical and magnetic properties of magnetic nanoparticles (MNPs) (Duan *et al*., 2017).

2.13.3 Molecular confirmation of *Salmonella* strains

Several molecular methods have been developed to ease the identification of *Salmonella* genus. The most widely used DNA based technique is the polymerase chain reaction (PCR) (Tennant *et al.,* 2010; Faik *et al*., 2014; Ghoddusi *et al*., 2015; Tehmina *et al*., 2015; Batista *et al*., 2016; Furukawa *et al*., 2017; El-Sebay *et al*., 2017). The assay is used to detect and confirm *Salmonella* pathogens by amplification of invasion protein A (*inv*A) gene product in various biological samples (Hossein *et al*., 2015; Aziz, 2016; Anejo-Okopi *et al*., 2016). The *inv*A gene is found in almost every *Salmonella* (Chiu and Ou, 1996), and is now widely used in PCR assays as an international standard for confirmation of genus *Salmonella* (Ohud *et al*., 2012; Kshirsagar *et al*., 2014; Smith *et al*., 2015b; Anukampa and Sivakumar, 2017; Karatug *et al*., 2018).

On the other hand, Biologists have employed a variety of PCR related methods to target 16S rRNA gene as a marker to discriminate between *Salmonella* and non-*Salmonella* species as

opposed to conventional biochemical methods (Chien-ku *et al*., 2004; Harmsen and Karch, 2004; Drancourt *et al*., 2004; Souii *et al*., 2012; El Allaoui *et al*., 2013; El-Tayeb *et al*., 2017). Most prokaryotes including bacteria have the three ribosomal RNA gene called the 5S, 16S and 23S rRNA. According to Janda and Abbott, (2007), 16S rRNA gene is the most common housekeeping (core) genetic marker possessed by bacteria, suggesting that the 16S rRNA gene contains a highly conserved as well as hypervariable nucleic acid sequences. The 16S rRNA gene is large enough (1500 bp) to provide informatics that concerns genome divergence over evolution time, particularly in the hypervariable region (Patel, 2001).

The 16S rRNA molecular method is preferred for identification of isolates that do not conform to any of the commonly used biochemical profiles in commercial identification systems (Janda and Abbott, 2007). Similarly, 16S rRNA of the pathogen can be targeted in an event that culture poses a biosafety hazard, or when suitable fresh tissue is not available or difficult to culture (Cai *et al*., 2014). This makes PCR-16S rRNA assay more precise and reliable method for identifying the *Salmonella* pathogens (Clarridge, 2004). All being considered, hypervariable loci part of the 16S rRNA gene is the most preferred target to facilitate the identification of genus *Salmonella* species (Janda and Abbott, 2007). While 16S rRNA gene can be highly useful in the case of bacterial classification, this may not be the case in the determination of phylogenetic species level because the tool has poor discriminatory power for some genera (Bosshard *et al.*, 2006; Mignard and Flandrois, 2006).

There are other PCR molecular methods used for the diagnosis of bacterial infections from pathologic and clinical samples in animals including multiplex PCR (Fitzgerald *et al*., 2007; Pui *et al*., 2011b), using multilocus variable-number tandem repeat analysis (MLVA) (Kabayashi *et al.*, 2014), quantitative PCR (Piknova *et al*., 2005), real time PCR (Malorny *et al*., 2004), PCR-ribotyping (Esteban *et al*., 1993), PCR-enterobacterial repetive intergenic consensus (PCR-ERIC) methods (Saxena *et al*., 2003; Bhowmick *et al*., 2012), and microarray analysis PCR (Rasooly and Herold, 2008). The PCR methods have higher sensitivity, specificity, cost effective and can be used to identify different bacteria including *Salmonella* serotypes from clinical samples (Zahraei *et al*., 2006).

Recently, a method was developed for detection of *S.* Typhi in clinical blood samples, which uses a magnetic nanoparticle–based enrichment to target bacterial cells, followed by cell lysis and loop-mediated isothermal amplification (LAMP) of nucleic acids for signal augmentation along with concurrent measurement of signal via an *in-situ* optical detection system (Kaur *et al*., 2018). A PCR-based enzyme-linked immunosorbent assay (PCR-ELISA) has been developed for identification of *Salmonella enterica* somatic groups C1 and E1 (Gillespie *et al*., 2003)

2.14 C**haracterization of *Salmonella* strains**

Salmonella strains from different sources causing diseases in humans and animals can be characterized to determine their pathogenicity by using multiple typing methods including, biotyping, antimicrobial resistance profiles and genotyping.

2.14.1 Characterization of *Salmonella* by biotyping

Several biochemical methods have been employed to characterize *Salmonella* strains (Olsen *et al*., 1992; Yang *et al*., 2013). Biochemical systems of any form have one disadvantage, of being unable to identify *Salmonella* isolates into serogroups or serotypes. However, biotyping is used in epidemiological investigation to identify different strains within a serotype (Duguid *et al*., 1975; Barker and Old, 1989). Various tests (Table 2-9) show the ability of *Salmonella* strains to utilize various carbohydrates as a source of energy; mannitol, maltose, arabinose, xylose, trehalose, melibiose, and glucose to produce acid and gas; whereas lactose, sucrose, salicin, raffinose and cellobiose are not fermented (Grieg and Holt, 1984; Paccagnela, 2005). Exceptions have been observed that *Salmonella enterica* sub species IV (*houtane*), could utilize salicin, while the majority of *Salmonella enterica* sub species IIIb (*diarizonae*) do utilize lactose (Table 2-10). However, a typical biochemical reaction of *S. enterica* subspecies *enterica* (I) strain is glucose, dulcitol, mannitol, arabinose, maltose, trehalose, melibiose, mucate and L(+)-tartrate positive; while lactose, sucrose, raffinose, cellobiose and salicin are negative as shown in Table 2-9 and Table 2-10.

Table 2-9: Differential biochemical characterization of the *Salmonella* subspecies (Krieg and Holt, 1984)

Substrate	*Salmonella* I	*Salmonella* II	*Salmonella* III	*Salmonella* IV
Glucose	+	+	+	+
Sucrose	-	-	-	-
D-Mannitol	+	+	+	+
Inositol	d	[-]	-	D
Arabinose	+	+	+	+
Raffinose	-	-	-	-
Maltose	+	+	+	+
Xylose	+	+	+	+
Trehalose	+	+	+	+
Cellobiose	-	-	-	[-]
Melibiose	+	+	+	+

Key: + = 90 – 100% positive; [+] = 76 – 89% positive; d = 26 – 75% positive; [-] = 11 – 25% positive; - = 0 – 10% positive.

Table 2-10: Biochemical characteristics of *Salmonella* genus (Paccagnela, 2005)

Characteristics	*Salmonella* genus						
	Salmonella enterica sub species						*S. bongori*.
	I *enterica*	II *salamae*	IIIa *arizonae*	IIIb *diarizonae*	IV *houtanae*	VI *indica*	V *bongori*
Dulcitol	+	+	-	-	-	D	+
ONPG	-	-	+	+	-	D	+
Malonate	-	+	+	+	+	+	-
Gelatinase	-	+	+	+	+	+	-
Sorbitol	+	+	+	+	+	-	+
Growth in KCN	-	-	-	-	+	-	+
L(+)-tartrate (a)	+	-	-	-	-	-	-
Galacturonate	-	+	-	-	+	+	+
Glutamyltransferase	+ (*)	+	-	+	+	+	+
Glucoronidase	d	d	-	+	-	d	-
Mucate	+	+	+	+	-	+	+
Salicin	-	-	-	-	+	-	-
Lactose	-	-	- (75%)	+ (75%)	-	d	-

(*) = *typhimurium*; + = 90% or more positive; - = 90% or more negative; d = 26 – 75% positive

2.14.2 Characterization of *Salmonella* using antimicrobial resistance profiles

The characterization of *Salmonella* pathogen virulence factors, including antimicrobial resistance profiles for typhoidal and NTS strains are of significance for assuring the safeguard of health in humans and animals under the "one health" concept. Antimicrobial resistance is constantly changing and the treatment of salmonellosis caused by MDR strains raises serious public health concerns (Krzyzanowski *et al*., 2014). Therefore, in order to counter the effect of the rapidly emerging MDR *Salmonella* isolates, it is imperative that antimicrobial resistance profile is performed through disck diffusion method (Bauer *et al*., 1966; Bauser *et al*., 1966). This method allows the establishment of the efficacy of an antibiotic by determining the diameter of the inhibition zone surrounding a respective antibiotic disc.

2.14.3 Molecular characterization of *Salmonella* species

Different molecular typing tools have been developed to determine the genetic variability of *Salmonella* strains that were previously confirmed by biochemical and serological tests (Wattiau *et al*., 2011). For instance PCR-restriction fragment length polymorphism (PCR-RFLP) was used to differentiate two biotypes namely *S.* Gallinarum and *S.* Pullorum (Cheraghchi *et al.*, 2014), while multiplex-PCR is used for discrimination of *Salmonella enterica* subsp *enterica* serovars (Paiao *et al.,* 2013; Shimizu *et al.*, 2014). Though multiplex PCR can discriminate strains into serotypes with ease, it is reported to have its own limitations of failing to resolve the differences between variants of the same serotype due to antigenic alterations or subtle point mutation in H1/H2 antigens responsible for loss of flagella expression (Hong *et al*., 2008). The real-time PCR has been used to identify *Salmonella enterica* subspecies *arizonae* and *S. enterica* subspecies *diarizonae* (Hopkins *et al*., 2009); single-Gene Target PCR for diagnosis of *S.* Typhi specific genes (Yuan *et al*., 2016). The work by Baratto *et al*. (2012), identified and differentiated *Salmonella* serotypes, based on 5 primers by random amplified polymorphic DNA (RAPD), enterobacterial repetitive intergenic consensus (ERIC-PCR), amplification of rDNA internal spacer analysis (RISA), and amplified ribosomal DNA restriction analysis (ARDRA) of 16S-23S rRNA internal spacer region (ISR) cleaved with *Arthrobacter luteus* I (*Alu* I) and *Haemophillus hymolyticus* I (*Hha* I) restriction enzymes. Some of these protocols utilize serotype-specific primers to identify *Salmonella enterica* species (Skiskaroly *et al*., 2017).

Several other methods have been developed for rapid identification of *Salmonella* serotypes based on pulsed-field gel electrophoresis (PFGE), useful for fingerprinting of strains in outbreak situations (Liebana *et al*., 2001b; Wen *et al*., 2012). Restriction fragment length polymorphism (RFLP) (Paiva *et al*., 2009a; Cheraghchi *et al*., 2014), ribotyping (Esteban *et al*., 1993) and multiple genetic fingerprinting (Liebana *et al*., 2001a), may be used to differentiate *Salmonella* species. Common food-borne such as *Salmonella*, *Escherichia coli*, *Shigella* and *Listeria monocytogens* can be identified by a platform that uses electrospray ionization mass spectroscopy (ESI-MS) to detect the base composition of short PCR amplicons (Pierce *et al*., 2012).

2.14.4 Molecular characterization of *Salmonella* virulence genes

There are several virulence genes found in different bacteria species but below we describe only two genes; *inv*A and *spv*ABC.

2.14.4.1 Prevalence of invasion protein A gene

*Salmonella inv*A gene contains a unique molecular marker for the identification of *Salmonella* to genus level (Rahn *et al.,* 1992; Malorny *et al*., 2003). The gene is highly conserved in almost all *Salmonella* strains and thereby used as a target gene in PCR methods (Hur, *et al*., 2011; Shanmugasamy *et al*., 2011).

Chromosomal *inv*A gene is a member of a set of several inner membrane proteins that form T3SS and plays a key role in the functioning of the T3SS (Lilic *et al.,* 2010). Therefore the presence of *inv*A gene on the SPI-1 (Zou *et al*., 2011), is suggestive of pathogenicity. In our literature review, distribution of *inv*A gene detected by PCR methods from various *Salmonella enterica* serotypes isolated from different sources in selected countries is shown in Table 2-11 and 2-12.

Table 2-11: Distribution of *inv*A gene in *Salmonella* strains isolated from different sources in selected countries in Africa and Asia

Region/Country	Host	No. of isolates	*Salmonella enterica* subspp.	% of *inv*A positive strains	Amplified fragment size (bp)	Reference
Africa						
Nigeria	Food samples	76	*Salmonella* strains	69.70	389	Smith *et al*., 2015
	Food samples	76	*Salmonella* strains	96.10	284	Smith *et al*., 2015
	Food samples	76	*Salmonella* strains	33.00	244	Smith *et al*., 2015
Egypt	Chicken meat	166	*Salmonella* strains	100.00	284	Abd-Elghany *et al*., 2015
Asia						
India	Pork	13	*S.* Enteritidis	100.00	284	Chaudhary *et al*., 2015
	Slaughter house	24	*S.* Typhimurium	100.00	284	Chaudhary *et al*., 2015
India	Poultry	2	*S.* Gallinarum	100.00	-	Susmita *et al*., 2017
Iran	Poultry	22	*S.*Typhimurium	100.00	-	Fazl *et al*., 2013
Iran	Clinical isolates	44	*S.* Enteritidis	100.00	243	Fardsanei *et al.*, 2018
Turkey	Chicken	34	*S. enterica.* subspp	94.12	521	Karatug *et al*., 2018

Table 2-12: Distribution of *inv*A gene in *Salmonella* strains isolated from different sources of selected countries in North America and South America

Region/Country	Host	No. of isolates	*Salmonella enterica* subspp.	% of *inv*A positive strains	Amplified fragment size (bp)	Reference
North America						
North Carolina	Human	425	*S.* Enteritidis	99.3	-	Zou *et al*., 2012
South America						
Brazil	Food	31	*S.* Enteritidis	100.00	284	Oliveira *et al*., 2003
Brazil	Broiler carcass	22	*S.* Enteritidis	100.00	284	Oliveira *et al*., 2003
Brazil	Poultry environment	21	*S. E*nteritidis	100.00	284	Oliveira *et al*., 2003
Brazil	Human	17	*S.* Enteritidis	100.00	284	Oliveira *et al*., 2003
Brazil	Pig	11	*S.* enteritidis	100.00	284	Oliveira *et al*., 2003
Brazil	Foodborne isolates	237	*S.* Enteritidis	100.0	-	Rowlands *et al*., 2014.

2.14.4.2 Prevalence of *Salmonella* plasmid virulence gene

The accessory virulence genes *spv*A, *spv*B and *spv*C are known to be associated with extra-intestinal infection (Guiney *et al*., 1995; Fluit, 2005; Rychlik *et al*., 2006). The *spv* genes are contained on virulence plasmids and suggestive of pathogenicity (Guiney *et al*., 1995). PCR methods have been described to detect these genes using uniplex pair of primers (Del Cerro *et al*., 2003; Huang *et al*, 2005; Swamy *et al*., 1996; Anukampa *et al*., 2017) respectively.

In our literature review, distribution of *spv*ABC genes detected by PCR methods from various *Salmonella enterica* serotypes isolated from different sources in selected countries is shown in Table 2-13 and 2-14.

Table 2-13: Distribution of *spv* genes from *Salmonella* serotypes isolated from various sources in selected countries of Africa and Asia

Region/ Country	Host	No. of strains	*Salmonella enterica* subspp.	Frequency of *spv genes* (%)			Reference
				A	B	C	
Africa							
Nigeria	Food samples	76	*Salmonella* spp.	0.0	0.0	-	Smith *et al*., 2015b
Egypt	Chicken meat	166		-	-	25.30	Abd-Elghany *et al.*, 2015
Asia							
Tehran	Food	34	*S.* Enteritidis	-	-	34.0	Fardsanei *et al*., 2017
India	Poultry	15	*S.* Enteritidis	-	46.6	93.3	Derakhshandeh *et al.*, 2013
India	Poultry	9	*S.* Typhimurium	-	22.2	77.7	Derakhshandeh *et al.*, 2013
India	Poultry	5	*S.* Infantis	-	20.0	20.0	Derakhshandeh *et al.*, 2013
India	Pork	13	*S.* Enteritidis	-	-	0.0	Chaudhary *et al*., 2015
India	Slaughter house (pig)	24	*S.* Typhimurium	-	-	0.0	Chaudhary *et al*., 2015
Iran	Poultry	68	*S.* Enteritidis	86.6	86.6	86.6	Kumarss *et al*., 2010
Iran	Bovine	13	*S.* Enteritidis	100.0	100.0	100.0	Kumarss *et al*., 2010
Iran	Human	20	*S.* Enteritidis	90.0	90.0	90.0	Kumarss *et al*., 2010
Iran	Clinical animal and human	64	*Salmonella* serotypes	65.6	-	-	Firouzi *et al*., 2014

Key: - = No test was conducted

Table 2-14: Distribution of *spv* genes from *Salmonella* serotypes isolated from various sources in selected countries of Europe and South America

Region/ Country	Host	No. of strains	*Salmonella enterica* subspp.	Frequency of *spv genes* (%)			Reference
				A	B	C	
Europe							
Poland	Raw milk	16	*S.* Enteritidis	100.0	56.25	62.50	Wiszniewska-Laszczych *et al*., 2018
South America							
Brazil	Various sources	102	*S.* Enteritidis	-	-	90.2	Oliveira *et al*., 2003

Key: - = No test was conducted

2.15 Sequencing of *Salmonella* strains

The most widely used DNA based technique is PCR, which utilizes genus specific primers targeting various genes (El-Sebay *et al*., 2017). Bacteria are generally identified by 16S rRNA sequencing, because the rRNA or rDNA are the most conserved (least variable) gene in all cells (Momen, 2016). These genes have been widely applied to determine taxonomy, phylogeny and to estimate rates of species divergence among bacteria (Momen, 2016). Moleculer typing methods are also used to determine the genetic variability of *Salmonella* isolates that were previously identified by biochemical tests. For instance, sequencing of *inv*A gene of *Salmonella* isolates has been used in previous studies to determine the genetic diversity of the gene (Suna *et al.*, 2010; Pal *et al*., 2017; El-Sebay *et al*., 2017; Kadry *et al*., 2019).

CHAPTER THREE

MATERIALS AND METHOD

3.1 Study areas

The study included seven districts of Southern Province (SP) (Mazabuka, Monze, Pemba, Choma, Kalomo, Zimba and Kazungula); three National Parks (Kafue National Park, Lochnivar National Park, and Lusaka Central Park); and Lusaka City (Figure 3-1).

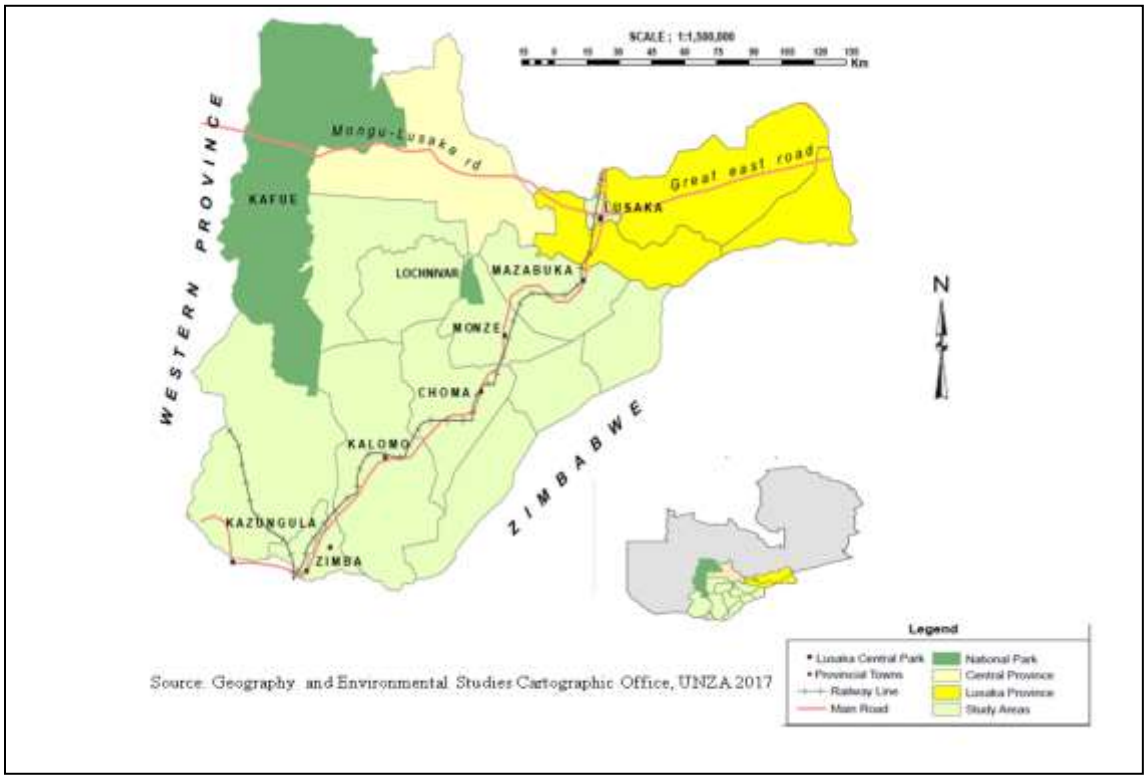


Figure 3-1: Map of KNP, LNP, Southern and Lusaka Provinces of Zambia (insert), the study

3.2 Study design

The study was cross-sectional in nature and therefore, the samples were drawn from various wildlife and domestic animals, and those that were submitted for routine diagnostic tests at the School of Veterinary Medicine, University of Zambia and an independent privately owned diagnostic Laboratory, Lusaka..

3.2.1 Sampling process

The samples were collected from June 2013 to December 2014 from wildlife and domestic animals as well as various locations. Sampling process and the collection of samples was based on the convenience of the availability of different animal species that are possible reservoirs of the pathogen.

3.2.1.1 Collection of faecal samples from cattle and horses

Freshly dropped faecal samples of about 5 grams each from apparent healthy cattle were conveniently and aseptically picked from the grazing pasture and cattle kraals and were placed in a 150 x 100 mm polythene self-adhesive bag. Samples that appeared to have normal faecal consistency of loose to thick state were picked up and kept in ice cooled box before transportation to the School of Veterinary Medicine, Department of Disease Control, University of Zambia, Lusaka.

3.2.1.2 Collection of faecal samples from free-ranging wildlife

Faecal samples were collected from free-range wildlife in three different areas namely Kafue National Park (KNP), Lochnivar National Park (LNP) and Lusaka Central Park (LCP). The faecal samples from seemingly freshly dropped were sampled. About two grams faeces of each animal species were conveniently and aseptically picked from the environment. Identification of the faecal samples was done by Game Scouts. All faecal samples included in the study were part of wider research activities investigating various diseases of wild animals. Faecal samples from KNP were provided by Zambia Carnivore Program in Conjunction with Montana State University, United States of America (USA). The collected samples were shipped to the School of Veterinary Medicine, Department of Disease Control, University of Zambia, Lusaka, Zambia. The faecal samples from LNP were from the rectum of apparently healthy hunter harvested baboons (*Papio anubis*). These samples were pre-determined by what had been authorized by then Zambia Wildlife Authority (ZAWA) for research quota and were kept at -20^{o}C before delivery to the Laboratory. In the case of samples from LCP, two grams of freshly voided faecal droppings were aseptically collected from the environment, and were delivered in a none-cooled container to the Microbiology Laboratory within 8 hours of collection.

3.2.1.3 Bacterial strains from Veterinary Diagnostic Institutions

Between 2012 and 2014, suspected *Salmonella* bacterial strains isolated from diseased samples of domestic animal and wildlife submitted for routine diagnostic tests at the School of Veterinary Medicine, Department of Disease Control, University of Zambia and the independently privately owned Veterinary diagnostic Laboratory, Lusaka, were included in the study. These *Salmonella* strains were identified by bacteriological methods according to standard methods of culture and isolation of *Salmonella*. All suspected *Salmonella* strains isolated from diseased animal samples were kept in 10% glycerol (v/v) in peptone water broth at -20^{o}C for further identification.

3.3 Culture of *Salmonella*

Samples were processed at the School of Veterinary Medicine, Department of Disease Control, University of Zambia, using conventional culture techniques recommended by International Organizations for Standardization (ISO-6579, 2002), and those recommended by the Global *Salmonella* Surveillance (GSS) and National Health Services for Wales (NHS) (Hendriksen, 2003). Isolation involved three stages; firstly, 1 gm or equivalent of faecal sample was pre-enriched with 9 ml of buffered peptone water (BPW) (HIMEDIA Laboratory Pvt, Ltd, M614, Mombai, India) and incubated for 24 hour at 37°C. Secondly, a portion (1.0 ml) of the pre-enriched broth culture was transferred to 10 ml selective enrichment Rappaport Vassiliadis Soyabean Meal Broth (RVSM) (Himedia M1448) and incubated at 42°C for 24 hours. Thirdly, from the RVSM broth, a loop full of the inoculum was transferred and streaked onto Xylose Lysine Deoxycholate (XLD (HIMEDIA, M031)) agar and Brilliant Green Agar (BGA (HIMEDIA, M016)) plates then incubated at 37°C for 24 hours according to the International Organization for Standardization [ISO] protocol (ISO 6579, 2002). Suspect colonies from each sample were sub-cultured onto selective and non-selective agars to ensure that possible contaminants were absent. For a definitive *Salmonella* confirmation, the isolates were subjected to various serological and biochemical tests. Pure cultures and bacterial isolates were kept frozen at -80^{o}C in 10% (v/v) glycerol peptone water broth. *Escherichia coli* (ATCC 25922) was used as quality control organism.

3.4 Phenotypic characterization of *Salmonella* strains

Different phenotypic systems were used to determine diversity among *Salmonella* strains from domestic animals and wildlife. The methods used included serogrouping, serotyping, fermentative grouping, and resistotyping according to Biljana *et al.,* (2009).

3.4.1 Identification of *Salmonella* strains by biochemical tests

Suspected isolates were inoculated onto composite media including triple sugar iron agar (TSI), urea, lysine iron agar (LIA), voges-proskauer (VP), methyl red (MR) (HIMEDIA), and indole tests (HIMEDIA) (Barrow and Feltham, 1993). Test cultures were inoculated in tubes covered with loose caps to maintain aerobic condition and incubated at 37^{o}C for 18 hour. Suspect colonies on composite media which produced alkaline (red) slant and acid (yellow) butt, with or without production of H_2S (Blackening of agar) in TSI and also produced an alkaline (purple) reaction in LIA butt and urease negative were retained as potential *Salmonella* isolates (Ewing, 1986). All suspected *Salmonella* colonies were further identified using Analytical Profile Index (API) 20 E systems (Biomerieux SA, Marcy-1 Etoile France). Metabolic properties, such as the ability to utlilize lactose, citrate, and urea, are considered as plasmid-encoded characteristics (Mayer, 1988).

3.4.2 Serological characterization of *Salmonella* strains

The biochemically identified strains from wildlife and domestic animals were screened for *Salmonella* strains using different serological methods.

3.4.2.1 Serogrouping of *Salmonella* strains

Salmonella strains were characterized into serogroups based on the presence of distinctive "O" antigenic factors. They were first examined with O polyvalent antiserum for A-S group antigen by latex agglutination test, followed by specific monovalent antiserum (group O2-O9) or (group B, C1, C2, D, E, and G) (MAST ASSURES, SA) according to manufacturer's protocol for identification of surface antigens and their differentiation into serogroups. Briefly, a drop of the appropriate antiserum was placed onto a clean microscopic slide. A single colony of overnight culture on nutrient agar was picked and emulsified in the antiserum drop to obtain a thoroughly

mixed suspension. The slide was gently rocked forward and backwards/side wards for 1 minute. Agglutination or clumping within 1-10 seconds was considered a positive reaction.

3.4.2.2 Serotyping of *Salmonella* Strains

Salmonella strains were differentiated into serotypes by serotyping analysis (Lin-Hui and Cheng-Hsun, 2007). In this study all the strains of *Salmonella* were referred to Deltamune (Pty) Laboratory, a South Africa, SANAS Accredited Veterinary Laboratory, for confirmation and serotyping. Characterization was done using the method described in the Microbiological Manual and serotyping (10.2:1995 CCFRA) based on White-Kauffman-Le Minor Scheme (WHO Collaborating Centre). The scheme discriminated serotypes on the basis of their somatic (O), flagella (H) and capsular (Vi) antigens present on the surface of *Salmonella* (Brenner *et al*., 2000).

3.4.3 Fermentative grouping of *Salmonella* strains

Biotyping has been widely used to characterize the biochemical heterogeneity among *Salmonella* strains from different sources (Ewing, 1986) and classify strains into sero-fermentative groups. In the present study *Salmonella* strains from different sources were tested for sugar utilization using phenol red broth (HIMEDIA) prepared according to the instructions of the manufacturer. The broth contained one of the following sugars or alcohol; dissacharides (maltose, trehalose, melibiose and cellobiose), hexoses (glucose, mannose and galactose), pentoses (arabinose, xylose, and rabitol), polyhydric alcohol (inositol, adonitol, mannitol, dulcitol and salicin) and trisaccharides (D-(+)-raffinose). The media was filtered through a 0.22μm pores and dispensed in 3 ml aliquots in capped sterile tubes. To a tube of phenol red broth, containing either one percent (w/v) of sugar or alcohol were inoculated with a single strain of *Salmonella* using a sterile straight wire. The broth tubes were incubated at 30±0.5^{o}C for 48 hours and the results were recorded positive if the production of acid condition induced a change in the phenol red indicator, from pink to yellow.

3.4.4 Antimicrobial resistance profiles of *Salmonella* strains

The antimicrobial resistance of *Salmonella* strains from wildlife and domestic animals was tested to 17 antimicrobials agents belonging to eight antibiotic classes (Beta-lactams, macrolides,

fluoroquinolones, tetracyclines, aminoglycosides, sulphanomides, nitrofurantoin and phenicols) based on different mechanism of action (Neu, 1992). These antibiotics constituted four modes of action; inhibition of cell wall synthesis, inhibition of protein synthesis, inhibition of DNA synthesis and inhibition of dihydropepteroate synthases needed for folic acid synthesis. Mueller-Hinton agar was used following Kirby-Bauer diffusion method. Each pure bacterial culture was emulsified in 5 ml sterile physiological saline (0.85 percent NaCl) to make a bacterial suspension compared with a barium chloride standard (0.5 McFarland). Prior to bacterial inoculation, the surface of Mueller-Hinton agar plates were dried at 37^{o}C. This was followed by dipping a sterile swab into the bacterial suspension, removed excess fluid by pressing the swab against the wall of the test tube and applied the swab contents evenly on to the surface of the agar. All antibiotic discs were products of Himedia Laboratories (Mumbai, India). Test culture plates were incubated at 37^{o}C for 24 hour and followed by determination of susceptibility or resistance profiles according to the breakpoints as described in the guidelines of the Clinical and Laboratory Standards Institute (CLSI, 2015). *Escherichia coli* (ATCC 25922) was used as a control organism. The antimicrobial agents and concentration tested are shown in Table 3-1.

Table 3-1: Antibiotic agents tested and their concentrations

Serial No.	Antibiotic agent	Abbreviation	Concentration µg/ml
1	Ampicillin	AMP	10
2	Azithromycin	AZM	15
3	Ceftazidime	CAZ	30
4	Cefotaxime	CTX	30
5	Ceftriaxone	CTR	30
6	Chloramphenicol	C	30
7	Ciprofloxacin	CIP	5
8	Co-Trimozoxale	COT	25
9	Erythromicin	E	15
10	Gentamicin	GEN	10
11	Levofloxacin	LE	5
12	Nalidixic Acid	NA	30
13	Nitrofurantoin	NIT	300
14	Norfloxacin	NOR	10
15	Penicillin G	PG	10 units
16	Streptomycin	S	300
17	Tetracycline	TE	30

3.4.4.1 Determination of multiple antibiotic resistances indexing (MARI)

The multiple antibiotic resistance index was calculated as follows; a/b, where 'a' represents the number of antibiotics to which the particular isolate was resistant and 'b' the number of antibiotics to which the isolate was exposed. MARI values >0.2 are considered significant indicating that the strains could have originated from sources where antibiotics are often used (Tambekar and Datil, 2006; Jayaraman *et al.*, 2012). The MARI value <0.2 suggests the strains originate from animal sources which are less frequent exposed to antibiotics or never at all (Krumperman, 1983). On the other hand, the MAR index of commonly used antimicrobial agents is calculated as follows; No. of resistant isolates to antibiotic (a) divide by No. of antibiotics used (b) x No. of isolates (c) (a ÷ b x c). The higher the index suggests that the antimicrobial agent is commonly used in that particular area.

3.4.4.2 Detection of extended spectrum beta-lactamases (ESBLs) in *Salmonella* strains

Detection of extended spectrum cephalosporinase producing isolates among the *Salmonella* strains isolated from wildlife and domestic animal sources was determined according to the method described by Rayamajhi *et al.* (2008). A pure culture of each *Salmonella* strain was streaked on freshly prepared MacConkey agar (HIMedium) containing 2 mg/L of cefotaxime (Sigma-Aldrich, Munich, Germany) for the purpose of screening the presence of ESBL producing bacteria. Culture plates were incubated at 37^{o}C for overnight and were examined for the presence of growth.

3.5 Molecular characterization of *Salmonella* strains

All biochemically identified *Salmonella* strains (n = 34) from wildlife and domestic animals, were confirmed by PCR for detection of 16S rRNA, *Salmonella* invasion protein A gene (*inv*A), and accessory virulence genes called *Salmonella* plasmid virulence A, B, and C (*spv*ABC) genes.

3.5.1 DNA preparation

Salmonella strains isolated from wildlife and domestic animals were kept at -80^{o}C for storage. These strains were sub-cultured on nutrient agar (HIMEDIA) and incubated at 37°C for 24 hours for the purpose of DNA extraction.

3.5.2 Extraction of bacterial DNA

The bacterial genome was extracted from each *Salmonella* strain using DNeasy Blood and Tissue Kit (Qiagen) as recommended by the manufacturer of the product. Briefly, a single colony was picked and suspended in 200µl of distilled water in 1.5 ml micro centrifuge tube and then vortexed. About 200 µl of DNAzol was added to the bacterial suspension and incubated at room temperature for 5 days. Thereafter, 800µl of DNAzol was added and incubated for 30 minutes at RT. The bacterial suspension was centrifuged at 10,000 *xg* for 10 minutes at 4^{o}C. About 750µl of the supernatant was transferred into a new 1.5 ml tube, while the rest was discarded. About 375µl of absolute Ethanol (100 percent) was added to the supernatant to make a total volume of 1,125µl and was mixed thoroughly by vortex. The mixture was centrifuged at 12,000 *xg* for 10 minutes at 4^{o}C. The supernatant was aspirated and the pellet was washed with 800µl of 75 percent Ethanol followed by centrifugation at 12,000 *xg* for 10 minutes at 4^{o}C. Further washing was done by adding 500µl of 75 percent ethanol and centrifuged at 12,000 *x*g for another 10 minutes followed by aspiration of the supernatant. The pellet in the micro-centrifuge tube was left to dry completely at room temperature for 10 minutes. To the pellet, 200µl of 8Mm NaOH was added and incubated for half a day at 4^{o}C, followed by centrifugation at 12,000 *xg* for 10 minutes at 4^{o}C. About 200µl of the supernatant was transferred into a new 1.5 ml tube with the addition of 2µl of HEPES. The DNA extract was kept at -30^{o}C till further use.

3.5.3 Selection of DNA primers

The forward and reverse primers at specific molecular size were used for detection of the presence of a stable genetic region of 16S rRNA and *inv*A genes of *Salmonella* following the protocol described by Weisburg *et al*. (1991), and *inv*A (Rahn *et al.,* 1992). Similarly, the choice of primers for virulence plasmids was based on the reported works; *spv*A (Del Cerro *et al*., 2003), *spv*B (Huang *et al*., 2005), and *spv*C (Swamy *et al*., 1996). The working concentration of each primer was 10.0µM and the oligonucleotide sequences are shown in Table 3-2.

Table 3-2: Choice of uniplex set of Oligonucleotides (primers) used for PCR amplicons

Primer	Target oligonucleotide sequence	Length	Reference
*inv*A F	5'-GTGAAATTATCGCCACGTTCGGGCAA-3'	284 bp	Rahn *et al*., 1992
*inv*A R	5' – TCATCGCACCGTCAAAGGGAACC-3'		
Fd1 (16S rRNA)	5′- AGAGTTTGATCCTGGCTCAG-3′	1400 bp	Weisburg *et al*., 1991
Rp2 (16S rRNA)	5′-ACGGCTACCTTGTTACGACTT-3′.		
*spv*A F	5' – GTCAGACCCGTAAACAGT-3'	604 bp	Del Cerro *et al*., 2003
spv A R	5' – GCACGCAGAGTACCCGCA-3'		
spv B F	5' – ATGTTGATACTAAATGGTTTTTCA-3'	1,776 bp	Huang *et al*, 2005
spv B R	5' – CTATGAGTTGAGTACCCTCATGTT-3'		
spv C F	5'-CGGAAATACCATCTACAAATA-3'	669 bp	Swamy *et al*., 1996
spv C R	5' – CCCAAACCCATACTTACTTACTCT-3'		

3.5.4 Polymerase Chain Reaction Detection of the 16S rRNA gene

The PCR amplification of the products were conducted in an individual reaction tube with a total volume of 25µl master mix containing; nuclease free water (18.375 µl), dNTPs (2.0 µl), 10X Buffer (2.5 µl), forward fd1 primer (0.5 µl), reverse rp2 (0.5 µl) (Thermo Fisher Scientific, Tokyo, Japan), Ex taq polymerase (0.125 µl) and template DNA (1.0 µl). The master mix was prepared on ice and the mixture was quickly transferred to a thermocycler preheated to the denaturation temperature (95°C). PCR conditions used are as shown in Table 3-3.

3.5.5 Polymerase Chain Reaction Detection of the invasion protein A (*inv*A) gene

PCR amplification of the product was performed using the primers as described by Rahn *et al*. (1992). The PCR master mix contained; nuclease free water (6.75 µl), 10X buffer (1.0 µl), primer *inv*A forward (0.2 µl, *inv*A reverse (0.2 µl) (Inqaba Biotechnical Industries (Pty) Limited), dNTPs (0.8 µl), Ex taq polymarase (0.05 µl) and DNA template (1.0 µl). The total volume of reaction was 10.0µl. The Master Mix was prepared on ice and the mixture was quickly transferred to a thermocycler preheated to the denaturation temperature (95°C). PCR conditions are shown in Table 3-3.

Table 3-3: PCR amplification conditions for 16S rRNA, *inv*A and *spv* genes

Stages	Conditions of application for each gene				
	16SrRNA	*inv*A	*spv*A	*spv*B	*spv*C
Initial denaturation (95^oC)	for 1 min				
Denaturation (95^oC) during thermocycling	20 sec	30 sec	30 sec	30 sec	30 sec
Annealing (30 sec)	55^oC	64^oC	60^oC	55^oC	43^oC
Extension (72^oC)	1 min 15 sec	30 sec	30 sec	2 min	1 min
Final extension ($72\ ^oC$)	for 5 min				
Cycling number	35				
Holding temp	4^oC				
Reference	Yongjin *et al.*, 2014	Rahn *et al.*, 1992	Del Cerro *et al*., 2003	Huang *et al*., 2005	Swamy *et al*., 1996

3.5.6 Detection of pathogenic *Salmonella* strains by detecting *Salmonella* plasmid virulence A, B, and C (*spv*ABC) genes using PCR method

The reactions were performed in PCR tubes with a final volume of 10µl. PCR master mix contained; nuclease-free water (6.75 µl), 10x buffer (1.0 µl), dNTPs (0.8 µl) (Takara, Japan), Ex taq polymarase HS (0.05 µl) (Takara, Japan), "forward" and "reverse" primer sets (0.2 µl) (provided by Inqaba Biotechnical Industries (Pty) Limited) and DNA (1.0 µl each). All reagents contained in the master mix were prepared on ice and were quickly transferred to a thermocycler preheated to the denaturation temperature (95^oC). The working concentrations of each primer were 10.0µM and the oligonucleotide sequences are shown in Table 3-2. PCR conditions are shown in Table 3-4.

