MOLECULAR CHARACTERISATION OF*SALMONELLA ENTERICA* SEROVARSISOLATED AT THE UNIVERSITY TEACHING HOSPITAL, LUSAKA

By

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A Dissertation Submitted to the University of Zambia in Partial Fulfilment of the Requirements for the Degree of Master of Science in Medical Microbiology

The University of Zambia

Lusaka
June 2014

Declaration

I, Annie Kalonda, declare that this is my own work. It is being submitted for the Degree of Master of Science in Medical Microbiology at the University of Zambia, Lusaka. It has notbeen submitted for any degree at this or any other university.

30th day of June, 2014

Annie Kalonda

Certificate of Approval

Dissertation Title:Molecular Characterisation of*Salmonella enterica* serovar Typhi isolated at the University Teaching Hospital, Lusaka

This dissertation of **Annie Kalonda**has been approved in partial fulfilment of the requirements for the degree of Master of Science in Medical Microbiology at the University of Zambia.

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Abstract

*Salmonellaentericas*pecies are important food-borne pathogens that cause gastroenteritis and enteric fever, and are responsible for a huge global burden of morbidity and mortality. In addition, *Salmonellaenterica* has been associated with multiple drug resistance and this complicates disease management. Since November 2010, Zambia has been experiencing a rise in the number of *Salmonella* infections but the antibiotic susceptibility pattern andmolecular epidemiologyremain unknown. Therefore, the main objective of this study was to determine the molecular characteristics of *Salmonella enterica* serovars isolated at the University Teaching Hospital in Lusaka, Zambia.This was a laboratory-based cross-sectional study on the molecular characterisation of *Salmonella* serovar obtained from the typhoid fever outbreaks. Antibiotic susceptibility testing was performed by the microbroth dilution method, and the presence of drug resistance genes were confirmed by Polymerase Chain Reaction and DNA sequencing. Additionally, Whole Genome Sequence Typing was performed to determine the genetic relatedness of *Salmonella* Typhi strains. Of the 127 *Salmonella* isolates analysed, 55.9% were *Salmonella* Typhi, 21.3% *Salmonella* Paratyphi B, 1.6% *Salmonella* Senftenberg and 21.3% were other non-typhoidal *Salmonella*. Multidrug resistance was alarmingly high, with 100% *Salmonella* Paratyphi B and Senftenberg, 85.9% *Salmonella* Typhi and 55.6% non-typhoidal *Salmonellae* exhibiting this trend.A few*Salmonella* Typhi isolates (2.8%) were nalidixic acid-resistant and this was due to single mutations in the *gyrA* gene at codons Ser83 and Asp87. Thirty three randomly selected *S.* Typhi revealed that all but one, the pan-susceptible isolate harboured*str*A, *str*B, Δ*aadA*1and $Bla_{\text{TEM-1}}$,*sul1, sul2, catA1, dfrA7 and dfrA14* genes. Class 1 integron, harbouring an 800bp integron containing the *dfrA7* gene, was detected in both *Salmonella* Typhi (29/44, 65.8%) and *Salmonella* Paratyphi B (22/27, 81.5%). Single Nucleotide Polymorphism analysis revealed a profound clustering among the isolates separating individual strains from the nearest neighbour with 2 to 62 single nucleotide polymorphisms.Phylogenetic analysis and deletion content clearly indicated that the *Salmonella* Typhi isolates involved in the outbreak were distantly related, suggesting that multiple clones and lineages were responsible for the outbreak. Hence regular monitoring and surveillance should be doneto prevent further emergence of drug resistance strains and spread of these global pathogen.

Dedication

I dedicate this work to my mother, Domitilla Kalonda, for her love, patience, encouragement, emotional and spiritual support during the time of my study

Acknowledgements

First and foremost, I would like to express my sincere gratitude to my primary Supervisor, Dr James C. L, Mwansa, for the remarkable supervision, material and moral support he rendered throughout the research work and write up of this dissertation. His guidance helped me in all the time of the research and writing of this dissertation, even during tough times in the pursuit of my master's degree.

I am heartily thankful to my Co-supervisor and the Head of the Department of Biomedical Sciences at the University of the Zambia, Dr Geoffrey Kwenda, for his patience, motivation, enthusiasm, vast scientific knowledge and continuous support for my studies. His guidance helped me in all the time of the research and writing of this dissertation, even during tough times in the pursuit of my master's degree. I could not have imagined having a better supervisor and mentor for my study.

I am thankful to Dr T. Kaile, the Head of the Department of pathology and Microbiology, School of Medicine, for giving me an opportunity to pursue my studies in his department, for having confidence in me and for supporting my research activities. I am also indebted to the Assistant Dean for Postgraduate Studies in the School of Medicine, Dr Selestine H. Nzala, for his encouragement and support.

I am grateful to the funding sources that made my work possible. My course and part of the research work was supported by the Staff Development Office of the University of Zambia. I also very much appreciate the support of the Medical Education Partnership Initiative Project at the School of Medicine for purchasing reagents for my molecular biology work.

My gratitude also goes to the academic and technical staff in the Department of Pathology and Microbiology and the Department of Biomedical Sciences, at the School of Medicine, for their invaluable assistance and technical support; members of staff of the Bacteriological laboratory of the Department of Pathology and Microbiology at the University Teaching Hospital for accommodating me in their busy laboratory and permitting me to use human *Salmonella* isolates from their storage bank.

Furthermore, I wish to thank Dr Rene S. Hendriksen of the Technical University of Denmark in Denmark for providing me with positive control samples for the integron PCR and his technical support.

Lastly, I would like to thank my family for their understanding and support during the years of my studies. Your patience, love and encouragement haveupheld me. My special gratitude should also goto my mother for her love and unwavering support. I also thank my colleagues, course-mates and friends for the roles they played towards the successful completion of this dissertation.Above all, I thank The Almighty Godfor the gift of life.

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List of Abbreviations

AIDS	Acquired Immune Deficiency Syndrome
ATCC	American Type Culture Collection
CDC	Centre for Disease Control and Prevention
CGE	Centre for Genomic Epidemiology
CLSI	Clinical and Laboratory Standards Institute
CIDRAP	Centre for Infectious Disease Research and Policy
CRAMP	Cathelicidin –Related Antimicrobial Peptides
DC	Dendritic Cells
E-Test	Epsilometer Test
EUCAST	European Committee on Antimicrobial Susceptibility Testing
HIV	Human Immunodeficiency Virus
IFN	Interferon
IL	Interleukin
INSARG	Indian Network for Surveillance of Antimicrobial Resistance Group
MAC	MacConkey
MDR	Multidrug Resistant
MLST	Multilocus Sequence Typing
NaCl	Sodium Chloride
NCBI	National Centre for Biotechnology Information
NTS	Non-typhoidal *Salmonella*
PCR	Polymerase Chain Reaction
PFGE	Pulsed-Field Gel Electrophoresis

QRDR Quinolone Resistant Determining Region

ROI Reactive Oxygen Intermediates

RNI Reactive Nitrogen Intermediates

SPI *Salmonella* Pathogenic Island

SNP Single Nucleotide Polymorphism

TTSS Type III Secretion System

TSI Triple Sugar Iron

UTH University Teaching Hospital

VNTR Variable Number Tandem Repeat

WGST Whole Genome Sequence Typing

XLD Xylose Lysine Deoxycholate

Chapter 1

Introduction

1.1 Background

*Salmonella enterica*is a common cause of human gastroenteritis and bacteraemia worldwide and is a major contributor to morbidity and economic costs (Stevens*et al.,* 2008; Ammari *et al.,* 2009; Tajbakhsh *et al.,* 2012). Human infections with *S. enterica* results in two major groups of diseases: gastroenteritis and enteric fever. A wide variety of animals, particularly food animals, have been identified as reservoirs fornon-typhoid *Salmonella* (NTS) responsible for gastroenteritis(Gordon*et al.,* 2011; Tajbakhsh *et al.,* 2012). The serovars most often isolated worldwide are *Salmonella enterica* serovar Enteriditis and *Salmonella enterica* serovar Typhimurium (Cormican*et al.,* 2002; Ammari *et al.,* 2009). In contrast, enteric fever is a human-specific disease caused by*Salmonella enterica* serovar Typhi or *Salmonella enterica*serovar Paratyphi A, B or C (Chart *et al.*, 2007;Gordon *et al.,* 2011; Zhang *et al.*, 2011).

Entericfever is transmitted through contaminated water and food(Kanungo *et al.,* 2008).It is endemic in low and middle income countries, where clean water is lacking and sanitation and hygienic standards are inadequate (Kanungo *etal.*, 2008; Demczuk *et al.*, 2010; Neil *et al.*, 2012). In industrialised countries, the incidence of enteric fever is low and invariably associated withchronic carriers, food handlers or acquired during travel to endemic regions (Demczuk *et al.*, 2010).In high-income countries, NTS predominantly cause self-limiting diarrhoeal illness in healthy individuals in whichbloodstream or focal infection is rareand mainlyhappens in individuals with

specific risk factors (Feasey*et al.,* 2012; Monack, 2012; Shi*et al.,* 2013). In contrast, in sub-Saharan Africa, NTSare consistently the most common bacterialbloodstream isolates in both adults and children presenting with fever (Feasey*et al.,* 2012;Strugnell*et al.,* 2014).

The most recentglobal burden of disease for enteric fever have been estimatedto be approximately 27 million cases, with 216 000 deaths annually (Crump *et al.*, 2004). In contrast, studies on the global burden of NTS causing gastroenteritis have estimatedthat, 93.8 million cases of gastroenteritis occur globally every year, resulting in 155,000 deaths (Majowicz *et al.,*2010). Of these, 80.3 million cases are foodborne-related (Tajbakhsh*et al.,* 2012). The data on which these estimates are based is limited, and come from isolated studies from countries with healthcare structures capable of assessing the burden of *Salmonella* infection. Accurate figures are compounded by limitations of the currently available diagnostic tests (Baker *et al.*, 2010; Crump and Mintz, 2010; Franklin*et al.,* 2011).

In addition to the burden of the disease, the emergence of*Salmonella*species resistant to nearly all commonlyavailable drugs exacerbates the morbidity and mortality (Bhutta, 2008;Gonzalez-Escobedo *et al.,* 2011; Tajbakhsh *et al.,* 2012). Factors contributing to the emergence of drug resistance include overuse, misuse and inappropriate antibiotic prescribing practices (Singh, 1991; Zhang *et al.,* 2011).The world at large is now experiencing widespread resistance to multiple first-line antimicrobial drugs such as ampicillin, chloramphenicol, streptomycin, sulfadiazine, tetracycline and trimethoprim (Demczuk *et al.*, 2010).This increase in antimicrobial resistance has reduced the effective treatmentoptions and subsequently increased the

treatment costs andthe risk of complications and death (Kariuki, 2008).Therefore, antimicrobial susceptibility profiles and molecular characterization of resistance determinants are useful epidemiologicaldata which can be used to determine the occurrence, prevalenceand spread of resistance genes (van Leeuwen, 2009; Tajbakhsh*et al.,* 2011).

1.2 Statement of the Problem

Salmonella infectionsare a life threatening infectious diseasesrequiring antibiotic intervention to control (Bhunia *et al.,* 2009; Bayram *et al.,* 2011). However, theemergence of multidrug-resistantstrains of *Salmonella* specieswith increased virulence, transmissibility, and survival has led to increased morbidity and mortality and has further complicated the management (Hamid and Jain, 2008).Recently, Zambia experienced major typhoid fever outbreaks in Lusaka, Western and Copperbelt Provinces in which 558 suspected cases were recorded with 298 confirmed cases in 2010 (MOH, 2011). From November 2011 to February, 2012, a further 4396 suspected cases of typhoid fever were reported including 9 deaths (Anonymous, 2012) although confirmed cases were not documented.Most of the samples from these cases were analysed at the University Teaching Hospital (UTH) but the characteristics of the *Salmonella* isolates were not determined due to lack suitable diagnostic tools. In addition UTH has recently recorded a high frequency in the number of suspected *Salmonella*(UTH Laboratory Records). However, despite the increase of *Salmonella* isolation at the UTH and outbreaks in the country the serovars, drug resistance patterns and molecular characteristics remained unknown.

1.3 Justification of the Study

Effective management of *Salmonella* infections requires administration of antibiotics to reduce morbidity and mortality. However, the inappropriate use of antimicrobial drugs has led to treatment failure and also the emergence and spread of drug resistance strains. The development of multidrug resistant *Salmonella* species is currently one of the greatest challenges in the effective treatment and management of these infections by both epidemiologists and clinicians. This study will contribute to the understanding of the common serovars, antimicrobial resistance profiles and the molecular epidemiology of *Salmonella*strains isolated at the UTH. This informationwill be important in the control of drug resistant strains and treatment of these important global pathogens. To the best of our knowledge, this was the first study of its kind in Zambia to utilise molecular tools in drug resistance profiling of *Salmonella* serovars and their molecular epidemiology.

1.4 Literature Review

1.4.1 General Characteristics of *Salmonella*

Salmonella are important food-borne pathogens that are responsible for serious cases of food-borne illness leading to millions of cases of diarrhoeal disease, thousands of hospitalizations and deaths annually worldwide (Ammari *et al.,* 2009; Feasey *et al.,*2012;Camarda*et al,* 2013). *Salmonella* is widely distributed in nature and cause a wide spectrum of diseases in man and animals (Agbaje *et al.*, 2011; Monack, 2012). In humans *Salmonella*causes several syndromes such as enteric fever, gastroenteritis, septicaemia, focal infectionsand, in the case of some typhoidal strains, an asymptomaticcarrier state (Agbaje *et al.*, 2011).In animals, *Salmonella* infection is manifested in four major forms,enteritis, septicaemia, abortion and asymptomatic

carriage (Agbaje *et al.*, 2011). However, some serovars like *S.* Typhi and *S.* Paratyphi A are host specific and only adapted to cause disease in humans and these are typhoid *Salmonella,*while NTS serovars, typically have abroad vertebrate host range(Todar, 2009; Agbaje *et al.*, 2011; Feasey *et al.,*2012).

Salmonellae arenoncapsulated, nonsporulating, Gram-negative, facultative and rod-shapedbacterium belonging to the family *Enterobacteriaceae*, which include*Escherichia coli, Shigella* species, *Enterobacter* species, *Klebsiella* species, *Serratia* species, *Proteus* species among others (Sleigh and Duguid, 1989; Todar 2009).*Salmonella*are generally motile with peritrichousflagella (*Salmonella* Gallinarum and *Salmonella* Pullorum are the notableexceptions), aerogenic, non-lactose fermenting, oxidasenegative,urease-negative, citrate-utilizing, acetylmethylcarbinol-negative and potassium cyanide-negative (Sleigh and Duguid, 1989; Todar,2009).Most strains grow on nutrient agar as smooth colonies, 2-4 mm in diameter and are prototrophs, not requiring any growth factors. However, auxotrophic strains do occur, especially in host-adapted serovars such as Typhi and Paratyphi A (Todar, 2009).

1.4.2 Taxonomy and Phylogenetic Status of *Salmonella*

Salmonella is a genus of the family *Enterobacteriaceae* in the gamma-proteobacteria subdivision, in the order Enterobacterialesand phylum Proteobacteria (Ellermeier and Slauch, 2006; Todar, 2009). The genus *Salmonella*is composed of bacteria related to each other both phenotypically and genotypically. *Salmonella* has a DNA base composition of about 50-52 mol% G+Cin their genomic DNA (Todar, 2009).The genus is composed of two species namely *Salmonella enterica* and *Salmonella*

bongori(Baker and Dougan, 2007; Fookes *et al.,* 2011; Feasey *et al.,* 2012). *S. bongori* has been known to be a commensal of cold blooded animals, while *S. enterica* is a major human and animal pathogen (Sabbagh*et al.,* 2010;Fookes*et al.,* 2011). More than 2,500 *S. enterica* serovars have been identified throughout the world based on their somatic (O) and flagellar (H) antigens (CIDRAP, 2006; Hendriksen *et al.,* 2009; Sabbagh *et al.,* 2010).

The nomenclatureand taxonomy of *Salmonella*are complex, controversial and have changed over the years and are still evolving (Brenner *et al.,* 2000; Sabbagh *et al.,* 2010; Agbaje*et al.,* 2011). Thisis because the original taxonomy of the genus was not based onDNA relatedness, but rather on clinical considerations (Todar, 2009). Over the last few decades, a number of tests and techniques have been developed foridentifying, characterising and classifying *Salmonella*, leading to the generation of ahuge amount of information.Thus, there was a great need for consolidating and standardising this information, especially that pertaining to classification. A variety of approaches have been used to characterise the systematics and taxonomy of*Salmonella.* In the past decades*Salmonella* classification was based on epidemiology, host range, clinical manifestation, biochemical reactions andsurface antigenic patterns (Farmer, 2003; Agbaje*et al.,* 2011). However, classification of the genus *Salmonella*has evolved over timefrom the initial one serotype-one species concept proposed by Kauffmann (1966) on the basis of somatic (O), flagellar (H) and capsular (Vi) antigens(Euzéby, 1999; Miller and Pegues, 2000; Agbaje *et al.,* 2011).When serological analysis was adopted into the Kauffmann-White Scheme in 1946, each *Salmonella* serovar was considered as aseparate species. Since the host-specificity suggested by some of these earlier names does not exist (e.g., *S. typhi-*

murium, S. cholerae-suis are in fact ubiquitous), names derived from the geographical origin of the first isolated strain of the newly discovered serovars were next chosen, such as,*S. london, S. panama, S. Stanleyville* (Brenner *et al.,* 2000; Su and Chiu, 2007).

Kauffman proposed designation of each serovar as a species (Kauffmann, 1966), and*Salmonella* serovars identified after 1966 were predominantly designated by their antigenic formula and numerous species within the genus were identified.In the early development of taxonomic scheme, biochemical reactions were used to separate *Salmonella* into subgroups and the KauffmannWhitescheme was the first to attempt to systematically classify *Salmonella* usingscientific parameters(Kauffmann, 1966; Brenner *et al.,* 2000; Su and Chiu, 2007).Thus, the effort culminated into development of fivebiochemically defined subgenera (I to V) where, individual serovars were designatedstatus of a species (Kauffmann, 1966; Brenner *et al.,* 2000; Su and Chiu, 2007).Subsequently, because of the complexity of multiple *Salmonella* species, three species nomenclature system was proposed using 16discriminating tests to identify *S. Typhi*, *S. Choleraesuis,* and*S. Enteritidis* and later the scheme recognized member of Arizona group as adistinct genus (Su and Chiu, 2007; Evangelopoulou*et al.,* 2010).

Despite immense efforts that had gone into classical*Salmonella* taxonomy, the defining moment for *Salmonella*nomenclature came in the early 1970s, when nucleotide sequence relatedness, DNA-DNA hybridization experiments and other molecular analysis demonstrated that all serovars and subgenera I, II, and IV of*Salmonella*and all serovars of “Arizona” were related at the species level(Brenner

and Falkow, 1971; Brenner *et al.,* 1972; Crosa *et al.,* 1973, Brenner *et al.,* 2000). Theonlyexception was *S. bongori*,previously described as subspecies V, but by DNA-DNA hybridization it has been found to be adistinct species (Reeves *et al.,* 1989; Boyd *et al.,* 1996; Brenner *et al.*, 2000).

According to the International Committee on Systematic Bacteriology, the genus *Salmonella* consists of two species, *S. bongori and S. enterica* (Tindall *et al.,* 2005; McQuiston *et al.*, 2008; Agbaje *et al.,* 2011). This is based on phenotypic traits, such as carbon source utilization and this has also been validated to a considerable extent by DNA-DNA hybridization (McQuiston *et al.*, 2008). Subspecies determination is based on presence or absence of 11 biochemical traits, which include dulcitol,ortho-Nitrophenyl-β-galactoside, gelatinase, Growth with potassium cyanide, γ-glutamyltransferase, ß-glucuronidase among them (Nataro *et al.,* 2007; McQuiston *et al.*, 2008).*S. enterica* is further divided into six subspecies that were categorized by Tindall and colleagues (2005) as follows: *S. enterica* subsp. *enterica* (subsp. I), *S. enterica* subsp. *salamae* (subsp. II), *S. enterica* subsp. *arizonae* (subsp. IIIa), *S. enterica* subsp. *diarizonae* (subsp. IIIb), *S. enterica* subsp. *houtenae* (subsp. IV), and *S. enterica* subsp. *indica* (subsp. VI). Subspecies VII was later described by Boyd and co-workers (1996).

In addition to the taxonomic classification of subspecies,*Salmonella* is further subdivided by serovars using a subtypingmethod based on surface antigens (Brenner *et al.*, 2000). Thismethod has been invaluable in understanding the epidemiologyof *Salmonella*. The combination of the subspecies, 46 Ogroups, and 114 H antigens accounts for all recognised serotypesof *Salmonella* and the most frequently

encountered subspecies is *S. enterica*subsp. Ifound primarily in mammals (Popoff *et al.*, 2003, 2004; McQuiston *et al.*, 2008;Tajbakhsh*et al.,* 2011).This subspecies is themost common cause of human disease(Tajbakhsh *et al.,* 2011). The other six subspeciesarefound primarily in nonhuman hosts and rarely cause disease in humans (Popoff *et al.,* 2000; Dieckmann*et al.,* 2008). Table 1.1 below shows the current taxonomic position and nomenclature of *Salmonella*.

Table 1.1 Current *Salmonella* nomenclature

Genus (capitalised, italicised)	**Species (not capitalised, italicised)**	**Subspecies (symbol) (not capitalised, italicised)**	**Serovar name (with examples) (capitalised, Roman)**
Salmonella	*enterica*	*enterica* (subspecies I)	Choleraesuis, Enteritidis, Paratyphi, Typhi, Typhimurium
		salamae (subspecies II)	9,46:z:z39
		arizonae (subspecies IIIa)	43:z29:-
		diarizonae (subspeciesIIIb)	6,7:l,v:1,5,7
		houtenae (subspecies IV)	21:m,t:-
		indica (subspecies VI)	59:z36:-
Salmonella	*bongori*	(Subspecies V)	13,22:z39:-

Adapted from Su and Chiu(2007)

In recent years there has been an emphasis on the development of molecular tools which have led to the profound modifications in the classification and methods of identification of *Salmonella.* These methods, utilising one or several appropriate genes, are gaining importance due to the fact that they yield quick and in most cases, unequivocal results(Ferdinand *et al.*, 2004). The genes being utilised include the 16S/23S rRNA, housekeeping genes and invasion genes (Fukushima*et al.,* 2002). In particular, the 16S rRNA sequences have been widely used to construct bacterial phylogeneticrelationships or to detect pathogenic bacteria.The 16S rRNA is an approximately 1500bp sequence encoded by the 16S ribosomal DNA. The use of the

16S rRNA in bacterial phylogenetic analysis has become popular because these sections of RNA are universally present, highly conservedand easy to amplify and sequence(Fukushima *et al.,* 2002).

However, classification of closely related species of bacteria such as *Shigella* species, *E. coli*and in particular distinguishing among *Salmonella*subspecies *enterica*is difficult to achieve through the analysis of 16S rRNA (Fukushima *et al.,* 2002; Leekitcharoenphon,*et al.,* 2012). As alternatives to 16S rRNA analysis, genes such as *rpoB*, *sodA*and *gyrB* have been suggested as substitutes and have shown improved efficacy in species identification (Fukushima *et al.,* 2002; Leekitcharoenphon, *et al.,* 2012).The*gyrB*region is also useful in analysing the phylogenetic relationship among*Salmonella.*Nevertheless, it remains unlikely that a single gene can always reflect the subtledifferences between genomes of the same species or subspecies. Therefore, this limitations of using a single gene may be improved by the simultaneous analysis of multiple genes.

1.4.3 Epidemiology of *Salmonella*

Salmonella infectionsoccur worldwide in both developed and developing countries posing a constraint in economic growth (Feasey *et al.,*2012;Camarda*et al.,* 2013). However, the occurrence of these infections vary widely depending on the *Salmonella* serovars involved (Sanchez-Vargas *et al.*, 2011).Enteric fever, caused by *S.* Typhi and *S.* Paratyphi, primarily affects communities in developing nations, while NTS infections tend to be self-limiting and affect communities worldwide (Sanchez-Vargas *et al.*, 2011).*Salmonella* is frequently isolated from environmental sources that serve as a relay for the bacteria and play a major role in its spread

between different hosts(Ammari *et al.*, 2009). Globalisation and increased volume of internationaltrade involving animal food products have facilitatedthe introduction of new *Salmonella* serovars into importingcountries (Hohmann, 2001; Agbaje *et al.,* 2011).

1.4.3.1 Typhoidal *Salmonella*

It has been estimated that enteric fever causes approximately 216 000 deaths and 27 million cases annually (Crump *et al.,* 2004;Crump and Mintz, 2010). However, the incidence of enteric fever varies substantially between countries with the highest incidence estimates (more than 100 cases per 100 000 inhabitants per year) calculated in south-central Asiaand south-east Asia while low incidence (less than 10 cases per 100 000 per year) wasreported in Europe, Australia,New Zealand and North America (Ochiai*et al.,* 2008; Gil Prieto*et al.,* 2009; Sanchez-Vargas *et al.*, 2011). In the United States, United Kingdom and Canada the incidence of enteric fever is infrequent and is related to travel, either, travellers returning from developing countries, or foreigners traveling to these countries(Kothari *et al.,* 2008; Sanchez-Vargas *et al.*, 2011).

South America, Latin America, and South and South-East Asia are amongst the most common regions affected by enteric fever, although the incidences vary substantially(Kanungo *etal.*, 2008; Crump and Mintz, 2010).Asia, India and Pakistan are the worst hit with the highest typhoid fever incidences (Kanungo *et al.,* 2008). About 400 000 cases of enteric fever occur annually in Africa, translating into an incidence of 50 cases per 100 000 persons per year (Kariuki, 2008; Kothari *et al.,* 2008).However, the burden of enteric fever is poorly characterized in many

developing countries, especially sub-Saharan Africa, because of limited availability of resources for diagnosis, surveillance tools and consequently epidemiologic data (Buckle *et al.,* 2010; Sanchez-Vargas *et al.*, 2011). Although, there is limited data in sub-Saharan Africa,typhoid fever is common in Nigeria, Mali, Ethiopia and Kenya (Karuiki, 2008). However in Zambia little is known about the epidemiology of enteric fever.

1.4.3.2 Non-typhoidal *Salmonella*

NTS illnesses continue to impose a significant burden on the population's health in industrialized and underdeveloped countries. It is estimated that 93.8 million cases of *Salmonella* gastroenteritis occur worldwide leading to 155,000 deaths annually (Majowicz *et al.,* 2010; Elhadi *et al.,* 2013; Tadesse, 2014). Data from the World Health Organization (WHO) Global Foodborne Infections Network (GFN) indicate that *S.* Typhimurium and *S.*Enteritidis account for nearly 80% of all human isolates reported globally (Sanchez-Vargas *et al.,* 2011; Haeusler and Curtis, 2013). In industrialised countries the increasing incidence of NTS has become a public health concern (Sanchez-Vargas *et al.,* 2011). In the United States approximately 1.4 million cases of NTS gastroenteritis, 15 000 hospitalisation and 500 deaths occur annually (Begum *et al.,* 2010; Fricke *et al.,* 2011). In Europe the incidence of NTS illness is estimated to be 690 per 100,000 persons annually,varying between regions from 240 per 100,000 in Western Europe to 2390 per 100,000 person annually in Central Europe (Majowicz*et al.,* 2010; Sanchez-Vargas *et al.,* 2011).

NTS have been shown to be among the leading causes of bacterial bloodstream infections in sub-Saharan Africa (Reddy *et al.,* 2010; Feasey *et al.,* 2012; Lunguya *et*

al., 2013). NTS bacteraemia mainly affects immunocompromised hosts and young children in whom they are associated with high mortality rates up to 27% (Lunguya *et al.,* 2013). In addition, invasive strains of NTS have emerged as a prominent cause of bloodstream infection in African adults and children, with an associated case fatality of 20–25% (Feasey *et al.,* 2012). The incidence of invasive non-typhoidal disease is very high in Africa, with rates of about 200/100, 000 per year and an increased numbers of cases of at least 10-fold in human immunodeficiency virus (HIV) positive individuals (Strugnell *et al.,* 2014). Generally, *S. enterica* problem in Africa and elsewhere, is understated because of limited bacteriological support for clinical diagnoses of pyrexia(Kanungo *et al.,* 2008; Crump and Mintz, 2010; Strugnell *et al.,* 2014).