Table 3-4: PCR amplification conditions for plamid encoded accessory virulence genes

Serial No.	Stages	Cycle	Temperature			Duration		
			*spv*A	*Spv*B	*spv*C	*spv*A	*spv*B	*spv*C
1	Initial denaturation	1	95	95	95	1 min.	1 min.	1 min.
2	Denaturation	35	95	95	95	30 sec.	30 sec.	30 sec.
	Annealing		60	55	43	30 sec.	30 sec.	30 sec.
	Extension		72	72	72	30 sec.	2 min.	1 min.
3	Final extension	1	72	72	72	5 min.	5 min.	5 min.
	Holding Temp		4	4	4	infinity	infinity	infinity

3.5.7 Electrophoresis of amplified PCR products

The amplified PCR products; 16SrRNA (1400 bp), *inv*A (284 bp), *spv*A (604 bp), *spv*B (1,776 bp), *spv*C (669 bp) were separated by electrophoresis in 1.0 or 1.5 percent agarose gels in Tris-Acetate –EDTA (TAE) buffer for 25 minutes run at 100 volts before staining with ethidium bromide. Loading Quick 100–bp and 1000-bp DNA ladder (Yokobo, Japan) were used as molecular markers accordingly. The amplified DNA fragment was visualized by UV trans-illuminator (Benchtop 3UV-UVP, CA, USA). The amplified products were stored at 4^{o}C for further analysis. *Escherichia coli* ATCC 25922 was used as a negative control.

3.5.8 DNA purification of PCR 16S rRNA and *inv*A gene amplified products

The purification of PCR products (16SrRNA and *inv*A) was done using Wizard SV Gel and PCR PromegaClean-Up system (Promega) Kit" according to the manufacturer's instructions as explained below;

After electrophoresis, PCR amplicon products were cut from the gel and placed into 1.5ml micro-centrifuge tube (a). The weight of sliced gel (b) was calculated by subtracting weight of the empty micro-centrifuge tube (c) (i.e. a-c = b). To the weight of sliced gel (1.0 mg), 1.0 ml

Membrane Binding solution was added at 1:1 ratio, followed by vortex and then incubation at 50-60^{o}C until the gel completely dissolved.

A volume of 7.0 µl of PCR amplicon product was added to an equal volume of Membrane Binding solution in a 1.5ml micro-centrifuge tube. Then the mixture was applied to the SV Mini-column assembly and incubated for 1 minute at room temperature. The mixture was centrifuged at 16,000 *xg* for 1 minute to bind DNA and the flow through in the collection tube was discarded while the Mini-column was reinserted into the collection tube.

The Mini-column assembly was washed by adding 700 µl Membrane Washing Solution and again centrifuged for 1 minute at 16,000 *xg*. The flow through was discarded and the unit was re-inserted. Washing was repeated twice with 500µl Membrane Washing Solution by centrifugation for 5 minutes at 16,000*xg* and discarding the supernatant each time. A Mini-column assembly was carefully transferred to a clean 1.5ml micro-centrifuge tube. To the assembly, 50 µl of nuclease –free water was added and followed by incubation at RT for 1 minute before centrifugation at 16, 000 *xg* for 1 minute. The Mini-column was discarded and the DNA elute was stored at -30^{o}C for further PCR sequencing. The concentration of PCR product for *Salmonella inv*A gene and 16S rRNA were measured by using a NanoDrop ND-1000 UV-spectrophotometer (Thermo-Fisher Scientific Inc., USA) at a concentration of 4.00ng/ µl (200-500 bp), while 16S rRNA was at 30.00ng/ µl (1400 bp) respectively, and then followed by PCR sequencing reaction.

3.6 PCR sequencing of 16S rRNA and invasion protein A (*inv*A) genes of local *Salmonella* strains

The PCR amplified products of both 16S rRNA-1400 bp and invA-284 bp genes were obtained by using a set of specific primers fd1/rp2 and f*inv*A/r*inv*A (Yongjin *et al.*, 2014; Biljana *et al*., 2009). The total volume of the PCR reaction was 20 µl consisting of the following components; nuclease free water (13.5 µl), 5x buffer (3.5 µl) Applied Biosystem, Warrington UK), specific primer sets (1.0 µl)Inqaba Biotechnical Industries (Pty), Big dye terminator v3.1 (1.0 µl) Applied Biosystem, Warrington UK) and purified DNA (1.0 µl). Assembly of the components was performed on ice. The sequencing condition for thermal cycler Applied Biosystem AB-Veriti)

was programmed at 96^{o}C for 1 minute for initial denaturation, followed by 35 cycles consisting of 96^{o}C for 10 seconds, 50^{o}C for 5 seconds, with final extension at 60^{o}C for 4 minutes for 25 cycles.

3.6.1 Precipitation of PCR sequence products using Ethanol/EDTA/Sodium Acetate method.

To each PCR sequence reaction product, 2.0µl of 125mM EDTA and 2.0µl of 3M Sodium acetate was added, followed by 90µl 100 percent Ethanol and incubation at room temperature (RT) for 10 minutes. The mixture was centrifuged at 15,000 *xg* for 20 minutes at 4^{o}C. This was followed by removal of supernatant before adding 200µl 70 percent Ethanol to the pellet and left to stand without mixing. Mixture was centrifuged at 15,000 *xg* for 5 minutes at 4^{o}C and this was repeated twice. The pellet was covered in aluminum foil and dried in the vacuum dryer for 10 minutes. To the pellet, 20µl of formamide was added followed by vortex. The product was denatured at 95^{o}C for 2 minutes and stored at 4^{o}C.

3.6.2 Sequencing and construction of the phylogenetic tree

A volume of 20µl formamide of each sample was transferred to each well of the sequencing plate. The amplified 16S rRNA (1400 bp) and invA-284 bp PCR products were resolved by a Genetic Analyzer 3130 sequencer (Applied Biosystem-Hitachi, Model No. 627-0040) following the manufacturer's instructions. The sequencer ran on four capillaries, thus loading four samples per run and each run was 2 hr. The sequences were collected, assembled and edited using ATGC software. Genetix was used to BLAST the assembled sequences of 16S rRNA and *inv*A genes which were analyzed and compared with other *Salmonella* reference strains on the NCBI Gen-Bank.

In order to understand the diversity among the local *Salmonella* strains isolated from wildlife and domestic animals, a phylogenetic tree was constructed based on nucleotide and amino acid sequences of the *Salmonella* fragment *inv*A -284 bp gene region. Therefore, evolutionary history of the randomly selected nucleotides and amino acids of the local isolates with reference strains from the GenBank data were inferred using the Maximum Likelihood method based on the

Tamura-Nei model (Jones *et al*., 1992), while the evolutionary analyses were conducted in MEGA7 version software (Kumar *et al*., 2016).

3.7 Data Storage and Analysis

Data was analyzed by STATA® statistical package version 12. A *p*-value of <0.005 was considered indicative of a statistically significant difference for all study statistical analysis. Data was established and stored in Microsoft Excel spread sheets, for handling, cleaning and visualization, while STATA® statistics package was applied such as summary histogram, measures of data spread, trends of central tendency, etc. The main areas of data analysis were biological, such as culture results data, antimicrobial profiles data, PCR data, and genetic analysis.

3.8 Ethical Consideration of the study

There were no ethical issues to be considered as samples were collected from freshly voided faecal samples of both domestic animals and wildlife from their naturally grazing areas. No animal life was sacrificed for the study and their welfare was not disturbed. Clinical samples were those that were submitted for routine diagnosis to Microbiology Laboratory at the School of Veterinary Medicine and the privately owned Veterinary Diagnostic Laboratories.

CHAPTER FOUR

RESULTS

4.1 Isolation of *Salmonella* from domestic animals and wildlife

A total of 1248 samples from domestic animals (1008) and wildlife (240) were obtained for *Salmonella* examination.

4.1.1 Samples analyzed from domestic animals

Of the domestic animal samples, 659 (65.4%) were faecal samples from apparently healthy animals (cattle = 632; horses = 27), while 349 (34.6%) were samples from diseased animals submitted for routine microbiological diagnosis. Of the diseased samples, 311 (89.1%) were from chickens, 36 (10.3%) dogs, and 1 (10.29%) from horse. The specimens comprised intestinal contents, faeces, organs, tissue, and swabs. Cattle samples were collected from seven districts of Southern Province; Mazabuka (n = 118), Monze (n = 126), Pemba (n = 126), Choma (n = 125), Kalomo (n = 50), Zimba (n = 28), and Kazungula (n = 59). All the faecal samples from horses were collected from Lusaka Province (Figure 1).

4.1.2 Samples analyzed from wildlife

A total of 240 faecal samples representing 17 free-ranging wildlife spp were collected from KNP (n = 210), LNP (n = 21) and LCP (n = 9). Of the 9 samples, one (11.1 percent) was from a diseased animal submitted for routine microbiological diagnosis. All samples were stored in a cooled box before transportation to the Microbiology Laboratory for analysis. The distribution of samples among the wildlife spp is shown in Table 4-1.

Table 4-1: Distribution of faecal samples of wildlife (*n* = 240) from KNP, LNP and LCP.

Host	Scientific name	KNP	LNP	LCP
Baboon	*Papio anubis*	0	21	0
Lion	*Panthera leo*	14	0	0
Porcupine	*Erethizon dorsaum*	1	0	0
Puku antelope	*Kobus vardonii*	59	0	0
Water buck	*Kobus ellipsiprymnus*	16	0	0
Cheetah	*Acinonyx jubatus*	1	0	0
Impala	*Aepyceros melampus*	42	0	0
Warthog	*Phacochoerus africanus*	3	0	0
Wild dogs	*Lycaon pictus*	10	0	0
Leopard	*Panthera pardus*	6	0	0
hartebeest	*Alcelaphus buselaphus*	2	0	0
Hippopotamus	*Hippopotamus amphibious*	9	0	0
Lechwe	*Kobus leche kafuensis*	29	0	0
Elephant	Loxodonta africana	10	0	0
Roan antelope	*Hippotragus equines*	2	0	0
Buffalo	*Bubalus bubalis*	6	0	0
Sable antelope	*Hippotragus niger*	0	0	9
Total		210	21	9

4.1.3 Isolation of *Salmonella* from different sources:

A total of 34/1248 (2.7%) *Salmonella* strains were isolated and identified from domestic animals and wildlife samples (Table 4-2). *Salmonella* was isolated from different sources i.e. faecal samples of apparently healthy cattle (0.32%; 2/632); free-ranging wildlife (0. 83%; 2/240) and from samples of sick animals (8.6%; 30/349). Of the 30 isolates from sick animals, 29 (96.7%) were from domestic animals, while one (3.3%) was from a wildlife sample (sable antelope). Among 29 isolates, 26 (89.7%) were from chickens, while 2 (6.9%) and one (3.4%) were from dogs and horse respectively. The two isolates from free-ranging wildlife were each from leopard (16.7%; 1/6) and impala antelope (2.4%; 1/42).

Table 4-2. Frequency of isolation of *Salmonella* spp. from domestic animals and wildlife ($n = 1248$).

General Host	Specific Host	Apparently healthy animals (Field samples)		Animals with clinical disease	
		No. of samples	No. of isolates	No. of samples	No. of isolates
Domestic animals ($n = 1008$)	Cattle	632	2	0	0
	Horse	27	0	1	1
	Dog	0	0	36	2
	Chicken	0	0	311	26
Wildlife* ($n = 240$)	Sable	9	0	1	1
	Leopard	6	1	0	0
	Impala	42	1	0	0

* = Some animals did not yield *Salmonella*

4.2 Biochemical analysis of bacterial strains isolated from domestic animals and wildlife

All 34 *Salmonella* suspect isolates produced alkaline (red) slant and acid (yellow) butt, with production of H_2S (blackening of agar) in Triple Sugar Iron (TSI), while; an alkaline (purple) reaction was produced in the LIA butt. All the isolates were; urease negative, citrate positive, tryptophanase (indole) negative, Voges-Proskauer (VP) negative, Methyl red (MR) positive, and oxidase negative. Further, confirmation of *Salmonella* species was done using the Analytical Profile Index (API) 20 E systems (Biomerieux SA, Marcy-1 Etoile France) and the API web software.

4.3 Serological characterization of isolated *Salmonella* strains

The slide agglutination test, revealed that of the 34 *Salmonella* strains, 94.1 percent produced positive agglutination reactions with *Salmonella* somatic "O" polyvalent A-S group antiserum, except two strains isolated from sable antelope (SI-I) and impala antelope (I-213) respectively (Table 4-3). All the strains were further characterized into sero-groups and serotypes.

4.3.1 Characterization of *Salmonella* strains based on phenotypic expression of somatic O Antigen (Serogroups)

Using specific monovalent antisera slide agglutination test, 34 *Salmonella* strains were grouped into four distinct sero-groups; B (O: 4), C1 (O: 6, 7), D1 (O: 9) and non-reactive group (NR) isolated from different animal species ($p < 0.001$, 95 percent CI) (Table Table 4-3). The study revealed that *Salmonella* strains belonging to serogroup D1 (O: 9) were the most predominant (50.0 percent, 17/34) in circulation and were exclusively isolated from clinical samples of domestic animals with 94.1 percent (16/17) from chickens, and 5.9 percent (1/17) from a horse. This was followed by *Salmonella* strains classified under non reactive serogroup with 29.4 percent (10/34), which were defective to the monovalent agglutination test. Other serogroups were O: 4 and O: 7, comprising 5.9 percent (2/34) and 14.7 percent (5/34) strains each respectively. The serogroups were significantly different ($p < 0.001$, 95 percent CI) and were from various animal species (Table 4-2). Of the 10 strains belonging to non-reactive group, 80 percent were from chicken clinical cases, while the rest were each from the sable antelope and impala antelope respectively. The five *Salmonella* strains belonging to serogroup O: 7 were isolated from both clinical samples (dog = 2, chicken = 2) and one non-clinical isolate from the leopard. The clinical strain isolated from sable antelope was found non-reactive. None of the strains tested in this study were found to belong to specific monovalent serogroups; O: 2; O: 3; O: 5 and O: 8. *Salmonella* strains from chickens' were represented by three monovalent serogroups (O: 7, O: 9 and non-reactive). The detailed distribution of *Salmonella* serogroups are presented in Table 4-3.

Table 4-3: Distribution of *Salmonella* strains by serogroups (*n* = 34)

Animal species	No. of strains	Positive polyvalent	No. of *Salmonella* strains per serogroup			
			O: 4 (B)	O: 7 (C1)	O: 9 (D1)	NR
Horse	1	1	0	0	1	0
Chicken	26	26	0	2	16	8
Cattle	2	2	2	0	0	0
Dog	2	2	0	2	0	0
Leopard	1	1	0	1	0	0
Impala antelope	1	0	0	0	0	1
Sable antelope	1	0	0	0	0	1
Total (%)	34 (100.0)	32 (94.1)	2 (5.9)	5 (14.7)	17 (50.0)	10 (29.4)

NR = none reactive

4.3.2 Characterization of *Salmonella* strains isolated from domestic animals and wildlife based on phenotypic expression of O and H Antigens (Serotypes)

Twelve distinct serotypes were identified among the 34 strains of *Salmonella* isolated from domestic animals and wildlife (p = 0.002, 95 percent CI) (Table 4-4). Of the 12 serotypes, 75 percent (9/12) were isolated from domestic animals (*S.* Heidelberg, *S.* Enteritids, *S.* Ruanda, *Salmonella* spp., *S.* Stockholm, *S.* Chardan, *S.* Sendai, *S.* Mbandaka and *S.* Hadar), while 25 percent (3/12) serotypes were isolated from wildlife (*S.* Garoli, *S.* Pomona and *S.* Roan). The predominant serotype was *S.* Enteritidis (47.1 percent, 16/34) accounting for almost one-half of the serotypes identified in the study, followed by *S.* Mbandaka (8.8 percent, 3/34), *S.* Heidelberg, *S.* Ruanda, *S.* Stockholm, *S.* Chardans, *Salmonella* spp. at 5.9 percent (2/34) each and *S.* Sendai, *S.* Hadar, *S.* Garoli, *S.* Pomona and *S.* Roan at 2.9 percent each. Of the 16, *S.* Enteritidis serotype, 93.8 percent were from chicken source.

Table 4-4: Distribution of *Salmonella* serotypes isolated from domestic animals and wildlife

Serotype	No. of serotypes						
	Domestic animals				Wildlife		
	Cattle	Horse	Chicken	Dog	Leopard	Sable	Impala
S. Heidelberg	2	0	0	0	0	0	0
S. Enteritidis	0	1	15	0	0	0	0
S. Garoli	0	0	0	0	1	0	0
S. Pomona	0	0	0	0	0	1	0
S. Roan	0	0	0	0	0	0	1
S. Ruanda	0	0	2	0	0	0	0
Salmonella spp*	0	0	2	0	0	0	0
S. Hadar	0	0	1	0	0	0	0
S. Stockholm	0	0	2	0	0	0	0
S. Chardan	0	0	2	0	0	0	0
S. Sendai	0	0	1	0	0	0	0
S. Mbandaka	0	0	1	2	0	0	0
Total	2	1	26	2	1	1	1

$p = 0.002$; * isolate could not be serotyped into *Salmonella enterica* sub spp.

The majority, 76.5 percent (26/34) serotypes were isolated from chicken source. On the other hand, 85.3 percent (29/34) of the serotypes were isolated from Lusaka region. The finding revealed that *S.* Enteritidis and *S.* Ruanda serotypes constituted the most predominant sero-group O: 9 (50.0 percent, 17/34), isolated from horse and chicken sources. Further observation revealed that five *Salmonella* serotypes comprising *S.* Mbandaka ($n = 3$), *S.* Hadar ($n = 1$) and *S.* Garoli ($n = 1$) belong to serogroup O: 7. The 10 *Salmonella* strains that were identified non-reactive to both polyvalent O serum and monovalent antiserum belong to rough serotypes; *S.* Pomona, *S.* Roan, *S.* Enteritidis, *S.* Sendai (with one each), *Salmonella* spp., *S.* Stockholm, and *S.* Chardan with two each.

4.4 Characterization of *Salmonella* serotypes isolated from domestic animals and wildlife based on phenotypic expression of fermentative groups

All 34 *Salmonella* strains demonstrated significant variability in their utilization pattern of substrates (Table 4-5). All 34 *Salmonella* serotypes without exception utilized trehalose, maltose, melibiose, galactose, glucose, mannose, xylose, mannitol and dulcitol to produce acid which was marked by colour change of the phenol red indicator from pink to yellow due to acidic condition

(p = 0.01, 95 percent CI). In contrast, all strains were exclusively negative for utilization of rabitol and raffinose.

Table 4-5: Frequency of utilization of substrate by *Salmonella* strains

S/N	Substrate	Frequency of *Salmonella* strains		Cumulative (%)
		Positive	Negative	
1	Cellobiose	7 (20.6)	27 (79.4)	100.0
2	Trehalose	34 (100.0)	0	100.0
3	Maltose	34 (100.0)	0	100.0
4	Melibiose	34 (100.0)	0	100.0
5	Galactose	34 (100.0)	0	100.0
6	Glucose	34 (100.0)	0	100.0
7	Mannose	34 (100.0)	0	100.0
8	Arabinose	7 (20.6)	27 (79.4)	100.0
9	Xylose	34 (100.0)	0	100.0
10	Rabitol	0	34 (100.0)	100.0
11	Inositol	9 (26.5)	25 (73.5)	100.0
12	Mannitol	34 (100.0)	0	100.0
13	Dulcitol	34 (100.0)	0	100.0
14	Salicin	1 (2.9)	33 (97.1)	100.0
15	Raffinose	0	34 (100.0)	100.0

Variable fermentation reactions were observed in the following substrates; arabinose, cellobiose, inositol and salicin (Table 4-6). Among the substrates with variable biochemical activities, inositol was the highest utilized substrate at 26.5 percent (9/34) by *Salmonella* strains belonging to five different serotypes; *Salmonella* spp, *S.* Hadar, *S.* Stockholm, *S.* Sendai and *S.* Mbandaka isolated from chicken and dog sources. This was followed by utilization of cellobiose and arabinose at 20.6 percent (7/34 each) respectively, by *Salmonella* serotypes from both domestic animals and wildlife sources. Salicin substrate, a polyhydric alcohol was the least utilized substrate only by *S.* Chardans 2.9 percent (1/34), from chicken. *Salmonella* Garoli, *S.* Enteritidis

and *S.* Ruanda were negative for utilization of cellobiose, arabinose, inositol and salicin as a source of energy. Further, observed that these serotypes were also in possession of *spv* genes.

Table 4-6: Identification of biotypes among *Salmonella* serotypes based on biochemical activities

Fermentative group.	Strain No.	Serotype	Variable fermentation activities of sugars and polyhydric alcohols			
			Arabinose	Cellobiose	Inositol	Salicin
-	-	Classic *S. enterica* subsp. reaction	+	-	D	-
I	3	*S.* Garoli	-	-	-	-
	B10	*S.* Enteritidis	-	-	-	-
	WF-SPL2	*S.* Enteritidis	-	-	-	-
	WF1-3	*S.* Enteritidis	-	-	-	-
	Para 18	*S.* Enteritidis	-	-	-	-
	Para 13	*S.* Enteritidis	-	-	-	-
	Para 13c	*S.* Ruanda	-	-	-	-
	Para 1	*S.* Enteritidis	-	-	-	-
	Para 6	*S.* Enteritidis	-	-	-	-
	Para 11A	*S.* Enteritidis	-	-	-	-
	Para 11B	*S.* Enteritidis	-	-	-	-
	Para 3	*S.* Enteritidis	-	-	-	-
	B3	*S.* Enteritidis	-	-	-	-
	B4	*S.* Enteritidis	-	-	-	-
	B6	*S.* Enteritidis	-	-	-	-
	Para 21B	*S.* Enteritidis	-	-	-	-
	Para cm	*S.* Enteritidis	-	-	-	-
	Para 4	*S.* Enteritidis	-	-	-	-
	Para 12	*S.* Ruanda	-	-	-	-
II	Para 19	*Salmonella spp.*	-	-	+	-
	B1	*S.* Hadar	-	-	+	-
	B2	*S.* Stockholm	-	-	+	-
	Para 21A	*S.* Sendai	-	-	+	-
	Para 14	*Salmonella spp.*	-	-	+	-
III	670	*S.* Heidelberg	+	+	-	-
	671	*S.*Heidelberg	+	+	-	-
	SI-I	*S.* Pomona	+	+	-	-
IV	D7	*S.* Mbandaka	-	+	+	-
	D8	*S.* Mbandaka	-	+	+	-
V	B5	*S.* Stockholm	+	-	+	-
	Para 5	*S.* Mbandaka	+	-	+	-
VI	B8	*S.* Chardan	+	+	-	+
VII	I213	*S.* Roan	-	+	-	-
VIII	B7	*S.* Chardan	+	-	-	-
Total			7	7	9	1

Key: + = positive reaction; - = negative reaction; d = 26 – 75% positive

The majority of the strains in this study complied with classic utilization of substrates by *S. enterica* subsp. I that include; dulcitol^{+}, salicin^{-}, mannitol^{+}, xylose^{+}, cellobiose^{-}, melibiose^{+}, glucose^{+}, arabinose^{+}, and raffinose^{-}. In contrast another variant of *S. enterica* subsp. I was identified which reacted as follows: salicin^{+} (2.9 percent, 1/34) strain; cellobiose^{+} (20.6 percent, 7/34) strains; while arabinose (79.4 percent, 27/34) strains. A symbol + denotes for a positive reaction, while – is a negative reaction.

All *Salmonella* strains were biochemically grouped into eight (I-VIII) fermentative profiles, based on their ability to utilize the four substrates. Bio-group I variant was the most predominant with 55.9 percent (19/34) of *Salmonella* strains from different animal hosts (leopard, horse and chickens). In this Group *Salmonella* strains were characterized by failure to utilize the following substrates; arabinose, cellobiose, inositol and salicin. This was followed by Bio-group II with 14.7 percent (5/34) *Salmonella* strains exclusively from chicken source; Bio-group III with 8.8 percent (3/34); and IV and V with 5.9 percent (2/34 each) from cattle, dogs and chickens respectively. Bio-groups VI, VII, and VIII profiles were represented by single (2.9 percent, 1/34) strain isolated from chickens, and impala antelope respectively.

All *S.* Enteritidis strains isolated from chickens and horse shared a common fermentation profile. In contrast, *S.* Mbandaka strains from dog and chicken were in two separate Bio-groups IV and V respectively. It was observed that *Salmonella* strains in the former Bio-group are not capable of utilizing arabinose as a source of energy but cellobiose, while the opposite is the case for the strain in the latter group. The overall difference between the strains belonging to Bio-group VI and other Bio-groups (I, II, III, IV, V, VII, and VIII) was the ability of Bio-group VI to utilize salicin. There was no marked difference in the ability to utilize the substrates between rough strains (*S.* Pomona and *S.* Roan) isolated from wildlife and other *Salmonella* strains that reacted positive to polyvalent "O" antisera. The study observed that three *Salmonella* strains isolated from wildlife, *S.* Garoli (leopard), *S.* Pomona (sable antelope) and *S.* Roan (impala antelope) were each classified into different fermentative groups I, VII and VIII respectively, despite *S.* Garoli and *S.* Roan having originated from the same area (KNP).

The study identified biotype variants among *S.* Chardan serotype strains i.e., biotype 1 (arabinose^{+}, cellobiose^{-}, inositol^{-}, salicin^{-}) and biotype 2 (arabinose^{+}, cellobiose^{+}, inositol^{-}, salicin^{+}); and *S.* Stockholm serotype strains i.e., biotype 1 (arabinose^{-}, cellobiose^{-}, inositol^{+}, salicin^{-}), and biotype 2 (arabinose^{+}, cellobiose^{-}, inositol^{+}, salicin^{-}). On the other hand, two biotypes were identified among the *S.* Mbandaka serotypes strains isolated from dogs and chicken i.e., two strains from dogs are represented by biotype 1 (arabinose^{-}, cellobiose $^{+}$, inositol^{-}, salicin^{-}), while the strain from the chicken is biotype 2 (arabinose^{+}, cellobiose^{-}, inositol^{+}, salicin^{-}).

4.5 Characterization of *Salmonella* strains isolated from domestic animals and wildlife based on phenotypic expression of antimicrobial resistance profile

The present study aimed at highlighting the current status of antimicrobial resistance in *Salmonella* strains isolated from domestic animals and wildlife of Zambia. The study showed, 60 percent (20/34) strains were susceptible, while 28 percent (10/34) and 12 percent (4/34) were completely resistant and intermediate respectively, to the seventeen tested antibiotics used in this investigation as per CLSI standard guidelines (Figure 4-1). There was no association between *Salmonella* serotype and antimicrobial resistance to the set of antibiotics used (p = 0.1, 95 percent CI). The results revealed that none of the 34 *Salmonella* strains was a carrier of an extended broad-spectrum beta-lactamases (ESBL) phenotype to third generation cephalosporins due to bacteria growth inhibition on MacConkey agar containing 2 mg/L of cefotaxime.

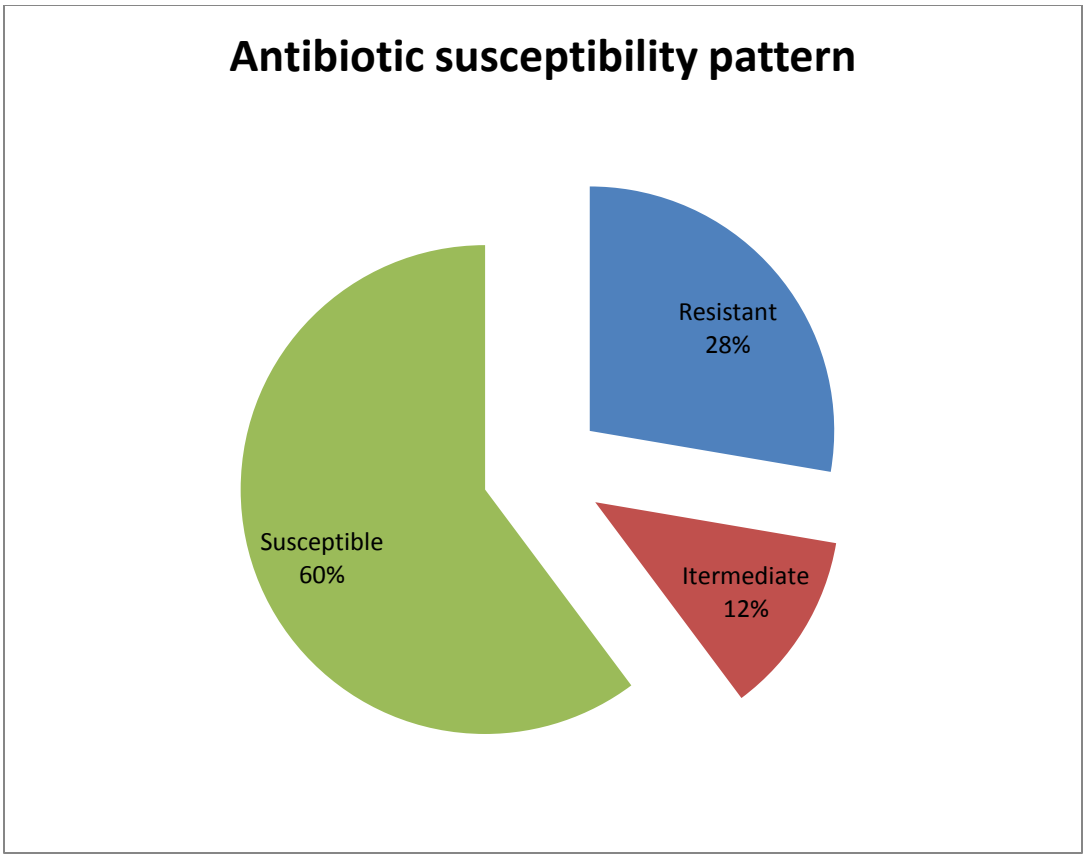


Figure 4-1: Overall susceptibility of 34 *Salmonella* isolates to 17 different antimicrobial agents

However, antibiotic-resistant *Salmonella* strains displayed resistance against seven antimicrobial agents; erythromycin, penicillin G (100 percent, 34/34 each), followed by ampicillin, gentamicin (88.3 percent, 30/34 each), nitrofurantoin (85.3 percent, 29/34), azithromycin (5.9 percent, 2/34) and ciprofloxacin (2.9 percent, 1/34) (Figure 4-2). In addition, intermediate resistance was observed against nalidixic acid (64.7 percent, 22/34), azithromycin (52.9 percent, 18/34), streptomycin (44.1 percent, 15/34), ampicillin and nitrofurantoin (11.8 percent, 4/34 each), chloramphenicol (8.8 percent, 3/34), tetracycline (5.9 percent, 2/34), ciprofloxacin, and levofloxacin (2.94 percent, 1/34 each). None of *Salmonella* strains was resistant to third generation cephalosporins antibiotics (ceftazidime, ceftriaxone, cefotaxime), sulfonamide (cotri-trimoxazole) and fluoroquinolome (norfloxacin). The incidences of resistance for all the isolates tested are presented in Figure 4-2 and Table 4-7.

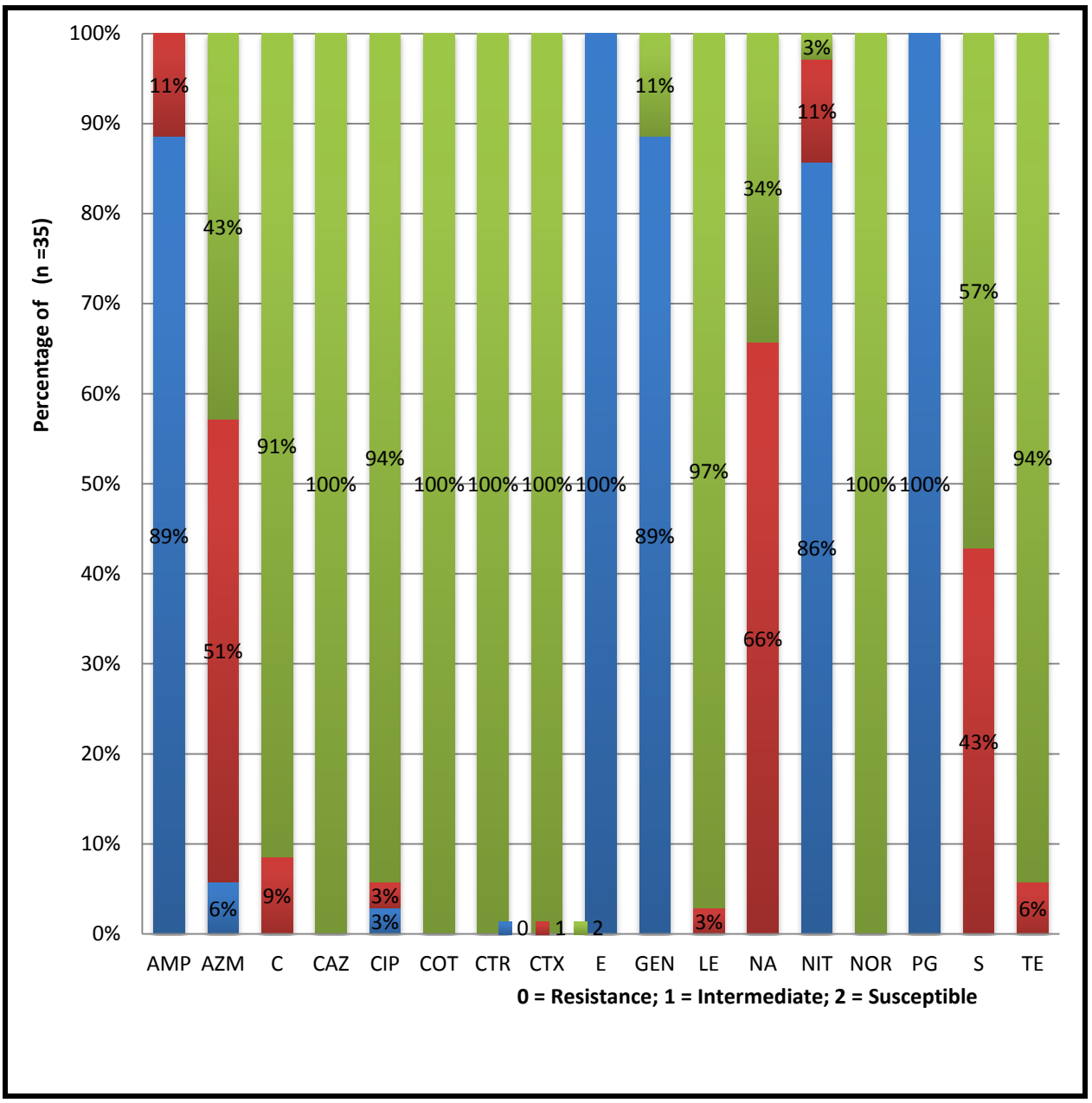


Figure 4-2: Frequency (%) of resistance of *Salmonella* strains from domestic and wild animals to 17 antibiotics.

Table 4-7: Distribution of antibiotic resistance of *Salmonella* strains from domestic animals and wildlife (*n* = 34).

No. of resistance strains						
Antibiotic	Domestic animals (*n* = 31)			Wildlife (*n* = 3)		
	Resistance	Intermediate	Susceptible	Resistance	Intermediate	Susceptible
Ampicillin	28	3	0	2	1	0
Azithromycin	2	17	12	0	1	2
Chlorampenicol	0	3	28	0	0	3
Ceftazidime	0	0	31	0	0	3
Ciprofloxacin	1	1	29	0	0	3
Cotrimoxazole	0	0	31	0	0	3
Ceftriaxone	0	0	31	0	0	3
Cefotaxime	0	0	31	0	0	3
Erythromycin	31	0	0	3	0	0
Gentamycin	27	0	4	0	0	3
Levofloxacin	0	1	30	0	0	3
Nalidixic Acid	0	19	12	0	3	0
Nitrofurantoin	26	4	1	3	0	0
Norfloxacin	0	0	31	0	0	3
Penicillin G	31	0	0	3	0	0
Streptomycin	0	14	17	0	1	2
Tetracycline	0	2	29	0	0	3

The distribution of antimicrobial resistance of *Salmonella* serotypes is shown in Table 4-8. Of 15 *S.* Enteritidis serotype isolated from chickens, 86.7 percent displayed a high antimicrobial resistance to ampicillin, gentamicin and nitrofurantoin, and low resistance to azithromycin (12.5 percent, 2/16). On the other hand, the only *S.* Enteritidis strain from the horse was resistant to ampicillin, azithromycin, gentamycin, and nitrofurantoin. Of the two strains resistant to azithromycin, one (50.0 percent) was from the horse and the other from the chicken source respectively. The study identified a single strain of *S.* Mbandaka (2.9 percent, 1/34) as the only

strain resistant to ciprofloxacin which was isolated from dog. Serotypes *S.* Heidelberg (cattle), *S.* Enteritidis (horse), *S.* Mbandaka (dog and chicken), *S.* Sendai, *S.* Hadar, *S.* Ruanda isolated from chickens; and *S.* Roan (impala antelope), *S.* Pomona (sable antelope) and *S.* Garoli (leopard) were resistant to nitrofurantoin.

Table 4-8: Distribution of antimicrobial resistance phenotypes of *Salmonella* serotypes from various animal sources (n = 34)

Serotype	Animal source	No. of isolates	No. of *Salmonella* strains resistant (%)				
			Amp	Azm	Cip	Gen	Nit
S. Enteritidis	Horse	1	1 (100.0)	1(100.0)	0	1 (100.0)	1 (100.0)
S. Mbandaka	Dog	2	2 (100.0)	0	1 (50.0)	2 (100.0)	2 (100.0)
S. Heidelberg	Cattle	2	2 (100.0)	0	0	1 (50.0)	2 (100.0)
S. Garoli	Leopard	1	1 (100.0)	0	0	1 (100.0)	1 (100.0)
S. Pomona	Sable	1	1 (100.0)	0	0	1 (100.0)	1 (100.0)
S. Mbandaka	Chicken	1	1 (100.0)	0	0	1 (100.0)	1 (100.0)
S. Enteritidis	Chicken	15	13 (86.7)	1 (6.7)	0	13 (86.7)	13 (86.7)
S. Hadar	Chicken	1	1 (100.0)	0	0	1 (100.0)	1 (100.0)
S. Sendai	Chicken	1	1 (100.0)	0	0	1 (100.0)	1 (100.0)
*S.*Ruanda	Chicken	2	2 (100.0)	0	0	1 (50.0)	1 (100.0)
S. Stockholm	Chicken	2	2 (100.0)	0	0	2 (100.0)	2 (100.0)
Salmonella. spp.	Chicken	2	1 (50.0)	0	0	2 (100.0)	2 (100.0)
S. Roan	Impala	1	0	0	0	1 (100.0)	1 (100.0)
S. Chardans	Chicken	2	2 (100.0)	0	0	2 (50.0)	0
Total		34	30	2	1	30	29

Amp = ampicillin, Azm = azithromycin, Cip = Ciprofloxacin, Gen = Gentamicin, Nit = Nitrofurantoin.

Multidrug resistance (MDR) phenotypes were observed in all 34 *Salmonella* strains isolated from both domestic animals and wildlife (Table 4-9). Multi Drug Resistance pattern was defined as resistance to antibiotics belonging to at least three or more classes of antibiotics. In the present

study antimicrobial agents were selected from eight different classes (Beta-lactams, macrolides, fluoroquinolones, tetracyclines, aminoglycosides, sulphanomides, nitrofurantoin and phenicols) based on different mechanisms of action. The study revealed that among 34 *Salmonella* strains; two (5.9 percent) were resistant to 6 antimicrobial agents, 22 (64.7 percent) strains to 5, and 10 (29.4 percent) strains to 4 antimicrobial agents. Among all *Salmonella* serotypes, *S.* Enteritidis and *S.* Mbandaka from horse and dog displayed the highest number of antibiogram.