In rural Kenya, the estimated minimum incidence of bacteraemia was 505 cases per 100,000 person-years in the age group of less than 5 years old, of which 88 cases per 100,000 person-years were NTS bacteraemia (Morpeth *et al.,* 2009). In rural Mozambique, the incidence of childhood bacteraemia was 425 cases per 100,000 person-years among children aged less than 15 years, and within this category, NTS incidence accounted for 120 cases per 100,000 person-years (Sigauque *et al.,* 2009), while Gambia had an incidence of NTS bacteraemia of 262 cases per 100,000 person-years among children aged less or equal to 29 months (Enwere *et al.,* 2006). In the Democratic Republic of Congo (DRC), a rural children's hospital showed that 62.1% of all bloodstream infections in children were caused by NTSwith *S.* Typhimurium and *S.*Enteritidis accounting for 60.5% and 22.3% of the cases, respectively (Vandenberg *et al.,* 2010).A study carried out in Lusaka, Zambia, to detect *Salmonella* from human samples, showed that out of the 200 clinical stool

samples, 9 (4.5%) were found to be bacteriological culture positive for *Salmonella* (Hang'ombe *et al.,* 2011). Inanother study, it was reported that of 124 adults and 105 children with persistent diarrhoea in Zambia, 6 (5%) and 21 (20%) were infected with NTSspecies (Mwansa *et al.*, 2002). These data demonstrate *Salmonella* to be an important pathogen in Zambia with potential of causing serious impact on human health leading to morbidity and/or death.

1.4.4 Genomics of *Salmonella*

The increasing number of available bacterial genome sequenceshas led to the realisation that the genetic variation within bacterial species is greater than previously predictedwhen these genomes are compared (Leekitcharoenphon*et al.,* 2012). Sequencing of these genomes allow investigators to gain a better understanding of the mechanisms by which closely related organisms have evolved and to look in more detail at the genomes of individual species, subspecies, serovars, and evendifferent isolates within the same serovar (Baker and Dougan, 2007; Leekitcharoenphon *et al.,* 2012). Therefore, the genetic blueprint of such bacteria can be examined andsimple comparisons on genomes of bacteria that share certain phenotypic characteristics can be made.

Molecular studies on the genome of *S.* Typhi suggest that this organism evolved recently and is younger than other *S. enterica* serotypes (Kidgell *et al.*, 2002) andcomplete genome sequence from multiple *Salmonella* strains are available (de Jong *et al.,* 2012).Multilocus Sequence Typing (MLST), with a seven housekeeping gene scheme, has shown that *S.*Typhi is genetically homologous (Kidgell *et al.*, 2002) and is considered to be clonal in distribution across the world with a few

clones circulating globally (Kubota *et al.*, 2005; Song *et al.*, 2010; de Jong *et al.,* 2012).The *S.* Typhi type strain Ty2, the multidrug resistant (MDR) isolate CT18, and the *S.* Typhimurium strainLT2 are composed of 4.79 (Ty2), 4.86 (CT18), and 4.81 (LT2)megabases, respectively(de Jong *et al.,* 2012). In addition, a comparison of *S.* Typhi CT18 and Ty2 has revealed a remarkable conservation of their genomes with only a few differences which include an additional cluster of a few genes in *Ty2* that might be a novel pathogenicity island and a P4-like phagedeterminant (Baker and Dougan, 2007).The 4.8-Mb complete genome sequence of *S.* Typhi strain Ty2, was compared with the genome sequence of recently isolated *S.* Typhi strain CT18 showed that 29 of the 4,646 predicted genes in Ty2are unique to thisstrain, while 84 genes are unique to CT18. Both genomes contain more than 200 pseudogenes; 9 of these genes inCT18 are intact in Ty2, while 11 intact CT18 genes are pseudogenes in Ty2. A half-genome interreplichore inversion in Ty2 relative to CT18 was confirmed. The two strains exhibit differences in prophages, insertion sequences, and island structures. While CT18 carries two plasmids, one conferring multiple drug resistance, Ty2 has no plasmids and is sensitive to antibiotics (Deng *et al.,* 2003).

Other molecular studies have revealed that if the DNA sequences of genes in the core genome of different enteric bacteria are compared, *Escherichia coli* and *S. enterica* are found to differ by about 10%, and *Salmonella* serovars within *S. enterica* differ by about 1% (de Jong *et al.,* 2012). This 10% divergence between the core sequences of *E. coli* and *S. enterica* most likely represents evolutionary drift over the 100 million years since the 2 species are separated from a common ancestor (Baker and Dougan, 2007; de Jong *et al.,* 2012). The homology of the 200 pseudogenes in *S.enterica* which are functionally disrupted or inactive, most are functional in *S.*

Typhimurium. This, in part, contributes to the host restriction in *S.* Typhi and in NTS serovars may be explained by differences in genome expression leading todifferences in host-pathogen recognition (de Jong *et al.,* 2012).

About 90% of the genes in *S.* Typhi and *S.*Typhimurium serovars are identical and 10% of genes that differ includevirulence factors, which determine their pathogenic potential(de Jong *et al.,* 2012). Comparative genomics performed on 17 NTS which were recently sequenced against the 11 previously sequenced NTS revealed that phenotypic and genotypic data comparisons in the phylogenetic species context suggest that the evolution of known *S. enterica* sublineages is mediatedmostly by two mechanisms: the loss of coding sequences with known metabolic functions, which leads to functional reduction and the acquisition of horizontally transferred phage and plasmid DNA, which provides virulence and resistance functions and leads to increasing specialisation (Fricke*et al.,* 2011).

Various molecular methods have been developed to facilitate the detection of genetic variations amongst phylogenetically-related bacteria and these include techniques that are usually used in evolution and epidemiological studies. These methods have successfully segregated closely related strains and they include Pulsed-Field Gel Electrophoresis (PFGE) (Thong *et al.*, 1996; Hosoglu *et al.*, 2003; Ben-Saida*et al.*, 2007), ribotyping (Navarro *et al.*, 1996), sequencing of 16S rRNA genes and Variable Number of Tandem Repeat (VNTR) typing (Liu *et al.*, 2003). More recently Single Nucleotide Polymorphism (SNP) typing has proven valuable in both phylogeneticand epidemiological studies (Ramisse*et al.*, 2004; Roumagnac *et al.*, 2006; Octavia and Lan, 2007).

1.4.5 Mode of Infection and Immune Response of *Salmonella*

S. enterica is a frequent gastrointestinal pathogen withthe ability to cause diseases ranging from local gastrointestinal inflammationand diarrhoea to life-threatening typhoid fever (Fierer and Guiney, 2001; Jantsch*et al.,* 2011).It infects various cell types of the host and can survive and proliferate in different populations of immune cells (Jantsch *et al.,* 2011).Enteric fever is transmitted through the faecal-oral route, usually through the consumption of contaminated food or water(Demczuk *et al.*, 2010; Jantsch*et al.,* 2011) whileNTS transmission to humans can occur by consumption of animal food products, non-animal food products, contaminatedwater, or by contact with animals (Sanchez-Vargas *et al.,* 2011). Food products,mass production and distribution disseminates pathogensrapidly to communities (Sanchez-Vargas *et al.,* 2011).Chronic carriers are the reservoirs for the spread of infection and disease (Monack, 2012).The infectious dose of *S.*Typhi in volunteers varies between 1000 and 1 million organisms(Kaur and Jain, 2012). However, the disease outcome is mainly dependent on the serotype of *S. enterica* encountered. *S.* Typhi and, to a lesser extent, *S.* Paratyphicause systemic infections that are major health issues indeveloping countries and among HIV infected individuals (Haeusler and Curtis, 2013;Wain*et al.,* 2013).

Pathogenic *Salmonella* species possess an array of invasion genes that produce proteins secreted by a specialized type III secretion system (Jones and Falkow, 1996). These proteins are used by the bacteria to penetrate the intestinal mucosa by invading and destroying specialized epithelial M cells of the Peyer's patches(Jones and Falkow, 1996). This in turn deposits the bacteria directly within the confines of

the reticuloendothelial system(Jones and Falkow, 1996). The host responds to these actions with nonspecific phagocytic cells and an inflammatory response as well as by activating specific cellular and humoral immune responses (Jones and Falkow, 1996).*Salmonella* evokes both innate and adaptive immune mechanisms.

1.4.5.1 Innate Immunity

Innate immunity provides the primary line of defence by inducing a variety of inflammatory and antimicrobial responses. This play an essential role in the early responses to *Salmonella* and in most subclinical infections may be enough to control progression to disease (Hughes and Galan, 2002). One of the initial obstacles faced by *Salmonella* is to cross the thick layer of mucus which is one of the major innate immune defences, covering the gastrointestinal tract (GIT) epithelium to gain direct access to the epithelium (Broz *et al.,* 2012). In addition to mucus secreted by goblet cells, the cells of the GIT secrete antimicrobial peptides which are capable of disrupting the cell integrity of the invading microorganisms (Broz *et al.,* 2012). However, *Salmonella* is better adapted to counter these host defences (Broz *et al.,* 2012).

Macrophages andpolymorphonuclear neutrophils are important cells in the early response to *Salmonella* infection (Hughes and Galan, 2002). Once *Salmonella* crosses the M cells or enterocytes, it encounters macrophages and dendritic cells which are involved in phagocytosis of *Salmonella* (Jones, 1997; Hughes and Galan, 2002; Broz *et al.,* 2012; Kaur and Jain, 2012).These inflammatorycells produce cytokines such as TNF-α, IFN-γ, IL-1, IL-2, IL-6 andIL-8(Hughes and Galan, 2002; Broz *et al.,* 2012; Kaur and Jain, 2012). In addition,there is a great body of evidence

that reactive oxygen intermediates (ROI) and reactive nitrogen intermediates (RNI)participate in killing of*Salmonella* species (Jantsch *et al.,* 2011; Ruby *et al.,* 2012). Regarding *Salmonella,* it has been shown that ROI mediate rapid clearanceof the pathogen. This may not exclusively be explained by adirect ROI-dependent action on intracellular *Salmonella*, butmay also involve an ROI-dependent induction of anti-microbialpeptides such as cathelicidin-related antimicrobial peptide (CRAMP)(Jantsch *et al.,* 2011).However, experimental evidence suggests that *Salmonella* has evolved mechanisms to circumvent or delay the killing activity of these mechanisms (Jones, 1997).

1.4.5.2 Adaptive Immunity

Successful immunity against *Salmonella* infections is dependent on the generation of T cells particularly$CD4^+$ T helper cells and to a lesser extent on antibody production and $CD8^+$T cells(Hughes and Galan, 2002).$CD4^+$T-helper (T_H) cells play a central role in the productionof cytokines during *Salmonella* infection (Ruby *et al.*, 2012).$CD4^+$ helper T cells (T_H) are divided into two types, T_H1 cells produce IFN-γ and TNF-α and activate cellular immunity and inflammation, while T_H2 cells which produce IL-4,IL-5, and IL-13 and induce B cell activation and differentiation (Hughes and Galan, 2002). Studies have shown that *Salmonella*infectionsresults in the induction of a T_H1 response(Hughes and Galan, 2002;Kaur and Jain, 2012). However, dendritic cells (DC) and B-cells are involved in the initiation and development of T-cell immunity to*Salmonella*(Kaur and Jain, 2012). Interaction between B and T-cells is needed for the development of antibody response to *Salmonella* proteins and for isotype switching of antibody response against lipopolysaccharide antigens (Kaur and Jain, 2012).

1.4.6 Pathogenesis of *Salmonella*

Following ingestion of contaminated food or water, *Salmonellae* reach the stomach where they survive the gastric acidity and out compete the resident normal microbiota (Giannella, 1996). *Salmonellae*travel from the stomach to the intestines where they are transported across the intestinal epithelium and they invade the phagocytic epithelial M-cells lining Peyer's Patches, as well as being phagocytosed by dendritic cells (Coburn *et al.*, 2007). Alternatively,*Salmonellae*may invade the cells by endocytosis, a process that involves the formation of large membraneruffles and cytoskeleton rearrangement (Francis *et al*., 1992).The organisms are then internalised within bound vacuoles through which they trancytosefrom the apical to the basolateral surface (Rathman *et al*., 1997).Through migration of infected macrophages to other organs of the reticuloendothelial system, the organisms are disseminated to other organ systems within the host.

The disease processinduced by *Salmonellae* depend on the coordinated function of various sets of virulence proteins encoded by gene clusters on the virulence plasmid or by specific chromosomal loci referred to as *Salmonella* pathogenicity islands (SPI)(Coburn *et al.,* 2007;Kaur and Jain, 2012). Five pathogenicity islands have been identified in *S. enterica*(Knodler*et al.,* 2002). Two of these pathogenicity islands, SPI-1 and SPI-2, encode type III secretion systems (TTSS), which are essential virulence determinants.SPI-1 is required for the invasion of non-phagocytic host cells and elicitation of diarrhoeal disease (Galan, 1999; de Jong *et al.,* 2012) while SPI-2 is essential for the intracellular survival and replication of the bacteria (Monack, 2012).During invasion of the gut, SipB protein,encoded by SPI-1, triggers the

activation of intracellular Caspase-1 within resident macrophages that induces apoptosis in the infectedmacrophages resulting in escape of *Salmonella* from these cells (Hersh *et al*., 1999). SPI1also encodes an effector protein SopB which is an inositol phosphate phosphatase and itsenzymatic activity results in activation of chloride channel in the membrane of epithelialtarget cells leading to the secretion of chloride and loss of fluid into the intestinal lumen(Norris *et al*., 1998).

Central to the functioning of SPI-1 and SPI-2, is the type III secretion system (TTSS)important in the pathogenesis of *S. enterica*.This is a specialised virulence device that involves indirect translocation of bacterial virulence proteins intothe host cell cytoplasm (Knodler*et al.,* 2002). TheSPI1-TTSS complex is an important inducer of effector proteins such as SipA, SopB, SopD, and SopE2 which persist in the infected cells. These effector proteins are toxin-like virulence factors that induce the reorganisation of the host cell actin cytoskeleton, leading to macropinocytosis (Kaur and Jain, 2012).

The SPI2-TTSS is required to protect the pathogens within the *Salmonella* containing vacuole (SCV) against the effectors functions of innate immunity. This is achieved by preventing the localisation of the phagocyte oxidase and the inducible nitric oxide synthetases to the SCV (Vazquez-Torres *et al*., 2000; Chakravortty *et al*., 2002). The complete pathogenesis of typhoid fever is not clearly understood.

1.4.7Clinical Presentation of Salmonellosis

Salmonellosis can manifest as self-limiting gastroenteritis or systemic diseasecharacterisedby septicaemia (Jantsch*et al.*, 2011; Haeusler and Curtis,

2013).Onoccasion,salmonellosiscanalsoproduce bacteraemia andbacteriacanbeisolatedfromtheblood,oftenresultinginhighfevers(distinguishablefro mtyphoidfevers,which continues throughout theinfection),andisquicklyclearedfromthehost (Jantsch *et al.*, 2011; Haeusler and Curtis, 2013).

1.4.7.1 Typhoidal Infection

Enteric fever is a severe systemic form of salmonellosis. The symptoms begin after an incubation period of 10 to 14 days after ingestion of contaminated food or water (Giannella, 1996). Enteric fever may be preceded by gastroenteritis, which usually resolves before the onset of systemic disease. The symptoms of enteric fever are nonspecific and include fever, anorexia, headache, myalgia, and constipation (Bhunia *et al.,* 2009; Bayram *et al.,* 2011).

Initially, there is low fever that risesprogressively, and by the second week it is often high(39-40˚C)and sustained (Kaur and Jain, 2012).The fever occurs in more than 80% of patients (Khan *et al.,* 1998).The classical disease description includes bacteraemiaand fever during the first week, as well as nonspecificsymptoms such as chills, headache, anorexia, sore throat, unproductive cough,constipation following diarrhoea, myalgia,psychosis and mental confusion in 5–10% of the cases (Demczuk *et al.,* 2010; Kaur and Jain, 2012). Acoated tongue, tender abdomen, hepatomegaly, and splenomegalyare common. In the second week, a few rose spots, blanchingerythematous maculopapular lesions, approximately 2–4 mm indiameter, appear in 5–30 percent of cases. These usually occuron the abdomen and chest and more rarely on the back, arms,and legs. A relative bradycardia in relation to fever,

intestinal constipationor diarrhoea in smaller number of patients (mainly inyoung children and adults with HIV infection) may occur(Bayram *et al.,* 2011; Kaur and Jain, 2012). Withouttreatment or correct diagnosis, the typhoid fever may prolongto the third week and the inflammatory lesions become intensein Peyer's patches and intestinal lamina propria (Kaur and Jain, 2012).

Ileocaecal ulceration and necrosis (cellulardeath), with subsequent gastrointestinal bleeding or intestinalperforation may occur. Further complications in 10–15% of patients may result in death, afterthe third week of disease.The fever declines in the fourth week of disease in 90% of the survivors, without antibiotic therapy. However,weakness and weight loss may persist for many months(Kaur and Jain, 2012). About 3–5% of those infected progress to a chronic carrier state (Demczuk*et al.,* 2010).*Salmonella* carriage is defined as asymptomatic excretion following acute infection and can be divided into convalescent carriage and chronic carriage (Haeusler and Curtis, 2013).

1.4.7.2 Non Typhoid *Salmonella* Infection

NTS usually cause an acute self-limiting gastroenteritisalthough in immunocompromised individuals, serious complications can occur (Boyle*et al.,* 2007).Clinical manifestations of NTS can be broadlydivided into four groups:acute gastroenteritis, extra-intestinal infection, non-infectious sequelae and *Salmonella* carriage (Haeusler and Curtis, 2013).

Gastroenteritisusually results in profuse and usually non-bloody diarrhoea which is usually self-limiting (Kariuki*et al.,* 2006; Sanchez-Vargas *et al.,* 2011). However,

there are some other associated symptoms which may occur and these aremyalgia, headache,fever, abdominal cramping, nausea and vomiting (Giannella, 1996;Pegues and Miller, 2010; Haeusler and Curtis, 2013).Fluid and electrolyte disturbances are the most frequent complication of NTS gastroenteritis. The duration of fever and diarrhoea varies, but is usually 2 to 7 days. Asymptomatic gastrointestinal infection can also occur.However, given the rate of convalescent NTS excretion following acute infection, the true incidence is unknown.

Extra-intestinal infections have been reported in NTS and are common in immunoincompetent individuals with HIV, diabetes mellitus and those on steroid therapy (Feasey*et al.,* 2012; Haeusler and Curtis, 2013). NTS bacteraemia is reported in up to9% of patients with acute gastroenteritis (Haeusler and Curtis, 2013). Bacteraemia may result in focal NTS infection at any site, including the central nervous system (Haeusler and Curtis, 2013).

1.4.8Diagnosis of Salmonellosis

Several options exist for diagnosing salmonellosis: clinical signs and symptoms, serological markers, bacterial culture, antigen detection, and *Salmonella*DNA detection (Wain and Hosoglu, 2008). However, clinical diagnosis of salmonellosis is difficult because the manifestations of the disease are diverse (Wain and Hosoglu, 2008). The diagnosis mainly depends on the isolation of *Salmonella* from the patients using appropriate clinical specimens(Giannella, 1996;Wain and Hosoglu, 2008). The diagnostic methods of *Salmonella*can broadly be classified into phenotypic and genotypic methods.Phenotypic methods are used to detect visible characteristics of

the organism colonial morphology and biochemicalreactions. Bycontrast, genotypic methods detect the genetic characteristics of the organism.

1.4.8.1 Phenotypic Methods

*Salmonella*grow at an optimum temperature of 37˚C on culture and produces greyish, transparent to opaque, glistening colonies, usuallymore than 1 mm in diameter on blood agar (Perilla*et al.,* 2003).*Salmonella* also grows on differential solid media like MacConkey agar (MAC) and Xylose Lysine Deoxycholate (XLD) agar. Blood specimens are usually inoculated on blood agar, chocolate agar and MAC agar whilst faecal specimens are inoculated on MAC and XLD agar. On MAC agar the colonies appear colourless and opaque, because they are non-lactose fermenters and appear red with or without a black centre on media containing ferrous sulphate like XLDand TSI due hydrogen sulphide production (Mangels, 2012).

The presumptive identification of *Salmonella* involves the use of biochemical tests. The main biochemical tests used for *Salmonella* identification include production of hydrogen sulphide, gas production from glucose, citrate consumption and carbohydrate fermentation(Wain and Hosoglu, 2008).Confirmation of identification of *Salmonella* can be performed by antigenic analysis of O and H antigens using polyvalent and specific antiserathrough serotyping (Giannella, 1996;Wain and Hosoglu, 2008).Serotyping of *Salmonella* uses the agglutination of the somatic “O” and flagella “H” antigen based on the Kauffmann-White scheme (Popoff *et al.,* 2004). Full serotyping to define *Salmonella* serovars is notalways easy to performand it is predominantly performed in reference laboratories, dueto the limitation of

acquiring antisera and the difficulty in detection of phase 2 “H”antigen(Popoff *et al.,* 2004).

1.4.8.2 Genotypic Methods

Over the last two decades a number of molecular techniques have been developed for the detection and identification of*Salmonella* speciesto replace or complement traditional serotyping methods. These methods are now providing rapid and accurate results. Thesetechniques are mainly based on the amplification or hybridisation of nucleic acids and enzyme restriction.These include ribotyping (Esteban*et al.,* 1993), ribosomal DNA intergenic spacer amplification (Jensen and Hubner, 1996), random amplification of DNA polymorphism (Shangkuan andLin, 1998), IS*200* analysis (Uzzau*et al.,* 1999), real-time PCR(Hoorfar*et al.,* 2000), PCR–single-strand conformation polymorphism analysis(Nair*et al.,* 2002), amplified fragment length polymorphism (Torpdahl and Ahrens, 2004), sequenceanalysis (Mortimer*et al.,* 2004), multiplex PCR (Alvarez*et al.,* 2004), and DNA microarrays (Porwollik *et al.*, 2004).

Multiplex PCR technique has been developed to differentiate between the most common clinical serovars of *S. enterica* (Kim *et al.,* 2006). In a study conducted in the United States 6 genetic loci from *S.* Typhimurium and four from *S.* Typhi were used to create an assay consisting of two five-plex PCRs. The assay can be easily performed on multiple samples with final results in less than 5 hours (Kim *et al.,* 2006). The other multiplex PCR utilises three sequential PCR to identify the three classical pathogens that cause enteric fever, *S.* Typhi, *S*. Paratyphi A, and *S.*Paratyphi

B (Abdissa *et al.,* 2006; Levy *et al.,* 2008). This PCR is based on an O grouping multiplex PCR which identifies groups A, B, and D, an H typing multiplex developed for identification of phase 1 H types "a," "b," and "d" and a third PCR identifies serovar Paratyphi B biovar *Java* that ferments d-tartrate (dT) (Hirose *et al.,* 2002; Malorny *et al.,* 2003; Levy *et al.,* 2008). The other type of PCR amplifies the 16S-to-23S spacer region of bacterial rRNA genes has been used to detect polymorphisms in bacterial species and to identify strains of *S.*Typhimurium associated with disease outbreaks (Pritchett *et al.,* 2000).

Definitive genetic identification of food-borne pathogens is done using DNA sequencing methods. DNA Sequencing is regarded as the "gold" standard for the identification and confirmation of all microorganisms, including *Salmonella*.The most popular PCR target is the 16S-23S rRNA spacer region, also known as the internal transcribed spacer (ITS), a hypervariable region specific for each bacterial species (Pritchett *et al.*, 2000). Once the DNA sequences have been determined, they are compared to a library of known sequences in the GenBank (http://www.ncbi.nlm.nih.gov/BLAST). Whole genome sequencing has also been used in the identification of *Salmonella* species (Leekitcharoenphon *et al.,* 2012). However, the cost and time involved in performing this technique are major constraints for its routine use (Leekitcharoenphon *et al.,* 2012).

1.4.9 Typing of *Salmonella*

Microbial typing is employed for epidemiological studies to determine the source and routes of infections, confirm or rule out outbreaks,trace cross-transmission of healthcare-associated pathogens, recognize virulent strains and evaluate the

effectivenessof control measures (Tenover *et al.,* 1997; Maccannell, 2013; Perez-Losada*et al.,* 2013).Clonally related organisms are members of the same species that share virulence factors, biochemical traits, and genomic characteristics. For epidemiological and evolutionary studies, different typing methods have been used for typing*Salmonella.*These are divided into phenotypic and genotypic typing methods (Maslow *et al*., 1993; Ranjbar *et al.,* 2014).

1.4.9.1 Phenotypic Typing of *Salmonella*

The earliest methods that were used to identify and typeorganisms were based upon their phenotypic characteristics (Singh *et al.,* 2006).Thesetyping methods, such as antibiotic susceptibility testing, biotyping, serotyping andphage typing have occasionally been useful indescribing the epidemiology of infectious diseases(Hopkins *et al.,* 2004;Ranjbar *et al.,* 2014). Historically these methods have provided datato be used for short-term epidemiological studies,assessment of epidemiological trends inwell-defined geographical areas and comparison between different countries (Tenover *et al.,*1997; Maccannell, 2013). However, phenotypicmethodshave drawbacks that limit their utility for highly discriminatory typing of microorganisms such as low discriminatory power, poorreproducibility, and labour intensive and less typeability properties (Singh *et al.,* 2006;Maccannell, 2013;Ranjbar *et al.,* 2014).

Serotyping uses a series of antibodies to detect antigens onthe surface of bacteria that have been shown to demonstrateantigenic variability. Serotyping methods have been used for decades for the taxonomic grouping of a numberof bacterial pathogen species and remain important for typing*Salmonella*and has shown to have

epidemiologicvalue in differentiating strains within species (Singh *et al.,* 2006).Serological analysis usually remains the first step in an epidemiological investigation of *Salmonella* and may be sufficient for epidemiological investigations associated with uncommon serotypes (Winokur, 2003).

Biotyping is often used to help determine the species of microorganisms based upon their abilities to utilize components in different growth media and carry out certain chemical reactions, but it can also be used to separate members of a particular species due to biochemical differences among the organisms (Singh *et al.,* 2006).Biotyping like most phenotyping methods has only modest reproducibility because the organism can alter unpredictable expression ofmany cellular products (Tenover *et al*., 1995).

Antibiotic susceptibility testing is technique used to group *Salmonella* serotypes according to resistance profiles or R-types (Tenover *et al*., 1997). In this method the results are quite reproducible within and between laboratories. However, in most epidemiological studies the antibiogram has limited value because isolates that are not genetically and epidemiologically related may have the same susceptibility pattern (Singh *et al.,* 2006). Thus, antimicrobial resistance is not one of the most stable epidemiological markers for the outbreak analyst.

Phage typing is a traditional method that has been employed over many years to differentiate further within the serovars of *Salmonella*. It provides a rapid, accurate and cheap method of investigating *Salmonella* strains for epidemiological purposes (Rabsch, 2007). *Salmonella* strains within a particular serovar may be differentiated into a number of phage types by their pattern of susceptibility to lysis by a set of

phages with different specificity(Rabsch, 2007). Characterization based on the pattern of phage lysis of wild strains isolated from different patients, carriers, or other sources is valuable in epidemiological study. Specialized typing schemes are applied to differentserovars within the *S. enterica* species (Pickard *et al.,* 2008).For *S.* Typhi, it is based on the detection of the Vi capsule by specific phages that target this antigen as receptoror virulence-associated polysaccharide capsular antigen normallypresent on the surface of clinical isolates (Levine and Noriega, 1995; Pickard *et al.,* 2008).While this is a fairly robust and discriminating typing approach, phage typing is only limited to reference laboratories due to difficulty in maintaining all the phages (Pickard *et al.,*2008;Baggesen*et al.,* 2010). Furthermore, Phage typing is a phenotypic method that is labour intensive and dependsvery much on the experience of the individual laboratoryand on support from the reference centre that coordinates the maintenance of phages and the updatingof the system (Singh *et al.,* 2006; Baggesen *et al.,* 2010).