Table 4-9: Distribution of antimicrobial resistance profiles of *Salmonella* strains by serotype and host

Serotype	Host	Antimicrobial resistance profile	No. of antibiotics	No. (%) of strains
S. Enteritidis	Horse	Amp, Azm, E, Gen, Nit, P	6	1(2.94)
S. Mbandaka	Dog	Amp, Cip, E, Gen, Nit, P	6	1(2.94)
S. Heidelberg	Cattle	Amp, E, Gen, Nit, P	5	1(2.94)
S. Garoli	Leopard	Amp, E, Gen, Nit, P	5	1(2.94)
S. Pomona	Sable	Amp, E, Gen, Nit, P	5	1(2.94)
S. Mbandaka	Dog	Amp, E, Gen, Nit, P	5	1(2.94)
S. Mbandaka	Chicken	Amp, E, Gen, Nit, P	5	1(2.94)
S. Enteritidis	Chicken	Amp, E, Gen, Nit, P	5	10(29.41)
S. Hadar	Chicken	Amp, E, Gen, Nit, P	5	1(2.94)
S. Sendai	Chicken	Amp, E, Gen, Nit, P	5	1(2.94)
S. Ruanda	Chicken	Amp, E, Gen, Nit, P	5	1(2.94)
S. Stockholm	Chicken	Amp, E, Gen, Nit, P	5	2(5.88)
Salmonella. spp.	Chicken	Amp, E, Gen, Nit, P	5	1(2.94)
S. Enteritidis	Chicken	Azm, E, Gen, Nit, P	5	1(2.94)
S. Enteritidis	Chicken	Amp, E, Gen, P	4	1(2.94)
S. Chardans	Chicken	Amp, E, Gen, P	4	2(5.88)
S. Enteritidis	Chicken	Amp, E, Nit, P	4	2(5.88)
S. Ruanda	Chicken	Amp, E, Nit, P	4	1(2.94)
S. Heidelberg	Cattle	Amp, E, Nit, P	4	1(2.94)
S. Enteritidis	Chicken	E, Gen, Nit, P	4	1(2.94)
S. Roan	Impala	E, Gen, Nit, P	4	1(2.94)
Salmonella spp.	Chicken	E, Gen, Nit, P	4	1(2.94)
Total				100.0

See Table 3-1 for abbreviations of antibiotics

Antimicrobial resistance of 34 *Salmonella* strains were grouped into seven distinct MDR resistotype profiles (I – VII) (Table 4-10 and Table 4-11).

Table 4-10: Distribution of multidrug resistance pattern among *Salmonella* strains from domestic animal and wildlife sources

Resistotype	Resistance profile	No. of antibiotic	No. of *Salmonella* strains resistant			
			Domestic animal	Wildlife	No. of strains	%
I	Amp, Azm, E, Gen, Nit, P	6	1	0	1	2.94
II	Amp, Cip, E, Gen, Nit, P	6	1	0	1	2.94
III	Amp, E, Gen, Nit, P	5	19	2	21	61.8
IV	Azm, E, Gen, Nit, P	5	1	0	1	2.94
V	Amp, E, Gen, P	4	3	0	3	8.82
VI	Amp, E, Nit, P	4	4	0	4	11.8
VII	E, Gen, Nit, P	4	2	1	3	8.82
Total			31	3	34	100.0

These resistotypes were determined based on the ability of *Salmonella* strains to confer resistance to a single or group of antibiotics. Resistotype group III was the most predominant group designated by five antibiotics, ampicillin, erythromycin, gentamicin, and nitrofurantoin and penicillin G (Amp-E-Gen-Nit-P) phenotype, and was represented by 61.8 percent (21/34) *Salmonella* strains isolated from domestic animals and wildlife. Of the 21 *Salmonella* strains, 19 (90.5 percent) were from domestic animal sources, while 2 (9.5 percent) were from wildlife. Among 19 *Salmonella* strains, 89.5 percent were from chicken sources. *Salmonella* strains isolated from chicken sources were represented in five (71.4 percent) of the seven MDR resistotypes (III-VII) (Table 4-11).

Table 4-11: Distribution of antimicrobial resistant among *Salmonella* strains from domestic animals and wildlife based on resistotypes

Resistotype	No. of strains	No. of resistant *Salmonella* strains (*n* = 34)						
		Chicken	Horse	Cattle	Dog	Sable	Leopard	Impala
I	1	0	1	0	0	0	0	0
II	1	0	0	0	1	0	0	0
III	21	17	0	1	1	1	1	0
IV	1	1	0	0	0	0	0	0
V	3	3	0	0	0	0	0	0
VI	4	3	0	1	0	0	0	0
VII	3	2	0	0	0	0	0	1
Total	34	26	1	2	2	1	1	1

Salmonella strains belonging to resistotype I were resistant against ampicillin, azithromycin, erythromycin, gentamicin, nitrofurantoin and penicillin G (Amp-Az-E-G-N-P); while strains in resistotype group II were resistant against ampicillin, erythromycin, ciprofloxacin, gentamicin, nitrofurantoin and penicillin G (Amp-E-Cip-G-N-P) phenotypes. Among the 16 *Salmonella* Enteritidis strains isolated from domestic animals, were detected into 6 resistotypes (Table 4-12). Group III was the most predominant with 10 (62.5 percent) strains showing a resistance phenotype against five antimicrobial agents; Amp-E-G-N-P.

Table 4-12: Distribution of multidrug resistance of the most predominant *Salmonella enteritidis* serotype in domestic animals (*n* = 16)

Group	Antimicrobial resistance phenotypes	No. of *Salmonella* strains			
		Chicken	Horse	Total	%
I	Amp, Azm, E, Gen, Nit, P	0	1	1	6.25
II	Amp, Cip, E, Gen, Nit, P	0	0	0	0.0
III	Amp, E, Gen, Nit, P	10	0	10	62.50
IV	Azm, E, Gen, Nit, P	1	0	1	6.25
V	Amp, E, Gen, P	1	0	1	6.25
VI	Amp, E, Nit, P	2	0	2	12.50
VII	E, Gen, Nit, P,	1	0	1	6.25
Total		15	1	16	

4.5.1 Characterization of *Salmonella* strains based on multiple antibiotic resistance index (MARI)

Salmonella Enteritidis and *S.* Mbandaka serotypes isolated from horse and dog respectively, showed the highest MAR index of 0.35 (Table 4.13). These strains were isolated from clinical samples of domestic animals around Lusaka Province. All strains in the study were multiple antibiotic resistant with a minimum MAR Index > 0.02. A moderate proportion of *Salmonella* serotypes, 35.3 percent (12/34) exhibited MAR index 0.29. Of these serotypes, 96.7 percent were isolated from domestic animals (cattle = 1, chicken = 10), while 8.33 percent (1/12) were from wildlife (leopard = 1).

Table 4-13: MAR index analysis of the *Salmonella* serotypes from domestic animals and wildlife (*n* = 34):

Serotype	Host	Antimicrobial resistance profile	No. of resistant antibiotics (a)	No. of antibiotic used (b)	MAR Index (a/b)
S. Enteritidis	Horse	Amp, Azm, E, Gen, Nit, P	6	17	0.35
S. Mbandaka	Dog	Amp, Cip, E, Gen, Nit, P	6	17	0.35
S. Heidelberg	Cattle	Amp, E, Gen, Nit, P	5	17	0.29
S. Garoli	Leopard	Amp, E, Gen, Nit, P	5	17	0.29
S. Pomona	Sable	Amp, E, Gen, Nit, P	5	17	0.29
S. Mbandaka	Dog	Amp, E, Gen, Nit, P	5	17	0.29
S. Mbandaka	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
S. Enteritidis	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
S. Hadar	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
S. Sendai	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
S. Ruanda	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
S. Stockholm	Chicken	Amp, E, Gen, Nit, P	5	17	0.29
Salmonella. spp.	Chicken	Amp, E, Gen,Nit, P,	5	17	0.29
S. Enteritidis	Chicken	Azm, E, Gen, Nit, P	5	17	0.29
S. Enteritidis	Chicken	Amp, E, Gen, P	4	17	0.24
S. Enteritidis	Chicken	Amp, E, Nit, P	4	17	0.24
S. Enteritidis	Chicken	E, Gen, Nit, P,	4	17	0.24
S. Stockholm	Chicken	Amp, E, Gen, P,	4	17	0.24
S. Ruanda	Chicken	Amp, E, Nit, P	4	17	0.24
S. Roan	Impala	E, Gen, Nit, P	4	17	0.24
S. Heidelberg	Cattle	Amp, E, Nit, P	4	17	0.24
S. Chardans	Chicken	Amp, E, Gen, P	4	17	0.24
Salmonella spp.	Chicken	E, Gen, P	3	17	0.24

4.5.2 Characterization of *Salmonella* strains based on multiple antibiotic resistance index (MARI)

The study, revealed MAR index of erythromycin (100 percent, MARI 0.059), penicillin G (100 percent, 0.059), ampicillin, (88.2 percent, MARI 0.052), gentamicin (88.2 percent, MARI 0.052), and nitrofurantoin (85.3 percent, MARI 0.052) (Table 4-14).

Table 4-14: MAR index analysis of the antibiotic against *Salmonella* serotype

Antibiotics	No. of resistant strains	No. of Susceptible strains	No. of Intermediate	MAR Index
Ampicillin	30	0	4	0.052
Azithromycin	2	14	18	0.003
Ceftazidime	0	34	0	0
Cefotaxime	0	34	0	0
Ceftriaxone	0	34	0	0
Chloramphenicol	0	31	3	0
Ciprofloxacin	1	32	1	0.002
Co-trimoxazole	0	34	0	0
Erythromycin	34	0	0	0.059
Gentamicin	30	4	0	0.052
Levofloxacin	1	33	0	0.002
Nalidixic Acid	0	12	22	0
Nitrofurantoin	29	1	4	0.052
Norfloxacin	0	34	0	0
Penicillin G	34	0	0	0.059
Streptomycin	0	19	15	0
Tetracycline	0	32	2	0

4.6 Genotypic characterization of *Salmonella* strains based on *inv*A, 16S rRNA, and *spv* locus genes

This study characterized chromosomal *inv*A and *spv* gene with a view to establishing genetic diversity among the strains of domestic animals and wildlife origin.

4.6.1 Characterization of *Salmonella* strains based on the (*inv*A) gene product

The PCR analysis detected the *inv*A-284bp amplicon in all 34 (100 percent) strains and amplification products are shown in Figure 4-3.

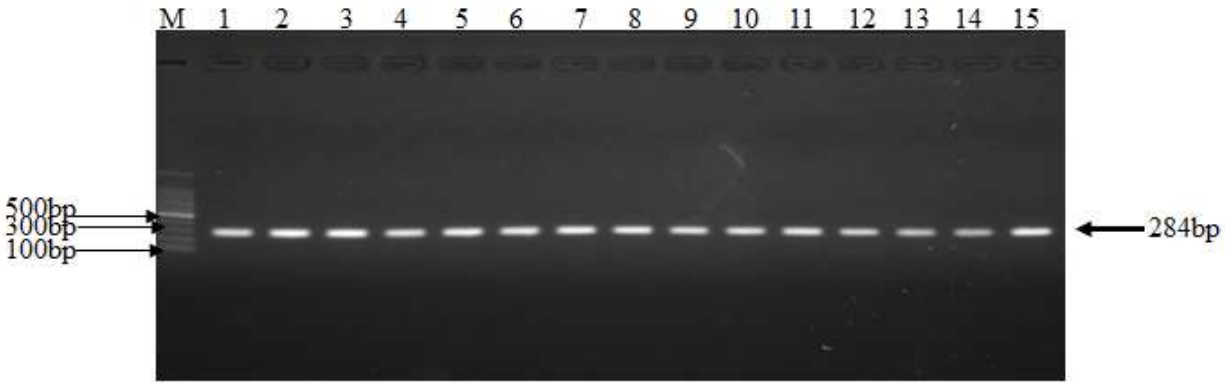


Figure 4-3: Gel picture showing 284bp *inv*A gene amplicon in all 34 *Salmonella* isolates which are represented by 15 *inv*A amplicon products. M; Molecular marker with Lane 1 to 15 as samples showing a positive amplicon at 284bp.

4.6.2 Characterization of *Salmonella* strains based on the 16SrRNA

All *Salmonella* strains from domestic animals and wildlife that tested positive for *inv*A gene were further investigated for the presence of the 16S rRNA product specific for *Salmonella* species (Figure 4-4 and Table 4-15). Of the 34 *Salmonella* strains, 97.1 percent (33/34) strains were positive for 16S rRNA amplification product except one strain number D8 (*S.* Mbandaka) from dog. The molecular weight of the DNA amplicon was 1400 bp as estimated by the standard DNA ladder as shown Figure 4-4.

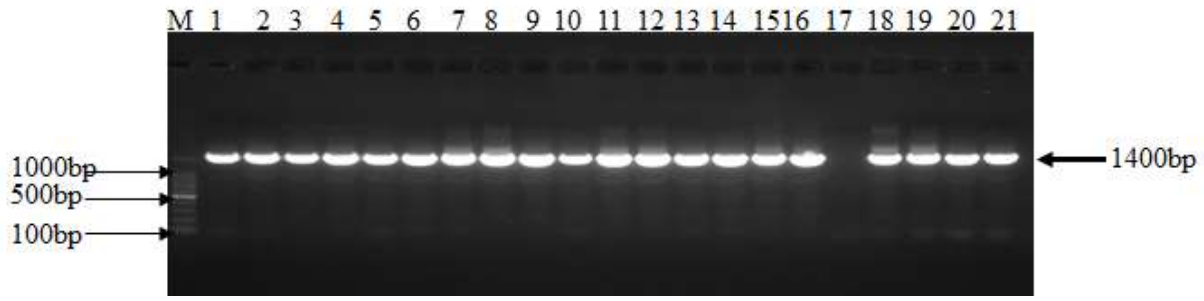


Figure 4-4: Gel picture showing 1400bp 16SrRNA gene amplicon in 33 *Salmonella* isolates which are represented by 20 16SrRNA amplicon products of *Salmonella* isolates. M; molecular marker, Lane 1 to 21 are sample DNA, with Lane 17 indicating a negative result.

Table 4-15: Distribution of *inv*A and 16S rRNA genes by *Salmonella* serotype in animal species

Source/Serotype	Total No. of *Salmonella* strains	No. of positive *Salmonella* strains	
		*inv*A	16S rRNA
Cattle			
S. Heidelberg	2	2	2
Leopard			
S. Garoli	1	1	1
Sable antelope			
S. Pomona	1	1	1
Impala antelope			
S. Roan	1	1	1
Horse			
S. Enteritidis	1	1	1
Poultry			
S. Enteritidis	15	15	15
S. Ruanda	2	2	2
Salmonella spp.	2	2	2
S. Hadar	1	1	1
S. Stockholm	2	2	2
S. Chardans	2	2	2
S. Sendai	1	1	1
S. Mbandaka	1	1	1
Dog			
S. Mbandaka	2	2	1
Total	34	34	33

4.6.3 Genotypic characterization of *Salmonella* strains based on *spv*A, *spv*B and *spv*C genes

The expected fragment length of respective PCR screening products for *spv*A (604 bp), *spv*B (1,776 bp) and *spv*C (669 bp) were detected in strains belonging to *S.* Enteritidis (O: 9), *S.* Garoli (O: 7), *S.* Ruanda (O: 9) and *S.* Stockholm (non-reactive) as shown in Figures 4-5 to 4-7. The carriage of plasmid encoded *spv* genes among the strains was at the following rates 58.8 percent (20/34), 55.9 percent (19/34) and 58.8 percent (20/34) for *spv*A, *spv*B and *spv*C, respectively (P=0.001, 95 percent, CI). Of the *spv* positive strains, 95.0 percent (19/20) serotypes were in possession of the three *spv* genes concurrently, except for one strain *S.* Enteritidis serotype (Para 18) that lacked the *spv*B (Table 4-16). Conversely, no virulence genes were found in strains belonging to serogroup O: 4. On the other hand, out of five *Salmonella* strains belonging to serogroup O: 7, only 20.0 percent (1/5) strain isolated from the leopard yielded *spv*ABC genes. Of 30 *Salmonella* strains from various clinical samples of domestic animals and wildlife, 19 (63.3 percent) were in possession of the three virulence *spv*ABC genes (Table 4-16). All strains (16) belonging to *S.* Enteritidis serotype from horse and chicken sources contained *spv*A (100 percent), *spv*B (93.8 percent) and *spv*C (100 percent). The *spv* genes were absent from serotypes *S.* Heidelberg (cattle); *S.* Pomona (sable antelope); *S.* Mbandaka (dog and chicken); *S.* Sendai, *S.* Hadar and *Salmonella* spp. from chickens (Figure 4-5 to 4-7). The distribution of the detected three virulence *spv*ABC genes from different serotypes is presented in Table 4-16.

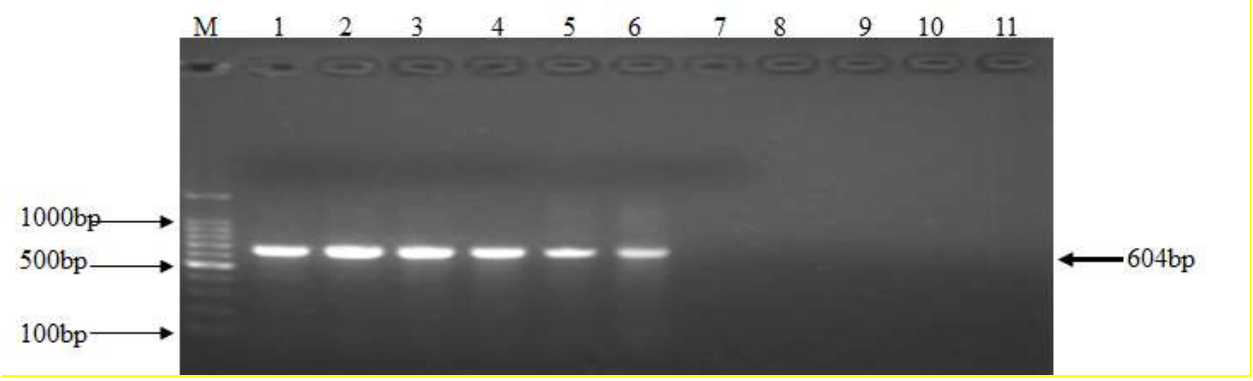


Figure 4-5: Gel picture showing 604bp *spv*A gene amplicon in 20 *Salmonella* isolates which are represented by 6 *spv*A amplicon products of *Salmonella* isolates. M; molecular marker, Lane 1 to 11 are sample DNA, with Lanes 7 to 11 indicating negative results.

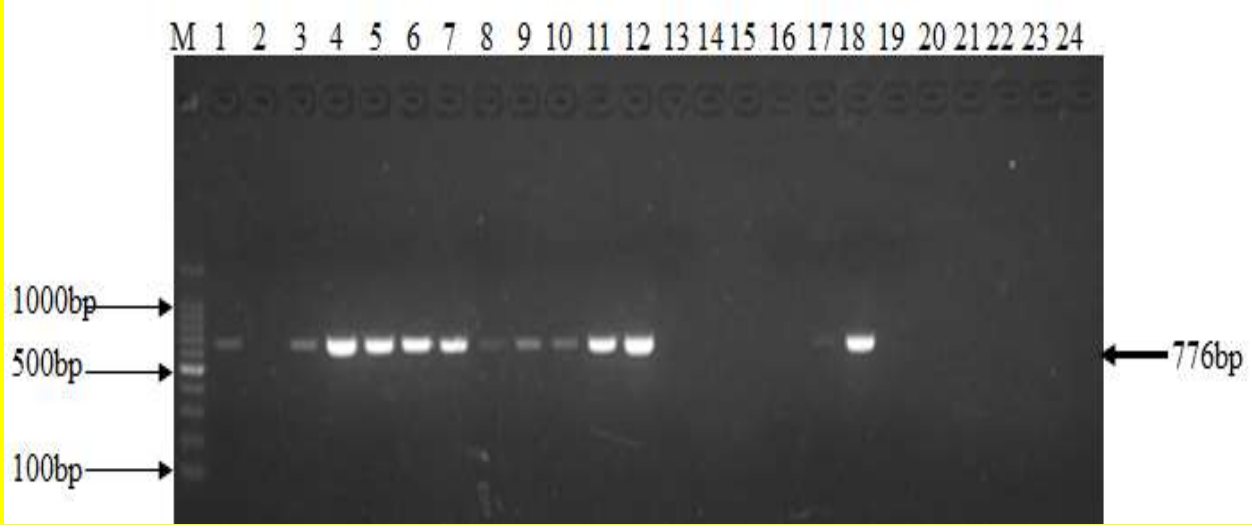


Figure 4-6: Gel picture showing 1776bp *spv*B gene amplicon in 19 *Salmonella* isolates which are represented by 11 spvB amplicon products of *Salmonella* isolates. M; molecular marker, Lane 3 to 12 and 18 are sample DNA indicating positive results.

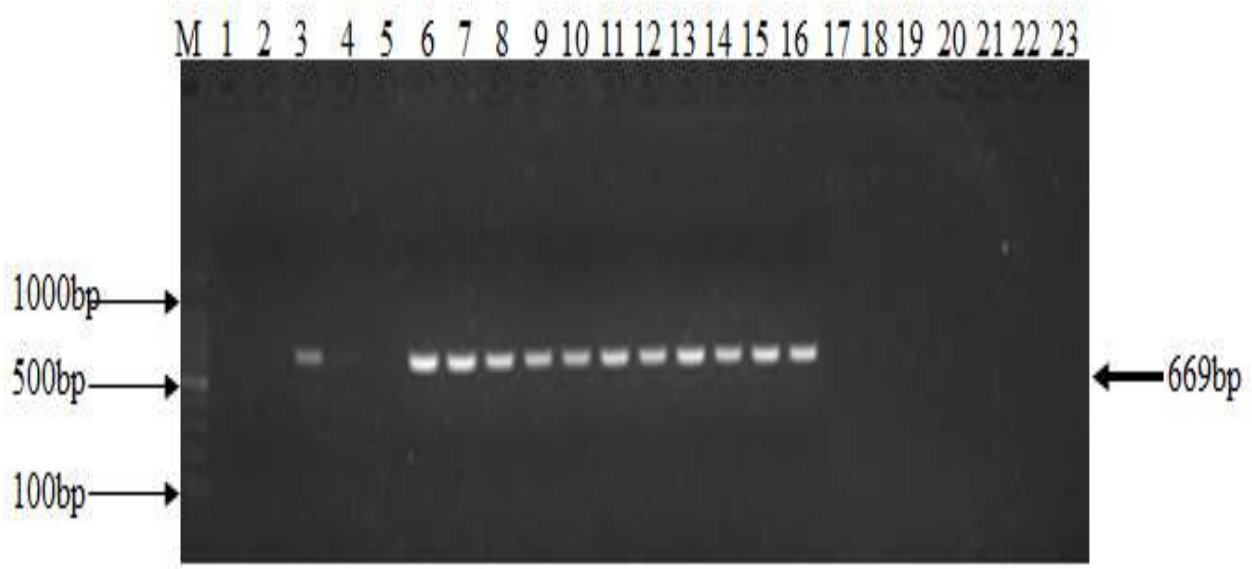


Figure 4-7: Gel picture showing 669bp *spv*C gene amplicon in 20 *Salmonella* isolates which are represented by 12 *spv*C amplicon products of *Salmonella* isolates. M; molecular marker, Lane 3, 6 to 16 are sample DNAindicating positive results.

Table 4-16: Distribution of *spv* genes by *Salmonella* serotype

Serotype	Serogroup	Total strains	No. of *Salmonella* strains carrying virulence genes		
			*spv*A	*Spv*B	*spv*C
S. Heidelberg	O: 4	2	0	0	0
S. Garoli	O: 7	1	1	1	1
S. Pomona*	NR	1	0	0	0
S. Roan	NR	1	0	0	0
S. Enteritidis*	O: 9	15	15	14	15
S. Enteritidis*	NR	1	1	1	1
S. Ruanda*	O: 9	2	2	2	2
*Salmonella spp**	NR	2	0	0	0
S. Hadar*	O:7	1	0	0	0
S. Stockholm*	NR	2	1	1	1
S. Chardan*	NR	2	0	0	0
S. Sendai*	NR	1	0	0	0
S. Mbandaka*	O: 7	3	0	0	0
Total		34	20	19	20
P' value			*P*=0.001	*P*=0.001	*P*=0.001

NR = Non-reactive, * = Clinical isolate

4.6.4 Identification of isolated *Salmonella s*trains to species level based on the specific 16S rRNA Nucleotide Sequence

Thirty–two (97 percent) of 33 of PCR 16S rRNA 1400 bp amplification products were sequenced except one strain *S.* Enteritidis from chicken which was negative (Table 4-17). These sequences were confirmed to be *Salmonella enterica* following a BLAST search by comparing with the sequences in the GenBank database. The percentage similarity of nucleotide sequences of strains from domestic animals and wildlife sequences with those from the GeneBank, revealed that 62.5 percent (20/32) strains were ≥ 97 percent identity, and 37.5 percent (12/32) strains yielded a similarity score ≥ 90.0 but ≤ 96 percent.

Table 4-17: 16S rRNA sequences of *Salmonella* strains from domestic animals and wildlife compared with accession numbers of corresponding sequences on GenBank

S/ No.	Isolate No.	Serotype	Animal host	Accession No.	Bacterial spp identity	% Identity
1	670	*S.* Heidelberg	Cattle	CP0075402.1	*S. enterica*	98.0
2	671	*S.* Heidelberg	Cattle	CP007540.2	*S. enterica*	96.0
3	3	*S.* Garoli	Leopard	CP022116.1	*S. enterica*	95.0
4	SI-1	*S.* Pomona	Sable	CP019415.1	*S. enterica*	95.0
5	I213	*S.* Roan	Impala	CP007534.1	*S. enterica*	96.0
6	B10	*S.* Enteritidis	Horse	GU826685.1	*S. enterica*	94.4
7	SPL-2	*S.* Enteritidis	Chicken	CP019649.1	*S. enterica*	97.0
8	WFI-3	*S.* Enteritidis	Chicken	KF928768.1	*S. enterica*	98.0
9	Para18	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
10	Para13	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
11	Para13C	*S.* Ruanda	Chicken	CP022003.1	*S. enterica*	98.0
12	Para1	*S.* Enteritidis	Chicken	DQ344532.1	*S. enterica*	96.0
13	Para6	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
14	Para11A	*S.* Enteritidis	Chicken	CP019649.1	*S. enterica*	98.0
15	Para11B	*Salmonella* spp	Chicken	JN615016.1	*S. enterica*	97.0
16	Para3	*S.* Enteritidis	Chicken	KJ620858.1	*S. enterica*	97.0
17	Para19	*Salmonella* spp	Chicken	CP022019.1	*S. enterica*	93.0
18	B1	*S.* Hadar	Chicken	KP313059.1	*S. enterica*	95.0
19	B2	*S.* Stockholm	Chicken	CP007540.2	*S. enterica*	94.0
20	B3	*S.* Enteritidis	Chicken	CP022003.1	*S. enterica*	99.0
21	B4	*S.* Enteritidis	Chicken	GU826685.1	*S. enterica*	99.0
22	B5	*S.* Stockholm	Chicken	CP022135.1	*S. enterica*	90.0
23	B6	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
24	B7	*S.* Chardans	Chicken	CP022135.1	*S. enterica*	99.0
25	B8	*S.* Chardans	Chicken	CP022135.1	*S. enterica*	99.0
26	Para21A	*S.* Sendai	Chicken	KT737738.1	*S. enterica*	90.0
27	Para21B	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
28	Paracm	*S.* Enteritidis	Chicken	CP022138.1	*S. enterica*	99.0
29	D7	*S.* Mbandaka	Dog	CP019405.1	*S. enterica*	99.0
30	Para5	*S.* Mbandaka	Chicken	CP019405.1	*S. enterica*	99.0
31	Para14	*Salmonella* pp.	Chicken	GU826683.1	*S. enterica*	90.0
32	Para12	*S.* Ruanda	Chicken	CP007262.1	*S. enterica*	99.0
	Control	*Escherichia coli*		CP012112.1	*Escherichia coli*	

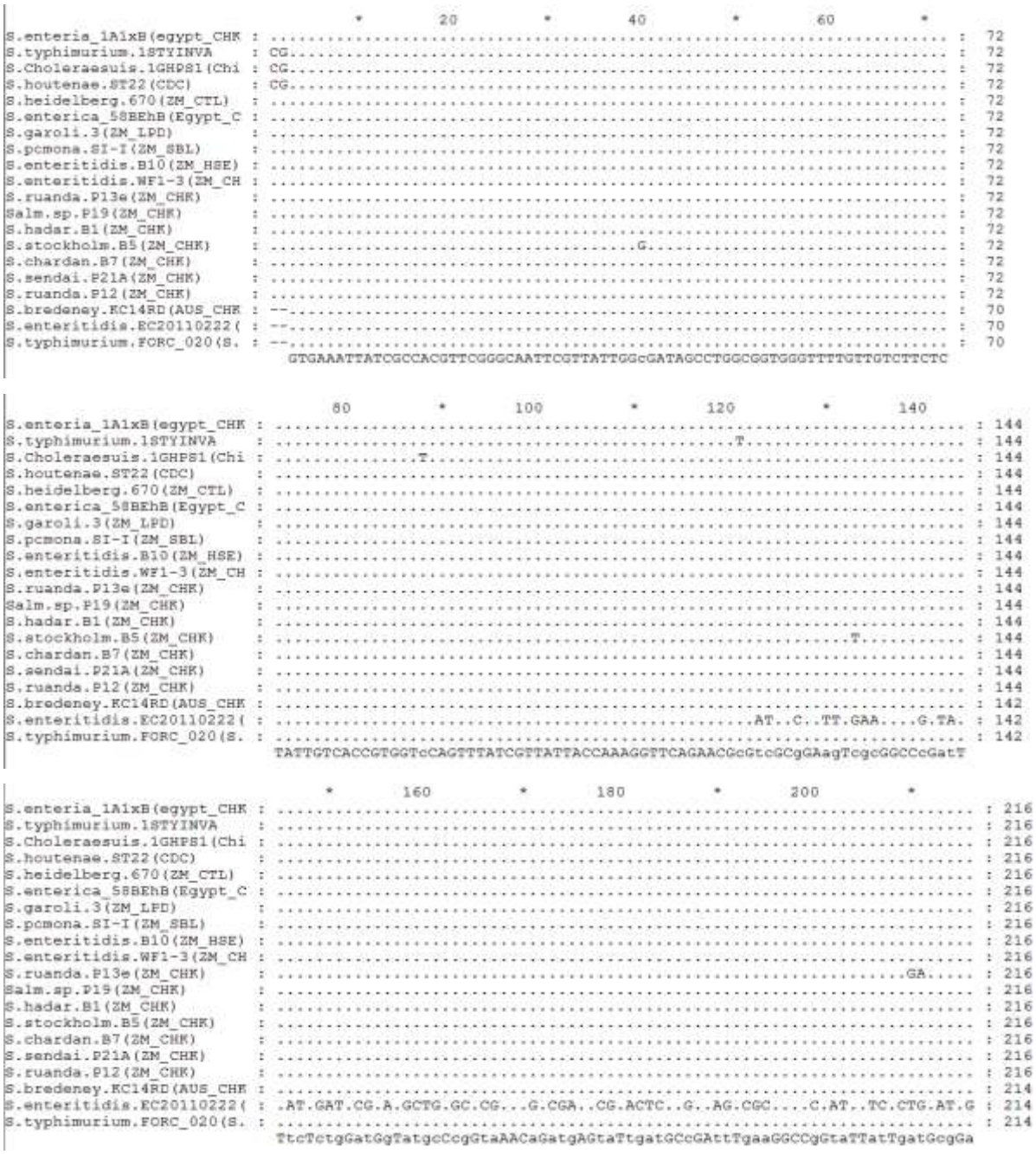
4.6.5 Detection of nucleotide sequence difference (NSD) between the *inv*A gene of local *Salmonella* strains and reference *Salmonella* strains

Twelve (80 percent) of 15 randomly selected *Salmonella inv*A 284 bp PCR amplification products were sequenced. These nucleotide sequences were from different *Salmonella* serotypes (*S.* Heidelberg, *S.* Enteritidis, *S.* Ruanda, *Salmonella spp.*, *S.* Hadar, *S.* Stockholm, *S.* Chardans, *S.* Sendai, *S.* Garoli and *S.* Pomona) and were compared with eight other published sequences [*S. enterica* subspp. *enterica* (KJ718884.1), *S. houtenae* (DQ644627.1), *S.* Choleraesuis (EU348367.1), *S.* Typhimurium (M90846.1), *S. enterica* subspp. *enterica* (KJ718878.1), *S.* Typhimurium (CP012144.1), *S.* Bredeney (KP279306.1), and *S.* Enteritidis (CP007323.1)] on GenBank (Table 4-18 and Figure 4-8).

Table 4-18: *Inv*A nucleotide sequence chains of reference *Salmonella* strains compared with the local *Salmonella* strains

Isolate No.	*Salmonella* sub spp	Animal host	Country	Accession No.	*Salmonella* spp
Reference	*S. enterica*	Chicken	Egypt	KJ718884.11AixB	*S. enterica*
Reference	*S. houtenae*	-	-	DQ644627.1	*S. enterica*
Reference	*S.* Choleraesuis	-	China	EU348367.1GHPS1	*S. enterica*
Reference	*S.* Typhimurium	-	-	M90846.1STYINVA	*S. enterica*
Reference	*S. enterica*	Chicken	Egypt	KJ718878.1BehB	*S. enterica*
Reference	*S.* Typhimurium	-	South Korea	CP012144FORC_020	*S. enterica*
Reference	*S.* Bredeney	Chicken	Australia	KP279306KC14BRD	*S. enterica*
Reference	*s.* Enteritidis	-	Canada	CP007323.1EC20110222	*S. enterica*
670	*S.* Heidelberg	Cattle	Zambia	Local strain	*S. enterica*
3	*S.* Garoli	Leopard	Zambia	Local strain	*S. enterica*
SI-1	*S.* Pomona	Sable	Zambia	Local strain	*S. enterica*
B10	*S.* Enteritidis	Horse	Zambia	Local strain	*S. enterica*
WF1-3	*S.* Enteritidis	Chicken	Zambia	Local strain	*S. enterica*
Para13C	*S.* Ruanda	Chicken	Zambia	Local strain	*S. enterica*
Para19	*Salmonella* spp.	Chicken	Zambia	Local strain	*S. enterica*
BI	*S.* Hadar	Chicken	Zambia	Local strain	*S. enterica*
B5	*S.* Stockholm	Chicken	Zambia	Local strain	*S. enterica*
B7	*S.* Chardans	Chicken	Zambia	Local strain	*S. enterica*
Para21A	*S.* Sendai	Chicken	Zambia	Local strain	*S. enterica*
Para12	*S.* Ruanda	Chicken	Zambia	Local strain	*S. enterica*

- = Source not known


S.enteria_1A1xB(egypt_CHK : 72
S.typhimurium.1STYINVA : 72
S.Choleraesuis.1GHPS1(Chi : 72
S.houtenae.ST22(CDC) : 72
S.heidelberg.670(ZM_CTL) : 72
S.enterica_58BEhB(Egypt_C : 72
S.garoli.3(ZM_LPD) : 72
S.pomona.SI-I(ZM_SBL) : 72
S.enteritidis.B10(ZM_HSE) : 72
S.enteritidis.WF1-3(ZM_CH : 72
S.ruanda.P13e(ZM_CHK) : 72
Salm.sp.P19(ZM_CHK) : 72
S.hadar.B1(ZM_CHK) : 72
S.stockholm.B5(ZM_CHK) : 72
S.chardan.B7(ZM_CHK) : 72
S.sendai.P21A(ZM_CHK) : 72
S.ruanda.P12(ZM_CHK) : 72
S.bredeney.EC14RD(AUS_CHK : 70
S.enteritidis.EC20110222(: 70
S.typhimurium.FORC_020(S. : 70
GTGAAATTATCGCCACGTTCGGGCAATTCGTTATTGGcGATAGCCTGGCGGTGGGTTTTGTTGTCTTCTC
S.enteria_1A1xB(egypt_CHK : 144
S.typhimurium.1STYINVA : 144
S.Choleraesuis.1GHPS1(Chi : 144
S.houtenae.ST22(CDC) : 144
S.heidelberg.670(ZM_CTL) : 144
S.enterica_58BEhB(Egypt_C : 144
S.garoli.3(ZM_LPD) : 144
S.pomona.SI-I(ZM_SBL) : 144
S.enteritidis.B10(ZM_HSE) : 144
S.enteritidis.WF1-3(ZM_CH : 144
S.ruanda.P13e(ZM_CHK) : 144
Salm.sp.P19(ZM_CHK) : 144
S.hadar.B1(ZM_CHK) : 144
S.stockholm.B5(ZM_CHK) : 144
S.chardan.B7(ZM_CHK) : 144
S.sendai.P21A(ZM_CHK) : 144
S.ruanda.P12(ZM_CHK) : 144
S.bredeney.EC14RD(AUS_CHK : 142
S.enteritidis.EC20110222(: 142
S.typhimurium.FORC_020(S. : 142
TATTGTCACCGTGGTcCAGTTTATCGTTATTACCAAAGGTTCAGAACGcGtcGCgGAagTcgcGGCCcGatT
S.enteria_1A1xB(egypt_CHK : 216
S.typhimurium.1STYINVA : 216
S.Choleraesuis.1GHPS1(Chi : 216
S.houtenae.ST22(CDC) : 216
S.heidelberg.670(ZM_CTL) : 216
S.enterica_58BEhB(Egypt_C : 216
S.garoli.3(ZM_LPD) : 216
S.pomona.SI-I(ZM_SBL) : 216
S.enteritidis.B10(ZM_HSE) : 216
S.enteritidis.WF1-3(ZM_CH : 216
S.ruanda.P13e(ZM_CHK) : 216
Salm.sp.P19(ZM_CHK) : 216
S.hadar.B1(ZM_CHK) : 216
S.stockholm.B5(ZM_CHK) : 216
S.chardan.B7(ZM_CHK) : 216
S.sendai.P21A(ZM_CHK) : 216
S.ruanda.P12(ZM_CHK) : 216
S.bredeney.EC14RD(AUS_CHK : 214
S.enteritidis.EC20110222(: 214
S.typhimurium.FORC_020(S. : 214
TtcTctgGatGgTatgcCcgGtaAACaGatgAGtaTtgatGCcGAttTgaaGGCCgGtaTTatTgatGcgGa

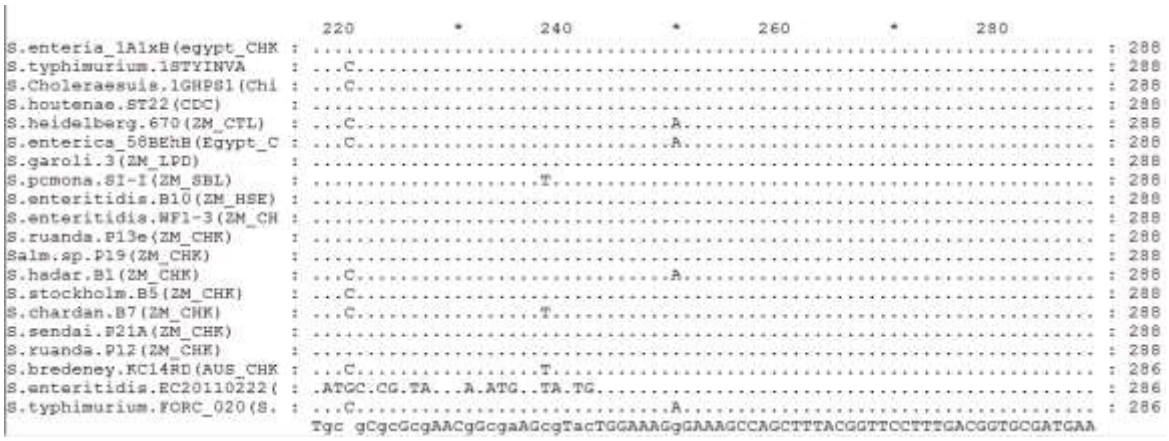


Figure 4-8: Multiple alignment of nucleotide sequences of *inv*A-284 bp gene of local *Salmonella* serotypes strains isolated from domestic animals and wildlife compared with *Salmonella* strains published sequence on GenBank.

Analysis of multiple alignment for *inv*A nucleotide sequences among the strains isolated from domestic animals, wildlife and reference strains showed variations between base pair number 40 and 250 bp of the 284 bp *inv*A fragment gene (Figure 4-8, Table 4-19) or between 407-619 bp (hot spot) from the start codon when compared with a complete *S.* Typhimurium *inv*A 2176 bp gene (Figure 4-9). Further analysis revealed nine distinct *inv*A sequence types among the 20 *Salmonella* strains (Table 4-19).

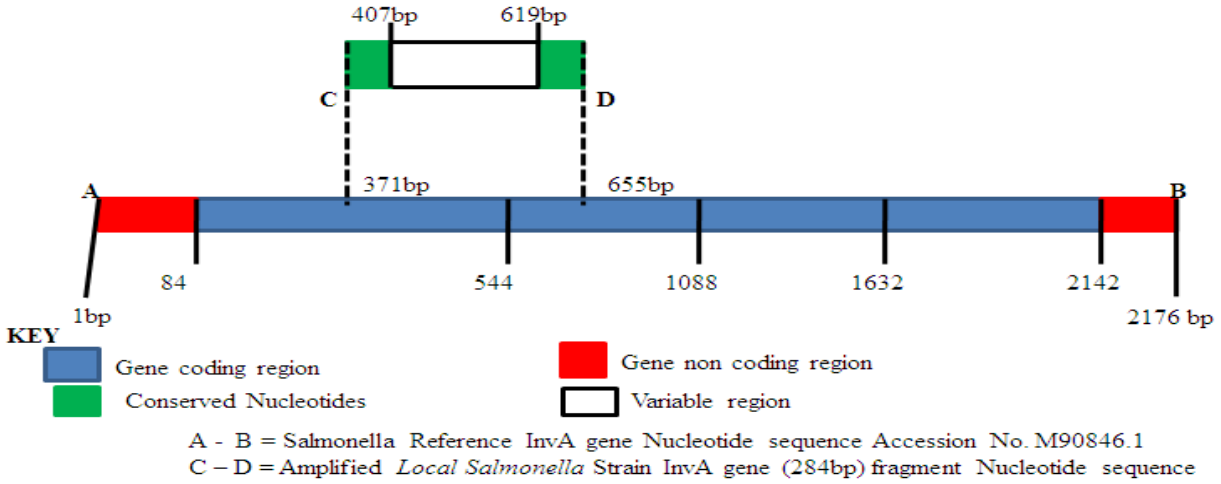


Figure 4-9: Diagrammatic representation of the location of the amplified *Salmonella inv*A-284 bp gene sequences compared with a complete *S.* Typhimurium *inv*A gene (Accession number M90846.1).

Table 4-19: Comparison of nucleotide substitution in the *inv*A-284bp gene fragment of local *Salmonella* strains with *Salmonella* strains from GeneBank

Sequence type	Serotype	Host	Positions of the nucleotide mutations in *inv*A gene sequences								
			40	88	121	133	210	211	220	238	250
1	Consensus sequence		C	C	C	C	A	T	T	C	G
	*S. enterica*_1A1XB (Egypt_CHK)	Chicken	C	C	C	C	A	T	T	C	G
	S. houtenae.ST22 (CDC)	Chicken	C	C	C	C	A	T	T	C	G
	S. Enteritidis.B10 (ZM_HSE) *	Horse	C	C	C	C	A	T	T	C	G
	S. Garoli.3 (LPD) *	Leopard	C	C	C	C	A	T	T	C	G
	S. Enteritidis.WF1-3 (ZM_CHK) *	Chicken	C	C	C	C	A	T	T	C	G
	Salmonella spp.P19 (ZM_CHK) *	Chicken	C	C	C	C	A	T	T	C	G
	S. Sendai.P21A (ZM.CHK) *	Chicken	C	C	C	C	A	T	T	C	G
	S. Ruanda.P12 (ZM_CHK) *	Chicken	C	C	C	C	A	T	T	C	G
2	*S.* Heidelberg.670 (ZM.CTL) *	Cattle	C	C	C	C	A	T	C	C	A
	*S. enterica*_58BEhB (Egyt_CHK)	Chicken	C	C	C	C	A	T	C	C	A
	*S.*Hadar.B1 (ZM_CHK) *	Chicken	C	C	C	C	A	T	C	C	A
	S. Typhimurium.FORC_020 (S.KOREA)	NK	C	C	C	C	A	T	C	C	A
3	*S. chardan*.B7 (ZM_CHK) *	Chicken	C	C	C	C	A	T	C	T	G
	S. bredeney.KC14RD (AUST_CHK)	Chicken	C	C	C	C	A	T	C	T	G
4	*S. typhimurium*.1STYINVA	NK	C	C	T	C	A	T	C	C	G
5	*S. choleraesuis*.1GHPS1 (China_CHK)	Chicken	C	T	C	C	A	T	C	C	G
6	*S. pomona*.SI-I (ZM_SBL) *	Sable	C	C	C	C	A	T	T	T	G
7	*S. ruanda*.P13e (ZM_CHK) *	Chicken	C	C	C	C	G	A	T	C	G
8	*S. stockholm*.B5 (ZM_CHK) *	Chicken	G	C	C	T	A	T	C	C	G
9	*S. enteritidis*.EC20110222 (CANADA)[#]	NK	C	C	C	G	T	G	G	T	G

Key: A=Adenine, C=Cytosine, G=Guanine, T=Thymine, CHK=Chicken, CTL=Cattle, HSE=Horse, LPD=Leopard, NK=Not known, *=Local *Salmonella* strains, # = several point mutations than indicated in the Table 4-18.

On the other hand, *Salmonella* strains isolated from domestic animals and wildlife were represented in six sequence types (Table 4-20).

Table 4-20: Distribution of *inv*A nucleotide sequence types among the local *Salmonella* strains isolated from domestic animals and wildlife (*n* = 12)

Sequence Type	Serotype	Positions of the nucleotide mutations in *inv*A gene sequences								
		40	88	121	133	210	211	220	238	250
	Consensus sequence	C	C	C	C	A	T	T	C	G
1	*S.* Garoli.3 (LPD) * *	C	C	C	C	A	T	T	C	G
	S. Enteritidis.B10 (ZM_HSE) **	C	C	C	C	A	T	T	C	G
	S. Enteritidis.WF1-3 (ZM_CHK) **	C	C	C	C	A	T	T	C	G
	Salmonella spp.P19 (ZM_CHK)	C	C	C	C	A	T	T	C	G
	S. Sendai.P21A (ZM.CHK)	C	C	C	C	A	T	T	C	G
	S. Ruanda.P12 (ZM_CHK) * *	C	C	C	C	A	T	T	C	G
2	*S.* Heidelberg.670 (ZM.CTL)	C	C	C	C	A	T	C	C	A
	S. Hadar.B1 (ZM_CHK)	C	C	C	C	A	T	C	C	A
3	*S.* Pomona.SI-I (ZM_SBL)	C	C	C	C	A	T	T	T	G
4	*S.* Ruanda.P13e (ZM_CHK) **	C	C	C	C	G	A	T	C	G
5	*S.* Stockholm.B5 (ZM_CHK) **	G	C	C	T	A	T	C	C	G
6	*S.* Chardan.B7 (ZM_CHK)	C	C	C	C	A	T	C	T	G

Key: A=Adenine, C=Cytosine, G=Guanine, T=Thymine, CHK=Chicken, CTL=Cattle, HSE=Horse, LPD=Leopard, NK=Not known, **= Local *Salmonella* strains in possession of *spv* genes.

Observed that 50 percent of the local strains (*S.* Garoli, *S.* Enteritidis.B10, *S.* Enteritidis.WF1-3, *Salmonella* spp, *S.* Sendai and *S.* Ruanda) were designated sequence type 1, with 100 percent conserved sequence chain. These strains were indistinguishable from the reference strains with accession no. KJ718884.1 (*S. enterica*), and DQ644627.1 (*S. houtenae*.ST22) from Egypt and CDC respectively.

The sequence variations were detected in 50 percent *Salmonella* strains isolated from the local domestic animals (n = 5) and wildlife (n = 1). In Table 4-20, shows transition substitution along the sequence chains belonging to five local *Salmonella* serotypes at the base pairs number; C 133 T (*S.* Stockholm), T 220 C (*S.* Heidelberg), T 220 C (*S.* Hadar), T 220 C (*S.* Stockholm), T 220 C (*S.* Chardan), C 238 T (*S.* Pomona), and G 250 A (*S.* Heidelberg). While transversion substitution was identified in two *Salmonella* serotypes at base pairs numbers; C 40 G (*S.* Stockholm), A 210 C (*S.* Ruanda.13e), T 211 A (*S.* Ruanda.13e).

Homology percentage of nucleotide sequences of the *inv*A gene of *Salmonella* serotypes revealed nucleotide similarities of 98~100 percent among the 12 local strains isolated from domestic animals and wildlife (Table 4-21).

Table 4-21: Homology percentage of nucleotide sequence of *inv*A-284bp of *Salmonella* serotypes isolated from domestic, and wildlife animals in Zambia.

Isolate No.	Isolates from domestic animals and wildlife (*n* = 12)											
	5	7	8	9	10	11	12	13	14	15	16	17
5	X	99	98	99	99	98	99	100	98	99	99	99
7	99	X	99	100	100	99	100	99	98	99	100	100
8	98	99	X	99	99	98	99	98	98	99	99	99
9	99	100	99	X	100	99	100	99	98	99	100	100
10	99	100	99	100	X	99	100	99	98	99	100	100
11	98	99	98	99	99	X	99	98	98	98	99	99
12	99	100	99	100	100	99	X	99	98	99	100	100
13	100	99	98	99	99	98	99	X	98	99	99	99
14	98	98	98	98	98	98	98	98	X	98	98	98
15	99	99	99	99	99	98	99	99	98	X	99	99
16	99	100	99	100	100	99	100	99	98	99	X	100
17	99	100	99	100	100	99	100	99	98	99	100	X

Key: Local strains

5	*S.* Heidelberg.670 (ZM.CTL) *	12	*Salmonella* spp.P19 (ZM_CHK) *
7	*S.* Garoli.3 (LPD) *	13	*S.* Hadar.B1 (ZM_CHK) *
8	*S.* Pomona.SI-I (ZM_SBL) *	14	*S.* Stockholm.B5 (ZM_CHK) *
9	*S.* Enteritidis.B10 (ZM_HSE) *	15	*S.* Chardan.B7 (ZM_CHK) *
10	*S.* Enteritidis.WF1-3 (ZM_CHK) *	16	*S.* Sendai.P21A (ZM.CHK) *
11	*S.* Ruanda.P13e (ZM_CHK) *	17	*S.* Ruanda.P12 (ZM_CHK) *

Sequence comparison of the *inv*A gene of local *Salmonella* strains with eight reference strains (*S. enterica* subspp. *enterica* (KJ718884.1), *S. houtenae* (DQ644627.1), *S.* Choleraesuis (EU348367.1), *S.* Typhimurium (M90846.1), *S. enterica* subspp. *enterica* (KJ718878.1), *S.* Typhimurium (CP012144.1), *S.* Bredeney (KP279306.1), and *S.* Enteritidis (CP007323.1) from the GenBank showed 74.0~100.0 percent identity (Table 4-22).

Table 4-22: Homology percentage of nucleotide sequence of invA-284bp gene of local *Salmonella* strains isolated from domestic animals and wildlife in Zambia in comparison with other published *Salmonella* strains from the GeneBank.

Isolate No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	X	98	98	99	99	99	100	99	100	100	99	100	99	98	99	100	100	98	75	98
2	98	X	99	99	98	98	98	98	98	98	97	98	98	98	98	98	98	98	74	98
3	98	99	X	99	98	98	98	98	98	98	97	98	98	98	98	98	98	98	74	98
4	99	99	99	X	98	98	99	98	99	99	98	99	98	98	98	99	99	98	75	98
5	99	98	98	98	X	100	99	98	99	99	98	99	100	98	99	99	99	98	74	99
6	99	98	98	98	100	X	99	98	99	99	98	99	100	98	99	99	99	98	74	99
7	100	98	98	99	99	99	X	99	100	100	99	100	99	98	99	100	100	98	75	98
8	99	98	98	98	98	98	99	X	99	99	98	99	98	98	99	99	99	98	75	98
9	100	98	98	99	99	99	100	99	X	100	99	100	99	98	99	100	100	98	75	98
10	100	98	98	99	99	99	100	99	100	X	99	100	99	98	99	100	100	98	75	98
11	99	97	97	98	98	98	99	98	99	99	X	99	98	98	98	99	99	97	75	97
12	100	98	98	99	99	99	100	99	100	100	99	X	99	98	99	100	100	98	75	98
13	99	98	98	98	100	100	99	98	99	99	98	99	X	98	99	99	99	98	74	99
14	98	98	98	98	98	98	98	98	98	98	98	98	98	X	98	98	98	98	74	98
15	99	98	98	98	99	99	99	99	99	99	98	99	99	98	X	99	99	99	75	98
16	100	98	98	99	99	99	100	99	100	100	99	100	99	98	99	X	100	98	75	98
17	100	98	98	99	99	99	100	99	100	100	99	100	99	98	99	100	X	98	75	98
18	98	98	98	98	98	98	98	98	98	98	97	98	98	98	99	98	98	X	75	99
19	75	74	74	75	74	74	75	75	75	75	75	75	74	74	75	75	75	75	X	75
20	98	98	98	98	99	99	98	98	98	98	97	98	99	98	98	98	98	99	75	X

Key:

1 *S. enterica*_1A1XB (Egypt_CHK)
2 *S.* Typhimurium. 1STYINVA
3 *S.* Choleraesuis. 1GHPS1 (China_CHK)
4 *S. houtenae*.ST22 (CDC)
5 *S.* Heidelberg.670 (ZM.CTL) *
6 *S. enterica*_58BEhB (Egyt_CHK)
7 *S.* Garoli.3 (LPD) *
8 *S.* Pomona.SI-I (ZM_SBL) *
9 *S.* Enteritidis.B10 (ZM_HSE) *
10 *S.* Enteritidis.WF1-3 (ZM_CHK) *
11 *S.* Ruanda.P13e (ZM_CHK) *
12 *Salmonella* spp.P19 (ZM_CHK) *
13 *S.* Hadar.B1 (ZM_CHK) *
14 *S.* Stockholm.B5 (ZM_CHK) *
15 *S.* Chardan.B7 (ZM_CHK) *
16 *S.* Sendai.P21A (ZM.CHK) *
17 *S.* Ruanda.P12 (ZM_CHK) *
18 *S.* Bredeney.KC14RD (AUST_CHK)
19 *S.* Enteritidis.EC20110222 (CANADA)#
20 *S.* Typhimurium.FORC_020 (S.KOREA)

4.6.6 Amino acid sequences of *inv*A gene of the *Salmonella* strains isolated from domestic animals and wildlife

The nucleotide substitution resulted into amino acid substitution or silent changes or non-significant changes (Figure 4-10).

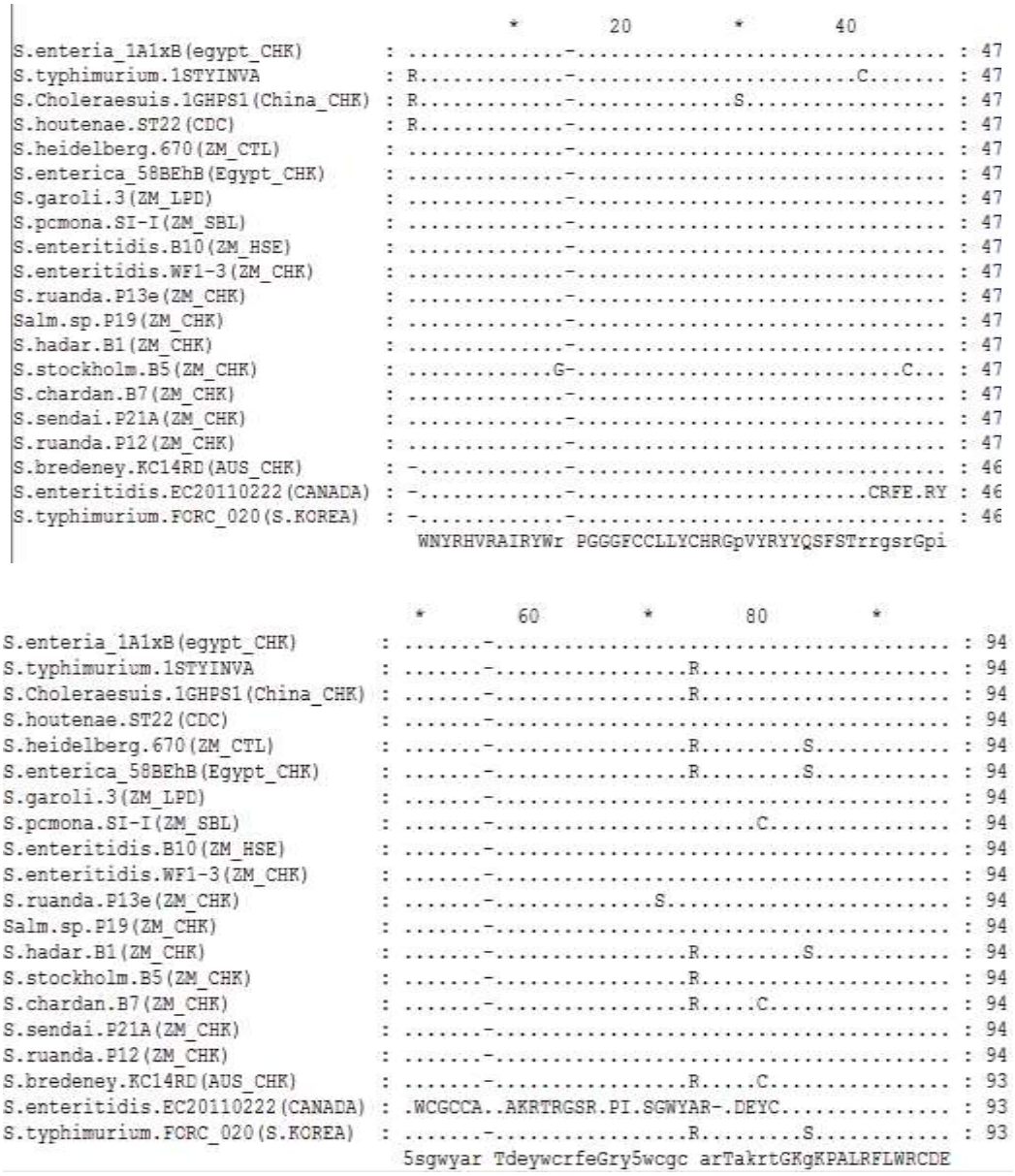


Figure 4-10: Multiple alignment of amino acid sequences of *inv*A-284 bp sequences of local *Salmonella* serotypes strains isolated from domestic animals and wildlife compared with *Salmonella* strains published sequence on GenBank.

Amino acid substitution was detected between nucleotide numbers 14 to 84 on the *inv*A 284 bp chain in both local and reference strains (Table 4-23). For instance significant changes were observed at the position A 74 R (*S.* Heidelberg, alanine to arginine), R 80 C (*S.* Pomona, arginine to cystein), A 74 R (*S.* Hadar, alanine to arginine), R 14 G (*S.* Stochholm.B5, arginine to glycine), R 45 C (*S.* Stockholm, arginine to cysteine), A 74 R (*S.* Stockholm, alanine to arginine), A 74 R (*S.* Chardan, alanine to arginine), R 80 C (*S.* Chardan, arginine to cysteine), C 71 S (*S.* Hadar, cysteine to serine), and A 74 R (*S.* Hadar, glutamine to arginine) for local strains. In the case of reference strains substitution was detected at position R 41 C (*S.* Typhimurium.ISTYIVA, arginine to cysteine), A 74 R (*S.* Typhimurium.ISTYINVA, alanine to arginine), P 30 S (*S.* Choleraesuis, proline to serine), A 74 R (*S.* Choleraesuis, alanine to arginine), A 74 R (*S. enterica*.58BEhb, alanine to arginine), A 74 R (*S.* Bredney, alanine to arginine), R 80 C (*S.* Bredney, arginine to cysteine), R 45 E (*S.* Enteritidis.EC20, arginine to glutamic acid), C 71 G (*S.* Enteritidis.EC20, cysteine to glycine), R 80 Y (*S.* Enteritidis.EC20, arginine to tyrosine), and A 74 R (*S.* Typhimurium.FORC_20, alanine to arginine). Whereas, other amino acids substitution yielded into silent substitution for local strains at position G 84 S (*S.* Heidelberg, glutamine to serine), C 71 S (*S.* Ruanda.13e, cysteine to serine), and G 84 (*S.* Hadar, glutamine to serine); for reference strains Q 84 S (*S. enterica*.58BEhb, glutamine to serine), Q 84 S (*S.* Typhimurium. FORC_20, glutamine to serine).

Table 4-23: Comparison of Amino acid substitution in *inv*A-284bp gene sequences of local *Salmonella* strains with *Salmonella* strains from GeneBank

Serotype	Host	Positions of the Amino acid in *invA* gene sequences							
		14	30	41	45	71	74	80	84
Consensus sequence	-	R	P	R	R	C	A	R	Q
S. enterica_1A1XB (Egypt_CHK)	Chicken	R	P	R	R	C	A	R	Q
S. Typhimurium.1STYINVA	NK	R	P	C	R	C	R	R	Q
S. Choleraesuis.1GHPS1 (China_CHK)	Chicken	R	S	R	R	C	R	R	Q
S. houtenae.ST22 (CDC)	Chicken	R	P	R	R	C	A	R	Q
S. Heidelberg.670 (ZM.CTL) *	Cattle	R	P	R	R	C	R	R	S
*S. enterica*_58BEhB (Egyt_CHK)	Chicken	R	P	R	R	C	R	R	S
S. Garoli.3 (LPD) *	Leopard	R	P	R	R	C	A	R	Q
S. Pomona.SI-I (ZM_SBL) *	Sable	R	P	R	R	C	A	C	Q
S. Enteritidis.B10 (ZM_HSE) *	Horse	R	P	R	R	C	A	R	Q
S. Enteritidis.WF1-3 (ZM_CHK) *	Chicken	R	P	R	R	C	A	R	Q
S. Ruanda.P13e (ZM_CHK) *	Chicken	R	P	R	R	S	A	R	Q
Salmonella spp.P19 (ZM_CHK) *	Chicken	R	P	R	R	C	A	R	Q
S. Hadar.B1 (ZM_CHK) *	Chicken	R	P	R	R	C	R	R	S
S. Stockholm.B5 (ZM_CHK) *	Chicken	G	P	R	C	C	R	R	Q
S. Chardan.B7 (ZM_CHK) *	Chicken	R	P	R	R	C	R	C	Q
S. Sendai.P21A (ZM.CHK) *	Chicken	R	P	R	R	C	A	R	Q
*S.*Ruanda.P12 (ZM_CHK) *	Chicken	R	P	R	R	C	A	R	Q
*S.*Bredeney.KC14RD (AUST_CHK)	Chicken	R	P	R	R	C	R	C	Q
*S.*Enteritidis.EC20110222 (CANADA)#	NK	R	P	R	E	G	A	Y	Q
*S.*Typhimurium.FORC_020 (S.KOREA)	NK	R	P	R	R	C	R	R	S

Key: R = Arginine, P = Proline, C = Cysteine, A = Alanine, Q = Glutamine, G = Glycine, S = Serine, NK = Not known, - = No structural change, CHK = Chicken, CTL = Cattle, HSE = Horse, LPD = Leopard, * = Local *Salmonella* strains, # = More protein structural changes than indicated in the Table.

The homology percentage of amino acid sequences showed 96~100 percent similarities among *Salmonella* strains isolated from domestic animals and wildlife (Table 4-24).

Table 4-24: Homology percentage of translated Amino Acid sequence of *inv*A-284bp gene in *Salmonella* serotypes isolated from domestic, and wildlife animals in Zambia.

Isolate No.	Isolates from domestic animals and wildlife (*n* = 12)											
	5	7	8	9	10	11	12	13	14	15	16	17
5	X	97	96	97	97	96	97	100	96	97	97	97
7	100	X	98	100	100	98	100	97	96	97	100	100
8	97	98	X	98	98	97	98	96	95	98	98	98
9	96	100	98	X	100	98	100	97	96	97	100	100
10	97	100	98	100	X	98	100	97	96	97	100	100
11	97	98	97	98	98	X	98	96	95	96	98	98
12	96	100	98	100	100	98	X	97	96	97	100	100
13	97	97	96	97	97	96	97	X	96	97	97	97
14	96	96	95	96	96	95	96	96	X	96	96	96
15	97	97	98	97	97	96	97	97	96	X	97	97
16	97	100	98	100	100	98	100	97	96	97	X	100
17	97	100	98	100	100	98	100	97	96	97	100	X

Key: Local strains

5	*S.* Heidelberg.670 (ZM.CTL) *	12	*Salmonella* spp.P19 (ZM_CHK) *
7	*S.* Garoli.3 (LPD) *	13	*S.* Hadar.B1 (ZM_CHK) *
8	*S.* Pomona.SI-I (ZM_SBL) *	14	*S.* Stockholm.B5 (ZM_CHK) *
9	*S.* Enteritidis.B10 (ZM_HSE) *	15	*S.* Chardan.B7 (ZM_CHK) *
10	*S.* Enteritidis.WF1-3 (ZM_CHK) *	16	*S.* Sendai.P21A (ZM.CHK) *
11	*S.* Ruanda.P13e (ZM_CHK) *	17	*S.* Ruanda.P12 (ZM_CHK) *

Whereas, 60~100 percent similarities were observed in *Salmonella* strains isolated from domestic animals and wildlife, compared with reference strains on the GenBank (Table 4-25). The strains *S.* Garoli.3 (ZM), *S.* Enteritidis.B10 (ZM), *S.* Enteritidis.WF1-3 (ZM), *Salmonella* spp. (ZM), *S.* Sendai (ZM), *S.* Ruanda (ZM), and the reference strain *S. enterica* accession no. KJ718884.1 (Egypt) showed 100.0% identical same as the nucleotide sequences.

Table 4-25: Homology percentage of Amino Acid sequences of *inv*A-284bp gene of local *Salmonella* strains isolated from domestic animals and wildlife in Zambia in comparison with other published *Salmonella* strains from the GeneBank.

Isolate No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20
1	X	96	96	98	97	97	100	98	100	100	98	100	97	96	97	100	100	96	61	96
2	96	X	97	97	96	96	96	95	96	96	95	96	96	95	96	96	96	96	60	96
3	96	97	X	97	96	96	96	95	96	96	95	96	96	95	96	96	96	96	60	96
4	98	97	97	X	96	96	98	97	98	98	97	98	96	95	96	98	98	96	60	96
5	97	96	96	96	X	100	97	96	97	97	96	97	100	96	97	97	97	96	60	98
6	97	96	96	96	100	X	97	96	97	97	96	97	100	96	97	97	97	96	60	98
7	100	96	96	98	97	97	X	98	100	100	98	100	97	96	97	100	100	96	61	96
8	98	96	95	97	96	96	98	X	98	98	97	98	96	95	98	98	98	97	61	95
9	100	96	96	98	97	96	100	98	X	100	98	100	97	96	97	100	100	96	61	96
10	100	96	96	98	97	97	100	98	100	X	98	100	97	96	97	100	100	96	61	96
11	98	95	95	97	96	96	98	97	98	98	X	98	96	95	96	98	98	95	61	95
12	100	96	96	98	97	96	100	98	100	100	98	X	97	96	97	100	100	96	61	96
13	97	96	96	96	100	100	97	96	97	97	96	97	X	96	97	97	97	96	60	98
14	96	95	95	95	96	96	96	95	96	96	95	96	96	X	96	96	96	95	60	95
15	97	96	96	96	97	96	97	98	97	97	96	97	97	96	X	97	97	96	61	96
16	100	96	96	98	97	97	100	98	100	100	98	100	97	96	97	X	100	96	61	96
17	100	96	96	98	97	97	100	98	100	100	98	100	97	96	97	100	X	96	61	96
18	96	96	96	96	96	96	96	97	96	96	95	96	96	95	96	96	96	X	61	95
19	61	60	60	60	60	61	61	61	61	61	61	60	60	60	61	61	61	61	X	60
20	96	96	96	96	98	98	96	95	96	96	95	96	98	95	96	96	96	95	60	X

Key:

1 *S. enterica*_1A1XB (Egypt_CHK)
2 *S.* Typhimurium. 1STYINVA
3 *S.* Choleraesuis. 1GHPS1 (China_CHK)
4 *S. houtenae*.ST22 (CDC)
5 *S.* Heidelberg.670 (ZM.CTL) *
6 *S. enterica*_58BEhB (Egyt_CHK)
7 *S.* Garoli.3 (LPD) *
8 *S.* Pomona.SI-I (ZM_SBL) *
9 *S.* Enteritidis.B10 (ZM_HSE) *
10 *S.* Enteritidis.WF1-3 (ZM_CHK) *
11 *S.* Ruanda.P13e (ZM_CHK) *
12 *Salmonella* spp.P19 (ZM_CHK) *
13 *S.*Hadar.B1 (ZM_CHK) *
14 *S.* Stockholm.B5 (ZM_CHK) *
15 *S.* Chardan.B7 (ZM_CHK) *
16 *S.* Sendai.P21A (ZM.CHK) *
17 *S.* Ruanda.P12 (ZM_CHK) *
18 *S.* Bredeney.KC14RD (AUST_CHK)
19 *S.* Enteritidis.EC20110222 (CANADA)#
20 *S.* Typhimurium.FORC_020 (S.KOREA)

4.6.7 Phylogenetic tree analysis based on the nucleotide sequences of *inv*A-284bp gene fragment of *Salmonella* strains from domestic animals, and wildlife

Twelve selected sequences of *Salmonella inv*A -284pb gene from domestic animals (*S.* Sendai, *Salmonella* spp., *S.* Stockholm, *S.* Heidelberg, *S.* Hadar, *S.* Chardan with one each; *S.* Ruanda, and *S.* Enteritidis with two each) and wildlife (*S.* Garoli and *S.* Pomona) were compared with 8 published sequences from the GeneBank; *S. enterica* subsp. (Accession no. KJ718884.1-Egypt), *S. houtenae* (Accession no. DQ644627.1-CDC), *S.* Choleraesuis (Accession no. EU348367.1-China), *S.* Typhimurium (Accession no. M90846.1), *S. enterica* subsp. (Accession no. KJ718878.1-Egypt), *S.* Typhimurium (Accession no. CP012144.1-South Korea), *S.* Bredeney (KP279306.1-Australia), and *S.* Enteritidis (CP007323.1-Canada).

Phylogenetic analysis of nucleotide sequences grouped *Salmonella* strains into two major clades, A and B, and clade A was divided into two sub clades I and II (Figure 4-11). The sub Clade I was further divided in six small groups Ia – If. Clade B was represented by the Canadian strain *S.* Enteritidis.EC2011022 (CP007323.1). Of 20 *Salmonella* strains sequences, 40 percent were detected in Clade Ia (consisting of six strains *S.* Sendai.P21A, *S.* Ruanda.P12, *Salmonella* spp.P19, *S.* Enteritidis.WF1-3, *S.* Enteritidis.B10, *S.* Garoli.3 from domestic animals and wildlife; and two reference strains *S. houtenae*-DQ644627.1-CDC, and *S. enterica* -KJ718884.1-Egypt). Clade Ia was characterized by strains that were highly conserved, followed by Clade If with 20 percent (4/20) strains (*S.* Heidelberg.670, and *S.* Hadar.B1, and two reference strains *S. enterica* subsp.-Accession no. KJ718878.1-Egypt, and *S.* Typhimurium Accession no. CP012144.1-South Korea) closely related (99~100 percent), and Clade IIb with 10 percent (2/20) strains *S.* Chardan.B7, and *S.* Bredeney.KC14RD from Australia. Interestingly sub Clades Ib, Ic and IIa were represented by single local strain *S.* Ruanda.P13e, *S.* Stockholm.B5 and *S.* Pomona respectively. However, the strain *S.* Enteritidis. EC20110222 from Canada belonging to Clade B is distantly related to other strains.

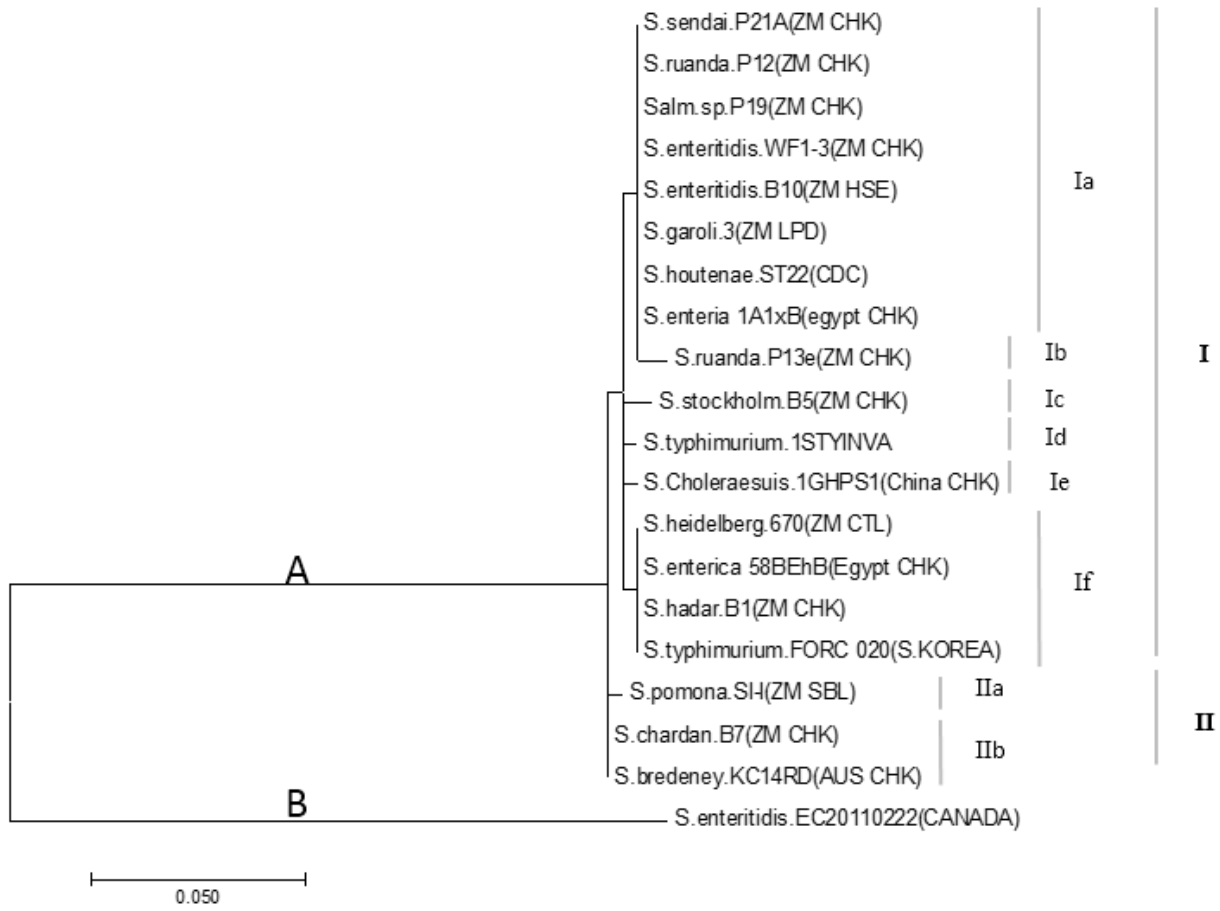


Figure 4-11: Molecular Phylogenetic analysis by Maximum Likelihood method of nucleotide sequences of *inv*A gene of *Salmonella* serotypes isolated from domestic animals and wildlife in Zambia compared with other published *Salmonella* strains from GeneBank.

4.6.8 Analysis of phylogenetic tree based on the amino acid sequences of *inv*A-284bp gene fragment of *Salmonella* strains from domestic animals, and wildlife B

The phylogenetic analysis of deduced amino acid (Figure 4-12), showed no significant difference from the nucleotide sequence.

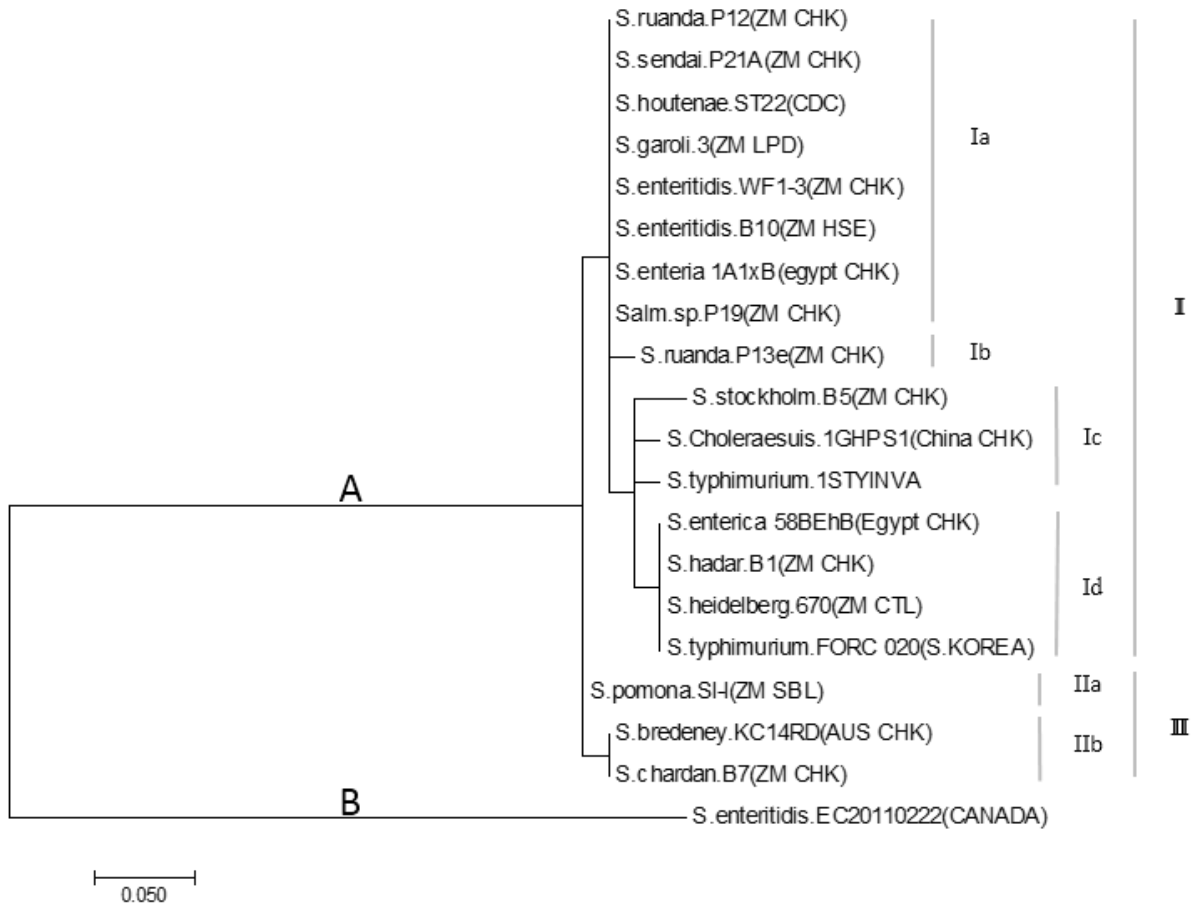


Figure 4-12: Molecular Phylogenetic analysis by Maximum Likelihood method of amino acid sequences of *inv*A gene of *Salmonella* serotypes isolated from domestic animals and wildlife in Zambia, compared with other published *Salmonella* strains from GeneBank.

CHAPTER FIVE

DISCUSSION

5.1 Isolation and identification of *Salmonella* from domestic animals and wildlife species

Non-typhoidal *Salmonella* has been implicated extensively in human invasive salmonellosis infections across the African regions (Muthumbi *et al*., 2015; Marks *et al.*, 2017). Although the risk factors of salmonellosis are known as being undernourishment in children, immunosuppression, malaria infections and other underlying health conditions such as cancer, the role of animal reservoirs is unknown. Our present study aimed to determine the prevalence of *Salmonella* from domestic animals and wildlife; characterize the strains into serogroups, serotypes, fermentative groups, resistotypes, beta-lactamase activity types, virulence gene types as well as to compare the *inv*A gene sequence of *Salmonella* strains with refence sequences on the Gene-Bank. Our study detected 34 *Salmonella* strains that belonged to 12 serotypes isolated from domestic animals and wildlife species (p = 0.002, 95 percent, CI). Further, our investigation identified that 34 *Salmonella* strains belonged to 4 distinct serogroups; B (O: 4), C1 (O: 6, 7), D1 (O: 9), and non-reactive (NR) (P<0.001, 95 percent, CI). The study showed, 60 percent (20/34) strains were susceptible, while 28 percent (10/34) and 12 percent (4/34) were resistant and intermediate respectively, to the 17 tested antibiotics used in this investigation (p = 0.1, 95 percent CI) with a minimum MAR Index > 0.02. None of the *Salmonella* strains was a carrier of an extended broad-spectrum beta-lactamases (ESBL) phenotype to third generation cephalosporins. The carriage of plasmid encoded *spv* genes among the strains was at the following rates 58.8 percent (20/34), 55.9 percent (19/34) and 58.8 percent (20/34) for *spv*A, *spv*B and *spv*C, respectively (p = 0.001, 95 percent, CI). The homology percentage of *inv*A nucleotide sequence (74.0 ~ 100.0) and amino acid sequence (60.0 ~ 100.0), showed genotypic differences between the strains isolated from domestic animals and wildlife, and the published sequences on the Gene-Bank. These finding suggested that there is a diversity of *Salmoella* strains circulating in domestic animals and wildlife.