1.4.9.2 Genotyping of *Salmonella*

Genotyping involves the use of molecular biological tools for subtyping of *Salmonella* serovars. The goal of genotyping studies is that epidemiologically related isolates collected during an outbreak of the disease are able to be linked to one another (Singh *et al.,* 2006). Hence, the use of strain typing in infection control decisions is based on several assumptions such as, isolates associatedwith the outbreak are recent progeny of a single clone, isolates will have the same genotype,and epidemiologically unrelated isolates will havedifferent genotypes (Singh *et al.,* 2006).Many techniques are routinely used to achieve differentiationbetween bacterial pathogens. However, not all methodsprovide

phylogenetic and genotypic information offeringa high degree of specificity, reproducibility and sensitivity (Holt *et al.,* 2010). For epidemiological studies, methods such as Pulsed-Field Gel Electrophoresis (PFGE), ribotyping and Variable Number of Tandem Repeat (VNTR) typing have successfully segregated closely related strains (Navarro *et al.*, 1996; Thong *et al.*, 1996; Hosoglu *et al.*, 2003;Ben-Saida *et al.*, 2007). More recently SNP typing has proven valuable in both phylogenetic and epidemiological studies (Ramisse *et al.*, 2004; Roumagnac *et al.,* 2006; Octavia and Lan, 2007). Among the molecular techniques, PFGE is currently considered one of the most reliable techniques for fingerprinting of *Salmonella* serovars and has become a method of choice.This technique is a well-established and highly effective epidemiological tool that has revealed considerable genetic diversity amongst human isolates of *S.* Typhi in different parts of the world (Ammari *et al.,* 2009).

A study done by Octavia and Lan (2010) the SNPs were used to type 71 global *S.* Typhi isolates and differentiated these isolates and the 19 genome sequenced strains into 25 SNP profiles. Phylogenetic analysis revealed that these SNP profiles were grouped into six major clusters. In another study in Kolkata, SNPs revealed that typhoid fever was caused by a diverse population of *S.* Typhi.However, H58 haplotypes dominate andwere associated with multidrug (Holt *et al.,* 2012). In South-East Asia the molecular characteristics of *S*. Typhi isolates from Malaysia, Thailand and Indonesia revealed multiple genetic variants of *S.* Typhi which were associated with sporadic cases of typhoid fever (Thong *et al.*, 1995).This implied that there was a movement of these strains within these three countries (Thong *et al.*, 1995). A similar study done in Indonesia on 33 isolates from different geographical areas

showed genomic diversity and the presence of more than one clone of *S*. Typhi strains (Moehario, 2009).

In Africa, there is a dearth of data on the genetic diversity of *S*. Typhi. Only a few countries have provided data on this. A case control study done in South Africa using PFGE and MLST, revealed common clusters of *S*. Typhi strains identified between 1993 and 2005, as well as between 2007 and 2009 (Keddy *et al*, 2011). All the *S*. Typhi strains isolated appeared to be related to strains obtained in 1993. This outbreak probably occurred in a non-immune population which consumed faecally contaminated water (Keddy *et al*, 2011). In Zambia, there is no data available on the molecular detection and characterisation of *Salmonella enterica* serovars circulating in our population despite numerous reports on typhoid fever outbreaks from various regions.

1.4.10Treatment of *Salmonella*Infections

*Salmonella*gastroenteritis caused by NTS in the absence of immune deficiency is usually mild, self-limiting and rarely requires intervention (Chiu*et al.,* 2004; Sanchez-Vargas *et al.,* 2011; Haeusler and Curtis, 2013).Antibiotic treatment of NTS gastroenteritis has been the subject of ameta-analysis, but questions regarding exactlywhich patients should be treated and the optimalregimen remain unanswered(Haeusler and Curtis, 2013). However, antimicrobial treatment is essential for enteric fever, invasive salmonellosis, and for patients at risk of extra-intestinaldisease (Sanchez-Vargas *et al,* 2011; Haeusler and Curtis, 2013).Chloramphenicol was first introduced to treat typhoid fever in 1948 (Woodward *et al.,*1948) and had been a drug of choice for more than 40years

(Sanchez-Vargas *et al.,* 2011; Das*et al.,* 2012) with ampicillin and cotrimoxazole (Mandal *et al.,* 2013; Baucheron *et al.,* 2014).However, resistance to chloramphenicol was reported in the 1970s and subsequently further resistance emerged to all first line drugs including ampicillin, trimethoprim andsulfamethoxazole (Butler *et al.,* 1991; Capoor and Nair, 2010) leading to emergence of multidrug resistant (MDR) *Salmonella*strains. MDR is defined as resistance to the traditional first-line antimicrobial agents ampicillin, chloramphenicol, and trimethoprim-sulfamethoxazole in *S. enterica* (Effa and Bukirwa, 2008; Holt *et al.,* 2012). Owing to resistance to these drugs, flouroquinolones (ciprofloxacin and ofloxacin) along with extended spectrum cephalosporins (ceftriaxone and cefixime) have become the treatment of choice in*Salmonella* infectionsand azithromycin as alternative treatments in uncomplicated typhoid fever(Crump and Mintz, 2010; Hassing *et al.,* 2011, 2013; Baucheron*et al.,* 2014).However, chloramphenicol, ampicillin and cotrimoxazole,remainappropriate for the treatment of typhoid fever in areasof the world wherethe bacterium is susceptible to these drugs (Mandal *et al.,* 2013).

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1.4.10.1 AntimicrobialDrug Resistance

Drug resistance in foodborne bacterial enteric pathogens is an almost inevitable consequence of the use of antimicrobial drugs in food-producing animals, and specifically in the developing countries by use of medicines in humans (Threlfall*,* 2000;van den Bogaard and Stobberingh, 2000). The emergence and spread of MDR *S.*Typhi have been reported in developing countries, particularly the Indian subcontinent and Southeast Asia(Das*et al.,* 2012). The first reported MDR outbreak occurred in Kashmir, India in 1988 (Kamili *et al.,* 1993). By 1990, there were reports

of MDR *S.* Typhi strains from India, Pakistan and the Arabian Gulf (Bhan *et al.,* 2005). Of concern is the global spread of the MDR strain of *S.* Typhimurium definitive type 104 (DT104) that is resistant to at least five antimicrobials (ampicillin, chloramphenicol, streptomycin, sulphonamides, and tetracyclines) in animals and humans since the 1990s (Le Hello *et al.*, 2013; Mather *et al.,* 2013). In *S.* Typhi the MDR phenotype is almost exclusively conferred by self-transmissible plasmids of the HI1 incompatibility type (IncHI1), although other plasmids are occasionally reported(Holt *et al.,* 2011). However, it remains unclear whether the increase in MDR typhoid is due to the exchange of resistance genes amongco-circulating *S.* Typhi or to the expansion of MDR *S.* Typhi clones(Holt *et al.,* 2011).

Antibiotic resistance genes can be propagated bymobile genomic cassettes, including integrons and transposons that can reside in thechromosome and on plasmids (Summers, 2002). Plasmid-associated integrons are frequently implicated in MDR*Salmonella*(Mulvey et *al.*, 2004; Al-Sanouri *et al.*, 2008;Holt *et al.*, 2011) and antibiotic resistance in several *S. enterica* serovars may also be due to a set of related genomic islands carrying a class 1 integron, which carries the resistance genes (Hall, 2010). Integrons are bacterial genetic elements able to promote acquisition and expression of genes embedded within gene cassettes (Stokes and Hall, 1989;Stalder *et al.*, 2012).Integrons are made of sequences of conserved DNA that contain an integrase gene *(IntI)* encoding the *IntI* integrase and cause transmission and incorporation of gene cassettes via site-specific recombination mechanisms (Mirnejad *et al.,* 2013). All the integrons consist of two conserved segments (5'CS) and (3'CS), the integrase gene, variable region and the cassette integration site (attI) (Stalder *et al.*, 2012;Mirnejad *et al.,* 2013).Several classes of integron have so far

been described with Class 1 integron being the most common and widely distributed among gram negative bacteria while others are class 2 and class 3 (Chen *et al.,* 2010; Stalder *et al.*, 2012;Mirnejad *et al.,* 2013).

Increasing multidrug resistance to the first line of drugs has been observed by several groups(Mirza *et al.*, 2000; Lee *et al.*, 2004; Tamang *et al.*, 2007). The administration of fluoroquinolones in treating *Salmonella* infections(Kariuki *et al.*, 2010) has led to a global increase in nalidixic acid (NAL)-resistant strains causing decreased ciprofloxacin susceptibility (Crump *et al.*, 2008; Kumar *et al.*, 2008; Medalla *et al.*, 2011). This decreased ciprofloxacin susceptibility may lead to longer fever clearance times and frequent treatment failures (Crump *et al.*, 2008; Capoor*et al.*, 2009).

Resistance to quinolones in *Salmonella* species is mostly attributed to point mutations in the quinolone resistance-determining regions (QRDRs) of the target genes *gyrA*, *gyrB*, *parC*, and *parE* (Gaind *et al.*, 2006; Crump *et al.*, 2008; Dimitrov *et al.*, 2010;Baucheron *et al.,* 2014) or because of decreased permeability to the agents or over-expression of efflux pumps (Ruiz, 2003). More recently *qnr* genes, the products which inhibit quinolone action by binding to *gyrA* and *gyrB* subunits, have been reported (Nordmann and Poirel, 2005; Gay, 2006).Nucleotidechanges in the QRDR of *gyrA* in *Salmonella* are morecommon than mutations in *gyrB* or *topoisomerase* genes (Ruiz, 2003; Roumagnac *et al.*, 2006; Capoor*et al.*, 2009; Song *et al.*, 2010).In *S.*Typhi,nucleotide substitutions at Ser-83, Asp-87, Glu-133, Asp-76, Phe-72, Leu-55, and Gln-106 of*gyrA* gene have been previously reported, with mutation at codon 83 being the most common (Capoor*etal.*, 2009;Song*et al.*, 2010).A clonal expansion of NAL-resistant strains with or without MDR phenotype

has been observedby SNP analysis. These NAL resistant strains belong to a single haplotype H-58 and are circulating globally especially in Southeast Asia (Roumagnac *et al.*, 2006; L*eet al.*, 2007; Kariuki*et al.*, 2010).

In Canada, a study done by Demczuk and colleagues (2010)revealed 18%MDR*S.* Typhi strains and 26 resistance profiles. The most prevalent was resistance to nalidixic acid which accounted for 41% of the isolates. There was also a substantial increase in NAL-R *S.* Typhi during the period 2000-2006 from 41%-80%, though no ciprofloxacin resistance was observed. The ampicillin-chloramphenicol-cotrimoxazole pattern was observed in 17% of the isolates while ampicillin-chloramphenicol-streptomycin-sulfonamide-tetracycline was observed in 13% of *S.*Typhi isolates (Demczuk *et al.,* 2010).

In the United States33% of the isolates were classified as MDR *S.*Typhi and all these isolates were believed to have originated from individuals who travelled to South Asia(Kubota *et al.,* 2005).The other observation was that 3 resistance profiles were observed among the MDR isolates as follows chloramphenicol-cotrimoxazole-tetracycline-ampicillin-sulfisoxazole-streptomycin, chloramphenicol-cotrimoxazole-tetracycline-nalidixic acid-ampicillin- sulfisoxazole-streptomycin and chloramphenicol-cotrimoxazole-ampicillin-sulfisoxazole-streptomycin, (Kubota *et al.,* 2005). In Florida 31 isolates from 2007 to 2010, associated with travelwere investigated. Of these 51% of the strains were resistant to at least one antimicrobial, and five were MDR. Three (9.6%) of the MDR strains harboured a 750-bp integron containing the dihydrofolate reductase VII (*dfr7*) gene conferring resistance to trimethoprim. The three integron-positive strains had a common resistance profile of

AMP-CHL-STR-SXT and 93% of the resistant strains showed a decreased ciprofloxacin susceptibility (DCS) profile (Tatavarthy *et al.,* 2012).

A study of 156 antibiotic-resistant clinical isolates of *S*. Typhimurium and *S*. Enteritidis from Norwegian hospitals demonstrated the presence of integron class 1. Integrons were found in 97% of*S.* Typhimurium and 22.2% *S*. Enteritidis isolates (22·2 %) with the following sizes; 650, 1000, 1200, 1500, 1600, 1700, 2000 and 2100 bp. Theintegrons were further sequenced and the *aadA1, aadA2, aadA5, aadB, pse-1, catB3, oxa1, dfrA1, dfrA12* and *dfrA17* genes, as well as a fragment of the *sat1* gene, were found embedded in cassettes (Lindstedt *et al.,* 2003).

A study done by Bayram and collegues in Turkey revealed that all 91 *S.* Typhi isolates in this study were MDRs being resistant to ampicillin, ampicillinsulbactam, cefuroxime, trimethoprim–sulfamethoxazole, amikacin, gentamycin and chloramphenicol (Bayram*et al.,* 2008).

In Asia several studies have demonstrated the existence of *Salmonella* MDR strains. Among them, Tajbakhsh*et al.,* (2012) in Iran revealed that 71 *Salmonella* isolates were recovered from 1120 patients and were identified as: 17 Typhi, 14 Paratyphi C, 13 Enteritidis, 11 Paratyphi B, 10Paratyphi A and six Infantis.Most resistance was observed towards sulfamethoxazole (30%), tetracyclines (25%), nalidixicacid (22%), spectinomycin (17%), trimethoprim (15%),ampicillin (14%) and kanamycin (14%). The tetracycline resistance genes *tet*(A), *tet*(B), and *tet*(G) were found in 28%,14% and 6% of the tetracycline resistant isolates, respectively.The genes *aadA, aadB,strA, strB and aphA1*-Iab were presentin 83%, 55%, 34%, 1% and 17% of the

aminoglycosideresistant isolates, respectively. Additionally, *bla*$_{PSE}$ and *bla*$_{TEM}$β-lactamase genes were detected in 63% and 18% of the ampicillin-resistant isolates. The 23 sulphonamide resistantisolates harboured *sul1* and *intI1* genes, typical toclass 1integrons. Nine of these isolates also yielded amplicons for*intI2* (class 2 integrons).