5.1.1 Prevalence of *Salmonella* in faeces of wildlife

The overall pooled recovery rate (0. 83 percent) of *Salmonella* in the faecal samples of seemingly healthy free-ranging wildlife species was slightly lower than reported by Renter *et al*. (2006) who detected one percent occurrence rate of *Salmonella* in free-ranging white-tailed deer (*Odocoileus virginianus*) in Nebraska; 1.6% in faecal samples collected from free-ranging California Sea Lions (*Zalophus californianus*) along the California Coast (Berardi *et al*., 2014). The lower recovery rate of *Salmonella* in the present study might be due to variariations in methods used to process the environmental faecal samples. In other studies, 25 percent recovery rate was reported in captive maned wolves (*Chrysocyon brachyurus*, Illiger 1815) in the State of São Paulo, Brazil (Gilioli and Silva, 2000); 21.1 percent was recovered in free-ranging small Asian mongooses (*Herpestes Javanicus*) at the east-coast site and 26.7 percent at the south-coast site of Barbodos, West Indies (Kamara *et al.,* 2014); 24.9 percent for faecal samples of different wild and exotic animal species in captivity in Ohio, USA (Farias *et al*., 2015); *Salmonella* organisms were isolated from 88 of 106 (83 percent) faecal samples of captive green iguanas (Burnham *et al*., 1998) and 78.2 percent (36/46), 75 percent (3/4) and 75 percent (3/4) from faecal samples of giraffes, cranes and raccoons respectively in captivity in Ohio, USA (Farias *et al*., 2015). However, high recovery rate of *Salmonella* in healthy Zoo animals can be attributed to shedding of the microbes in faeces and as such these animals may be acting as carriers for spreading the microbes (Awandkar *et al*., 2018). Further, one isolate of *Salmonella* was included in the present study isolated from a sick animal that was submitted for routine diagnosis. Salmonellosis infection has been reported in wildlife worldwide (Locke *et al*., 1974; van Andel, *et al.,* 2015; CDFW, 2016). Similarly, *Salmonella* spp have been isolated from a wide range of wildlife reservoirs (Percipalle *et al*., 2011; Chiari *et al*., 2014). Wildlife can transmit *Salmonella* to other animals and humans (Reeuwijk *et al*., 2009; Lal *et al.,* 2012).

5.1.2 Prevalence of *Salmonella* in faeces of domestic animals

The prevalence of *Salmonella* in the faecal samples of apparently healthy cattle (0.32 percent) in this study indicated a low rate of asymptomatic carriage of *Salmonella* among the population of pastoral cattle. Domestic animals can sometimes appear healthy even when they are carrying zoonotic pathogens that can make people sick. On the other hand, the prevalence rate of *Salmonella* strains from the faeces of cattle was higher (52 percent) in Burkina Faso (Kagambega

et al., 2013), 2.6 percent in cows in Nairobi (Nyabundi *et al*., 2017) and 9.1 percent was detected in animal feedlots in the United Sates (Dargatz *et al*., 2016). The levels of *Salmonella* in the faeces of cattle can vary depending on different factors such as the study design, geographical variation and the nature of the production system (Al-Saigh *et al*., 2004). For instance, the animals in the feedlot would be said to have a higher occurrence rate as they are kept under the intensive management system. Interestingly, none of the faecal samples from apparently healthy horses in the present study yielded *Salmonella* strains, which was at variance with a similar studies in the USA (Traub-Dargatz *et al*., 2000) and Southern Iran (Ranjbry *et al*., 2013). However, the only *Salmonella* isolate included in the present study was isolated from the diseased horse. This is in tandem with the isolation of *Salmonella* spp. isolates from horses exhibiting various clinical symptoms such as depression, anorexia and diarrheoa (Walker *et al*, 1991), abortion (Madic *et al*., 1997), septicemia (Schott *et al*., 2001), and colic infection (Jay-Russell *et al*., 2014).

5.1.3 Biochemical and molecular identification of *Salmonella*

In the present study, all 34 *Salmonella* isolates complied to the "Gold Standard Method"for biochemical identification of *Salmonella* (D'Aoust *et al*., 2007; Abulreesh, 2012). All *Salmonella* isolates were further confirmed by targeting *inv*A gene. Invasion protein A gene contains a unique nucleotide sequence that is targeted to identify the bacteria of *Salmonella* genus level as this gene is used as an international standard parameter and because of its specificity (Malorny *et al*., 2003; Sunar *et al*., 2010; Chaudhary *et al*., 2015). Our results revealed that all strains were positive for *inv*A virulence gene located on the *Salmonella* pathogenicity island-1 (SPI-1) (Table 4-15). This finding indicated their potential to penetrate the intestinal lining of their host and capable of causing disease in domestic animals and wildlife (Rahn *et al*., 1992; Hur *et al.*, 2011; Gole *et al.*, 2013; Oludairo *et al.*, 2013). Our results were in consonance with the work by other investigators, who indicated 100 percent presence of *inv*A gene in all *Salmonella* isolates they investigated (Al-Harthi *et al*., 2012; Karmi, 2013; Ikuno *et al*., 2014; Ezzat *et al*., 2014; Firouzi, *et al*., 2014; Abd-Elghany *et al*., 2015; Chaudhary *et al*., 2015; Susmita *et al*., 2017; Fardsanei *et al.*, 2018; Lan *et al*., 2018). Our findings also correlate with the detection of *inv*A gene in *Salmonella* isolates from captive wildlife (lion, stripped hyna, chimpanzee, African hawk eagle and peafowl) in the work conducted by Oludairo *et al.* (2013). Previous studies in Zambia, also

demonstrated the presence of *inv*A gene in *Salmonella* strains isolated from beef and poultry carcasses (Hang'ombe *et al.,* 2008). The detection rate of *inv*A gene in *Salmonella* isolates from different sources by other investigators varied from 33.0 to 100.0 percent (Table 2-11 and 2-12).

On the other hand, PCR results using the 16S rRNA primer set revealed that 97.1 percent strains yielded 16S rRNA 1400 bp amplicon, except one strain initially identified as *S.* Mbandaka from a dog was negative (Figure 4-4 and Table 4-15). The detection rate of 16S rRNA gene from *Salmonella* strains in the present study was lower than the work by Smith *et al*. (2015) who reported that 100 percent isolates were positive for 16S rRNA in Lagos, Nigeria. The lower detection rate in our study could have been attributed to technical error during the process of the test. The specific 16S rRNA gene is a widely used marker for identification of bacterial species (Amarantini *et al*., 2011; Taddele *et al*., 2011; Jeng *et al*., 2012; Anejo-Okopi *et al*., 2014; Srinivasan, *et al* 2015; Karatug *et al*., 2018).

Therefore, by integrating biochemical results of the strains with the presence of *inv*A, could lead to 100 percent *Salmonella* genus level identification. In this study, the PCR-*inv*A gene assay was considered more sensitive (Chaudhary *et al*., 2015; Fardsanei *et al.*, 2018) than the PCR-16S-rRNA method. The use of molecular assays in our study, reaffirms the importance of PCR method in terms of reliability as a diagnostic tool for confirmation of *Salmonella* genus (Oliveira *et al*., 2003; Nabil *et al*., 2008).

5.1.4 Detection of serogroups among *Salmonella* strains

The study revealed that 94.1 percent of 34 *Salmonella* strains reacted positively to polyvalent antiserum, except two strains from wildlife each from sable antelope and impala antelopes respectively, suggesting they were rough strains (Table 4-2). Further, our investigation identified that 34 *Salmonella* strains isolated from domestic animals and wildlife species belonged to 4 distinct serogroups; B (O: 4), C1 (O: 6, 7), D1 (O: 9), and non-reactive (NR) ($P<0.001$, 95 percent, CI) (Table 4-2), suggesting the diversity of *Salmonella* serogroups circulating in domestic animals and wildlife capable of transmitting the infection to humans or within animal community (Giannella, 1996; Fierer and Guiney, 2001; Eng *et al*., 2015). Our present study revealed *Salmonella* strains belonging to serogroup D1 (O: 9) were the most predominant (50.0

percent, 17/34) in circulation and were exclusively isolated from clinical samples of domestic animals with 94.1 percent (*n* = 16, chicken), and 5.9 percent (*n* = 1, horse). Serotypes carrying the O:4 and O:9 antigens are among the most prevalent NTS worldwide (Molla *et al*., 2003; Gunaydin, 2005; Elhadi *et al*., 2014; Naberhaus *et al.*, 2019).

According to Fierer and Guiney (2001), human infection are usually due to *Salmonella* strains that belong to serogroups; A, B, C, D and E. In the USA, serogroup C (O:6,7, O:6,8, or O:8 epitopes) is the most common serogroup, and the prevalence of serovars from this serogroup has been increasing in Europe and the United States over the last decade (Fuche *et al*., 2016). Further, serogroup C consists of highly diverse organisms among which 37 serotypes account for the majority of human cases (Fuche *et al*., 2016). In Saudi Arabia, 25.3 percent of human NTS cases, belonged to serogroup D1 and was the most prevalent, followed by serogroup B (19.6%), and serogroup C1 (18.9 percent) (Elhadi *et al*., 2013). Another study in Uganda revealed a prevalence rate of 52 percent serogroup D strains isolated from humans and animals (Kagirita *et al.*, 2017).

In our investigations, we report the detection of *Salmonella* strains belonging to serogroups B (*S.* Heidelberg), C1 (*S.* Mbandaka, *S.* Garoli), and D1 (*S.* Enteritidis) which have been incriminated in human salmonellosis (Lubwana, 1985; Evans *et al*., 2014; CDC 2014c; Gieraltowski *et al*., 2016; CDC, 2018b). In Africa, humans, domestic animals and wildlife tend to live in close vicinity, such that hygiene control of the environment as well as the food chain of animal origirin is not often guanteed. In light of this, animals can pose a serious public health risk in human-domestic animal-wildlife interface areas in Zambia.

5.1.5 Detection of serotypes among *Salmonella* strains

The Kauffmann-White serotyping scheme is the conventional method for primary characterization of *Salmonellae* into serotypes (Le Minor and Popoff, 1992). On the other hand, it is considered to be inappropriate method for determination of strain relatedness. Our investigation revealed 12 *Salmonella* serotypes circulating in domestic animals: (*n* = 9) (*S.* Heidelberg, *S.* Enteritids, *S.* Ruanda, *Salmonella* spp., *S.* Stockholm, *S.* Chardan, *S.* Sendai, *S.* Mbandaka and *S.* Hadar), and from wildlife: (*n* = 3) (*S.* Garoli, *S.* Pomona and *S.* Roan) (Table 4-

4). These findings, suggested that there is a diversity of serotypes circulating in domestic animals and wildlife. Further, these *Salmonella* serotypes were allocated into 7 categories based on the origin of the sample. Our finding revealed that serotypes were not distributed evenly between the sample categories. However, *S.* Enteritidis was common to two of sample categories from domestic animals: chicken (*n* = 15) and horse (*n* = 1). Futher, the study showed that *S.* Enteritidis was the most predominated, and was isolated from domestic animals. Based on our findings it seems that domestic animals can share the same *Salmonella* serotypes and potentially transmit some of them to humans.

Salmonella is a host adapted and non host adapted organism (Evangelopoulou *et al.,* 2013). None of the *Salmonella* serotype observed in this study was host specific, meaning they can infect a wide range of animal hosts including humans (Blanco, 2018). Currently there are more than 2500 different serotypes recognized (Feng *et al.,* 2012; Evangelopoulou *et al.,* 2013), and some can cause illnesses ranging from severe to milder illnesses (Roer *et al*., 2016).

5.1.6 Detection of *Salmonella* fermentative groups

Biotyping is considered to be an important method for separating strains of a species based on differences in the utility of substrates. This method has been widely used in the context of its application in studying the epidemiology of *Salmonella* infection (Poppe *et al*., 1993; Old *et al.,* 1999). In the present study, all 34 *Salmonella* isolates without exception utilized trehalose, maltose, melibiose, galactose, glucose, mannose, xylose, mannitol and dulcitol which is a typical biochemical reaction of *S. enterica* subspecies *enterica* (I) as described by Paccagnela, (2005), and Krieg and Holt, (1984) (Table 2-9 and Table 2-10 respectively). In contrast, all strains were exclusively defective towards utilization of rabitol and D-(+)-raffinose. However, incompatibilities were observed in the fermentation of arabinose, cellobiose, inositol and salicin; yielding decreased arabinose (20.6 percent, 7/34), and increased cellobiose (20.6 percent, 7/34), and salicin (2.9 percent, 1/34) (Table 4-5). On the other hand utilization of inositol is within the acceptable range (Table 2-8) **(**Krieg and Holt, 1984).

In the present study, we examined biochemically, these strains for phenotypic differences in the ability to utilize four substrates; arabinose, cellobiose, inositol and salicin. Therefore, we

describe fermentative groups and biotypes of the *Salmonella* strains within serotypes isolated from domestic animals and wildlife. Our findings revealed 8 different metabolic strategies (fermentative groups I-VIII) for utilization and acquisition of energy (Table 4-6). There was no significant association between *Salmonella* isolates and utilization of substrates (p = 0.01, 95 percent CI). Our results indicate that there is a diversity of unrelated serotypes among *Salmonella* strains isolated from domestic animals and wildlife. Overall, fermentative group I variant was the most predominant with 55.9 percent (19/34) of *Salmonella* strains from different animal hosts (leopard, horse and chickens), suggesting that these strains are more likely to be related. Further in this group, almost all serotypes in possession of *spv*ABC genes *S.* Enteritidis (n = 16), *S.* Ruanda (n = 2), *S.* Garoli (n = 1), failed to utilize arabinose, cellobiose, inositol, and salicin substrates in phenol red broth (Table 4-6), except one strain of *S.* Stockholm serotype, utilized arabinose and inositol. This finding suggested that they could have genes which encode the release or block specific hydrolyzing enzymes and the metabolic system become nonfunctional. On the other hand, a typical biochemical reaction of *S. enterica* sub spp. I is cellobiose negative and arabinose positive (Krieg and Holt, 1984). Further, our finding suggests that failure to utilize these substrates could be a useful marker for detection of epidemiologically related strains in possession of *spv* genes.

On the contrary, *S.* Stockholm strain that did not carry *spv* gene was unable to utilize arabinose, cellobiose, inositol and salicin substrates, suggesting a possible association between metabolic activity of arabinose and inositol substrates and the carriage of *spv*ABC genes. However, this observation may not be plausible, since only one strain was tested. Therefore, we recommend that further studies be done, to establish the potential value of biotyping as a discriminatory tool for the identification of *Salmonella* strains in possession of virulence plasmids. The method may help less funded laboratories in developing countries to at least make a tentative diagnosis but also to reduce on the costs of running expensive molecular diagnostic assays such as the PCR assays.

5.1.7 Detection of antimicrobial resistance profiles of *Salmonella* strains

In the present study, variable antimicrobial resistance (AR) rates were observed against 7 antimicrobial agents used. The resistance rate ranged from 2.94 percent to 100 percent (Table 4-7

and Figure 4-2). Therefore, based on the antimicrobial resistance profiles, this study documented seven (I-VII) distinct MDR resistotypes (Table 4-10 and 4-11). The resistotypes of these strains did not coincide with characteristics of fermentative groups. However, the resistotypes in the present study were low in comparison with the work by Nouichi *et al*. (2018), who detected 17 different antimicrobial resistance patterns, among *Salmonella* strains isolated from bovine and ovine samples in slaughterhouses in Algiers, Algeria. Our investigation revealed that all *Salmonella* serotypes were exclusively resistant to erythromycin, and penicillin G (Figure 4-2; Table 4-7). This was followed by ampicillin and gentamicin (88.2 percent each), and nitrofurantoin (85.3 percent). Our investigation indicate that there was no association between *Salmonella* serotype and antimicrobial resistance to the set of antibiotics used in the investigation (p = 0.1, 95 percent, CI). Our findings is in tandem with the work by Abd-Elghany *et al*. (2015), in which *Salmonella* isolates from poultry meat revealed 100 percent resistance to erythromycin and penicillin in Egypt. Similarly, our finding is congruent with the work by Rajagopal and Mini, (2013), who showed that the three *Salmonella* isolates from poultry were resistant to erythromycin.

According to Wasy and Hoszowski, (2004), *Salmonella* strains tend to have natural resistance towards erythromycin and penicillin. All *Salmonella* strains included in the present study were resistant to at least four antimicrobial agents and revealed MDR characteristics (Table 4-10). By definition all strains that exhibited antimicrobial resistance to at least two or more classes of antimicrobials agents are considered MDR (Schwarz *et al.,* 2010). In the present study, antimicrobial agents were selected from eight different classes (Beta-lactams, macrolides, fluoroquinolones, tetracyclines, aminoglycosides, sulphanomides, nitrofurantoin and phenicols) based on different mechanisms of action (Neu, 1992). Correspondingly, our findings demonstrated unique resistance phenotype to at least four of the antimicrobial classes; beta-lactams, macrolides, aminoglycosides and nitrofurantoin (Table 4-10). In this study, ampicillin, erythromycin, gentamicin, nitrofurantoin and penicillin G were the most frequent resistant antimicrobial agents. This indicated that MDR *Salmonella* strains are prevalent in both domestic animals and wildlife. The problem of NTS antimicrobial resistance is real and has been reported in several countries with each country exhibiting different antimicrobial resistance phenotype (Pan *et al*., 2010; Adzitey *et al*., 2015; Andoh *et al*., 2016; Moe *et al.,* 2017; Cortes *et al*., 2017;

Wang *et al*., 2017a; Wang *et al*., 2017b). Distribution of *Salmonella* antimicrobial resistance profiles from different animal sources and the environment in other countries is shown in Table 2-6.

The level of resistance to ampicillin in the present study was lower than 91.6 percent as reported by Abd-Elghany *et al*. (2015) in Egypt. In Ethiopia, *Salmonella* strains from raw meat and swab samples from butcher shops'were 88.7 percent resistant to ampicillin (Legesse *et al*., 2015).

Salmonella resistance in this study towards gentamicin and nitrofurantoin warrants public health concern due to their wider application in treatment of infections. For instance, gentamicin has been used in the treatment of *Staphylococcus* infections (Chambers and Pallagrosi, 1977), human plague (Mwengee *et al*., 2006), and human brucellosis (Roushan *et al*., 2006). On the other hand nitrofurantoin has been used in the treatment of human chronic urinary tract infections in patients with type 2 diabetes mellitus (Mozdzan *et al*., 2006). In the present study, none of the strains were resistant to tetracycline, contrary to reports of other studies who have indicated that tetracycline was the most common resistant antimicrobial drug followed by sulfamethoxazole (Johnson *et al*., 2005; Andoh *et al*., 2016; Nyabundi *et al*., 2017; Moe *et al.,* 2017; Cortes *et al*., 2017).

Our investigation revealed the overall 28 percent (10/34) and 12 percent (4/34) were resistant and intermediate to 17 antimicrobial agents tested (Figure 4-1). Observed resistance against these antimicrobial agents in this study could also indicate a possible reduction to the easily affordable drugs. The overall *Salmonella* resistance prevalence rate observed in this study was comparatively lower than reported elswhere (Adzitey *et al*., 2015; Andoh *et al*., 2016; Nyabundi *et al*., 2017; Moe *et al.,* 2017; Cortes *et al*., 2017), but higher than the resistance rate reported in Alberta, Canada (Johnson *et al*., 2005). However, *Salmonella* antimicrobial resistance rates ranging from 11.6 to 60.6 percent have been reported in different countries such as Ghana (Adzitey *et al*., 2015; Andoh *et al*., 2016), Kenya (Nyabundi *et al*., 2017), Canada (Johnson *et al*., 2005), and Columbia (Cortes *et al*., 2017).

The MAR index calculated for each antibiotic against 34 *Salmonella* strains in this study, were in the range of 0.0 - 0.059, suggesting a multi-faceted antimicrobial resistance patterns (Table 4-14). All antibiotics that exhibited MAR index >0.002 were considered highly resistant to a set of *Salmonella* strains tested against. This finding has far reaching consequences to the majority of poor patients in Zambia, given that they may not be able to afford to pay for the alternative and relatively expensive drugs.

Furthermore, no extended spectrum β-lactamases (ESBLs) producers were identified among the strains screened against cefotaxime. Our results are in agreement with the work by Brooks *et al*., (2015), and therefore, it can be inferred that the MDR antimicrobial resistance phenotype exhibited by *Salmonella* in the present study were due to different types of resistant mechanisms. Our findings could also indicate that not many NTS serotypes circulating in the study areas are producers of the ESBLs (Carattoli *et al*., 2008; Michael and Schwarz, 2016). On the contrary, several investigators have reported the occurrence of ESBL-producing *Enterobactericeae* in human and veterinary medicine worldwide (Huijbers *et al*., 2013; Stefani *et al*., 2014; Tellevik *et al*., 2016; Seni *et al*., 2016; Jaramillo *et al*., 2017; Gay *et al*., 2018). Extended spectrum β-lactamases cephalosporin antibiotics such as ceftriaxone (Basnyat, 2007; Mileva *et al*., 2016), ceftazidime (Chen *et al*., 2014), and cefotaxime (Kariuki *et al*., 2015b; Malet *et al*., 2016) are considered important therapeutic agents in the treatment of invasive *Salmonella* infections.

5.1.8 Detection of carriage of accessory *spv* genes

In the present study, the overall carriage rate of *spv* genes was 58.8 percent (20/34), 55.9 percent (19/34) and 58.8 percent (20/34) for *spv*A, *spv*B and *spv*C respectively (Table 4-16), suggesting that these virulence genes could be discriminatory of the pathogens responsible for extra-intestinal salmonellosis from those causing gastro-intestinal infections (Guiney *et al.*, 1995; Libby *et al*., 1997; Heithoff *et al.*, 2008; Nhidza *et al.*, 2012; Shah *et al*., 2011; Feng *et al*., 2012; Nuccio and Baumler, 2014). Of the 20 *Salmonella* strains carrying *spv* genes, 95 percent (19/20) strains were from clinical samples of domestic animals, while 5 percent (1/20) was from a none-clinical sample of wildlife sources.

Our study revealed that *S.* Enteritidis was the serotype most frequently found to carry *spv* genes isolated from the diseased domestic animal sources (horse = 1, and chicken = 15), followed by *S.* Ruanda, and *S.* Stockholm, suggesting that any *Salmonella* serotype has the potential to *spv* genes. In the present study, all *S.* Enteritidis (*n* = 16) were concurrently in possession of *spv*A, *spv*B and *spv*C genes except one strain from chicken which lacked *spv*B gene, which constituted a distribution frequency of 100 percent (16/16), 93.3 percent (15/16) and 100 percent (16/16) respectively (Table 4-16). Our finding regarding the detection of *spv* genes in *S.* Enteritidis from animals is consistent with other studies; for instance in Iran, *spv*A, *spv*B and *spv*C were present in 100 percent *S.* Enteritidis from bovine sources (Amini *et al*., 2010). In Brazil, 91.2 percent of *S.* Enteritidis isolated from humans, domestic animals, and food were carriers of *spv*C gene (de Oliveira *et al*., 2003). Whereas, the study by Ikuno *et al*., (2014), detected 48.1 percent prevalence rate of *spv*C in *Salmonella* strains isolated from food sources between 1993 and 2007, in Brazil; in Zimbabwe, 79.5 percent of *S.* Enteritidis from animal origin clinical samples were positive for *spv* genes (Nhidza *et al*., 2012). Similarly, other investigators have also described high detection and distribution rate of *spv* genes in *S.* Enteritidis strains isolated from humans, bovine and poultry sources (Kumarss *et al*., 2010). Our result is in agreement with the work by Olsen *et al*., (2004), who reported that not all strains of serotypes that typically possess virulence plasmids would possess the genes at all times. The abscence of *spv*B in one strain *S.* Enteritidis serotype in this study could be indicative of the strain's inability for systemic survival (Heithoff *et al*., 2008), and impotent to establish virulence phenotype (Lesnick *et al*., 2001).

Furthermore, the finding indicated that both *inv*A and *spv* genes could have been responsible for the causation of invasive infections in clinical samples. An association was observed between serotype and possession of *spv* genes (p = 0.001, 95 percent, CI), suggesting that the gene was likely to be found in certain serotypes compared to others. The widely known host-adapted serotypes that carry virulence plasmids in animals include; *S.* Gallinarum/Pullorum are biovars of the same serotype in poultry (Barrow *et al*., 1987; Barrow and Lovell, 1988), *S.* Cholerasuis in pigs (Danbara *et al*., 1992), *S.* Dublin (cattle), and *S.* Abortusovis (sheep) (Rotger and Casadesus, 1999; Feng, *et al.*, 2012; Derakhshandeh *et al*., 2013). These serotypes are capable of causing higher mortality rates in animal infections (Baumler *et al*., 1998). On the other hand, *S.* Paratyphi C is host-adapted in humans, although the role of its *spv* gene in infection is not well

elucidated (Boyd and Hartl, 1998). However, previous studies have reported occurrence of *spv* genes in serotypes that lack host specificity such as *S.* Enteritidis, *S.* Typhimurium, *S.* Rostock, *S.* Newport, and *S.* Moscow (Rotger and Casadesus, 1999; Chu *et al*., 1999). The occurrence rate of *spv*C genes in *Salmonella* serotypes was lower in the present study than the study by Derakhshandeh *et al.*, (2013), who reported occurrence rate of 73.3 percent in different *Salmonella* serotypes from various clinical specimens submitted for routine diagnostic in Iran. Further, the prevalence rate of *spv*B was also lower (43.3 percent) than the rate observed in the present study. On the other hand, Farahani *et al*. (2018) detected 61.1 percent of *spv*C from none clinical strains of *Salmonella* in Iran, which is higher than in the present study.

The detection of *spv* genes among the strains isolated from domestic animals and wildlife in the present study revealed a degree of diversity of serotypes in possession of *spv* genes. The distribution of of *spv* genes in *Salmonella* serotypes from different sources worldwide by different investigators; for instance serotype *S.* Enteritidis (Oliveira *et al*., 2003; Kumarss *et al*., 2010; Derakhshandeh *et al.,* 2013; Chaudhary *et al*., 2015; Fardsanei *et al*., 2017; Wiszniewska-Laszczych *et al*., 2018), *S.* Typhimurium (Derakhshandeh *et al.,* 2013; Chaudhary *et al*., 2015), and *S.* Infantis (Derakhshandeh *et al.,* 2013) (Tables 2-13 and 2-14).

Salmonella virulence factors are required to facilitate colonization of the small intestine, as well as to permit multiplication in the intestinal mucosa (Clements *et al*., 2001). According to Guiney and Fierer, (2011), *Salmonella* serotypes in possession of *spv* genes are linked to invasive systemic infections in immune compromised HIV patients, particularly those with low levels of Cluster of Differentiation 4 **(**CD4**)** T cells count as they cannot fight the infection. Of the 20 *Salmonella* strains carrying *spv*A gene, 95 percent exhibited MDR to at least 4 antimicrobial agents. In view of our finding, we are in agreement with the work by Gebreyes *et al*. (2009), who indicated that the presence of *spv*A genes in *Salmonella* isolates is significant and indicative that the isolates are more than three times as likely to be of clinical origin.

Interestingly, we detected *spv* genes in serotypes *S.* Garoli, *S.* Stockholm and *S.* Ruanda that are rarely carriers of these genes. Our findings demonstrate that these *Salmonella* strains that inhabit the intestinal tract of domestic animals and wildlife could be carrying *Salmonella* virulence

plasmids, capable of conferring disease in human beings or animals. Further, this could suggest that these serotypes could have acquired the genes through coexistence with other serotypes that are known to harbour virulence genes within the ecosystem (Baumler, 1997; Skippington and Ragan, 2011).

5.2 Diversity of wildlife as reservoirs of *Salmonella*

Wildlife are known as long-term asymptomatic reservoirs for zoonotic pathogens, such as *Salmonella* (Vigo *et al*., 2011; Hidalgo *et al.,* 2013; Lopez *et al*., 2015). In the present study, the distribution of three *Salmonell*a strains isolated from wildlife were each from Leopard, Sable antelope and Impala antelope respectively. Furthermore, the observed diversity of putative potential animal reservoirs signals public health implications requiring those mandated over the welfare of game animals to be cautious. *Salmonella* can contaminate the environment such as the pastures and water point sources. Such a possibility perpetuates the survival of *Salmonella*. Previous studies have implicated wildlife in zoonotic transmission of human salmonellosis (Hilbert *et al.,* 2012; Chiari *et al*., 2013; Berardi *et al*., 2014; Iovine *et al*., 2015; Afema and Sischo, 2016). In a set up, where wildlife is utilized for human consumption, the results of the present study are important as some *Salmonella* strains identified may be pathogenic to humans.

5.2.1 Diversity of seroroups among *Salmonella* strains isolated from wildlife

In the present study, of the 3 *Salmonella* strain isolated from wildlife; 33.3 percent (1/3) belonged to serogroup C isolated from leopard, while the two strains were non-reactive, with each from Sable antelope and Impala antelope respectively. In contrast, all *Salmonella* strains belonged to D serogroup isolated from wild captive herbivores In Iran (Koochakzadeh *et al.*, 2015).

5.2.2 Diversity of serotypes among *Salmonella* strains isolated from wildlife

Our investigations identified three distinct *Salmonella* serotypes circulating in wildlife; *S.* Pomona, *S.* Garoli, and *S.* Roan (Table 4-4). Some of these *Salmonella* serotypes isolated from wildlife faeces were potential zoonotic pathogens, suggesting that wildlife may serve as a reservoir for *Salmonella* infections in humans. There were several reports about distribution of *Salmonella* serotypes isolated from different wildlife sources. In Rio de Janeiro, one strain of *S.*

Typhimurium and two strains of *S.* Panama serotypes were isolated from Temminck's seedeater (*Sporophila falcirostris*) and chestnut-capped blackbirds (*Chrysomus ruficapillus*) respectively (Matias *et al.*, 2016). In Ohio, *Salmonella* serotypes Braenderup, Senftenberg, Oranienburg, and Kentucky were isolated from faecal samples from various wildlife species admitted to two rehabilitation centers (Steffani *et al*., 2007). In summer 2000, *S.* Newport and *S.* Enteritidis were isolated from 8.9 percent of southern giant petrels (*Macronectes giganteus*) (Vigo *et al*., 2011). In Uttar Pradesh and Delhi (India), *Salmonellae* serotypes were isolated from a variety of Zoo animals including *S.* Typhimurium, from a leopard and a wild cat, *S.* Enteritidis from two black and white rats and a strain of *Salmonella* group E1 from a leopard (Sharma and Singh, 1980). In Iran, the most predominant serotypes were *S.* Typhimurium and *S.* Enteritidis (9 and 8 cases each, respectively), whereas only 1 serotype belonged to S. Montevideo, isolated from house sparrows (Mirzaie *et al*., 2010). In Barbodos, West Indies, four serotypes were isolated from free-ranging small Asian mongoose: *S.* Rubislaw, *S.* Kentucky, *S.* Javiana, and *S.* Panama (Kamara *et al*., 2014).

The majority of outbreaks of salmonellosis in captive and free-ranging wildlife in most countries are caused by different serotypes; in USA, an outbreak of salmonellosis occurred in an open-air zoologic exhibit and *S.* Typhimurium, was isolated from four dead lorikeets and lories (*Trichoglossus, Lorius* and *Eos* spp.) during August 2001 (Ward *et al*., 2003); in another study, *S.* Typhimurium was one of several factors responsible for losses among young herons being held at the Patuxent Wildlife Research Center in the USA (Locke *et al*., 1974); in Buenos Aires, Argentina, *S.* Typhimurium was isolated from adult chinchillas (*Chinchilla lanigera*) that had suddenly died in a commercial farm in 2012 (Churria *et al*., 2014); in North Eastern Italy, *S.* Choleraesuis, was isolated from dead wild boars between 2012 and 2013 (Conedera *et al*., 2014). In the present study, *S.* Pomona was first isolated from a clinical sample of sable antelope, which was characterized by profuse diarrhea; while the rest were from none clinical samples. In contrast, *S.* Pomona was isolated from apparently healthy pig in South Africa (Mathole *et al*., 2017). In Mexico, *S.* Pomona was isolated from a captive keel-billed toucan (*Ramphastos sulfuratus*) in an Aquarium (Silva-Hidalgo *et al*., 2014). In Ethiopia, *S.* Pomona was among the aetiological agents causing febrile and diarrhoeic illness in children (Beyene *et al*., 2011). Turtles

and other reptiles are considered the natural reservoirs of *S.* Pomona serotype and subsequently became major sources of human *Salmonella* infection (CDC, 2007; Gong *et al.*, 2014).

Salmonella Garoli was first isolated in the Belgian Congo from a patient with intestinal disturbance (Le Minor *et al*., 1953). On the other hand, *S.* Garoli was the most frequent serotype causing diarrhoeal symptoms in human patients from 1967-1970, and also caused an epidemic in 1969 at Mulago hospital in Uganda (Lubwana, 1985).

Several investigators have reported human cases of salmonellosis associated with wildlife (Hernandez *et al*., 2012; Becki *et al*., 2014; Bosch *et al*., 2016). In particular the human-livestock-wildlife interface areas where both domestic animals and wildlife may play a significant role in the transmission and maintenance of diseases to humans or vice versa (Skov *et al*., 2008; Wiethoeltera *et al*., 2015), or within Zoo animals (Paulsen *et al*., 2012; Paulsen *et al*., 2016). A point in case was in Denmark, where *Salmonella* was reported to have been transmitted from infected herds of animals (cattle and pigs) to wildlife within the vicinity of the farming area (Skov *et al*., 2008). Therefore, the occurrence of serotypes; *S.* Pomona, and *S.* Garoli, in wildlife should be a source of concern as these serotypes have been reported to have a relatively greater propensity to cause human invasive salmonellosis (Diao *et al*., 2014; Gong *et al.*, 2014). For instance *S.* Pomona which was once considered a rare serotype but recently was found to be highly pathogenic, caused severe human salmonellosis especially in children (Diao *et al*., 2014; Gong *et al.*, 2014).

Our investigation revealed that there was no cross infection of circulating *Salmonella* serotypes or strains between domestic animals and wildlife, because *Salmonella* serotypes isolated from domestic animals and those from wildlife were distinctive of each group respectively. We agree with the submission by other investigators that captive and free-ranging wildlife are potential carriers of *Salmonella* which could be transmitted as pathogens to humans (Chethan *et al*., 2013; More *et al*., 2017; Lourdes *et al*., 2017; Elmberg *et al.,* (2017). Infection can be acquired through direct or indirect contact with faeces of wildlife or consumption of improperly cooked meat from an infected hunted game (Paulsen *et al*., 2012; Paulsen *et al*., 2016; Cheyenne *et al*., 2017). Sable antelopes, leopards and impala antelopes are potential reservoirs that is likely to contaminate the

ecosystems through faecal droppings which in turn may facilitate the transmission of *S.* Pomona and *S.* Garoli to humans. Therefore, monitoring of almonellosis in wildlife is advised. Game viewers, game scouts and/or persons living in the domestic animal/wildlife interface areas are high risk. To our knowledge this is the first report to describe these serotypes isolated from the respective wildlife species.

5.2.3 Diversity of *Salmonella* fermentative groups isolated from wildlife

The study observed that three *Salmonella* strains isolated from wildlife, *S.* Garoli (leopard), *S.* Pomona (sable antelope) and *S.* Roan (impala antelope) were each classified into different fermentative groups I, III and VII respectively, despite *S.* Garoli and *S.* Roan having originated from the same area (KNP) (Table 4-6). Our finding indicate that that serotype *S.* Garoli is more likely to be related to *S.* Enteritidis (horse, chicken) and *S.* Ruanda (chiken) than to serotypes *S.* Roan and *S.* Pomona from Impala antelope and Sable antelope respectively. Further, we observed a relationship between serotypes *S.* Heidelberg and *S.* Pomona isolated from cattle and Sable antelope respectively, as all biotypes found in fermentative group III were able to utilize cellobiose and not arabinose, inositol and salicin, suggesting that the clones could be related despite their differences in serotype and source. The usefulness of biotyping of *Salmonella* strains isolated from wildlife has been reported by other investigators (Ishiguro and Sato, 1981; Mubita *et al*., 2020).

5.2.4 Diversity of antimicrobial resistance profiles among *Salmonella* strains isolated from wildlife

In the present study, the 3 *Salmonella* strains from free ranging wildlife were resistant to four or five antibiotics (Table 4-10). *Salmonella* strains from wildlife were represented in only two resistotypes (III and VII). Resistotype group III was the most predominant group designated by five antibiotics, ampicillin, erythromycin, gentamicin, nitrofurantoin and penicillin G (Amp-E-Gen-Nit-P) phenotype and was represented by 66.7 percent (2/3) *Salmonella* strains isolated from Sable and leopard (Table 4-11). Increasing prevalence of antibiotic resistance in *Salmonella* serotypes in wildlife to ampicillin, erythromycin, gentamicin and penicillin G has been reported by other investigators (Hidalgo *et al.,* 2014; Lopez, *et al*., 2015; Matias *et al*., 2016).

However a worrisome scenario was observed in this study regarding the AR *Salmonella* strains isolated from wildlife with no history of previous exposure to antimicrobial agents in Kafue NP (North-Park). The antimicrobial resistance in this case indicates a reflection of how we may not understand extrinsic factors that lead to drug resistance of pathogens in nature. In the same manner, another understanding of this resistance may be attributed to poor treatment of effluents or pollutants/wastes discharged from the Copperbelt industries, residential areas and farms along the Kafue River, and are deposited into Kafue NP which acts as a sieving bed from which the environment becomes contaminated. As the Kafue River approaches the Busanga plains in the Kafue NP, it splits up into branches under the swamps and and this is where the actual filtration and screening of these pollutants could occur. Consequently, pollutants are likely to deposit compounds of antibiotic residues and clinical organisms from the environment which in turn contaminate the grazing pastures and drinking water holes in the Kafue NP in response to the anthropogenic use of antibiotic compounds. Therefore, this becomes part of the basis in the rising concern that the global propagation of antibiotic resistance is also associated with environmental reservoirs that are linked to anthropogenic activities (Tripathi and Cytryn, 2017).

Domestic animals are also major players in the contribution to environmental contamination through excretion of faeces and urine which contains residues of antibiotics and subsequently washed down the streams into the river (Sorum *et al*., 2002; Sarmah *et al.*, 2006). Whereas, the work done by Mariano *et al*., (2009), reported that free-ranging impala antelope drinking water from contaminated rivers that flow through the Kruger National Parks, South Africa, were found to have high frequency of tetracycline resistant *Escherichia coli* among the commensal flora in their intestine than do the unexposed impala. According to Olobatoke, (2017), this type of resistance attracts public health concerns due to its ability to propel circulation of resistance within the population or environment. In aquatic environment, *Salmonella* strains can survive for long duration (Iovine *et al*., 2015), as a result the wet Northern part of KNP would provide conducive environment for *Salmonella* to thrive. This can be affirmed by the study of Costa *et al*., (2013), who suggested that wildlife can acquire resistant bacterial strains and residual antimicrobial products that have been disseminated in the environment through effluents from urban areas and animal production units. Hence, occurrence of MDR serotypes in wildlife may

pose a challenge to the epidemiologic control of *S.* Pomona and *S.* Garoli serotypes that have potential to cause human infection.