In Pakistan,58.7% of *S.* Typhi isolates were MDR and the commonresistance pattern (13/80) was ampicillin-chlorampenicol-streptomycin-tetracycline-cotrimoxazole-sulfamethoxazole-trimethoprim, which is the most frequent type observed in India and Pakistan. The most common drug resistant genes were *bla*TEM-1, *cat*, *strA-strB*, *tetB*, *sul1*, *sul2,* and *dfrA7.* Among the detected genes, only *dfrA7* was found to be associated in the form of a single gene cassette within the class 1 integrons (Afzal*et al.,* 2013). In North India, multidrug resistance sequentially increased from 34% in 1999 to 66% in 2005. Increasing resistance was also observed to the other antibiotics, especially to the cephalosporins. In addition 8% of the *S.* Typhi isolates were found to be presumptive extended spectrum β-lactamaseproducers. There was a gradual development of resistance to fluoroquinolones over the seven years.No resistance was observed to fluoroquinolones in 1999, while in 2005, 4.4% resistance wasobserved to sparfloxacin, 8.8% resistance to ofloxacin and a high resistance, 13%, tociprofloxacin. This is an alarming development and it is of paramount importance to limitunnecessary use of fluoroquinolones and third generation cephalosporins so that their efficacyagainst *Salmonella* is not jeopardized further(Kumar *et al.,* 2008)

In a study done on isolates from Malawi-Mozambique border all 42 isolates tested were MDRs, resistant to ampicillin, chloramphenicol, and trimethoprim-

sulfamethoxazole and four isolates were also resistant to nalidixic acid (Lutterloh*et al.,* 2012).A study conducted in Lusaka, Zambia showed that *S*. Typhimurium was resistant to amoxicillin, ampicillin and erthyromycin but susceptible to chloramphenicol and gentamicin (Degroote, 1997, personal communication). In another Zambian study, it was reported that *S.* Typhimurium was susceptible to chloramphenicol, gentamicin, furazolidone and tetracycline (Hang'ombe, 1999). Yet another study, demonstrated that human *Salmonella* isolates were sensitive to nitrofurantoin (68%) and amoxicillin (48%), while resistance was observed with tetracycline (52%), co-trimoxazole (56%), gentamycin (60%), ampicillin (68%), vancomycin and erythromycin (Ulaya, 2012).

1.5 Research Questions

What *Salmonellaenterica*serovars were isolated at the University Teaching Hospital? What were the antimicrobial resistance patterns of these *Salmonella enterica* serovars? What was the genetic relatedness of *S.* Typhi in the outbreak?

1.6 Objectives

1.6.1General objective

To determine the molecular characteristics of *Salmonella enterica* serovars isolated at the University Teaching Hospital.

1.6.2 Specific objectives

1.6.2.1 To identify the *Salmonella serovars* isolated at the UTH.

1.6.2.2 To determine the drug resistance patterns of *Salmonella serovars* isolated at the UTH.

1.6.2.3 To determine the genetic relatedness of *S.* Typhi strains obtained from the UTH.

Chapter 2

Materials and Methods

2.1 Study Design

This was a laboratory-based retrospective cross-sectional study on *Salmonella* isolates collected during the 2010-2012 salmonellosis outbreaks.

2.2 Study Site

This study was conducted at the University Teaching Hospital (UTH) located in Lusaka, the Capital City of Zambia. It offers specialised inpatient and out-patient care. The hospital is a tertiary referral and teaching hospital with a bed capacity of approximately 2000 and comprises of several clinics and admission wards. It is the largest referral hospital and the centre for all microbiology diagnostic work in Zambia.

2.3 Sampling Frame

Convenience sampling was employed. One hundred and twenty seven archived clinical isolates of *Salmonella* stored in the Bacteriology Laboratory during at the UTH 2010-2012 were analysed.

2.3.1 Inclusion Criteria

Single patient *Salmonella* isolates from clinical specimens from 2010 to 2012 from both children and adults of all ages.

2.3.2 Exclusion Criteria

Multiple isolates from the same specimen or contaminated were excluded from the study.

2.4Identification of *Salmonella*Serovars

2.4.1 Specimen Collection and Processing

Salmonella isolates used in this study were archival isolates obtained from the blood and stool specimens which were part of the routine specimen collection by the Bacteriology Laboratory at the UTH.

2.4.2 Bacterial Strains and Growth Conditions

Culture of bacteria from archives was carried out by standard culture methods for suspected *Salmonella*.This was inoculated on MacConkey agar and Xylose Lysine Deoxycholate (XLD) agar(Merck, Hamburg, Germany) and incubated for 18-24hours at 37˚C. The sizes and morphological characteristics of the resulting colonies were observed and recorded.The identification of the *Salmonella* isolates were identified by standardbiochemical tests, serotyping and identity confirmed by *Salmonella* specific PCR (Rahn *et al.*, 1992).

2.4.3 Biochemical Identification

The biochemical profiles of the isolates were obtained through conversional methods using Triple Sugar Iron (TSI) agar, Lysine Iron Agar (LIA), urease agar, citrate agar and motility test. Pure colonies were used for the inoculation of test media and were incubated for 18 to 24 hours at 37ºC. The motility test was conducted by observing

the growth of the bacteria in a semi-solid Bacto motility test medium (Becton Dickinson, Sparks, USA).

2.4.4Serotyping

All the *Salmonella* isolates were serotyped by a slide agglutination serological test using a mixture of monoclonal mouse antibodies (Enteroclon Anti-*Salmonella* A-67, omnivalent, specific for O and Vi antigens andEnteroclon Anti-*Salmonella* poly-H phase 1 & 2 sera [SIFIN, Berlin, Germany]) according to the manufacturer's protocol. Briefly, using a wire loop, a small amount of bacteria was picked from a well-isolated colony, transferred onto a glass slide and mixed with a drop of either poly-H or poly-O antiserum. The homogenous, slightly milky suspension was tilted back and forth for less than 20 times on the glass slide and the reaction read on a dark surface by naked eye within 1min from the time of mixing. A drop of sterile physiological saline (0.85% sodium chloride) was used as a negative control and the test performed simultaneously with the test sample.

2.4.5 Identification of *Salmonella* by PCR

2.4.5.1 DNA Extraction andEstimation

DNA was extracted on the easyMag instrument (bioMérieux Inc, Durham, NC, USA) according to the manufacturer's protocol. A loopful of bacteria was emulsified in nuclease free water and lysed using the "Off-board lysis" protocol, after which 400µl of the lysed sample was transferred to the sample strip well. The sample strips were then loaded onto the easyMAG machine and the extraction process was performed according to the manufacturer's protocol. DNA was eluted in a final volume of 50µl.

The concentration of DNA was estimated by ultraviolet spectroscopy at 260nm. A DNA sample with an optical density (OD) of 1 at 260nm corresponded to a DNA concentration of 50µg/ml of double-stranded DNA. The purity of the DNA was determined by a DNA/protein absorbance ratio of 260nm/280nm. The DNA was stored at -20°C until required.

2.4.5.2*Salmonella* Specific PCR

The isolates were subjected to a sequential PCR in order to identify the three classical pathogens that cause enteric fever, *S.* Typhi, *S.*Paratyphi A, and*S.* Paratyphi B. This multiplex PCR method was used due to its consistent, high-throughput in typing aetiological agents (Abdissa *et al.,* 2006; Levy *et al.*, 2008). An O grouping multiplex PCR identifies groups A, B, and D, an H typing multiplex developed for this work identifies phase 1 H types "a," "b," and "d" and a third PCR to identify serovar Paratyphi B biovar *Java* that ferments *d*-tartrate (dT) were used (Malorny *et al.,* 2003; Levy *et al.*, 2008). The primers used are listed in Table 2.1 below.

PCR was performed in 1X PCR buffer, 3.5 mM $MgCl_2$, 0.2mM of deoxynucleoside triphosphates, and 0.2 U of Invitrogen *Taq* DNA polymerase (final volume of 25 µl) on a Mastercycler (Eppendorf North America, Westbury, NY). Primers were combined at a final concentration of 0.2µM, except for the positive control primers (*oriC*) that were used at a final concentration of 0.14 µM in the H-antigen mix. For each PCR, 1.0µl of mix was used per reaction. The cycling conditions of the PCRs were as follows. The O grouping multiplex PCR consisted of denaturation at 95°C for 2 min, followed by 35 cycles at 95°C for 30s, 55°C for 30s, 72°C for 30s, and a final step of 72°C for 5 min. The H typing multiplex PCR comprised a denaturation

step of 2 min at 95°C, followed by 35 cycles of the following two steps: 95°C for 30s and 55°C for 15s. The dT fermentation PCR consisted of a denaturation step of 95°C for 2 min, followed by 35 cycles of 95°C for 30s and 60°C for 30s. PCR products were separated on 2% (wt/vol) agarose gels, stained with ethidium bromide and visualised on a UV transilluminator. *S.* Typhi (ATCC 19430), *S.* Paratyphi A (ATCC 54388) and *S.* Paratyphi B were used as a positive controls. A negative control without DNA was always included.

Table 2.1:Primers used in the multiplex PCR assays and the expected amplicons

Primer	Primer sequence (5-3)	Amplicon size (bp)	Reference
O serogroup			
rfbJ-F	CCAGCACCAGTTCCAACTTGATAC	662	Lim *et al.,* 2003
rfbJ-R	GGCTTCCGGCTTTATTGGTAAGCA		
tyv-F	GAGGAAGGGAAATGAAGCTTTT	614	Hirose *et al.,* 2002
tyv-R	TAGCAAACTGTCTCCCACCATAC		
vi-F	GTTATTCAGCATAAGGAG	439	Hirose *et al.,* 2002
vi-R	CTTCCATACCACTTTCCG		
prt-F	CTTGCTATGGAAGACATAACGAACC	256	Hirose *et al.,* 2002
prt-R	CGTCTCCATCAAAAGCTCCATAGA		
H antigen typing			
H-F	ACTCAGGCTTCCCGTAACGC		
Ha-R	GAGGCCAGCACCATCAAGTGC	423	Levy *et al.,* 2008
Hb-R	GCTTCATACAGACCATCTTTAGTTG	551	Levy *et al.,* 2008
Hd-R	GGCTAGTATTGTCCTTATCGG	763 (d) or 502 (j)[b]	Levy *et al.,* 2008
dT fermentation			
dT-F	GTAAGGGTAATGGGTTCC	289	Malorny *et al.,* 2003.
dT-R	CACATTATTCGCTCAATGGAG		
Internal control[a]			
P1 (oriC)	TTATTAGGATCGCGCCAGGC	161	Widjojoatmodjo *et al.,* 1991
P2 (oriC)	AAAGAATAACCGTTGTTCAC		

[a] Internal controls were included in both multiplex mixes and monoplex PCRs.[b]Letters in parentheses indicate antigens.

2.5Determination of the Drug Resistance Pattern of *Salmonella* Isolates

2.5.1Antimicrobial Susceptibility Testing

Antibiotic resistance profiles were determined using a commercially prepared dehydrated panel (Sensititre; TREK Diagnostic Systems Ltd., East Grinstead,England) and was performed according to the manufacturer's protocol. One to two isolated colonies from freshly streaked plates were suspended in 5ml of demineralized water to obtain a 0.5 McFarland density. Ten microliters of the bacterial water suspension was added to 11 ml of Cation Adjusted Mueller-Hinton broth (CAMHB). A 50µl aliquot of the CAMHB cell suspension was dispensed into each well of a 96-well panel consisting of 16 antimicrobials. The panels were incubated at 35°C for 18hours and then read by an auto reader. The 16 antimicrobials tested included amoxicillin/clavulanic Acid (AMC), ampicillin (AMP), Apramycin (APR), ceftaxime (CTX), chloramphenicol (CHL), ciprofloxacin (CIP), colistin (COL), flofernicol (FFN), gentamicin (GEN), nalidixic acid (NAL), neomycin (NEO), spectomycin (SPE), streptomycin (STR), sulfamethoxazole (SMX), tetracycline (TET) and trimethoprim (TMP). Results were interpretedusing the Clinical and Laboratory Standards Institute (CLSI, 2012) clinical breakpoints, except for azithromycin, ceftiofur, colistin, florfenicol, neomycin, spectinomycin and streptomycin, where epidemiological cut-off values according to European Committee on Antimicrobial Susceptibility testing(EUCAST) recommendations was used (http://www.eucast.org). Apramycin was interpreted according to research results from Technical University of Denmark. Quality control was performed by using reference strain *E. coli* ATCC 25922 according to CLSI guidelines.

All the *Salmonella* isolates, including control strains *E. coli* ATCC 25922 and *Staphylococcus aureus* ATCC 25923, were also subjected to the Kirby-Bauer Disc

Diffusion method using the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2012). Isolates were tested for susceptibility to the following antibiotics with the respective disc concentrations: 25µg trimethoprim-sulfamethoxazole (TS), (Oxoid, UK Ltd). Briefly, a sterile, non-toxic swab was used to collect a well-separated colony from an overnight culture streaked on a nutrient agar plate. The bacteria were suspended into 4 to 5ml of sterile normal saline and the turbidity of the suspension was adjusted with sterile saline to obtain a suspension visually similar to that of a 0.5 McFarland standard. The turbidity was read against a standard card with black lines on a white background. A new swab was dipped into the suspension and, following removal of excess inoculum by pressing the swab gently against the wall of the tube, bacteria was spread evenly on the Mueller Hinton agar plate (Fort Richard Laboratory Ltd., Auckland, NZ). An automatic dispenser was employed to ensure discs were no closer than 24mm from centre to centre. Each disc was pressed gently with sterile forceps to ensure complete contact with the agar and the inoculated plates were incubated for 16 to 18 hours at 37˚C. The zones of inhibition were measured using a Vernier calliper and end points determined based on the areas showing no bacterial growth visible to the naked eye as shown in Figure 2.1A. Results were interpreted according to the CLSI guidelines (CLSI, 2012).

A B

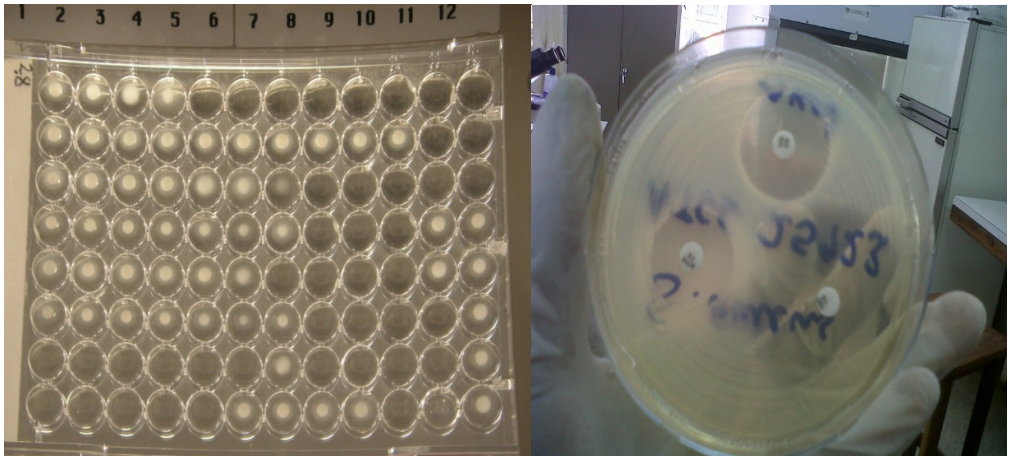

Figure 2.1:A) Microbroth dilution plate for determination of Minimum Inhibitory Concentrations (TREK Sensititre); B) The Kirby Bauer disk diffusion test.

2.5.2 Detection of Drug Resistance Genes

2.5.2.1 Integron Screening and DNA Sequencing

The isolates were screened for class 1 integrons by PCR as described previously (Levesque *et al.,* 1995). The following primers were used: Class 1 integron forward primer intF: GGCATCCAAGCAGCAAG and reverse primer intR: AAGCAGACTTGACCTGA. Amplification was performed in a final volume of 25µl. Each reaction mixture contained 5.5µl molecular grade water, 12.5µl PCRmaster mix 2X (0.05u/µl Taq DNA polymerase, reaction buffer, 4 mM MgCl2, 0.4 mM of each dNTP) (Thermo Fisher Scientific Inc,NYSE: TMO , USA), 2.5µl of each primer and 2µl of template bacterial DNA. Amplification reaction was carried on a GeneAmp System 2700 PCR thermocycler (Applied Biosystems, Foster City, CA, USA) with an initial denaturation at 94°C for 5 minutes, followed by 35 cycles of denaturation (94°C, 30 seconds), annealing (55°C, 30 seconds) and extension (72°C, 2 min 30 seconds) and a final extension for 10 minutes at 72°C. The amplified products were separated by gel electrophoresis on 1.5% agarose and were detected by comparison against a 100bp DNA ladder as a size marker under the visualisation

of UV light on a Biotop SC - 645Gel Documentation system (Biotech Co. Ltd, Shanghai China). Amplified products were then purified with the Wizard PCR prep DNA Purification System (Promega,Sunnyvale, CA, USA) according to the manufacturer's protocol.

Sequencing was performed using the above-named primers. Forward and reverse linear amplification was performed in 10µl using 2µl of the purified PCR product (about 20 to 200ng), 2µl BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA), 1µl BigDye Sequencing Buffer (Applied Biosystems, Foster City, CA, USA) 1mM of each primer. Linear amplification consisted of 25 cycles of denaturation at 96^{o}C for 10s, annealing at 60^{o}C for 30s and elongation at 72^{o}C for 60s using the iCycler Thermocycler (Bio-Rad, Hercules, CA, USA). Briefly, the entire extension products was transferred into 80µl of freshly prepared precipitation solution (3µl of 3M sodium acetate [pH 4.6], 62.5µl of non-denatured 95% ethanol and 14.5µl deionised water), incubated for at least 1hr at room temperature and centrifuged at 14000rpm for 20min. After carefully removing the supernatant, 250µl of 70% ethanol was added to the pellet, vortexed and the contents re-centrifuged at 14000rpm for 8min. The ethanol was carefully aspirated and the pellet air-dried for 15min at room temperature. The samples were analysed on an ABI PRISM 3730XL DNA analyser (Applied Biosystems, Foster City, CA, USA). The DNA sequence reads were edited using Ridom TraceEdit software (Ridom Bioinformatics GmbH, Würzburg, Germany) and used to search the National Center for Biotechnology Information (NCBI) RefSeq database using BLASTN

software (http://www.ncbi.nlm.nih.gov/BLAST). A distance score of 0.00% to less than 1.00% was used as the criteria for identification of the integrons.

2.5.2.2Whole Genome Sequencing

A subset of 33 *S.* Typhi isolates were conveniently selected for whole genome sequencing typing (WGST) to cover almost all antimicrobial resistance phenotypes, using the Illumina sequencing platform (Illumina, Inc., San Diego, CA). Five previously published genomic sequences of haplotype H58; AG3, E02-2759, ISP-04-06979, E03-9804, ISP-03-07467 were obtained from GenBank and Sanger Institute (accessed 5/4/2013). The AG3 genome was downloaded from GenBank with ID, CAAY00000000 while the other four genomes were retrieved from Sanger bacterial genome database (http://www.sanger.ac.uk/resources/downloads/bacteria/). The raw reads were assembled using the pipeline available on the Center for Genomic Epidemiology (CGE) (www.genomicepidemiology.org) which is based on Velvet algorithms for *de novo* short reads assembly (Zerbino and Birney, 2008).

2.5.2.2.1Screening for Mutations in DNA Gyrase and Topoisomerase IV Genes

Each genome was examined for mutation in *gyrA*, *gyrB*, *parC* and *parE* genes (Song *et al.,* 2010) by determining SNP from the position of those genes in *S*. Typhi str. CT18 (Parkhill*et al.,* 2001). Additionally, the *gyrA* sequences of quinolone resistant strains, were compared to the sequences of *S*. Typhi str. CT18 by using multiple alignments in CLC Bio Workbench to localize the QRDR nucleotide and the consequent and amino acid changes.

2.6 Determination of Genetic Relatedness of *S.* Typhi

2.6.1 Phylogenetic Analysis of *S.* Typhi Using Single Nucleotide Polymorphisms

Single nucleotide polymorphisms (SNPs) were determined using the pipeline available on the Center for Genomic Epidemiology (www.genomicepidemiology.org)(Leekitcharoenphon*et al.,* 2012). Fundamentally, each of the assembled genomes or contigs were aligned against the reference genome (*S.* Typhi str. CT18 (National Center for Biotechnology Information, accession: AL513382, length of 4,809,037 bp) using the application "Nucmer" of MUMmer version 3.23 (Delcher *et al.,* 2002). SNPs were identified from the alignments using "Show-snps" (using option "-Cl1rT") from MUMmer. Subsequently, SNPs were selected when meeting the following criteria: a minimum distance of 20 bps between each SNP, and all indels were excluded. The selected SNPs from assembled genomes were confirmed by SNPs being called by mapping raw reads to the reference genome using BWA (Li and Durbin, 2009) and SAMTools (Li *et al.,* 2009).

The qualified SNPs from each genome were concatenated to a single alignment corresponding to position of the reference genome using an in-house Perl script. In case SNPs were absent in the reference genome, they were interpreted as not being a variation and the relatively base from the reference genome was expected(Leekitcharoenphon *et al.,* 2012). The concatenated sequences were subjected to multiple alignments using MUSCLE from MEGA5 (Tamura *et al.,* 2011). The final phylogenetic SNP tree was computed by MEGA5 using the maximum likelihood method (Tamura amd Nei, 1993) of 1,000 bootstrap replicates (Felsenstein, 1985).

The non-synonymous SNP/synonymous SNP ratio (dN/dS) is a measurement of stabilizing selection (Holt *et al.,* 2008). A ratio of 1 is expected in the absence of selection, a low ratio (dN/dS<1) indicates stabilizing selection, while a high ratio (dN/dS>1) indicates positive selection (Rocha *et al.,* 2006). The dN/dS ratio, was calculated for each core gene using codeML from the package PAML (Yang, 2007). The approximation of the dN/dS ratio was an average of dN/dS from all core genes. The haplotypes from each genome were assigned based on biallelic polymorphisms positions (BiP) previously described (Roumagnac*et al.,* 2006). Additionally, node B of haplotype H58 was determined from SNP position 1,193,220 (Kariuki*et al.,*2010).

A BLAST atlas based on BLASTP (Hallin*et al.,* 2008) was used to predict putative deletions in a comparison of the genomes against the reference genome, CT18. The putative deletions were aligned against Zambian genomes using execrate (Slater and Birney, 2005).The hit score was calculated by multiplying percent identify with deletion's alignment length and dividing with deletion's sequence length. The presence of deletions in the Zambian genomes was confirmed based on the hit score with a threshold of at least 95%. The presence and absence of the deletions were finally visualized in a heatmap.

2.7Data Analysis

Descriptive data were analysed with GraphPad Prism Software Version 6.0 for Windows (GraphPad Software, San Diego, California, USA) to generate frequency tables and graphs. Ridom Trace edit software was used to edit the DNA sequences. SNPs were determined using the SNP analysis software, snp Tree 1.1.

(www.genomicepidemiology.org).The dN/dS ratio, was calculated for each core gene using codeML from the package PAML.

2.8Ethics Approval

This study was a laboratory-based study, with no direct contact with patients. Ethics approval for this study was obtained from the University of Zambia Biomedical Research and Ethics Committee.The ethics clearance letter reference number was013-09-12 (Appendix1).Permission to use the clinical bacterial isolates was obtained from the Head of the Department of Pathology and Microbiology at the University Teaching Hospital.

Chapter 3

Results

3.1 Identification of*Salmonella enterica* Serovars.

A total of 127 *Salmonella* isolates from the archives were analysed in this study. The identity of the isolates were confirmed by PCR. Out of these isolates, 71/127 (55.9%) were *S.* Typhi, 27/127 (21.3%) were *S.* Paratyphi B, 2/127 (1.6%) were *S.* Senftenberg and 27/127 (21.3%) were other*Salmonellae* as shown in Figure 3.1 below.

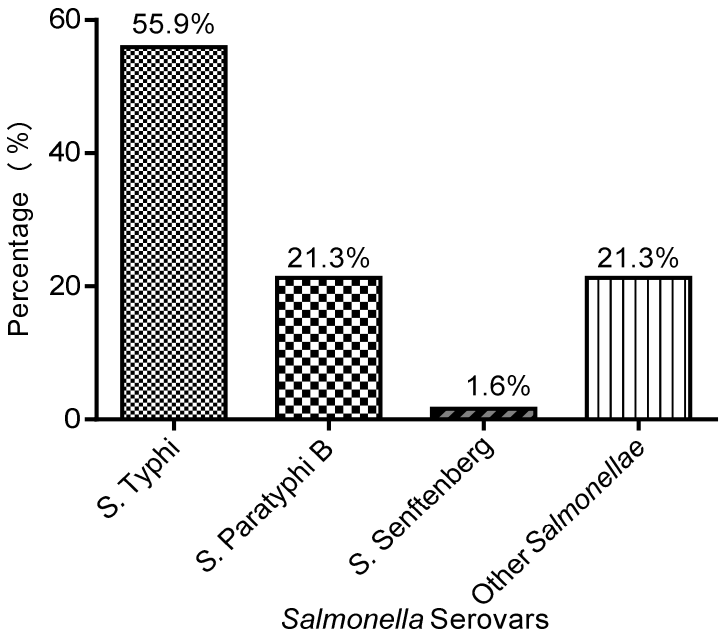

Figure 3.1: *Salmonella enterica* serovars isolated at the UTH from 2010-2012.

3.2 Determination of Drug Resistance Pattern of *Salmonella* serovars

3.2.1 Phenotypic Detection of Drug Resistance

The phenotypic results showed that *S.* Typhi was 98.6% resistant to ampicillin, cotrimoxazole, sulfamethoxazole, trimethoprim and streptomycin. Resistance was also observed against chloramphenicol (85.9 %), azithromycin (21.1%), ciprofloxacin (4.2%), nalidixic acid (2.8%), amoxicillin + clavulanic acid (1.4 %), tetracycline (1.4%) and spectinomycin (1.4%) (Figure 3.2A). *S.* Paratyphi B was resistant to the following drugs: ampicillin (100%), chloramphenicol (100%), cotrimoxazole (100%), sulfamethoxazole (100%), spectinomycin (100%), streptomycin (100%), amoxicillin + clavulanic acid (11.1%), colistin (11.1%), ciprofloxacin (7.4%), tetracycline (7.4%) and gentamicin (3.7%). (Figure 3.2B). Typhoidal *Salmonallae* were completely sensitive to apramycin, cefotaxime, florfenicol, gentamycin, neomycin, and ceftiofur. One (1.4%) of the *S*. Typhi isolate was pansusceptible. *S*. Senftenberg was 100% resistant to amoxicillin plus clavulanic acid, ampicillin, cefepime, cefotaxime, cefpodoxime, ceftazidime, ceftiofur, ceftriaxzone, chloramphenicol, ciprofloxacin, gentamicin nalidixic acid, neomycin, spectinomycin, streptomycin, sulfamethoxazole, tetracycline and trimethoprim (Figure 3.2C). In addition, one isolate of *S*. Senftenberg was also resistant to florfenicol as shown in Figure 3.2C. The two isolates were susceptible to apramycin, cefoxitin, colistin, imipenem, meropenem, and tigecycline. The other NTS were resistant to cotrimoxazole (100%), sulfamethoxazole (85.2%), ampicillin (77.8%), streptomycin (74.1%), trimethoprim (74.1%), chloramphenicol (55.6%), spectinomycin (40.7%), colistin (29.6%), tetracycline (25.9%), ciprofloxacin, gentamycin, nalidixic acid and neomycin (all at 3.7%) as shown in Figure 3.2D. These NTS were completely sensitive to amoxicillin plus clavulanic acid, apramycin,

cefotaxime, ceftiofur and florfenicol. Apramycin, florfenicol and tigecycline are only approved for veterinary use.