The multiple antibiotic index (MAR index) calculated for each *Salmonella* serotype was in the range of 0.24 - 0.29, suggesting these serotypes could have originated from the environment contaminated with antibiotic residues (Table 4-13). Overall, there was no much difference of MAR index between *Salmonella* strains from domestic animals and those from wildlife, suggesting they have all been exposed to the environment contaminated with antibiotic residues or any other natural resistance inducing substances (Tambekar *et al.,* 2006; Jayaraman *et al.,* 2012).

We therefore report the occurrence of MDR phenotype among the *Salmonella* strains circulating in wildlife (sable antelope, impala antelope and leopard) from two study locations Kafue National Park and Lusaka Central Park respectively. Based on the antimicrobial resistance profiles recorded in the present study, the *Salmonella* strains isolated from domestic animals and wildlife represent a potential public health concern which requires immediate attention in terms of utilization of game meat, especially during hunting and evisceration, and also keeping in mind that when cattle mix with wildlife there is a possibility of transmission of drug resistant *Salmonella*.

5.2.5 Diversity in carriage of accessory *Salmonella* plasmid virulence (*spv*) genes among *Salmonella* strains isolated from wildlife

Interestingly, a none clinical strain *S.* Garoli serotype isolated from a leopard was found in possession of the *spv*ABC genes concurrently, suggesting the strain could be a potential pathogen capable of causing invasive infection in humans or animals. Detection of *spv* genes in the present study, is to our knowledge being described here for the first time in *S.* Garoli serotype.

5.3 Diversity of domestic animals as reservoirs of *Salmonella*

The present study revealed that different animal reservoirs from apparently healthy (cattle) and diseased (horse, dog, and chicken) animal species were positive for *Salmonella* (Table 4-4),

suggesting their potential source and distribution vehicle (Ravary *et al*., 1998; Bagcigil *et al.*, 2007; Tsai *et al.*, 2007; Tarsitano *et al*., 2010; Langholz and Russell, 2013; Jajere *et al.*, 2014; Shu-Kee *et al*., 2015; Magwedere *et al*., 2015; Odoch *et al*., 2017). Animals play an important role in human lives such as for food, fiber, livelihoods, or companionship. Further, being in close contact with animals and their environments can facilitate opportunities for diseases to pass between animals and people. Our finding subscribes to the existing knowledge that *Salmonella* colonizes the intestinal tract of apparently healthy looking animals (Kagambega *et al*., 2013; Addis *et al*., 2011), but can also cause disease in animals (Gaffga *et al*., 2012; Kim *et al*., 2012; Makaya *et al*., 2012; Loharikar *et al*., 2013; Halimi *et al*., 2014; Lowden *et al*., 2015; Basler *et al*., 2016; Kiflu *et al*., 2017; Alegria-Moran *et al*., 2017; Valenzuela *et al*., 2017). The detection of *Salmonella* in food (cattle, chicken), companion (dog) and other (horse) animals demonstrates the need to improve in controlling and preventing *Salmonella* transmission.

5.3.1 Diversity of serogroups among *Salmonella* strains isolated from domestic animals:

Salmonella strains belonging to serogroup D1 (O: 9) were the most predominant strains (50. 0 percent), accounting for half the strains tested, and were isolated from clinical samples of domestic animals (Table 4-3). This result suggests that the strains may be from same clonal source as *Salmonella* in poultry may occur as an outbreak from the hatchery source, and/or farm contamination. The detection rate of *Salmonella* strains serogroup D1 isolated from domestic animals in the present study, was higher than the work by Kuang *et al*., (2015), who detected 3.5 percent prevalence rate in China. On the other hand, our result was congruent with the work by Kim *et al*. (2012) who described 57 percent prevalence rate of *Salmonella* strains belonging to serogroup D, isolated from chickens in Korea. In Turkey, 14 percent of *Salmonella* isolates were serogroup B, followed by 11 percent serogroup C1, 7 percent serogroup C2-C3, 7 percent sergroup E2, and 4 percent non-reactive isolates, all strains were isolated from poultry-derived food and avian (Ata *et al*., 2017).

5.3.2 Diversity of serotypes among *Salmonella* strains isolated from domestic animals:

In total, 9 different serotypes in domestic animals were documented; *S.* Enteritidis, *S.* Heidelberg, *S.* Ruanda, *S.* Stockholm, *S.* Chardans, *S.* Sendai, *S.* Hadar, *S.* Mbandaka, and *Salmonella* spp.: and of these 8 were from chicken. The serotypes on *Salmonella* from domestic animals are heavily inclined towards chikens; 83.9 percent (26/31) of isolates included in the study were from chickens. Some of these serotypes have been associated with human and animal salmonellosis worldwide (Diao *et al*., 2014; Walters *et al*., 2016; Sakaguchi *et al*., 2017). A similar study conducted in Zambia, reported isolation and identification of 14 serotypes among 806 *Salmonella* cultures isolated from poultry (Sharma *et al*., 1991). The occurrence of more serotypes in the previous study could be attributed to a high number of *Salmonella* isolates that were included in the study. However, by permutation our present study actually denotes a higher proportion of serotypes. In respect of the total number of circulating serotypes, the findings in the present study was lower compared with study by Guerin *et al*. (2005), who described 17 different serotypes from chicken samples. Of these, *S.* Heidelberg (21.6 percent) was the most predominant serotype, followed by *S.* Hadar (14.3 percent), *S.* Mbandaka (5.4 percent) and *S.* Enteritidis (1.9 percent). In Eastern China, *Salmonella* isolates from faecal samples of domestic animals (chicken, duck, goose and pig) yielded 15 different serotypes, out of which *S.* Senftenberg, *S.* Typhimurium, *S.* Pullorum and *S.* Enteritidis were the most common serotypes (Pan *et al*., 2010). In Japan, 27 serovars were identified; the predominant serovars were *S.* Infantis (17.9 percent), *S.* Kalamu (12.4 percent), and *S.* Schwarzengrund (9.5 percent) isolated in chicken meat (Iwabuchi *et al.*, 2011). However, in the present study, we detected more variants of serotypes circulating in chickens than the work by Osman and Waheed, (2017), who described only five serotypes namely *S.* Enteritidis, *S.* Typhimurium, *S.* Muenster, *S.* Anatum and *S.* Virchow from diseasesd and freshly slaughtered chickens in Egypt. In Equador, the most common serotype was *S.* Infantis, followed by *S.* Enteritidis and *S.* Corvallis isolated from broilers at slaughter agae (Vinueza *et al*., 2016). Our findings cement our study hypothesis that both domestic animals and wildlife are putative reservoirs of diverse *Salmonella* clones that have the potential to be transmitted to humans. Our investigation revealed that there is a wide diversity of well-adapted clones of *Salmonella* serotypes *S.* Enteritidis, *S.* Stockholm, *S.* Ruanda, *S.* Sendai, *S.* Hadar, *S.* Chardan, *S.* Mbandaka and *Salmonella enterica* species related to poultry

infections in the study areas. Similarly, the majority of outbreaks of salmonellosis in domestic animals in most countries are caused by different serotypes; in Midwestern Brazil, *S.* Dublin serotype was isolated from three outbreaks of septicemic salmonellosis in calves (Guizelini *et al*., 2020); in Europe, *S.* Stockholm was the cause of an outbreak of salmonellosis in a Swiss cattle herd (Brawand *et al*., 2019); and in Wisconsin, MDR *S.* Heidelberg was the cause of salmonellosis in calves (VSCEAH, 2018).

In the present study, *S.* Enteritidis serotype was the most predominant strain, accounted for 47.1 percent of domestic animal isolates, and was isolated from diseased horse and chicken sample categories, followed by *S.* Mbandaka from dog and chicken, suggesting the possibility of cross infection between animal species (Table 4-4). The isolation of *S.* Enteritidis serotype from diseased chickens was in agreement with the findings of other investigators (Braden, 2006; Betancor *et al*., 2010; Nagwa *et al*., 2012; Moussa *et al.,* 2013). Similarly, *S.* Enterititidis was the most prevalent serotype (55.7 percent), isolated from turkey and broiler carcasses between 2004 and 2006 in Southern Brazil (Palmeira *et al*., 2016). In contrast, *S.* Enteritidis strains were among the most common serotypes isolated from apparently healthy domestic poultry meat and products in Africa (Orji *et al*., 2005; Makaya *et al*., 2012), and Asia (Soomro *et al.,* 2010; Kim *et al*., 2012; Makaya *et al*., 2012; Lu *et al*., 2014; Irfan *et al*., 2015). In Eastern China, *S.* Enteritidis was among the most prevalent serotype isolated from faecal samples of domestic animals during 008-2009 (Pan *et al*., 2010). *Salmonella* Enteritidis was among the most dominant serotype in human infection across the world and accounted for the majority of human salmonellosis (Hendricksen *et al.,* 2011).

Other studies have associated human and animal salmonellosis to be caused by *S.* Enteritidis (Laupland *et al*., 2010; Nagwa *et al*., 2012; Bronowski *et al*., 2013; CFSPH, 2013). *Salmonella* Enteritidis is usually the cause of extra-intestinal infection in humans (Galanis *et al.,* 2006; Gordon *et al.,* 2008; Takem *et al.,* 2014; Ao *et al*., 2015). For instance, *S.* Enteritidis strain is one of the most prevailing NTS serotype, responsible for bactereimia in adults and children across African region (Kingsley *et al.,* 2009; Feasey *et al*., 2010; Muthumbi *et al*., 2015; Uche *et al*., 2017; Garcia *et al*., 2018). In Zambia, *Salmonella* Enteritidis has previously been reported from different sources (Hang'ombe *et al*., 1999b; Munangandu *et al.,* 2012).

Human infection is largely attributed to consumption of contaminated foods especially of animal origin such as poultry, eggs and dairy products are the most common vehicles of salmonellosis (Chai *et al*., 2012; Hale *et al*., 2012; Hilbert *et al*., 2012; De Knegt *et al*., 2014; Mughini *et al*., 2014) and direct or indirect contact with *Salmonella* pathogens through shedding in faeces. By and large, horses have a limited number of diseases that can be transmitted to humans under natural conditions (Dwyer, 2014). However, there are fewer recorded cases of human salmonellosis that have been linked to consumption of horse meat (Espie *et al*., 2005) or being in close contact with hospitalized horses in veterinary hospitals (Morse *et al*., 1978). Perhaps some of the horse infection may have been associated with *Salmonella* serotypes rarely linked to human infection.

The isolation of *S.* Mbandaka from clinical samples of dogs in the present study, should also be a source of worry considering that companion animals have been implicated in human salmonellosis (Sato *et al*., 2000; Clark *et al*., 2001; CDC, 2006a). The infection due to *S.* Mbandaka was common in particular children (Sato *et al*., 2000) who are fond of playing with dogs or treat dogs as their close interaction pets. In 1996, *S.* Mbandaka infected 15 persons in South Australia (Scheil, *et al*., 1998). Other studies have indicated that *S.* Mbandaka was isolated from clinically sick cattle on three dairy farms after the animals had consumed feed containing vegetable fat supplement which was contaminated with *S.* Mbandaka (Jones *et al*., 1982). In Uganda, *S.* Mbandaka and *S.* Hadar were isolated from the faecal samples of apparently healthy poultry (Odoch, *et al*., 2017**)**. A recent report indicate that about 73 people from 31 States in the USA, were infected with *S.* Mbandaka outbreak strain after consuming Kellogg's Honey Smacks cereal (CDC, 2018b).

In the present study, the prevalence rate of *S.* Heidelberg isolated from faecal samples of apparently healthy cattle was 0.32 percent, so were the findings in the study by Guerin, *et al*., (2005), who reported a prevalence rate of 0.24 percent. In Zambia, *S.* Heidelberg was isolated from cattle (Ngoma *et al*., 1993) and human patient (Dube and Bhagwat, 1983). *Salmonella* Heidelberg has been linked to human foodborne salmonellosis infection (Foley and Lynne, 2008; CDC, 2009; Evans *et al*., 2014; CDC, 2014b; Gieraltowski *et al*., 2016). Further, it can cause devastating clinical manifestations more than other serotypes (Clothier and Byrne, 2016).

Further, in the study, we observed circulation of other serotypes (*S.* Stockholm, *S.* Hadar, *S.* Chardans, *S.* Sendai, *S.* Ruanda and *Salmonella* spp.) in domestic animals sources. *Salmonella* Stockholm was first reported in Sweden as a new *Salmonella* serotype by Lagergren and Osterling (1951). Similarly, a study by Agarwal *et al.* (1999) reported isolation of *S.* Stockhlm serotype for the first time in India from beef collected from slaughter house. In 2006, *S.* Stockholm caused the largest outbreak of salmonellosis in 25 years in Stockholm City after persons had a meal from a restaurant (Jong *et al*., 2007). On the other hand, *S.* Hadar was reported as one of the 20 most frequently isolated serovars from human clinical sources in the USA (CDC, 2009). In England and Wales *S.* Hadar was the second commonest serotype isolated from patients with cases of food poisoning in the 1970's (Rowe *et al*., 1980). Turkeys were considered the main reservoirs of these pathogens (Rowe *et al.,* 1980). Outbreaks caused by this serotype were often associated with food of animal origin such as meat and poultry products (Bisbini *et al*., 2000). In our literature review, there is paucity of information regarding the prevalence of Sendai serotype in poultry. However, this serotype is considered a rare and human-restricted (Parry *et al*., 2002; Feng *et al*., 2019).

Our study further revealed that majority (66.7 percent) of serotypes were isolated from clinical chicken samples, suggesting that a wide range of *Salmonella* serotypes have predilection for chicken host which in turn incriminates them as potential sources of human salmonellosis (CDC, 2009; Nagwa *et al*., 2012; Bronowski *et al*., 2013; CFSPH, 2013). Further, the observed diversity of serotypes circulating in poultry could be a reflection of a wide practice in the poultry industry of sourcing day old chicks from different poultry breeders either within or outside the country whereby, these birds bring with them new *Salmonella* strains (Osman *et al*., 2010; Osman *et al*., 2014). However, the Zambian government through the Ministry of Livestock imposes stringent sanitary measures with an emphasis on bio-security considerations to control importation of poultry products (AGRIPROFOCUS ZAMBIA, 2015).

On the other hand, distribution of *Salmonella* serotypes revealed that the majority 66.67 percent (8/12) of *Salmonella* serotypes prevailed around Lusaka region. Our considered view is that Lusaka region is a cosmopolitan area, thereby it attracts different livestock production activities, such as the commercial farmers and cooperate companies involved in poultry breeding, piggery

and dairy farming. The Lusaka region, also has the backyard, and small scale farmers (Munang'andu *et al.*, 2012; AGRIPROFOCUS ZAMBIA, 2015; Dumas *et al*., 2016). In 2015 alone, production of broiler chickens was pegged at 79 million chickens (Zambia Daily Mail, 2017), with most of the production likely to have been raised around Lusaka and surrounding areas (AGRIPROFOCUS ZAMBIA, 2015).

These poultry products are sold in markets managed by Local Councils or sometimes are displayed by the road side (AGRIPROFOCUS ZAMBIA, 2015). Often times these vendors use public transport to deliver their merchandize to selling points, exposing other passengers to possible *Salmonella* infection through contact with faecal droppings. The results in the present study point to the need to have increased public awareness about the possible dangers associated with food of animal origin in view of the fact that sources of human salmonellosis infections include consumption of contaminated poultry meat and their products worldwide (Antunes *et al*., 2016). In Zambia, consumption of poultry meat accounts for an estimated 50 percent of the total meat consumed because of its relatively low cost as a source of animal protein when compared with red meats (Ngosa, 2011). Therefore, diversity of serotypes circulating in chickens in the present study, sounds a warning that increased exposure to raw poultry meat and their products has the potential to transmit *Salmonella* to humans (Hedican *et al*., 2010; Foley *et al*., 2011; Aziz, 2013; Andino and Hanning, 2015; Gieraltowski *et al*., 2016; Antunes *et al*., 216; Huusko *et al*., 2017). This is in view of the increase of trade in the informal poultry market taken up by small scale producers who sell live birds, unpacked poultry meat and ungraded table eggs in Zambia.

Similarly, appreciable population of horses owned by individuals, societies and government institutions such as Ministry of Home Affairs (Zambia State Police) for various reasons are found in Lusaka region. Furthermore, majority of the family households that are capable of keeping dogs for security purposes and or as pets such as cats would live around Lusaka region, thereby creating conducive environment for proliferation of bacterial pathogens due to high population of domestic animals, in particular poultry (Foley *et al*., 2011). The study further revealed, that *S.* Mbandaka serotype were isolated from a dog as well as a chicken respectively, suggesting that this serotype may not be restricted to a particular animal host species (Rotger *et*

al., 1999; Kingsley *et al.,* 2013; Foley *et al*., 2013). Isolation of *Salmonella* from a wide range of domestic animal sources suggests that *Salmonella* is widespread in food animals (cattle, chicken), other livestock (horse) and companion animals (dog) which underlines the necessity for a coordinated surveillance and monitoring programs for salmonellosis and other major food borne zoonotic diseases in Zambia.

5.3.3 Diversity of fermentative groups among *Salmonella* strains isolated from domestic animals:

The present study showed that 31 *Salmonella* strains from domestic animals were represented in 7 fermentative groups, namely, biogroup I, II, III, IV, V, VI, and VIII. Fermentative group I constituted the majority of strains from domestic animals: horse (n = 1) and chicken (n = 17), suggesting clonal relationships in diverse serotypes. Furthermore, *Salmonella* strains from chickens showed a greater level of diversity in the strategies to exploit the four substrates as source of energy and are represented in 6 fermentative groups (I, II, IV, V, VI and VIII) Table (4-6). Interestingly *S.* Heidelberg and *S.* Pomona isolated from cattle and sable antelope respectively shared biogroup III, despite being from different geographical regions. On the other hand, *S.* Enteritidis strains from the horse and chicken displayed a consistent and identical fermentative profiles in all 15 substrates. Therefore, based on the limited and available substrates used, our investigation revealed that *S.* Enteritidis strains comprised a single biotype and thus could have originated from the same bacterial population. Similarly, the strains of *S.* Ruanda, and *Salmonella enterica* spp. serotypes were indistinguishable in the utilization of 15 substrates respectively. Failure to utilize salicin is a classic reaction of *Salmonella enterica* subspp I (Barrow and Feltham, 1993; Paccagnela, 2005), but our findings revealed that one strain belonging to *S.* Chardan serotype isolated from clinical samples of chicken was salicin positive. This finding suggested that this strain could have its origin from *Salmonella enterica* sub species IV (*houtanae*) (Paccagnela, 2005). Thus, could have diversified by mutation in the chromosal genes or by the loss or gain of phages and plasmids into two biotype strains over a period of time (Barker, 1986).

Accordig to Krieg and Holt, (1984), *Salmonella enterica* sub spp. *enterica* does not utilize cellobiose. However, the present study detected positive strain belonging to *S.* Chardan:

suggesting that this serotype could have evolved to gain a competitive advantage over other species by adapting to cellobiose as a source of energy. Therefore, analysis of utilization of four substrate (arabinose, cellobiose, inositol and salicin) by *Salmonella* strains in the present study, identified biotype strains within *S.* Stockholm, *S.* Chardan and *S.* Mbandaka serotypes. Biotype 1 strains of *S.* Stockholm was (positive for inositol^{+}, and negative for arabinose^{-}, cellobiose^{-}, salicin^{-}), *S.* Chardan (positive arabinose^{+}, and negative for cellobiose^{-}, inositol^{-}, salicin^{-}), and *S.* Mbandaka (positive cellobiose^{+}, inositol^{+}, and negative arabinose^{-}, salicin^{-}). Biotype 2 strains of *S.* Stockholm was (positive for arabinose^{+}, inositol^{+}, and negative cellobiose^{-}, salicin^{-}), *S.* Chardan (positive arabinose^{+}, salicin^{+} and negative cellobiose^{-}, inositol^{-}), and *S.* Mbandaka (positive arabinose^{+}, inositol^{+}, and negative cellobiose^{-}, salicin^{-}) (Table 4-6). Furthermore, biotyping results indicate that *S.* Mbandaka serotype isolated from clinical samples of two dogs and one chicken respectively were not epidemiologically related. The strain from the latter did not utilize cellobiose, while the opposite was the case for arabinose (Table 4-6). Furthermore, literature search showed that *Salmonella* biotypes from different sources have been reported by several investigators (Duguid *et al*., 1975; Barker and Old, 1989; Zhu *et al*., 2015). We report for the first time occurrence of *Salmonella* biotypes among *S.* Stockholm, *S.* Chardan and *S.* Mbandaka circulating in domestic animals (dogs and chicken) respectively around Lusaka region.

5.3.4 Diversity of antimicrobial resistance profiles among *Salmonella* strains isolated from domestic animals

Our finding showed that 31 *Salmonella* serotypes isolated from domestic animals were represented in all seven resitotypes (Tables 4-10 and 4-11), with a MAR index varying from 0.24 to 0.35 (Table 4-13). This finding suggested that these strains could have originated from a source where judicious use of antimicrobial agents for therapeutic or prophylactic purposes in animal husbandry management were not considered (Yang *et al*., 2010; Upadhyaya *et al*., 2013). *Salmonella* strains from chickens were represented in five of 7 resistotypes (Table 4-12), an indication that the strains could have originated from a source where antibiotics were over used. Resistotype group III was the most predominant phenotype group comprising of five antibiotics, ampicillin (88.2 percent), erythromycin (100 percent), gentamicin (88.2 percent), nitrofurantoin (85.3 percent) and penicillin G (100 percent) (Amp-E-Gen-Nit-P) phenotype. This group was

represented by 55.9 percent (19/34) *Salmonella* strains isolated from domestic animals: chicken (*n* = 17), cattle (*n* = 1) and dog (*n* = 1) (Table 4-11). In other studies, the dominant resistance profiles for NTS isolates from poultry were tetracycline, ciprofloxacin, sulphamethoxazole, trimethoprim, and ampicillin in Ghana (Andoh *et al*., 2016); in Kenya isolates from domestic animals were resistant to sulfamethoxazole, cotrimoxazole, streptomycin, and tetracycline (Nyabundi *et al*., 2017). While in Columbia, resistance to ampicillin, amikacin, tobramycin, cefazoline, cefoxitin, nitrofurantoin, trimethoprim-sulfamethoxazole, tetracycline, and ciprofloxacin were prevalent in isolates from chickens (Cortes *et al*., 2017). Our investigation demonstrates that there is a pool of clones of *Salmonella* strains belonging to different serotypes and isolated from different animal species, that are resistant to ampicillin-erythromycin-gentamicin-nitrofurantoin-penicillin G (Amp-E-Gen-Nit-P). Chickens are exposed to antibiotics through growth promoters (Chattopadhyay, 2014; Van Boeckel *et al.,* 2015; Costa *et al*., 2017; Gadde *et al*., 2018), treatment of infections (Lipsitch *et al*., 2002; Bengtsson and Greko, 2014), and/or use as routine prophylaxis (Diarra and Malouin, 2014; Mukerji *et al.,* 2017). Furthermore, literature search showed an increasing prevalence of antibiotic resistance in *Salmonella* serotypes in domestic animals to ampicillin, erythromycin, gentamicin and penicillin G (Dariusz and Andrzej, 2004; Salihu *et al*., 2014; Adzitey *et al*., 2015; Andoh *et al*., 2016; Lertworapreecha, *et al*., 2016). Studies have also shown that pathogenic Gram negative bacteria can disseminate MDR traits to none pathogenic strains of the same or different species through a highly mechanized system (Mukerji *et al.,* 2017).

In Zambia, ampicillin, gentamicin, and penicillin G are frequently used antimicrobial agents in treatment of various livestock infections or generally animal husbandry. As such it may not be surprising to record an upsurge of *Salmonella* resistant strains against these antibiotics (Lin *et al*., 2004). On the other hand, the observed resistance to ampicillin in the present study, could be attributed to the fact that the drug is more effective against Gram positive bacteria than it is for Gram negative bacteria (Adams, 1995). However, the concerns are precipitated by several reports that have indicated that antimicrobial resistance to bacteria of animal origin can be transmitted to humans through many routes including the food chain (Price *et al.,* 2005), or exposure to contaminated environment (Graham *et al.,* 2009) which could include to poor sanitation among others (Smith *et al.,* 2013; Fletcher, 2015).

On the other hand, there is scanty information regarding the use of nitrofurantoin in livestock production in Zambia, although the drug is widely used to treat infections and promote growth of livestock (Gustafson, 1986). However, in the present study, high frequency of resistance by isolates was observed against nitrofurantoin, and the findings were in agreement with the work by Wasy and Hoszowski, (2004), who recorded a high number of resistant *Salmonella* strains in Poland. Similarly, *Salmonella* strains isolated from faecal matter of domestic animals and animal products in Nairobi were 84 percent intermediate resistant to nitrofurantoin (Nyabundi *et al*., 2017). In Ethopia, 63.3 percent strains were found resistant to nitrofurantoin in dairy cattle (Tadesse *et al*., 2016). Nitrofurantoin has shown good efficacy against a broad range of bacteria responsible for urinary tract infection in humans (Mendoza *et al*., 2010, Fransen *et al.*, 2016). Therefore, high rate of resistance observed against this agent in the present study should be a source of public health concern. On the contrary, the study by Ulaya (2013), showed that *Salmonella* strains from dogs (94.4 percent) and chickens (100 percent), were susceptible to nitrofurantoin respectively, while *Salmonella* isolates from chickens were found susceptible to ampicillin and tetracycline (82.6 percent each) in the Lusaka District, Zambia. The difference in antimicrobial resistance profiles may be attributed to the use of different antimicrobial drugs by different veterinary health providers. In European Union (EU), the application of nitrofuran antibiotics for the treatment of bacterial diseases in livestock production was banned in 1995, following the concerns about the carcinogenicity of their residues in the edible tissue (Vass *et al*., 2008).

Overall, our investigations revealed that a panel of four antimicrobial agents consisting of erythromycin-gentamicin-nitrofurantoin-penicillin G (E-Gen-N-P) was the most common phenotype represented in 71.4 percent (5/7) of the resistotypes. Interestingly, resistotypes I, II, IV and VII were represented by single strains from domestic animal sources, suggesting that they were not epidemiologically related. The findings in the present study, should be a source of concern because some of the antimicrobial agents to which the isolates showed high percentage of resistance are considered important for the treatment of human infections (Mukerji *et al.*, 2017). Therefore, under these circumstances it is prudent that antibiotic resistance is continuously monitored to keep watch of any changes in drug resistance profiles.

Further analysis of antimicrobial resistance profiles of clinical strains (Table 4-11), revealed that *S.* Enteritidis and *S.*Mbandaka serotypes, each isolated from horse and dog respectively displayed profiles with the highest number of antimicrobial agents of six each. The former was against Amp-Azm-E-Gen-Nit-P, and the latter Amp-Cip-E-Gen-Nit-P. Each strain revealed a multiple resistance index (MARI $\leq$ 0.35) (Table 4-13) respectively, suggesting they were from a source where there is a heightened use of medically potent antibiotics to treat animal infections or used as growth promoters in animal feed (Bengtsson and Greko, 2014; Weese, 2015). This was followed by serotypes; *S.* Heidelberg (cattle) and *S.* Enteritidis (chickens) with each against 5 antibicrobial agents. The first global-scale genetic study of *S.* Enteritidis revealed that there are in fact three separate types; two are novel types found in Africa having an identical appearance, but were genetically different from the Western type (WTSI, 2016; Feasey *et al*., 2016). The African type is characterized by high level of antimicrobial resistance and manifests itself slightly differently from the type commonly found in the West. We therefore suggest, that *S.* Enteritidis strains isolated from the horse and poultry in the present study be further genetically characterized to determine their African type, and also to differentiate them from the Western type as well as to understand their animal reservoir.

In addition, the results revealed the emergence of two different *S.* Mbandaka strains from dogs with each displaying a distinctive pattern of antimicrobial resistance profile, suggesting they were not epidemiologically related (Table 4-13). The study observed that all clinical strains were represented in the seven resistotypes, with a MAR index > 0.2, indicating that they originated from a source where antimicrobial agents were over used (Tambekar *et al.,* 2006; Jayaraman *et al.,* 2012). In Nigeria, antimicrobial resistance pattern of *S.* Enteritidis isolates from commercial poultry were amoxicillin-clavulanic acid (100 percent), sulphamethoxazole (66.7 percent), ciprofloxacin (33.3 percent), chloramphenicoal (66.7 percent), ceftazidime (67.7 percent), ceftriazone (100 percent), streptomycin (100 percent) and ampicillin (100 percent) (Agada *et al*., 2014). These antimicrobial resistance pathogens are transmitted to humans through consumption of poultry meat or products, and as such *S.* Enteritidis has become a major cause of blood poisoning and death in Africa and food poisoning in the Western World (Phu *et al*., 2016; Feasey *et al*., 2016).

On the other hand, the lowest frequency of resistance among *Salmonella* serotypes were to azithromycin (5.9 percent, 2/34), and ciprofloxacin (2.9 percent, 1/34). This is almost in agreement with the study by Williamson *et al*. (2018), which showed that 4.9 percent of NTS strains in Australia were resistant to ciprofloxacin. On the contrast, all *Salmonella* isolates from meat samples in Ghana were susceptible to ciprofloxacin (Adzitey *et al*., 2015). We highlight concerns with MDR-*Salmonella* strains isolated from the horse and dog towards clinically important azithromycin and ciprofloxacin (Fluoroquinolones) drugs. Carriage of MDR *Salmonella* strain isolated by these animal species might serve as a warning to horse handlers and dog owners of the potential risks of exposure to bacterial pathogens through intimate relationships. Ciprofloxacin can be an appropriate drug in the treatment of invasive infections caused by MDR *Salmonella* (Chiu *et al*., 2002), and severe gastroenteritis (Pui *et al.,* 2011a), whereas azithromycin is used to treat wide variety of bacterial infections (Guchev *et al*., 2004; Ahmad *et al*., 2010; Imperi *et al.,* 2014; Euba *et al*., 2015), and also effective against Gram-positive bacteria (Powers, 1996).

In the light of the previously reported human MDR *Salmonella* infection in Zambia (Hendriksen *et al*., 2013; Chiyangi *et al.,* 2017), it can be inferred that the highly MDR *Salmonella* strains detected in horse and dogs faecal samples in the present study are likely to fuel the prevalence of antimicrobial resistance and thereby reducing further the choice of therapeutic agents in the event of human or animal infection. Therefore, both the horse and dog may contribute to the dissemination of MDR *Salmonella* strains in the environment through faecal contamination (Espie *et al*., 2005; Wright *et al.,* 2005; Dallap *et al*., 2010; Steneroden *et al*., 2010; Burges, 2015; Vangelis and Gousia, 2015; Burgess *et al*., 2018).

Infections caused by MDR *Salmonella* strains tend to take long to treat and manifests into severe illness unlike those infected with susceptible strains (Cook *et al*., 2009). Therefore, with the occurrence of MDR strains circulating in food of animal origin, it is important that patients due to salmonellosis can be subjected to laboratory tests to determine the appropriate and effective drugs. However, the concerns of antimicrobial resistance were tabled at a recent UN meeting attended by the Permanent Secretary, Ministry of Health. At that forum Zambia committed itself to tackling antimicrobial resistance comprehensively and multi-sectorally through improving

awareness of antimicrobial resistance (Lusakatimes.com, 2016). In line with the government initiative, this study provides the necessary public health information about the distribution of MDR *Salmonella* strains in various animal hosts and the likely trend of MDR to be encountered in the food of animal origin.

None of the *Salmonella* isolates was to be resistant to nalidixic acid antibiotic. This finding is considered a good indicator of susceptibility to fluoroquinolones such as ciprofloxaxin, levofloxacin and norfloxacin (Ochiai *et al*., 2008). In the present study none of the tested strains were found resistant to third generation cephalosporins (ceftazidime, ceftriaxone, cefotaxime), sulfonamide (cotri-trimoxazole) and fluoroquinolone (norfloxacin). Our findings, suggest that these antimicrobial agents are less abused in farm animal management or veterinary medicine in the study areas, and as such both cephalosporins and fluroquinolone antimicrobial agents could still be effective in the treatment of salmonellosis infections in domestic animals and humans, in spite of resistance that have been reported elsewhere (Crump and Mintz, 2010; Liakopoulos *et al*., 2016a; Liakopoulos *et al*., 2016b; Bhetwal *et al*., 2017; Klemm *et al*., 2018).

Our results were in tandem with the work done in Ghana (Andoh *et al*., 2016), China (Elhadi, 2014), and Ethopia (Legesse *et al*., 2015), in which all the *Salmonella* strains tested were found susceptible to cefotaxime, ceftazidime and cefoxitin. In contrast, the work by Hendriksen, *et al*, (2013) in Zambia, reported an extremely drug-resistant *S.* Senfteberg from human patients to cefotaxime, ceftazidime, ceftriaxone, chloramphenicol, ciprofloxacin, nalidixic acid, streptomycin and tetracycline among other drugs. These strains belonged to a multilocus sequence typing of sequence type 14 (MLST ST14) strains which contained various resistance genes, in addition to two mutations in gyrase (*gyr*A) and one mutation in DNA topoisomerase 4 subunit A protein (*par*C gene) which are responsible for high-level fluoroquinolone resistance (Hendriksen, *et al,* 2013). However, other studies have described resistance to fluoroquinolones (broad-spectrum antibiotics) which is used in medical practice for the treatment of resistant infections (Redgrave *et al*., 2014). In this study, domestic animals in particular chickens have been recognized as important reservoirs for the zoonotic food-borne transmission of resistant bacteria (Carattoli *et al*., 2008).

5.3.5 Diversity in carriage of accessory *spv* genes among *Salmonella* strains isolated from domestic animals

Our study revealed that *S.* Enteritidis was the serotype most frequently found to carry *spv* genes isolated from the diseased domestic animal sources (horse = 1, and chicken = 15), followed by *S.* Ruanda, and *S.* Stockholm, suggesting that any *Salmonella* serotype has the potential to *spv* genes.

Interestingly, all *S.* Enteritidis (*n* = 16) were concurrently in possession of *spv*A, *spv*B and *spv*C genes except one strain from chicken which lacked *spv*B gene, which constituted a distribution frequency of 100 percent (16/16), 93.3 percent (15/16) and 100 percent (16/16) respectively (Table 4-16). Our finding agrees with the existing knowledge that *S.* Enteritidis serotype worldwide harbour *spv* genes (Aminia *et al.*, 2015). Similarly, other investigators have also described high detection and distribution rate of *spv* genes in *S.* Enteritidis strains isolated from humans, bovine and poultry sources (Kumarss *et al*., 2010). Our result is in agreement with the work by Olsen *et al*., (2004), who reported that not all strains of serotypes that typically possess virulence plasmids would possess the genes at all times. The abscence of *spv*B in one strain *S.* Enteritidis serotype in this study could be indicative of the strain's inability for systemic survival (Heithoff *et al*., 2008), and impotent to establish virulence phenotype (Lesnick *et al*., 2001). Detection of *spv* genes in the present study, is to our knowledge being described here for the first time in *S.* Ruanda and *S.* Stockholm serotypes.

No significant correlation was observed between virulence profiles and antimicrobial reistance. However, a marginal association was observed between strains in possession of *spv*ABC genes and resistance to chloramphenicol antimicrobial agent ($p = 0.06$, 95 percent CI). The majority of strains in possession of *spv*ABC genes were susceptible to chloramphenicol and a few were resistant. In this study, all isolates possessing *spv*ABC genes were resistant to at least four antimicrobial agents, suggesting clinical significance (Wondwossen *et al*., 2009). A combination of MDR traits and possession of virulence genes can be potential catalyst for human invasive salmonellosis (Gebreyes *et al*., 2009). The results suggest the need to be wary of the possible sources of invasive *Salmonella* serotypes circulating in domestic animals and wildlife in Zambia. However, we could not confirm the virulence traits of the *Salmonella* strains such as *S.*

Stockholm, *S.* Ruanda and *S.* Garoli rarely known to carry *spv* genes in mouse or chicken infection models as this was beyond the scope of the present study. According to Clements *et al*. (2001), possession of genes encoding virulence factors does not guarantee effective infection. Therefore, we recommend further investigation to ascertain the phenotypic virulence traits of these serotypes.

In the present study, none of the serotypes *S.* Sendai, *S.* Hadar, *S.* Chardan, *S.* Mbandaka or untyped *Salmonella enterica* spp. yielded *spv* genes despite being clinical isolates that were characterized by MDR traits. Our finding is incongruent with the previous studies; for instance in Poland, no *spv* genes were detected in *S.* Hadar strain isolated from human diarrhoeal faecal samples (Trafny *et al*., 2006), suggesting that there could be other mechanisms that trigger disease causation. There is scanty information on the involvement of *S.* Garoli in human systemic infections, but the closest report linking *S.* Garoli were outbreaks of diarrhoeal diseases in Uganda (Lubwana, 1985). By inference, the presence of *spv* genes detected in non host-adapted serotypes such as *S.* Garoli, *S.* Ruanda and *S.* Stockholm in the present study, demonstrates evolution of possible expansion of serotypes carrying specific virulence genes which were not known to carry virulence genes. Our findings further revealed that more than half of the strains tested in the present study, possess virulenced genes and are capable of causing infection either through consumption of undercooked meat of food animals origin or being in contact with faeces from domestic animals or wildlife.

5.4 Sequencing of 16 rRNA and *inv*A genes of *Salmonella* strains isolated from domestic animals and wildlife

In the present study, 16S rRNA sequencing results revealed that 32 strains were *S. enterica,* when compared with refence sequences in the GenBank (Table 4-20). Almost, all serotypes that cause disease in humans, and mammals including birds belong to *Salmonella enterica* (Silva *et al*., 2014). We conclude that sequencing of the 16S rRNA gene of *Salmonella* strains isolated from domestic animals and wildlife facilitated the confirmation of these strains as *S. enterica* (Srinivasan *et al*., 2015).

Furthermore, 12 local *Salmonella* strains were sequenced, and their nucleotide and amino acid sequences of the 284 bp PCR fragment of the *inv*A gene were compared with 8 other reference strains from the GenBank. According to El-Sebay *et al*., (2017), *inv*A gene is a very important diagnostic application which contains sequences unique to the genus *Salmonella* and therefore requires periodical evaluation of sequence variation. Based on the nucleotide sequence of *inv*A gene, *Salmonella* strains in the present study were analyzed to detect their genetic relationship (Suna *et al.*, 2010; El-Sebay *et al*., 2017).

Analysis of the multiple alignment of 20 nucleotide sequences comprising local (12) and published (8) strains, showed differences mainly in the region between 40 bp and 250 bp position of the nucleotide sequence of the *inv*A-284 bp gene fragment (Figure 4-8, Table 4-19), or between 407 bp and 618 bp when compared with a complete *Salmonella* Typhimurium *inv*A gene–2176 bp (Accession no: M90846.1) (Figure 4-9). This finding, is suggesting that this region (hot spot) could have a predilection for the integration of foreign DNA elements by substitution (Table 4-19). It is a fragile region in which most translocation of DNA frequently takes place. Further analysis, demonstrates that the nucleotide sequences of *inv*A-284 bp chain of the local strains and reference strains appeared stable in the region between 1~39 bp, and 251~284 bp positions (Figure 4-8 and Table 4-19), or between 371~406 bp and 619~655 bp on complete *inv*A gene, suggesting this region is highly conserved within *inv*A gene (Figure 4-9). A study by El-Sebay *et al*. (2017), showed multiple nucleotide substitution in the sequence chain of a local *S.* Typhimurium strain from position 208 bp~1813 bp of the 2058 bp *inv*A gene in Egypt. However, we observed multiple substitutions in the reference strains *S.* Enteritidis.EC20110222 and *S.* Typhimurium FORC020 from Canada and South Korea respectively. It was further observed that nucleotide substitution resulted in significant amino acid changes in both local (*S.* Heidlberg.670, *S.* Pomona.SI-1, *S.* Hadar, *S.* Stockholm.B5 and *S.* Chardan) and reference strains (*S.* Typhimurium.1STYINVA, *S.* Choleraesuis.1GHPS1, *S. enterica*.58BEhb, *S.* Bredney.KC14RD and *S.* Typhimurium.FORC020) (Table 4-23 and Figure 4-10). For instance nucleotide substitution changes in the local strain *S.* Heidelberg.670 at position 220 bp resulted in amino acid changes at the position A 74 R (from alanine to arginine), suggesting the occurrence of substitution in the *inv*A gene over a period of time. This phenomenon was observed in at least 45 percent (9/20) different *Salmonella* strains, suggesting that amino acid

(alanine) at the position 74 is likely to be substituted by another amino acid (e.g. arginine), while amino acid substitution at position 84 leads to silent substitutional changes (for instance glutamine to serine). In this case, substitution of a single DNA nucleotide within a protein-coding portion of a gene does not affect the sequence of amino acids that make up the gene's protein.

In the present study, 98~100 percent nucleotide similarity was observed among the local *Salmonella* strains (Table 4-21), suggesting that no significant difference exist between clinical and none clinical *Salmonella* strains. Nucleotide sequence of the local strains shared 74~100 percent identity when compared with the sequences of *inv*A gene of 8 reference *Salmonella* strains from the GenBank [*S. enterica*, No. KJ718884.1, (Egypt); *S.* Typhimurium, No. M90846.1, (unknown origin); *S.* Choleraesuis, No. EU348367.1, (China); *S. houtenae*,No. DQ644627.1, (unknown origin); *S. enterica*, No. KJ718878.1, (Egypt); *S.* Bredeney, No. KP279306.1 (Australia); *S.* Typhimurium, No. CP012144.1, (S. Korea); and *S.* Enteritidis, No. EC20110222, (Canada). However, a high similarity (98~99 percent) was observed between local strains (*S.* Heidelberg, *S.* Pomona, *S.* Ruanda.P13e, *S.* Hadar.B1, and *S.* Stockholm.B7), with the seven reference strains except *S.* Enteritidis, No. EC20110222, isolated from Canada, suggesting the protein *invasion* A is highly conserved in some *Salmonella* strains regardless of the diverse serotypes, geographic locations and the animal hosts. In a similar study, in North China, the *inv*A nucleotide sequences from 12 of 33 strains isolated from clinically symptomatic chickens were 72.9 to 97.6 percent identical, and they shared 78.9 to 97.2 percent identity to the sequences from reference *Salmonella inv*A genes from GenBank (Shi *et al*., 2012).

On the other hand, homology percentage of amino acid sequences showed 96~100 percent similarity among *Salmonella* strains isolated from domestic animals and wildlife (Table 4-24). But 60~100 percent similarity was detected when compared with other reference strains in GenBank (Table 4-22), suggesting a wider variations in the genomic structure of other strains across the world. A previous study in Egypty showed the homology percentage of nucleotide sequence (99.2~99.4 percent) and amino acid (99.6~99.9 percent) similarity between the local isolates and the other published sequences in the GenBank (El-Sebay *et al*., 2017), suggesting close relatedness. In China sequence comparison showed nucleotide identities of 72.9~97.6

percent among the *inv*A genes of *Salmonella* isolated from clinical samples of chickens (Shi *et al*., 2012). But they shared 78.9~97.2 percent identity with the sequences from reference *Salmonella* strains (*S.* Gallinurum, No. U43273; *S. enterica*, No. U43272; *S. bongori*, No. DQ644632; *S.* Typhimurium, No. M90846; and *S.* Choleraesuis, No. EU348367.

Interestingly, in the present study a comparison of the nucleotide and amino acid sequences exhibited 100 percent homology identity in six local *Salmonella* strains; *S.* Garoli.3 (leopard), *S.* Enteritidis.B10 (horse), *S.* Enteritidis.WF1-3 (chicken), *Salmonella* spp.P19 (chicken), *S.* Sendai.P21A (chicken), and *S.* Ruanda.P12 (chicken)], and one reference strain *S. enterica* accession no. KJ718884.1 isolated from chicken in Egypt. The finding indicates that the *inv*A gene is highly conserved in *Salmonella* strains, despite their differences in serotype and carriage of accessory virulence genes (Table 4-25 and Table 4-28). Further, we observed that *Salmonella* strains carrying *spv* genes tended to have an identical nucleotide sequence chain. On the contrary, *S.* Ruanda and *S.* Stockholm serotypes carrying *spv* genes revealed substitutional changes in their chains resulting in protein structure change from adenine to guanine (position 210) and adenine to thymine (position 133) respectively. Therefore, it is prudent that virulence parameters of these strains are assessed *in vivo* to establish their pathogenicity. However, similar changes were observed with reference strains (*S.* Typhimurium.1STYINVA, *S.* Choleraesuis.1GHPS1, *S. enterica*_58BEhB, *S.* Bredeney.KC14RD, *S.* Enteritidis.EC20110222 and *S.* Typhimurium.FORC_020 from other countries.

Nucleotide sequence comparisons of related serotypes, showed 75~99 percent similarity between *S.* Enteritidis from local strains and reference *Salmonella* strain from the GenBank. For instance *S.* Enteritidis strain (CP007323.1) from Canada revealed greater nucleotide sequence variation than the local *S.* Enteritidis strains from horse and chicken respectively, suggesting the strains are distantly related due to sequence changes that could have taken place over a period of time (Table 4-26 and Figure 4-9). In the present study, it has been proved that sequences of PCR *inv*A-284 bp amplicon products is useful for detecting the genetic diversity of *Salmonella* strains isolated from domestic animals and wildlife.

5.5 Phylogenetic analysis

The construction of phylogenetic tree based on nucleotide and amino acid sequence differences of *inv*A-284bp gene revealed two major clades, lineages A and B (Figure 4-11 and Figure 4-12). *Salmonella* Enteritidis (CP007323.1) isolated from unknown source in Canada formed lineage B and was distinct from the other *Salmonella* strains. Subgroup I consisted of the majority (10) local strains from different serotypes; *S.* Sendai, *S.* Ruanda, *Salmonella* spp., *S.* Enteritidis, *S.* Garoli, *S.* Stockholm, *S.* Heidelberg, *S.* Hadar), and these were not distinct from the six reference strains (accession no: KJ718884.1, DQ644627.1, EU348367.1, M90846.1, KJ718878.1, CP012144.1). Our investistigation found that isolates in possession of *spv* genes (*S.* Ruanda, *S.* Enteritidis, *S.* Garoli, and *S.* Stockholm) were distributed in three separate phylogenetic sub Clades (Ia, Ib and Ic), with the majority strains being in Ia.

Suffice to say that *inv*A gene is part of the loci that constitutes invasion genes which are responsible for invasion of epithelial cells of the host (Chiu and Ou, 1996; Darwin and Miller, 1999; Gole *et al.,* 2013). To our knowledge this could be the first report of genetic diversity of *Salmonella* strains isolated from domestic animals and wildlife in selected areas of Zambia.

5.6 Public health significance of the study

The strains of *Salmonella* identified in this study are zoonotic in nature, and therefore are of public health significance. These pathogens are capable of causing disease in man through direct consumption of contaminated food of animal origins, or water, or indirect through contact with animals or their faecal droppings. *Salmonella* Enteritidis is one of the most common cause of food poisoning in humans, from poultry meat, and poultry products. However the other serotypes *S.* Heidelberg, *S.* Pomona, *S.* Garoli and *S.* Sendai, have also been linked to human salmonellosis. Salmonellosis is a major cause of bacterial enteric illness in both humans and animals. Some serotypes have emerged to possess virulence genes such as the *S.* Enteritidis, *S.* Ruanda, *S.* Garoli and *S.* Stockholm indicative of the potential to cause extra-intestinal or invasive salmonellosis within the domestic animals, wildlife or humans. The study revealed that *S.* Enteritidis is the most predominant serotype, overwhelming the isolation rates of other serotypes.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

6.1.1 The study revealed that there is phenotypic diversity of *Salmonella* strains (sero-groups, serotypes, biotypes, and resistotypes) circulating among the domestic animals, captive and wildlife in selected areas of Zambia.

6.1.2 The study detected the presence of *spv* genes in isolates from domestic animals (*S.* Enteritidis, *S.* Stockholm *and S.* Ruanda) and wildlife (*S.* Ggaroli) belonging to serogroups O9 and O7 respectively, indicating that *spv* gene is not limited in distribution to specific serogroups, serotype and source of isolation. These strains are non-host adapted *Salmonella* serotypes and are rarely known to carry *spv* genes except *S.* Enteritidis. Therefore, the present study signals an insight into possible potential sources of invasive MDR-NTS strains circulating in domestic animals and wildlife in selected areas of Zambia.

6.1.3 Based on the sequence analysis of the *inv*A gene of *Salmonella* serotypes from domestic animals and wildlife, the study revealed nucleotide sequence variations exist among the local strains and strains from other regions.

6.1.4 Furthermore, the study has shown that domestic animals (cattle, horses, dogs and chickens) and wildlife animals (sable antelopes, leopard and impala antelopes) are potential zoonotic reservoirs of diverse NTS serotypes. In an area with high population of domestic and wildlife, this diversity of serotypes could signify a possible wide dissemination of NTS serotypes through environmental faecal contamination.

6.1.5 The study revealed that horses and dogs are possible animal reservoir for the transportation and dissemination of MDR *Salmonella* strains between hosts and also contamination of the environment through faecal matter.

6.1.6 Free-range wildlife in Zambia, are reservoirs of *Salmonella* pathogens that can contaminate the ecosystems through faecal droppings, which may result in human

infection in persons as the game scouts, game viewers and/or any other stakeholders through contact.

6.1.7 Based on biotyping, failure to ferment arabinose, cellobiose, inositol and salicin by isolates is a useful marker for identifying serotypes in possession of *spv* genes.

6.2 Recommendations

6.2.1 Although the occurrence of *Salmonella* species in the faeces of wildlife was low in this study, Game scouts, tourists and professional hunters of leopards, sable antelope and impala antelopes are cautioned to have minimal contact with excreta of the animals mentioned herein.

6.2.2 The antibiotic susceptibility profiles of *Salmonella* pathogens to commonly used antibiotics be monitored regularly.

6.2.3 Continuous studies are needed to address the data gaps on the association between multi drug resistance of some *Salmonella* isolates and virulence determinants found in some serotypes.

6.2.4 Farmers must be encouraged to submit clinical samples on suspected *Salmonella* cases for antibiotic resistance monitoring.

6.2.5 Domestic animal owners should be made aware that these animals may be possible carriers of *Salmonella*.

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Characterization of non-typhoid *Salmonellae* isolated from domestic animals and wildlife from selected areas of Zambia

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ABSTRACT

Domestic animals and wildlife are considered important reservoirs from which Non-Typhoid *Salmonella* serotypes have emerged to cause public health concerns worldwide. Identification of the zoonotic origin of such organisms is important to understand the risk factors associated with a diversity of host types. In this study, phenotypic and genetic properties of *Salmonella* strains isolated from domestic animals and wildlife sources were characterized to determine their potential for causing invasive salmonellosis. The isolates were characterized into serogroups, serotypes, fermentative groups, resistotypes, and beta-lactamase activity types. Further, *Salmonella* plasmid virulence genes responsible for extraintestinal manifestations were determined among *Salmonella* serotypes from domestic animals (bovine, horse, dogs, and chickens) and wildlife (Leopard, Sable and Impala). Thirty-four *Salmonella* strains belonging to four sero-groups; B, C1, D1, and a non- reactive group, were isolated and identified from the various animal hosts studied. The most prevalent sero-group was D1 (50.0%; 17/34) *Salmonella* strains, all from domestic animals followed by a non-reactive group (29.4%; 10/34) strains. Further, 12 serotypes, were detected among the strains. The most predominant serotype was *Salmonella enterica* serotype Enteritidis (47.1%; 16/34) isolated from horse and chicken, followed by *S.* Mbandaka (8.8%; 3/34) from dog and chicken. All *Salmonella* isolates were resistant to at-least four antimicrobial agents. Ampicillin, erythromycin, gentamicin, nitrofurantoin and penicillin G were observed as the most frequently resisted antimicrobial agents. *Salmonella* Enteritidis and *S.* Mbandaka isolated from the horse and dog respectively, each displayed multidrug resistance to 6 antimicrobial agents. All strains harboured *Salmonella* invasion A gene unique to the genus, while some strains possessed *Salmonella* plasmid virulence locus gene. In conclusion, the present study isolated and identified multi-drug resistant *Salmonella* serotype strains that possess virulence genes which are of potential public health concern.

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Introduction

Non-typhoid *Salmonella enterica* subspecies (NTS) are the cause of approximately 1.3 billion cases of human NTS salmonellosis annually worldwide [1]. A recent report indicates that approximately 550 million adults, including 220 million children of less than five years old develop diarrhoeal diseases as a result of ingestion of *Salmonella* contaminated food [2]. *Salmonella* can also cause disease in domestic animals which then become the source of zoonotic infection to humans [3]. However, the environmental reservoir from which these *Salmonella* strains are transmitted to people is still unknown [4]. Lack of such information makes more difficult, controlling and preventing the spreading of the infections. *Salmonella* pathogens are maintained in animal reservoirs, and infection in humans follows ingestion of contaminated animal products, farm produce contaminated with infected animal faeces, or in close contact with infected animals [5,6].

In Zambia, *Salmonella enterica* serotype Senftenberg (*S.* Senftenberg) was among the clinical serotypes that caused severe human infections for which there were very few treatment options [7]. Domestic animals and wildlife play a pivotal function of being zoonotic reservoirs capable of spreading different *Salmonella* serotypes to humans. The severity of salmonellosis in humans or animals is reflected by inherent pathogenesis properties of each serotype such as antigenic properties, virulence and antimicrobial phenotypes [8]. Among the virulence genes is included; Salmonella invasion A (invA) gene which is found in all the *Salmonella* serotypes [9] and is involved in the earliest stages of the invasion of intestinal epithelial cells by the facultative intracellular pathogenic *Salmonella* spp. [10]. A previous study indicated that a few *Salmonella* serotypes belonging to subspecies *enterica* carry specific large virulence plasmids (VPs) [11]. Virulence plasmids contain virulence genes (*spv*) associated with severe systemic infection by NTS serotypes [12]. Plasmid-encoded *spv* genes have been reported in the common broad-host-range serotypes such as *S.* Enteritidis, *S.* Paratyphi C, *S.* Rostock, *S.* Newport and *S.* Moscow [13,14]. Thus, it is against this back ground, that the present study characterized *Salmonella* strains isolated from domestic animals and wildlife in selected areas of Zambia, for the purpose of understanding the sero-groups, serotypes, and their virulence potential existing in the country.

Materials and methods

Study areas, sampling process and study design

During the period June 2012-December 2014, a total of 1248 samples from domestic animals (1008) and wildlife (240) were obtained for *Salmonella* examination. Of the 1008 domestic animal samples, 659 (65.4%) were faecal samples from apparently healthy animals (cattle = 632; horses = 27), while 349 (34.6%) were mixed samples from suspected sick animals submitted for routine microbiological diagnosis. Of the sick animal samples, 311 (89.1%) were from chickens, 36 (10.3%) dogs, and 1 each (0.3%) from horse and sable antelope respectively. The mixed samples comprised intestinal contents, faeces, organs, tissues, and swabs. Cattle samples were collected from seven districts of Southern Province; 1. Mazabuka (n = 118), 2. Monze (n = 126), 3. Pemba (n = 126), 4. Choma (n = 125), 5. Kalomo (n = 50), 6. Zimba (n = 28), and 7. Kazungula (n = 59). All the faecal samples from horses were collected from Lusaka Province (Fig. 1). All samples were stored in an ice cooled box before transportation to the Microbiology Laboratory, University of Zambia.

The wildlife faecal samples from 17 free-ranging wildlife spp. were collected from Kafue National Park (KNP (n = 210)), Lochnivar National Park (LNP (n = 21)) and Lusaka Central Park (LCP (n = 9)). All samples were stored in an ice cooled box before transportation to the Microbiology Laboratory for analysis. The distribution of samples among the wildlife spp. is shown in Supplementary Table 1.

Isolation and identification of Salmonella

One gram or equivalent of fresh sample was pre-enriched with 9 ml of buffered peptone water (BPW (HIMEDIA Laboratory Pvt, Ltd, M614, Mumbai, India)) and incubated for 24 h at 37 °C. A portion (1 ml) of the pre-enriched broth culture was transferred to 10 ml selective enrichment Rappaport Vassiliadis Soybean Meal Broth (RVSM (HIMEDIA M1448)) and incubated at 42 °C for 24 h. From the RVSM broth, a loop full of the inoculum was transferred and streaked onto Xylose Lysine Deoxycholate (XLD (HIMEDIA, M031)) agar and Brilliant Green Agar (BGA (HIMEDIA, M016)) plates then incubated at 37 °C for 24 h according to the International Organization for Standardization [ISO] protocol (ISO 6579:2002) [15]. Suspected colonies on XLD and BGA were selected for biochemical tests using composite media [16]. All isolates were further identified as *Salmonella* using Analytical Profile Index (API) 20 E systems (Biomerieux SA, Marcy-1 Etoile France) and the API web software. Pure bacterial isolates were kept frozen at −80 °C in 10% (v/v) glycerol peptone water broth. *Salmonella* Typhimurium (ATCC 14028) was used as quality control organism.

Detection of Salmonella *serogroups and serotypes*

Salmonella strains were characterized into serogroups based on the presence of distinctive “O” antigenic factors. They were first examined with O polyvalent antiserum for A-S group antigen by latex agglutination test, followed by specific monovalent antiserum (group O2-O9) or (group B, C1, C2, D1, E, and G) (MAST ASSURES, SA) according to manufacturer’s instructions. All *Salmonella* isolates were referred to the Deltamune (Pty) Laboratory, South Africa, for confirmation and

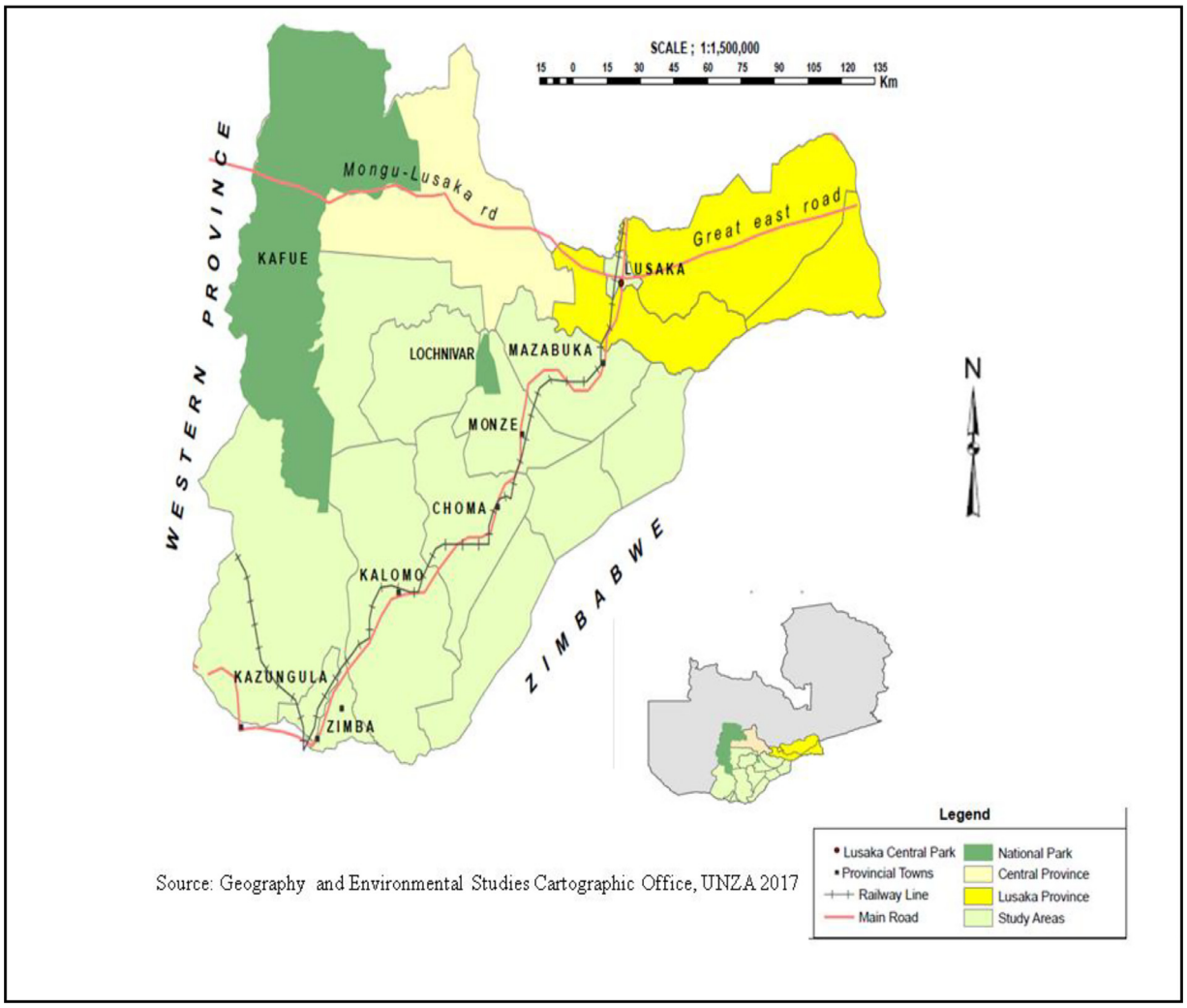

Fig. 1. Map of Southern Province (cattle faecal samples $n = 632$) and Lusaka Province (horse faecal samples $n = 27$; various samples from different diseased animals $n = 349$; wildlife faecal samples from LCP $n = 9$) Provinces, with deep green shading indicating KNP (wildlife faecal samples $n = 210$) and LNP (wildlife faecal samples $n = 21$) and insert – Map of Zambia.

serotyping. Serotyping was done using the method described in the Microbiological Manual and serotyping (10.2:1995 CCFRA) based on White-Kauffman-Le Minor Scheme (WHO Collaborating Centre).

Detection of extended spectrum beta-lactamases (ESBLs) in Salmonella *strains*

Detection of extended spectrum cephalosporinase producing isolates was accomplished using freshly prepared MacConkey agar (HIMEDIA) containing 2 mg/L of cefotaxime (Sigma-Aldrich, Munich, Germany) according to the method described in [17].

Detection of antimicrobial resistance

Kirby-Bauer diffusion method was used on Mueller-Hinton agar to test for antimicrobial resistance of *Salmonella* strains [18]. A panel of 17 antimicrobial agents (HIMEDIA) was used which included; ampicillin (Amp10 μg), azithromycin (Azm 15 μg), chloramphenicol (C 30 μg), ceftazidime (CAZ 30 μg), ciprofloxacin (CIP 5 μg), cotrimoxazole (COT 25 μg), ceftriaxone (CRO 30 μg), cefotaxime (CTX 30 μg), erythromycin (E 15 μg), gentamicin (Gen 10 μg), levofloxacin (LE 5 μg), nalidixic Acid (NA 30 μg), nitrofurantoin (Nit 300 μg), norfloxacin (NOR 5 μg), penicillin G (PG 10units), streptomycin (S 300 μ.g) and tetracycline (TE 30 μg). Susceptibility (S) or resistance (R) or intermediate (I) profiles were determined in millimetres according to the breakpoints as described in the guidelines of the Clinical and Laboratory Standards Institute [19].

Table 1
List of uniplex set of Oligonucleotides (primers) used for PCR amplification.

Primer	Target oligonucleotide sequence	Length (bp)	Reference
*inv*A F	5'-GTGAAATTATCGCCACGTTCGGGCAA-3'	284	[20]
*inv*A R	5' – TCATCGCACCGTCAAAGGGAACC-3'		
*spv*A F	5' – GTCAGACCCGTAAACAGT-3'	604	[21]
spv A R	5' – GCACGCAGAGTACCCGCA-3'		
spv B F	5' – ATGTTGATACTAAATGGTTTTTCA-3'	1776	[22]
spv B R	5' – CTATGAGTTGAGTACCCTCATGTT-3'		
spv C F	5'-CGGAAATACCATCTACAAATA-3'	669	[23]
spv C R	5' – CCCAAACCCATACTTACTTACTCT-3'		

Table 2
Frequency of isolation of *Salmonella* spp. from domestic animals and wildlife ($n = 1248$).

General Host	Specific Host	Apparently healthy animals (Field samples)		Animals with clinical disease	
		No. of samples	No. of isolates	No. of samples	No. of isolates
Domestic animals ($n = 1008$)	Cattle	632	2	0	0
	Horse	27	0	1	1
	Dog	0	0	36	2
	Chicken	0	0	311	26
Wildlife* ($n = 240$)	Sable	9	0	1	1
	Leopard	6	1	0	0
	Impala	42	1	0	0

* Some animals did not yield *Salmonella*

Detection of fermentative groups of Salmonella *isolates*

Utilization of sugars by *Salmonella* strains from different sources were tested using phenol red broth (M054-500G HI-MEDIA) prepared according to manufacturer's instructions. The broth contained one of the following sugars or alcohol; disaccharides (maltose, trehalose, melibiose and cellobiose), hexoses (glucose, mannose and galactose), pentoses (arabinose, xylose, and rabitol), polyhydric alcohol (inositol, adonitol, mannitol, dulcitol and salicin) and trisaccharides (raffinose).

Molecular characterization of Salmonella *isolates*

The bacterial genome was extracted from *Salmonella* culture on nutrient agar (HIMEDIA) by using a DNeasy Blood and Tissue Kit (Qiagen) as recommended by the manufacturer of the product. *Escherichia coli* ATCC 25922 was used as a negative control. All *Salmonella* isolates were examined for the presence of DNA sequence amplification for the following genes; *inv*A [20], *spv*A [21], *spv*B [22], and *spv*C [23]. The amplification conditions and the primers used were adapted as described by other workers (Table 1). Amplification products were considered positive based on the appropriate base pair size. The amplified PCR products; *inv*A, *spv*A, *spv*B and *spv*C of the *Salmonella* DNA were subjected to gel electrophoresis with ethidium bromide staining. The amplified DNA fragments were visualized by UV trans-illuminator (Benchtop 3UV-UVP, CA, USA).

Results

Isolation of Salmonella *from different sources*

A total of 34/1248 (2.7%) *Salmonella* strains were isolated and identified from domestic animals and wildlife samples (Table 2). *Salmonella* was isolated from different sources i.e. faecal samples of apparently healthy cattle (0.32%; 2/632); free-ranging wildlife (0. 83%; 2/240) and from samples of sick animals (8.6%; 30/349). Of the 30 isolates from sick animals, 29 (96.7%) were from domestic animals, while one (3.3%) was from a wildlife sample (sable antelope). Among 29 isolates, 26 (89.7%) were from chickens, while 2 (6.9%) and one (3.4%) were from dogs and horse respectively. The two isolates from free-ranging wildlife were each from leopard (16.7%; 1/6) and impala antelope (2.4%; 1/42).

Characterization of Salmonella *serogroups and serotypes*

The slide agglutination test (Table 3), revealed that 32 (94.1%) isolates produce positive agglutination reactions with *Salmonella* somatic "O" polyvalent A-S group antiserum, except two strains isolated from sable antelope (SI-I) and impala antelope (I-213) respectively. Serological analysis showed that all strains belonged to four distinct monovalent sero-groups; B (O: 4), C1 (O: 6, 7), D1 (O: 9) and a non-reactive group (NR) ($p < 0.001$, 95% CI) (Table 3). *Salmonella* strains belonging to serogroup D1 (O: 9) were the most predominant (50.0%; 17/34) and were exclusively isolated from samples of sick domestic animals with 16 (94.1%; 16/17) from chickens, and one (5.9%; 1/17) from a horse. This was followed by *Salmonella* strains

Table 3
Distribution of *Salmonella* serogroups and serotypes isolated from domestic animals and wildlife.

Serotype	Animal host	No. of serotypes	Positive so-matic poly-va-lent A-1	No. of positive serogroups B	C1	D1	NR
S. Garoli**	Leopard	1	1	0	1	0	0
S. Pomona*	Sable	1	0	0	0	0	1
S. Roan**	Impala	1	0	0	0	0	1
S. Heidelberg**	Cattle	2	1	2	0	0	0
S. Mbandaka*	Dog	2	3	0	2	0	0
S. Enteritidis*	Horse	1	1	0	0	1	0
S. Enteritidis*	Chicken	15	15	0	0	14	1
S. Ruanda*	Chicken	2	2	0	0	2	0
Salmonella spp.*	Chicken	2	2	0	0	0	2
S. Hadar*	Chicken	1	1	0	1	0	0
S. Stockholm*	Chicken	2	2	0	0	0	2
S. Sendai*	Chicken	1	1	0	0	0	1
S. Mbandaka*	Chicken	1	1	0	1	0	0
S. Chardan*	Chicken	2	2	0	0	0	2
Total		34	32	2	5	17	10

Key: NR = None-reactive;
* Clinical isolate
** None-clinical isolate.

Table 4
Distribution of *Salmonella* serotypes based on fermentative activities and occurrence of *spv* genes.

Host	Serotype/fermentative group.	No. of strains	Variable fermentation activities of sugars and polyhydric alcohols Arabinose	Cellobiose	Inositol	Salicin	Presence of *spv* gene *spv*A	*spv*B	*spv*C
Leopard	*S.* Garoli[I]	1	-	-	-	-	+	+	+
Horse	*S.* Enteritidis[I]	1	-	-	-	-	+	+	+
Chicken	*S.* Enteritidis[I]	14	-	-	-	-	+	+	+
Chicken	*S.* Enteritidis[I]	1	-	-	-	-	+	-	+
Chicken	*S.* Ruanda[I]	2	-	-	-	-	+	+	+
Chicken	*S.* Stockholm[II]	1	+	-	+	-	+	+	+
Total		**20**	**1**	**-**	**1**	**-**	**20**	**19**	**20**

Key: + = positive reaction; - = negative reaction; I – II = fermentative group

classified under the non-reactive serogroup with 10 (29.4%; 10/34), which were defective to the monovalent agglutination test (Table 3).

Twelve distinct *Salmonella* serotypes were identified among the 34 *Salmonella* strains with the prevalence of 35.3% ($p = 0.002$, 95% CI). The distribution of identified serotypes from each specific animal source is summarised in Table 3. The predominant serotype was *S.* Enteritidis (47.1%; 16/34) accounting for almost one-half of the serotypes identified in the study. This study further revealed that *S.* Enteritidis (44.1%; 15/34) was the most frequent isolated strain from chicken, followed by *S.* Ruanda, *S.* Stockholm, *S.* Chardan, *Salmonella* spp. (5.9%; 2/34 each), *S.* Harder, *S.* Sendai, and *S.* Mbandaka (2.9%; 1/34 each). Three serotypes belonging to *S.* Garoli, *S.* Pomona and *S.* Roan were identified from wildlife samples.

Characterization of Salmonella *sero-fermentative groups*

All *Salmonella* serotypes without exception utilized trehalose, maltose, melibiose, galactose, glucose, mannose, xylose, mannitol and dulcitol to produce acid ($p = 0.01$, 95% CI). In contrast, all strains were exclusively negative for utilization of rabitol and raffinose. On the other hand, variable fermentation reactions were observed in the following substrates; arabinose, cellobiose, inositol and salicin (Table 4). The majority of the strains in this study complied with classic utilization of substrates by *S. enterica* subsp. I that include; dulcitol^{+}, salicin^{-}, mannitol^{+}, xylose^{+}, cellobiose^{-}, melibiose^{+}, glucose^{+}, arabinose^{+}, and raffinose^{-} (+ = positive; - = negative reaction). *Salmonella* strains in possession of *spv*ABC genes were biochemically grouped into two (I–II) fermentative profiles, based on their ability to utilize the four (cellobiose, arabinose, inositol and salicin) substrates (Table 4). All strains belonging to fermentative group I (*S.* Garoli, *S.* Enteritidis and *S.* Ruanda) were negative for utilization of cellobiose, arabinose, inositol and salicin as a source of energy. There is an association between *spv*A, *spv*C genes and none utilization of substrate [arabinose ($p = 0.012$), cellobiose ($p < 0.001$), and inositol ($p < 0.001$)]. Similarly a relationship was observed between *spv*B gene and substrate [arabinose ($p = 0.028$), cellobiose ($p < 0.001$), and inositol ($p = 0.004$)].

Table 5
Distribution of antimicrobial resistance profiles of *Salmonella* strains by serotype and host.

Group	Resistotype	Serotype	No. of strains	Host
I	Amp, E, Gen, Nit, P	Heidelberg**	1	Cattle
		Garoli**	1	Leopard
		Pomona*	1	Sable
		Mbandaka*	1	Dog
		Mbandaka*	1	Chicken
		Enteritidis*	10	Chicken
		Hadar*	1	Chicken
		Sendai*	1	Chicken
		Ruanda*	1	Chicken
		Stockholm*	2	Chicken
		*Salmonella. spp.**	1	Chicken
II	Amp,	Enteritidis*	2	Chicken
	E,	Ruanda*	1	Chicken
	Nit,	Heidelberg**	1	Cattle
III	P,	Enteritidis*	1	Chicken
	Gen,	Roan**	1	Impala
	Nit,	*Salmonella spp.***	1	Chicken
IV	Amp,	Enteritidis*	1	Chicken
	E,	Chardan*	2	Chicken
V	Amp, Azm, E, Gen, Nit, P	Enteritidis*	1	Horse
VI	Amp, Cip, E, Gen, Nit, P	Mbandaka*	1	Dog
VII	Azm, E, Gen, Nit, P	Enteritidis*	1	Chicken
Total			**34**	

Key: Amp = ampicillin, Azm = azithromycin, Cip = Ciprofloxacin, E = erythromycin, Gen = Gentamycin, Nit = Nitrofurantoin, P = Penicillin G;
* Clinical isolate
** None clinical isolate.

Characterization of Salmonella *strains based on antimicrobial resistance profile*

All 34 *Salmonella* strains isolated in this study were none carriers of an extended broad-spectrum beta-lactamases (ESBL) phenotype to 3rd generation cephalosporins (ceftriaxone, ceftazidime, and cefotaxime), indicating that they are more likely to be susceptible also to other 3rd generation cephalosporins. This is confirmed by none-bacteria growth on MacConkey agar (HIMEDIA) containing 2 mg/L of cefotaxime (Sigma-Aldrich, Munich, Germany).

To determine the antimicrobial resistance phenotypes of the *Salmonella* strains as per CLSI standards, all 34 strains were tested against a panel of 17 antimicrobial agents. The incidences of resistance for all the isolates tested are presented in Supplementary Table 2. There is no association between *Salmonella* serotype and resistance to the set of antibiotics used ($p > 0.05$). The results revealed that 58.8% (20/34) strains were susceptible to different antibiotics, while 29.4% (10/34) and 11.8% (4/34) were resistant and intermediate respectively. *Salmonella* strains displayed multi-resistance against 7 antimicrobial agents regardless of its source; erythromycin and penicillin G (100%; 34/34 each), ampicillin, and gentamycin (88.3%; 30/34 each), nitrofurantoin (85.3%; 29/34), azithromycin (5.9%; 2/34) and ciprofloxacin (2.9%; 1/34). In addition, intermediate resistance was observed against nalidixic acid (64.7%; 22/34), azithromycin (52.9%; 18/34), streptomycin (44.1%; 15/34), ampicillin and nitrofurantoin (11.8%; 4/34 each), chloramphenicol (8.8%; 3/34), tetracycline (5.9%; 2/34), ciprofloxacin, and levofloxacin (2.94%; 1/34 each). There is an association between resistance to azithromycin and utilization of cellobiose ($p = 0.034$). All *Salmonella* strains were susceptible to sulfonamide (cotri-trimoxazole) and fluoroquinolone (norfloxacin).

Antimicrobial resistance profiles of 34 *Salmonella* strains were grouped into seven (I–VII) distinct MDR resistotype profiles (Table 5). These resistotypes were determined based on the ability of *Salmonella* strains to confer resistance to a single or group of antibiotics. Resistotype group I was the most predominant group designated by five antibiotics, Amp-E-Gen-Nit-P phenotype, and was represented by 21 (61.8%) *Salmonella* strains isolated from domestic animals and wildlife. Of the 21 *Salmonella* strains, 19 (90.5%) were from domestic animal sources, while 2 (9.5%) were from wildlife. Among the 19 *Salmonella* strains, 89.5% were from chicken source. *Salmonella* strains isolated from chicken source were represented in 5 (71.4%) of the 7 MDR resistotypes (I, II, III, IV, and VII).

The only *S.* Enteritidis strain from the horse was resistant to six antibiotics, Amp-E-Az-Gen-Nit-P. All the 16 *Salmonella* Enteritidis strains isolated from domestic animals, were identified into 6 resistotype groups (I, II, III, IV, V and VII) (Table 5). Group I was the most predominant with 10 (62.5%; 10/16) strains showing a resistance phenotype against five antimicrobial agents; Amp-E-Gen-Nit-P. The present study observed a single strain of *S.* Mbandaka, isolated from a dog as the only strain resistant to ciprofloxacin. On the other hand, the wildlife clinical isolate from sable antelope revealed antimicrobial resistance against five antibiotics, same as a none-clinical isolate from apparently healthy leopard.

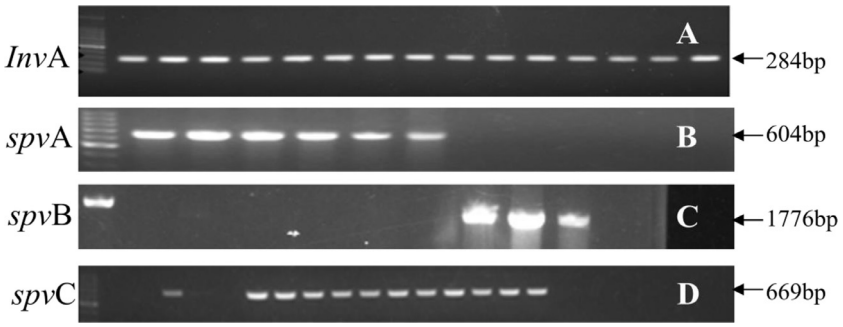

Fig. 2. PCR amplicon products of *Salmonella* genes; *inv*A ($n = 34$) (2A), *spv*A ($n = 20$) (2B), *spv*B ($n = 19$) (2C), and *spv*C ($n = 20$) (2D).

Confirmation of Salmonella *isolates by PCR*

The *Salmonella* isolates were confirmed by detection of the *inv*A-284bp amplicon product in all 34 isolates which are represented by 15 *inv*A amplicon products as shown in Fig. 2A.

Characterization of Salmonella plasmid virulence (spv) genes by PCR

The expected fragment length of respective PCR screening products for *spv*A (604 bp), *spv*B (1776 bp) and *spv*C (669 bp) were detected in strains belonging to *S.* Enteritidis ($n = 16$), *S.* Garoli ($n = 1$), *S.* Ruanda ($n = 2$) and *S.* Stockholm ($n = 1$) as shown in Table 4. Of the 34 *Salmonella* strains analysed, 19 (55.9%) were in possession of *spv*A, *spv*B and *spv*C genes. One strain had both *spv*A and *spv*C but lacked *spv*B gene. The carriage of plasmid encoded *spv* genes among the strains was at the following rates 58.8% (20/34), 55.9% (19/34) and 58.8% (20/34) for *spv*A, *spv*B and *spv*C, respectively ($p < 0.001$, 95 percent, CI). All *S.* Enteritidis strains ($n = 16$) from horse and chicken sources contained *spv*A (100%; 16/16), *spv*B (93.8%; 15/16) and *spv*C (100%; 16/16). The *spv* genes were absent from serotypes *S.* Heidelberg (cattle); *S.* Pomona (sable antelope); *S.* Mbandaka (dog and chicken); *S.* Sendai, *S.* Hadar and *Salmonella* spp. from chickens.

Of the 20 *Salmonella* strains carrying *spv* genes, 85.0% (17/20) belong to serogroup D1, 5.0% (1/20) to C1, followed by 10.0% (2/20) non-reactive serogroup. All strains were isolated from various samples of sick domestic animals except one strain belonging to serogroup C1 which was from an apparently healthy Leopard. On the other hand, no *spv* genes were detected in strains belonging to serogroup B. Similarly, out of five *Salmonella* strains belonging to serogroup C1, only 20.0% (1/5) strain yielded *spv*ABC genes.

Discussion

Domestic animals and wildlife are increasingly becoming important as possible reservoirs of Salmonellae for human infection as well as environmental contamination [24,25]. The present study describes the isolation and characterisation of Salmonella isolates from domestic animals and wildlife in Zambia. Twelve (12) different *Salmonella* serotypes (Enteritidis, Garoli, Ruanda, Stockholm, Mbandaka, *Salmonella* spp., Hadar, Sendai, Heidelberg, Chardan, Roan and Pomona) were identified from 34 strains ($p = 0.002$). One key finding in terms of serotype clustering can be postulated i.e there is a probable adaptability of serotypes to either domestic animals or to specifically wildlife. This is so as serotypes found in domestic animals were not present in wildlife (Table 3). This may also explain an existence of contiguous domestic animals and wildlife populations.

A similar study conducted in Zambia, reported isolation and identification of 14 serotypes among 806 *Salmonella* cultures isolated from poultry [26]. The occurrence of more serotypes in the previous study could be attributed to a high number of *Salmonella* isolates that were included in the study. However, by permutation our present study actually denotes a higher proportion of serotypes. Further the present investigations revealed that the serotypes belonged to various serogroups [(B, C1, D1 and non-reactive) ($p < 0.001$)], fermentative groups or resistotypes (Type I–VII). Human infections with *Salmonellae* are usually associated with serogroups A, B, C1, D1 and E [27]. We report the detection of serotypes belonging to serogroups B (*S.* Heidelberg), C1 (*S.* Mbandaka, *S.* Garoli), and D1 (*S.* Enteritidis, *S.* Ruanda) indicating their pathogenic nature and have been implicated in human salmonellosis [28,29]. This study did not identify any *Salmonella* belonging to serogroup A which comprises serotypes that are particular restricted to human infection such as *S.* Paratyphi A serotype [30]. Similarly, the study did not record *Salmonella* belonging to serogroup E.

Salmonella serotypes belonging to serogroup D1 have been documented worldwide as the most prevalent cause of human salmonellosis [31,32]. Among the *Salmonella* serotypes, some are known to be host-adapted [33,34]. None of the Salmonella serotypes observed in this study were host specific, implying they could infect a wide range of animal hosts including humans [35].

In domestic animals, 9 serotypes were isolated from both asymptomatic (Heidelberg) and symptomatic (Enteritidis, Ruanda, Stockholm, Mbandaka, *Salmonella* spp., Hadar, Sendai, Chardan) animal sources (Table 3). Of these, 7 serotypes were obtained from chicken source, an indication that a wide range of Salmonella serotypes have predilection for chicken hosts; a potential source of human salmonellosis [36]. This finding in chickens has significant ramifications for foodborne derived salmonellosis in humans, a public health significant aspect. As far as the authors are aware, the current report denotes the first report on circulation of *S.* Stockholm, *S.* Ruanda, *S.* Chardan, and *S.* Sendai in poultry in Zambia.

Further, in wildlife, 3 distinct *Salmonella* serotypes (*S.* Pomona, *S.* Garoli, and *S.* Roan) were isolated. To our knowledge this is the first report describing these serotypes in Sable antelope, Leopard and Impala in Zambia. *Salmonella* Pomona serotype has been reported as one of the causes of outbreaks of human salmonellosis [37]. Previous studies have isolated *S.* Pomona from diseased farmed American alligators [38]. *Salmonellae* from captive and free-ranging wildlife have been identified as sources of infection for humans [24]. Thus, the detection of 3 serotypes in wildlife raises public health concerns through direct or indirect contact with faeces or consumption of improperly cooked game meat [39].

All *Salmonella* isolates in the current study demonstrated the typical biochemical reaction of *S. enterica* subspecies *enterica* (I) as described by Krieg and Holt [40]. Of critical importance, are the findings of emergence of resistance to multiple antimicrobial agents. Worldwide, there has been an increase of public health concern over the occurrence of antibiotic-resistant strains of pathogenic bacteria, including *Salmonella* [41]. All *Salmonella* strains were resistant to at least four antimicrobial agents and exhibited MDR characteristics. The frequency of antimicrobial resistance of Salmonella strains from domestic animals was 31 (100%), 31 (100%), 28 (90.3%), 27 (87.10%), 26 (83.9%) and 2 (6.5%), while that from wildlife was 3 (100%), 3 (100%), 2 (66.7%), 0%, 3 (100%) and 0% against penicillin G, erythromycin, ampicillin, gentamicin, nitrofurantoin and azithromycin respectively. This study showed resistance against the antimicrobial agents (ampicillin, gentamicin, and penicillin G) that are frequently used in treatment of various livestock infections or generally animal husbandry in Zambia.

On the other hand antimicrobial resistance against the antibiotics that are less frequently used in animal husbandry was significantly low. A previous study [42] showed that *Salmonella* isolates from Brazilian broiler chickens exhibited higher resistance to ampicillin (100%), tetracycline (83%), streptomycin (12.5%) and chloramphenicol (4.2%). In contrast, a study in Ethiopia demonstrated a relatively lower rate (88.7%) of strains from raw meat and swab samples from butcher shops were resistant to ampicillin [43]. However, our investigation demonstrates that there is a pool of clones of *Salmonella* strains belonging to different serotypes and isolated from different animal species, that are resistant to ampicillin-erythromycin-nitrofurantoin-penicillin G (Amp-E-Nit-P) phenotype.

Further, we highlight concerns with MDR *S.* Enteritidis and *S.* Mbandaka strains, in horses and dogs, towards clinically important azithromycin and ciprofloxacin (Fluoroquinolones) drugs. We observed also that it is difficult to predict antimicrobial resistance based on the utilization of substrates except for azithromycin which is associated with cellobiose ($p = 0.034$).

Wildlife is perceived to have minimal exposure to antibiotics; however, the levels of MDR observed in wildlife could reflect unidentified extrinsic factors that lead to emergence of drug resistance of pathogens in nature.

The PCR technique has been widely used to confirm *Salmonella* isolates by detection of the *inv*A gene [44]. All the 34 isolates were positive for *inv*A virulence gene suggesting the potential for these pathogens to penetrate the intestinal lining of their host and capability to cause disease [45]. However, the findings of the current study are in consonance with the work by other investigators, who indicated 100% presence of *inv*A gene in all *Salmonella* isolates they investigated [46–48]. Our findings also correlate with the detection of *inv*A gene in *Salmonella* isolates from captive wildlife (lion, stripped hyena, chimpanzee, African hawk eagle and peafowl) [45].

The NTS serotypes in possession of *spv* genes are associated with severe systemic infection [49,50]. A combination of MDR traits and possession of *spv*ABC genes can be a potential catalyst for human invasive salmonellosis [50,51]. In this study, all isolates possessing *spv*A (58.8%), *spv*B (55.9%) and *spv*C (58.8%) genes were resistant to at least four antimicrobial agents, suggesting clinical significance. There is no statistically significant difference ($p > 0.05$) between strains in possession of *spv*ABC genes and resistance to a set of antimicrobial agents investigated. Our finding revealed that one strain possessed both *spv*A and C but lacked *spv*B gene. The carriage rate of *spv*B in the present study is higher compared to previous study [52], who reported 43.3% (*spv*B) and 73.3 % (*spv*C) genes in *Salmonella* strains isolated from different sources in Iran. In another study, *spv*A gene was detected in all isolates (100%), *spv*B in (56.25%) and *spv*C in (62.5%) in *Salmonella* isolates from raw milk [53]. The role of *spv*A is to promote the macrophage phase avoiding destruction by neutrophils [54]. The function of *spv*B is to encode an enzyme ADP-ribosylates which modifies actin cytotoxin required for systemic survival [55], and it is also responsible for the *spv* virulence phenotype [56]. On the other hand *spv*C has phosphothreonine lyase activity which inhibits mitogen-activated protein kinases signalling (MAPKs) [49].

The study also observed a relationship between fermentative group I and possession of *spv*ABC genes ($p < 0.001$). The failure to utilize arabinose, cellobiose, inositol and salicin substrates by 19 strains belonging to fermentative group I could be a useful marker for possession of *spv*ABC genes (Table 3). The only exception was *S.* Stockholm belonging to fermentative group II which was found to carry the *spv*ABC genes, in addition to partial utilization of the four substrates. The utilization of arabinose/inositol by the *S.* Stockholm suggests that these substrates could be markers for possession of *spv*ABC genes in the *S.* Stockholm serotype. A broader study to establish the potential value of biotyping as a discriminatory tool for the identification of *Salmonella* strains in possession of virulence plasmids may be required.

Salmonella virulence plasmid genes were identified in *S.* Enteritidis ($n = 16$), *S.* Ruanda ($n = 2$), *S.* Garoli ($n = 1$) and *S.* Stockholm ($n = 1$) isolated from domestic and wildlife sources (Table 4). *Salmonella* Enteritidis isolated from the sick domestic animal sources (horse and chickens) was the most frequent serotype found to carrying *spv*A (100%), *spv*B (93.8%)

and *spv*C (100%) genes isolated from the sick domestic animal sources (horse and chickens). Our findings are in agreement with the previous study that reported *S.* Enteritidis to be among the serotypes which carry *spv* genes [53]. Our results regarding the carriage rate of *spv* genes in *S.* Enteritidis serotype is higher compared to a previous study [57], which reported 86.6% each of *spv*A, *spv*B, and *spv*C genes. To the best of our knowledge and as well as based on literature review, there is, currently, no information showing or indicating the capacity of *S.* Ruanda (serogroup D1), *S.* Garoli (serogroup C1) and *S.* Stockholm (NR) to possess *spv* genes.

The absence of *spv* genes in some strains isolated from clinical sources, suggests the existence of other mechanisms that could trigger disease causation. Therefore, there is need for policy makers to consider extending the National surveillance for antimicrobial resistant *Salmonella* strains to include game hunted meats.

Conclusion

Findings of the current study indicate an emergence of MDR and possible development of clonal groups of pathogenic strains of public health importance in both humans and animals. Thus highlights the need for strengthening of surveillance in MDR and foodborne pathogens.

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Authors' contribution

CM conceived the study, investigations and drafted the manuscript; BH initiated the study topic and made major contributions to the design, review and validation of the study; BJM undertook statistical analysis, and contributed in the validation of the manuscript; GP, KS, NS and MM contributed in writing, review and editing; KM, and YQN undertook the analysis of molecular work in *Salmonellae* isolates.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationship that could be construed as potential conflict of interest.

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Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.sciaf.2020.e00345.

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ORIGINAL ARTICLE

Isolation of *Salmonellae* from Kafue lechwe (*Kobus leche kafuensis*) Antelope and Cattle Faeces in the Livestock/Wildlife Interface Areas of the Kafue Flats in Zambia

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Abstract

The study aimed to investigate the prevalence of *Salmonellae* in the Kafue lechwe and cattle sharing grazing areas and water points in the livestock/wildlife interface areas of the Kafue Flats of Zambia. The study was driven by interspecies transmission of diseases and outbreaks in the livestock/wildlife interface areas that are becoming more apparent with the increasing human encroachment into wildlife sanctuaries. Freshly voided faecal droppings from both Kafue lechwe and cattle were conveniently and aseptically picked up from the grazing areas. Additionally, compounded faecal contents of hunter-harvested Kafue lechwe carcasses were also collected. A total of 593 faecal samples were microbiologically processed for *Salmonellae*, of which 232 (39.1%) were from Kafue lechwe while 361 (60.8%) were from cattle. Pure isolates were phenotypically characterized and tested for the presence of virulence *invasion* A gene. A total of 89 *Salmonellae* isolates were isolated in the study from Kafue lechwe (n=59) and cattle (n=30) of the 59 isolates from Kafue lechwe, 19 tested positive on agglutination test with *Salmonella* polyvalent O antiserum, and of these 19, 26.3% (5/19) tested positive to serogroup O8 antisera of the 30 cattle isolates, 13 were positive to polyvalent O antiserum, and one was positive to serogroup O8. Further, 53 isolates randomly selected from Kafue lechwe (n=43) and cattle (n=10) were tested for the presence of *invasion* A gene, of which 39 (73.6%) were positive. The study concluded that the Kafue lechwe and cattle on the Kafue Flats in Zambia harbour pathogenic *Salmonellae* and could serve as potential vehicles for transmission to humans. Therefore, hygiene measures to protect human exposure to virulent *Salmonellae* contaminating food and water should be observed.

Keywords: *Salmonellae*-specific O8 antiserum, *Salmonellae-invasion* A (*inv*A) gene, Kafue lechwe, Cattle, Domestic/wildlife interface areas, Zambia.

1. Introduction

Salmonella is an important zoonotic bacterium, and little is known about the role that free-living animal carriers of this pathogen play in its epidemiology (Silva-Hidalgo *et al*., 2013). However, several reports have high-lighted zoonotic infections at the human-domestic-wildlife interface areas, and this has become a focus of public health concern worldwide (Siembieda *et al*., 2011; Singh and Gajadhar, 2014). Animal faeces are known to transmit salmonellosis and other diseases (Food Waste Recycling Action Plan, 2003). Salmonellosis in humans could be contracted by ingesting contaminated foods, water or beverages (Forshell and Wierup, 2006; Carlos *et al*., 2012). In the Kafue flood plains of Zambia (commonly called the Kafue Flats) interspecies transmission of diseases has been incriminated in the epidemiology of bacterial diseases (Siamudaala *et al.*, 2003). However, the extent as well as the role to which free-living animals such as Kafue lechwe (*Kobus leche kafuensis*) play has not been determined. The interaction of domestic and wildlife animals in the Kafue Flats is usually promoted by climatic factors such as drought and flooding (Siamudaala *et al.*, 2003). In times of flooding or rainy season, the semi-aquatic Kafue lechwe antelope move to higher ground inhabited by humans and their domestic livestock. Local livestock farmers in the Kafue Flats practice transhumance pastoralism and

every year, in the dry season for about six months their domestic animals move to the Kafue Flats for fresh vegetation and water. Arising from these movements, human settlements have tended to encroach into animal habitats with a possibility of pathogenic agents such as *Salmonellae* from either wildlife or pastoral cattle finding themselves in human populations and vice versa. Humans in the Game Management Areas (GMAs) surrounding the National Parks (NP) are likely to be exposed to the biological hazards through drinking of water contaminated with animal faecal matter and also the consumption of meat contaminated with bacterial pathogens. In the case of *Salmonellae*, the consequences arising thereafter could lead to a severe threat to public health with a likelihood of diarrheal disease outbreaks (Aljoudi *et al*., 2010). Further, pastoral cattle and Kafue lechwe are often slaughtered for consumption by communities living in the areas and by licensed and unlicensed game hunters (Siamudaala *et al.*, 2003). This is due to the absence of formal slaughter and carcass inspection facilities in these areas, which increases the risk of carcass contamination with *Salmonellae*. This then becomes a source of concern because consumers are likely to be exposed to the biological hazards in the absence of measures to certify the safety of the meat. It is important; therefore, that occurrence of *Salmonellae* in food-producing wild animals is well elucidated to create awareness on the risk associated with the consumption of unhygienically processed wildlife meat. There is evidence that some properties of these organisms in some parts of the world are usually indicative of some interactions between man and animals (Sanchez *et al*., 2002). This study aimed to isolate and characterize *Salmonellae* from the faeces of Kafue lechwe and cattle which share grazing areas in the interface of the Kafue Flats of Zambia.

2. Materials and Methods

2.1 Study Area

The study was conducted in the livestock/wildlife interface areas of the Lochinvar (410 km^2) and Blue-Lagoon (420 km^2) National Parks (NP) on the Kafue Flats in Zambia (Fig 1). The Kafue Flats are located in the southern part of the country covering the Kafue Game Management Area (GMA) (6000 km^2). The Kafue Flats are the only known natural habitat of the Kafue lechwe with an estimated population of approximately 40,000 (Chansa and Kampamba, 2010). The Blue-Lagoon and Lochinvar NP provide a rich lacustrine habitat for the Kafue lechwe. GMAs are interface areas where interaction between wildlife and humans is facilitated through transhumant livestock herding activities carried out by the surrounding livestock farmers, including fishing. The Kafue Flats were selected as the study site because they provide a unique interaction between livestock and wildlife as over 300,000 heads of cattle are moved from the upland areas to the wetlands during the drier months of the year. The Kafue Flats, like the rest of Zambia, has three distinct seasons; a warm wet rainy season from November-April, a cold, dry season from May-August and a hot, dry season before the rainy season.

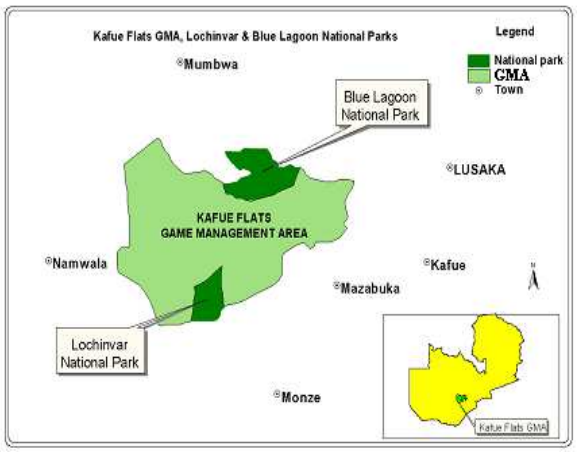

Fig 1: The study areas of Lochinvar and Blue-lagoon National Parks (Adapted from Milner, 2003).

2.2 Sample Collection

The study was conducted from July to December 2008-2015, in the Lochinvar and Blue-Lagoon NPs, which provide the interface areas of livestock and wildlife in the Kafue Flats. Faecal samples were collected from Kafue lechwe and cattle that shared grazing areas on the Flats. Samples from the lechwe were in two categories; the first category consisted of compounded samples obtained from the lechwe that were sacrificed for research purposes with authorization by the Zambia Wildlife Authority (ZAWA). These samples were obtained as part of a wider research project that involved the investigation of several diseases. The sample size was pre-determined by what had been authorized by ZAWA for a research quota under that particular special license. Details of other research activities are provided elsewhere (Munyeme *et al*., 2010; Kuroda *et al*., 2013; Mubita *et al*., 2015). In the present study, samples consisting of 2g of faecal contents from the rectal solid matter (R); swab contents from the lower small intestine (SI), the ileocaecal junction (IC) and the caecum (C) of each hunter-harvested lechwe were obtained from the study sites. The second category involved freshly voided, while grazing, Kafue lechwe

faecal samples of about 5g each. To avoid soil contamination, only those portions of the faecal matter not in direct contact with the soil were picked up for the study. Freshly voided faecal samples picked from the grazing pastures, and the rectum were each placed in a well-labelled 150 x 100 mm self-adhesive polythene bag and then stored at 4^{o}C till laboratory analysis. Sample analysis took no longer than 168 hours (7 days) after collection. Swab contents were stored in Carry and Blair (1964) transport medium (BBL - Becton Dickinson Co. Cockeysville, USA). In the case of pastoral cattle, freshly voided faecal samples were conveniently picked up from the grazing areas in the Lochinvar and Blue-lagoon National Parks.

2.3 Culture and Identification of *Salmonellae*

The faecal samples were cultured for the presence of *Salmonella,* using techniques recommended by the International Organization for Standardization (ISO-6579, 2000), and those recommended by the Global *Salmonella* Surveillance (GSS) (Hendriksen, 2003). One gram of faeces was added to 9 ml of *Salmonellae* enrichment Rappaport Vassiliadis Soybean Meal broth (RVSM broth) (Himedia Laboratories Pvt. Ltd. Mumbai-400086, India), whereas swabs were inoculated into 10 ml of RVSM and incubated at 37^{o}C for 18 hrs. A loop full of the bacterial culture was streaked on Xylose-Lysine Deoxycholate Agar (XLD Agar) (Himedia Laboratories Pvt. Ltd. Mumbai-400086, India) and incubated at 37^{o}C for 18 hrs. Between one to four suspicious *Salmonellae* colonies on XLD Agar from a positive faecal sample were sub-cultured on non-selective Nutrient Agar (NA) (Himedia Laboratories Pvt. Ltd. Mumbai-400086, India) and after that subjected to conventional biochemical identification to the genus of *Salmonellae* according to the standard procedures (Barrow and Feltham, 1993). Presumptive *Salmonellae* isolates were agglutinated using *Salmonellae* somatic polyvalent O antiserum (Denka Seiken Co. Tokyo, Japan) (Gyles, 1996; Edwards and Wing, 1972). Further, *Salmonellae* isolates were differentiated into serogroups with the commercially available *Salmonellae* antisera kit consisting of 14 specific panels (O 2, O 4, O 7, O 8, O 9, O 9, 46, O 3, 10, O 1, O 11, O 13, O 6, 14, O 16, O 18, O 21) (Denka Seiken Co. Tokyo, Japan). Non-reactive strains and weak agglutination were recorded as negative. A confirmed *Salmonella* isolate was used as positive control while an *E. coli* isolate ATCC25922 was the negative control. Following sero-grouping, isolates belonging to sero-group O8 and non-reactive were randomly selected for fermentation reaction tests to a range of 20 carbohydrates to determine the similarities of the isolates (Jane-Francis *et al*., 2009).

2.4 Confirmation of *Salmonella* Isolates from Kafue lechwe and Cattle Faeces by PCR

Biochemically identified *Salmonellae* isolates, were randomly selected and further investigated for the presence of the virulence *inv* A gene using PCR (Hamza, 2010; Oliveira and Rodenbusch, 2003).

2.5 DNA Extraction

DNA from the *Salmonellae* isolates was extracted and used to target the presence of *inv* A genes as follows: Test cultures were sub-cultured on Brain Heart Infusion agar (BHI) and incubated at 37^{o}C for 18 hrs. A loopful of a pure sub-culture was emulsified into 1 ml of sterile distilled water and heated at 95^{o}C in a dry block bath (Scinics Co. Japan) for 10 minutes. To 9 µl PCR mixture (5 µl Phusion flush mixture, 1.5 µl sterile distilled water, 2.5 µl primers (Forward and Reverse) was added 1 µl of bacterial DNA template. The PCR for 96 Wells (Finnzymes Piko) was performed using 10 µl total reaction volumes. The thermal cycling protocol was performed using the rapid cycle DNA amplification method, which consisted of initial denaturation step at 98^{o}C for 30 seconds, followed by 30 cycles of template denaturation at 98^{o}C, primer annealing at 60^{o}C and extension at 72^{o}C for 10 seconds. Finally, extensions stood at 72^{o}C for 2 minutes. The PCR products were viewed with ethidium bromide after electrophoresis through 3% agarose gels in TAE buffer.

2.6 Statistical Analysis

The database was established in Microsoft Excel spreadsheets, and for statistical analysis, it was transferred to STATA SE/11 for windows statistical package (Stata Corp. College Station, Texas. USA). A p-value of <0.05 was considered indicative of a statistically significant difference for all statistical survey analyses.

3. Results

3.1 Distribution of Faecal Samples

A total of 593 faecal samples were microbiologically processed for *Salmonellae,* of which 232 (39.1%) were from Kafue lechwe, while 361 (60.9%) were from cattle. Seventy-seven (33.2%) of the 232 samples were compounded faecal contents from hunter-harvested Kafue lechwe, while 155 faecal samples were from the faecal matter freshly voided while grazing. Out of the 77 hunter-harvested lechwes, 52 were from Lochinvar, while 25 were from Blue-lagoon NPs. On the other hand, the freshly voided faecal samples were from Lochinvar (n=110) and Blue-lagoon (n=45). In the case of cattle, 361 faecal samples

were collected, of which 261 were from Lochinvar and 100 samples from Blue Lagoon National Parks. The distribution of faecal samples by study area is shown in Table 1.

3.2 Isolation of *Salmonellae* from Kafue lechwe Faeces

Salmonellae were isolated from 6 (7.8%) of the 77 compounded faecal samples and 12 (7.7%) of the 155 faecal samples from grazing pasture. Details of the distribution of positive samples from Kafue lechwe by study area and specimen are provided in Table 1. Collectively, 18 positive faecal samples from Kafue lechwe yielded 59 *Salmonellae* strains. The isolation rate of *Salmonellae* strains was high in the faeces picked up from pasture - 45.8% (27/59), followed by rectum - 27.1% (16/59), small intestine - 13.6% (8/59), ileo-caecum - 8.5% (5/59) and caecum - 5.1% (3/59).

3.3 Isolation of *Salmonellae* from Pastoral Cattle Faeces

The recovery rate of *Salmonellae* from cattle faecal samples was; 1.5% (4/261) of samples from Lochinvar NP and 5.0% (5/100) of samples from Blue-lagoon NP, respectively (Table 1). A total of 30 *Salmonellae* isolates were collectively recovered from nine (2.5%) positive samples of the 361cattle faecal samples of these isolates, 15 (50.0%) were from Lochinvar NP and 50% from Blue-lagoon NP.

3.4 Characterization of *Salmonellae* Serogroups from Kafue lechwe

Nineteen (32.2%) of the 59 isolates were positive to *Salmonellae* polyvalent O antisera, while 40 isolates were serologically negative of the 19 *Salmonellae* isolates, 26.3% (5/19) were identified to belong to serogroup O8, while 73.7% (14/19) isolates did not react to the available panel of specific *Salmonellae* antisera groups. Out of the five isolates, 80% were from the faecal sample collected from the rectum, while 20% was from a sample collected from the pasture ($p<0.1$). A positive association was observed between agglutination tests with polyvalent 'O' antisera and source of isolate ($p<0.01$). Isolates from the Lochnivar NP agglutinated positively to polyvalent 'O' compared to those from the Blue-lagoon NP. Seven (36.8%) of the 19 isolates positive to polyvalent somatic antisera were from the rectum followed by the ileocaecal junction and the small intestine (each 21.1% (4/19)) and caecum (5.8% (3/19)) respectively ($p<0.01$). Further, 43.3% (13/30) isolates from cattle, tested positive on agglutination test with *Salmonellae* polyvalent 'O' antisera, whereas the rest of the isolates (56.7% (17/30)) were non-reactive. There was a significant difference ($p<0.01$) in the isolation frequency of *Salmonellae* isolates positive to polyvalent 'O' antisera between faecal samples collected from Lochinvar and Blue-lagoon NPs of the 13 serologically positive isolates, belonging to *Salmonellae* serogroup O8, 92.3% (12/13) were from the Lochinvar NP and 7.7% (1/13) from the Blue-lagoon NP.

3.5 Characterization of *Salmonellae* Biotypes

The biochemical profiles of 13 randomly selected *Salmonella* isolates are shown in Table 3 of these, four were from pastoral cattle and nine from Kafue lechwe. Among the isolates, three from cattle (Isolates 1, 3 and 5) and four from Kafue lechwe (Isolates 12, 14, 16 and 18) showed a similar pattern of fermentative reaction to 20 carbohydrates, suggesting they were from a common source. Similarly, *Salmonellae* strains belonging to serogroup O8 (Isolates 9, 9-1, 9-2, and 9-3) from faecal rectal contents of Kafue lechwe were related. In contrast, one strain belonging to serogroup O8 from faeces of pastoral cattle was not related with the isolates from the rectum of Kafue lechwe. However, three biotypes were identified among the isolates belonging to serogroup O8 namely; biotype 1 from cattle utilized arabinose and xylose; whereas from the Kafue lechwe, biotype 2 fermented arabinose but failed to utilize xylose, and biotype 3 was arabinose negative and xylose positive.

3.6 Characterization of *Salmonellae Inv* A Genes

Altogether, 53 *Salmonellae* isolates from Kafue lechwe (n=43), and pastoral cattle (n=10) were randomly selected and tested for the presence of *the inv* A gene. Thirty-nine (73.6%) *Salmonellae* isolates were positive to the *inv* A gene, as shown in Fig 2, where a 284bp amplicon product was detected. Thirty-one (79.5%) of the 39 isolates were from Kafue lechwe, and eight (20.5%) were from pastoral cattle (Table 4). Of the 53 isolates, 47.2% (25/53) were positive to polyvalent O antiserum, while the rest were non-reactive.

4. Discussion

The present study aimed to isolate and characterize *Salmonellae* from Kafue lechwe and cattle faeces in the domestic/wildlife interface areas of the Kafue Flats. There is a paucity of information on the prevalence of *Salmonellae* spp. in free-ranging wildlife in Zambia. In a previous study (Kuroda *et al*., 2013), a total of 120 faecal samples (Kafue lechwe = 60; Cattle = 60) were investigated for the presence of virulence genes (EAST1, *stx*-1and *stx*-2) of pathogenic *E. coli* –

Table 1: Distribution of *Salmonellae* positive faecal samples from Kafue lechwe and pastoral cattle by study area (n = 593)

Location	Sample Source	Kafue lechwe (*n* = 232)		Pastoral cattle (*n* = 361)	
		Total No. of faecal samples	No. of positive samples (%)	Total No. of faecal samples	No. of positive samples (%)
Lochinvar	Grazing area	110	7 (6.4)	261	4 (1.5)
	Compounded	52	4 (7.7)	0	0 (0)
Blue-lagoon	Grazing area	45	5 (11.1)	100	5 (5.0)
	Compounded	25	2 (8.0)	0	0 (0)
Total		232	18 (7.7)	361	9 (2.5)

Table 2: Distribution of polyvalent O positive *Salmonella* strains from faecal samples of Kafue lechwe by source and location (*n* = 59)

Source (No. of isolates)	*Salmonellae* isolates reaction to polyvalent O antiserum			
	Lochinvar National Park		Blue-lagoon National Park	
	Positive	Negative	Positive	Negative
Pasture (27)	1	15	0	11
Rectum (16)	7	0	0	9
Ileo-caecal-junction (5)	4	1	0	0
Caecum (3)	3	0	0	0
Small intestine (8)	4	4	0	0
Total (59)	19	20	0	20

Table 3: Comparison amongst *Salmonellae* biotypes from Kafue lechwe and pastoral cattle (*n* = 13) by the fermentative reaction

Test	Reaction of *Salmonellae* isolates												
	Isolates from pastoral cattle					Isolates from Kafue lechwe							
	1	3	5	7*	9*	9-1*	9-2*	9-3*	11*	12	14	16	18
Arabinose	-	-	-	+	-	-	-	-	-	-	-	-	-
Cellobiose	-	-	-	-	-	-	-	-	-	-	-	-	-
Dextran	-	-	-	-	-	-	-	-	-	-	-	-	-
Dulcitol	+	+	+	+	+	+	+	+	-	+	+	+	+
Galactose	+	+	+	+	+	+	+	+	+	+	+	+	+
Inositol	-	-	-	-	+	+	+	+	+	-	-	-	-
Maltose	+	+	+	+	+	+	+	+	-	+	+	+	+
Fructose	+	+	+	+	+	+	+	+	+	+	+	+	+
Raffinose	-	-	-	-	-	-	-	-	-	-	-	-	-
Resorcinol	-	-	-	-	-	-	-	-	-	-	-	-	-
Ribitol	-	-	-	-	-	-	-	-	+	-	-	-	-
Rhamnose	+	+	+	+	+	+	+	+	-	+	+	+	+
Trehalose	+	+	+	+	+	+	+	+	-	+	+	+	+
Xylose	+	+	+	+	+	+	+	+	-	+	+	+	+
Salicin	-	-	-	+	-	-	-	-	+	-	-	-	-
Sorbitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannitol	+	+	+	+	+	+	+	+	+	+	+	+	+
Mannose	+	+	+	+	+	+	+	+	+	+	+	+	+
Starch	+	+	+	+	+	+	+	+	+	+	+	+	+
Barbitol	-	-	-	-	-	-	-	-	-	-	-	-	-

Key (= isolates belonging to serogroup 8, - = negative reaction, + = positive reaction)*
Isolate numbers: 1 = Lmc 226-1, 3 = Lmc 227-1, 5 = Lmc 231-1, 7 = Sc 88-2, 9 = Lr = 65-1, 9-1 = Lr 65-2, 9-2 = Lr 65-3, 9-3 = Lr 65-4, 11 = Lp 260-2, 12 = Lr 79-4, 14 = Icj =59-1, 16 = C 67-2, and 18 = Si 69-1

Table 4: Summary of PCR results of *inv* A gene from *Salmonella* isolates (*n* = 53) and their serological characteristics

Host (Total No. of isolates)	Presence of *inv* A gene		Reaction to polyvalent 'O' antiserum		Reaction to antisera group O:8	
	Positive	Negative	Positive	Negative	Positive	Negative
Lechwe(43)	31	12	19	24	4	39
Cattle(10)	8	2	6	4	1	9
Total (53)	39	14	25	28	5	48

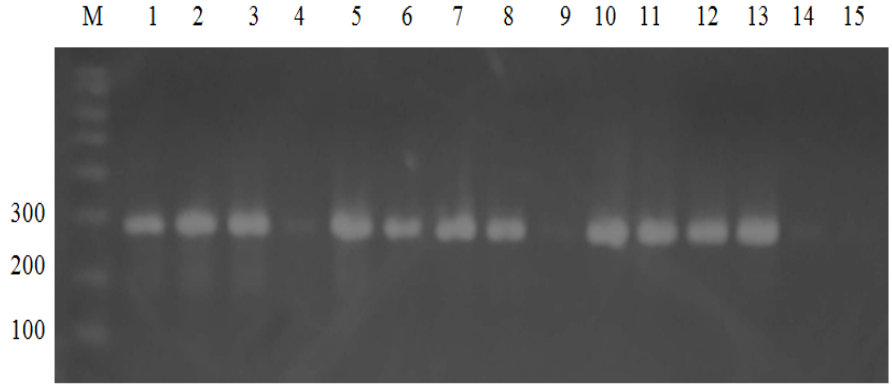

Fig 2: PCR products of *inv* A from *Salmonella* isolates. Lane M is the molecular marker while lane 1 to lane 14, are *Salmonella* isolates from pastoral cattle and Kafue lechwe. Lane 4, 9 and 14 are negative. Lane 15 is negative control-*Escherichia coli* (ATCC 25922).

and *inv* A of *Salmonella* strains in faecal samples of Kafue lechwe and cattle by multiplex polymerase chain reaction. The EAST1 gene was the most prevalent in faecal samples from both Kafue lechwe and cattle, while the *stx*-2 gene was detected in 20% of Kafue lechwe faecal samples and 55.1% of cattle respectively. On the other hand, the *Salmonella inv* A gene was detected in only one faecal sample from cattle. The present study determined that free-ranging Kafue lechwe had a higher prevalence of *Salmonellae* than pastoral cattle indicating that Kafue lechwe is more exposed to *Salmonellae.* This may be due to various factors; firstly, the aquatic environment which the Kafue lechwe normally inhabit has been found to act as a significant source of *Salmonella* transmission to hosts (Cherry *et al*., 1972). This theory may in part be supported by the fact that Kafue lechwe are semi-aquatic antelopes and may on occasion shed *Salmonellae* through faeces in the shallow waters of the lagoons where such pathogens remain viable for long periods, and this facilitates transmission to other susceptible hosts (Angulo *et al*., 2000; Pandey and Nambota, 2007). Another critical aspect under the ecological grid of interpretation is that the same environment is also shared with thousands of resident and migratory wild water fowls which are known carriers of *Salmonellae* (Nielsen *et al*., 2004; Foster *et al*., 2006). Further, Kafue lechwe live in large populations and are confined to a relatively small area, particularly in and around the Lochinvar and Blue-lagoon NPs (Siamudaala *et al.*, 2003), which may result in high levels of cross-contamination of the environment through faecal matter throughout the year. This situation may be made worse during the rainy season when most of the plains are flooded, and the lechwe congregates to graze on the few areas of land that are not flooded. This finding is in accord with other reports that indicate that transmission of *Salmonellae* is facilitated by tropical and subtropical conditions (Tessari *et al*., 2012), as well as areas where there is a large congregation of animals and people such as the Lochinvar and Blue lagoon NPs and GMA in Zambia. In the food chain, faecal droppings of Kafue lechwe are considered a source of manure, food for fish in the lagoons (Siamudaala *et al.*, 2003), and subsequently, these fishes and semi-aquatic wildlife may consume and shed the pathogens in the water of the lagoons through their faeces. In the present study, 73.5% (39/53) of the *Salmonellae* isolates from Kafue lechwe and cattle were found to harbour the *inv* A 284 bp amplicon genes, suggesting the potential of the pathogen to cause infection in humans or animals. In

the same context, the studies showed that 26.4% (14/53) of *Salmonella* isolates were negative to *inv* A gene and therefore are potentially non-pathogenic. This finding differs from an earlier study in which we reported the presence of *Salmonella inv* A gene in 1.6% (1/60) of faecal samples from pastoral cattle and none in faecal samples from Kafue lechwe (Kuroda *et al*., 2013). Further, results of the current study are in accordance with those of a study done in Teheran, Iran (Salehi *et al*., 2009) which showed, by PCR reaction, that *Salmonellae* isolates positive to polyvalent O antiserum were also positive to *inv* A gene. However, the detection of *inv* A from *Salmonellae* isolates negative to *Salmonellae* polyvalent O antiserum in the current study could be indicative of a *Salmonella* strain yet to be identified serologically, colonizing the Kafue lechwe. In Zambia, *Salmonellae* isolates from poultry and beef have been found to possess *sip* C and *inv* A genes by Dot Blot Hybridization method (Hang'ombe *et al*., 2008). Detection of *inv* A genes in *Salmonella* isolates is of paramount importance because the pathogenicity of *Salmonella* species is dependent upon the ability of these organisms to gain access to cells that are usually non-phagocytic (Ginocchio and Galan, 1995; Rychlik *et al*., 1998). Therefore, the findings in the present study indicate that Kafue lechwe is potential carriers of *Salmonellae* and may amplify transmission of the bacteria to pastoral cattle through faecal contamination of grazing areas and drinking water holes. Kafue lechwe comes into contact with pastoral cattle when they graze together on the fresh grass which grows as the floods recede (Pandey and Nambota, 2007). The study further revealed that *Salmonella* isolates from faeces of Kafue lechwe and cattle were more prevalent in the Blue-lagoon NP than in the Lochinvar. The reason for this is not very evident but probably could be accounted for by the fact that, generally, there are more cattle in the Blue-lagoon NP than in the Lochinvar NP, which could result in increased contamination of the grazing areas (Muma *et al*., 2006). This may also be attributed to the fact that cattle herds in the Blue-lagoon NP are more transhumant than in the Lochinvar NP because large herds cannot find sufficient pasture around the homesteads and tend to trek to the Kafue Flats in search of water and pasture. Conversely, the study has also shown that all isolates, positive to *Salmonellae* polyvalent somatic antiserum were from the Lochinvar NP. This finding suggests that the predilection of *Salmonellae* bacteria in the Kafue lechwe host is dependent on the area of sampling. Furthermore, there was a significant difference ($p<0.01$) between the frequency of detection of *Salmonellae* isolates positive to polyvalent O antiserum and source. Our results suggest that the recovery of serologically positive *Salmonellae* is somewhat high from Kafue lechwe rectal faeces than other sources. The non-reactive *Salmonella* isolates may be attributed to the presence of rough mutant strains which lack the specific side chains responsible for "O" specificity (Parker and Duerden, 1990). Studies, using molecular techniques such as the PCR and sequence analysis, of these non-reactive isolates would have yielded data that would have had an ascribed percentage likeness to the reactive isolates. Isolation of *Salmonellae* from pastoral cattle in this study conservatively suggests and is in congruence with the existing information that cattle are reservoirs of *Salmonellae* (McEvoy *et al*., 2003). Further, the results in this study correlate with the work by previous workers who isolated *Salmonellae* from cattle in Zambia (Ngoma *et al*., 1993). Another interesting finding was that strains belonging to serogroup O8 from the rectum of Kafue lechwe and cattle were not related because their biochemical profiles were not identical. However, this does not rule out the possibility that heightened interactions between wildlife and cattle could likely lead to interspecies bacterial transfer (Gilbreath *et al*., 2009). A comparison of *Salmonella* biotypes from humans living in the GMAs and NPs with those derived from wildlife and pastoral cattle may yield interesting results and could ascribe to possible zoonotic transfer. We thus recommend that more comprehensive surveillance to evaluate the actual burden of *Salmonellae* prevalence across wild waterfowls, cattle, lechwe and humans be conducted. This study has shown that PCR remains a relatively rapid and highly sensitive method in the diagnosis of non-typable *Salmonella* isolates (Singer *et al*., 2006).

5. Conclusion

Salmonella was present in the faeces of Kafue lechwe and pastoral cattle, and the recovery rate of *Salmonellae* was much higher among Kafue lechwe. Isolation of potentially pathogenic *Salmonella* isolates from Kafue lechwe is of food safety significance considering the unhygienic processing of meat for human consumption from this highly hunted antelope. The study concluded that the Kafue lechwe and cattle on the Kafue Flats in Zambia harbour pathogenic *Salmonellae* and could serve as potential vehicles for transmission to humans. Therefore, hygiene measures to protect human exposure to virulent *Salmonellae* contaminating food and water should be prevented.

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