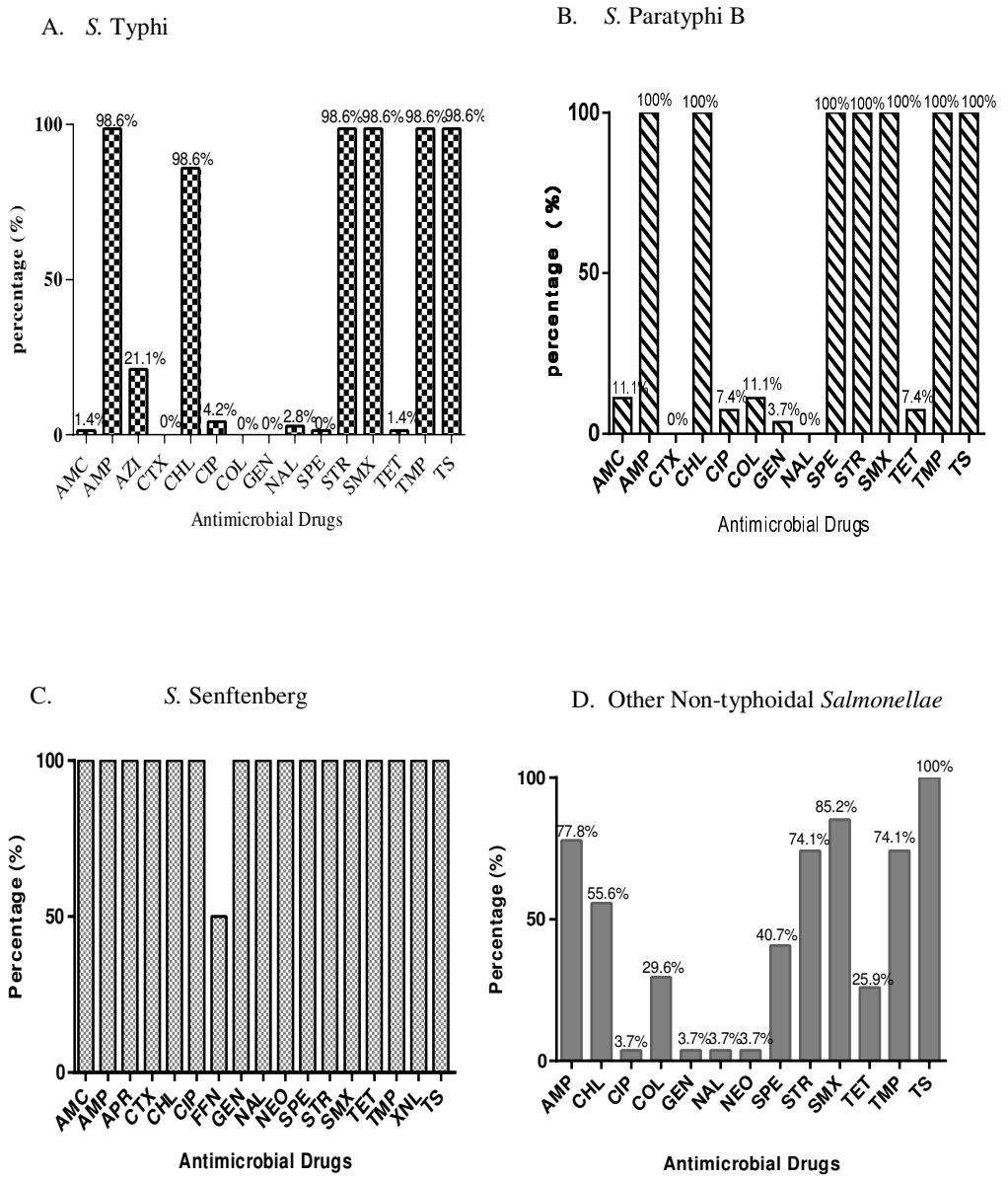

Figure 3.2. Antimicrobial Resistance profiles of *Salmonella serovars*isolated at UTH. (A). *S.* Typhi, (B). *S.* Paratyphi, (C). *S.* Senftenberg and (D). Other Non-typhoidal *Salmonellae.*AMC-amoxacillin+clavulanic acid, AMP-ampicillin, APR-apramycin, AZI-azithromycin, CHL-chloramphenicol, CIP-ciprofloxacin, COL-colistin, CTX-cefotaxime, FFN-florfenicol, GEN-gentamicin, NAL-nalidixic acid, NEO-neomycin, SMX-sulfamethoxazole, SPE-spectinomycin, STR-streptomycin, TET- tetracycline, TMP-trimethoprim, TS-cotrimoxazole and XNL-ceftiofur.

Table 3.1. Antimicrobial resistance patterns of *Salmonella* serovars isolated at the UTH.

Antimicrobial Resistance Patterns	**No. of Isolates (%)**
Salmonella Typhi	
AMC-AMP-AZI-CHL-SMX-STR-TMP-TS	1(1.4)
AMP-CHL-CIP-NAL-STR-SMX-TMP-TS	2(2.8)
AMP-AZI-CHL-SMX-STR-TET-TMP-TS	1(1.4)
AMP-AZI-CHL-SPE-STR-SMX-TMP-TS	1(1.4)
AMP-AZI-CHL-SMX-STR-TMP-TS	9(12.7)
AMP-AZI-STR-SMX-TMP-TS	1(1.4)
AMP-CHL-CIP-SMX-STR-TMP-TS	1(1.4)
AMP-AZI-SMX-STR-TMP-TS	2(2.8)
AMP-CHL-SMX-STR-TMP-TS	47(66.2)
AMP-SMX-STR-TMP-TS	6(8.5)
Total MDRs For *S.* Typhi	**61(85.9%)**
Salmonella Paratyphi B	
AMP-CHL-COL-SPE-SMX-STR-TMP-TET-TS	2(7.4)
AMP-CHL-COL-SPE-SMX-STR-TMP-TS	1(3.7)
AMP-CHL-GEN-SPE-SMX-STR-TMP-TS	1(3.7)
AMC-AMP-CHL-SPE-SMX-STR-TMP-TS	1(3.7)
AMP-CHL-SPE-SMX-STR-TMP-TS	20(74.1)
AMP-CHL-CIP-SPE-SMX-STR-TMP-TS	2(7.4)
Total MDRs For *S.* Paratyphi B	**27(100%)**
Salmonella Senftenberg	
AMC-AMP-AZI-FEP-CTX-CAZ-CPD-CRO-CHL-CIP-GEN-NAL-NEO-SPT-STR-SMX-TET-TMP	2(100)
Total MDRs For *S.* Senftenberg	**2(100%)**
Other Non-typhoidal *Salmonellae*	
AMP-CHL-COL-SPE-STR-SMX-TET-TMP	3(11.1)
AMP-CHL-CIP-NAL-STR-SMX-TMP	1(3.7)
AMP-CHL-COL-STR-SMX-TET-TMP	1(3.7)
GEN-NEO-SPE-STR-SMX-TMP	1(3.7)
AMP-CHL-COL-STR-SMX-TMP	2(7.4)
AMP-CHL-SPE-STR-SMX-TMP	5(18.5)
AMP-CHL-STR-SMX-TMP	2(7.4)
AMP-STR-SMX-TET-TMP	1(3.7)
AMP-CHL-SPE-STR-SMX	1(3.7)
AMP-STR-SMX-TMP	3(11.1)
SMX-TET-TMP	1(3.7)
AMP-SMX	1(3.7)
AMP	1(3.7)
COL	2(7.4)
SMX	1(3.7)
Total MDRs For Other NTS	**15(55.6%)**

AMC-amoxacillin+clavulanic acid, AMP-ampicillin, AZI-azithromycin, CHL-chloramphenicol, CIP-ciprofloxacin, CTX-cefotaxime, NAL-nalidixic acid, SPE-spectinomycin, SMX-sulfamethoxazole, STR-streptomycin, TET- tetracycline, TMP-trimethoprim, and TS-cotrimoxazole

Overall *S.* Typhi isolates were at least resistant to 5 or more antimicrobial drugs. The commonest resistant pattern was AMP-CHL-SMX-STR-TMP-TS, with about 47 (66.2%) isolates being resistant to 6 drugs, followed by 9 (12.7 %) of the isolates

being resistant to 7 antimicrobials with resistant pattern AMP-AZI-CHL-SMX-STR-TMP-TS (Table 3.2). The majority of *S.* Paratyphi B isolates were resistant to 7 or more antibiotics, with the AMP-CHL-SPE-SMX-STR-TMP-TS pattern being the commonest (20[74.1%]) (Table 3.2). About 61 (87.3%) of *S.* Typhi, 27(100%) of *S.* Paratyphi B, *S.* Senftenberg 2 (100%) and the other NTS 15(55.6%) isolates were multidrug resistant. These isolates were all resistant to ampicillin, chloramphenicol and cotrimoxazole and other antibiotics.

3.2.2 Molecular Detection of Drug Resistance

3.2.2.1 Screening of Integron and DNA Sequencing

Twenty nine (65.9%) out of the 44 *S*. Typhi isolates and 22/27(81.5%) of*S.* Paratyphi Bwere positive for integron class I (Figure 3.3).

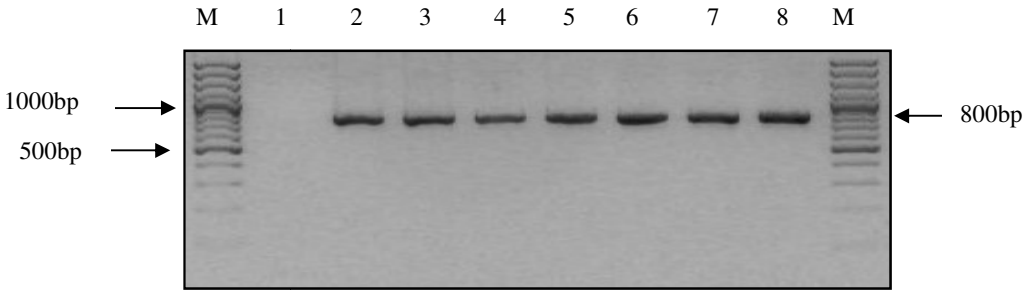

Figure 3.3. PCR Detection of Class 1 integron. M: 100bp Marker, Lane 1: Negative Control (Water), Lane 2: Positive Control, Lane 3-8 bacterial isolates of *S.* Typhi and *S.* Paratyphi B

DNA sequencing revealed the presence of the dihydrofolate reductase VII gene (*dfrA7*) conferring resistance to trimethoprim. Our sequence showed 99% similarity to the *Salmonella* Typhi class 1 integron *dfrA7* gene (GenBank accession number gi|312183497|gb|HQ132376.1|). Of the 29 integron positive *S.* Typhi, 77.8% had a

common resistance pattern of ampicillin-chloramphenicol-sulfamethoxazole-trimethoprim-cotrimoxazole, while the common resistance pattern for integron positive *S.* Paratyphi B 22/27(81.5%) was ampicillin-chloramphenicol-spectinomycin-sulfamethoxazole-trimethoprim-cotrimoxazole.

All but one isolate analysed by WGST contained the following genes; *str*A, *str*B, Δ*aadA*1 (aminoglycoside: streptomycin), and $bla_{\text{TEM-1}}$ (beta-lactam: ampicillin). Six(18.2%) isolates harboured the *Sul2* gene, 1 (3%) *Sul1* gene while 25/33 (75.8%) harboured both the *Sul1* and *Sul2* genes conferring resistance to sulfonamides. Genes conferring resistance to trimethoprim, *dfrA14* and *dfrA7*, were found in 5/33 (15.2%) and 27/33 (81.8%) of the isolates, respectively. Some of these isolates (5/33, 15.6%) also harboured the *cat*A1 gene.Representative data on WGST is shown in Appendix III.

All the 33 WGST isolates were also analysed for the presence of fluoroquinolones resistance associated with mutations in the Quinolone Resistance Determinant Regions (QRDR) of the gyrase and DNA topoisomerase IV genes; *gyrA*, *gyrB*, *parC*, and *parE*. Two (6.1%) isolates revealed a single mutations in *gyrA* QRDR, which led to an amino acid substitution at codon Asp87 (Asp-Asn) in one strain and codon Ser83 (Ser-Tyr) in another strain.

WGST of the two *S.* Senftenberg isolates revealed that both isolates harboured resistant genes against 9 classes of antibiotics as shown in Table 3.2 below.

TABLE 3.2.Distributions of antimicrobial resistance genes in *S.*Senftenberg isolates

	Resistance gene, class, and/or mutation in isolate ID no.:	
Antimicrobial agent(s)	**Isolate 1**	**Isolate 2**
Aminoglycoside	*aac(6=)-IIc* *aac(6=)-Iy* *aph(3=)-Ic* *aac(6=)-Ib-cr* *strA* *strB*	*aac(6=)-IIc* *aac(6=)-Iy* *aadA2* *aac(6=)-Ib-cr* *strA* *strB*
Beta-lactam	$bla_{\text{TEM-1}}$ $bla_{\text{CTX-M-15}}$ $bla_{\text{OXA-10}}$	*bla*TEM-1 $bla_{\text{CTX-M-15}}$ $bla_{\text{OXA-30}}$
Fluoroquinolone Low-level resistance High-level resistance	 *aac(6=)-Ib-cr* *gyr*(A) (S-83-Y and D-87-G) *par*(C) (S-80-I)	 *aac(6=)-Ib-cr* *gyr*(A) (S-83-Y and D-87-G) *par*(C) (S-80-I)
Macrolide-lincosamidestreptogramin	*ere*(A)	*ere*(A)
Phenicol	*catB3* *catA2* *cmlA1* *floR*	*catB3* *catA2*
Rifampin	*arr-2*	
Sulfonamide	*sul1* *sul2*	*sul1* *sul2*
Tetracycline	*tet*(A) *tet*(D)	*tet*(D)
Trimethoprim	*dfrA14* *dfrA18* *dfrA23*	*dfrA18*

Adapted from Hendriksen *et al.,* (2013)

3.3 Determination of Genetic Relatedness of *S.* Typhi

A phylogenetic SNPs tree with the inclusion of the available non-outbreak genomes from sub-Saharan Africa and Asia of haplotype H58 and the reference genome; CT18 belonging to haplotype H1 was reconstructed to investigate the evolutionary relationships as shown Figure 3.3A below.

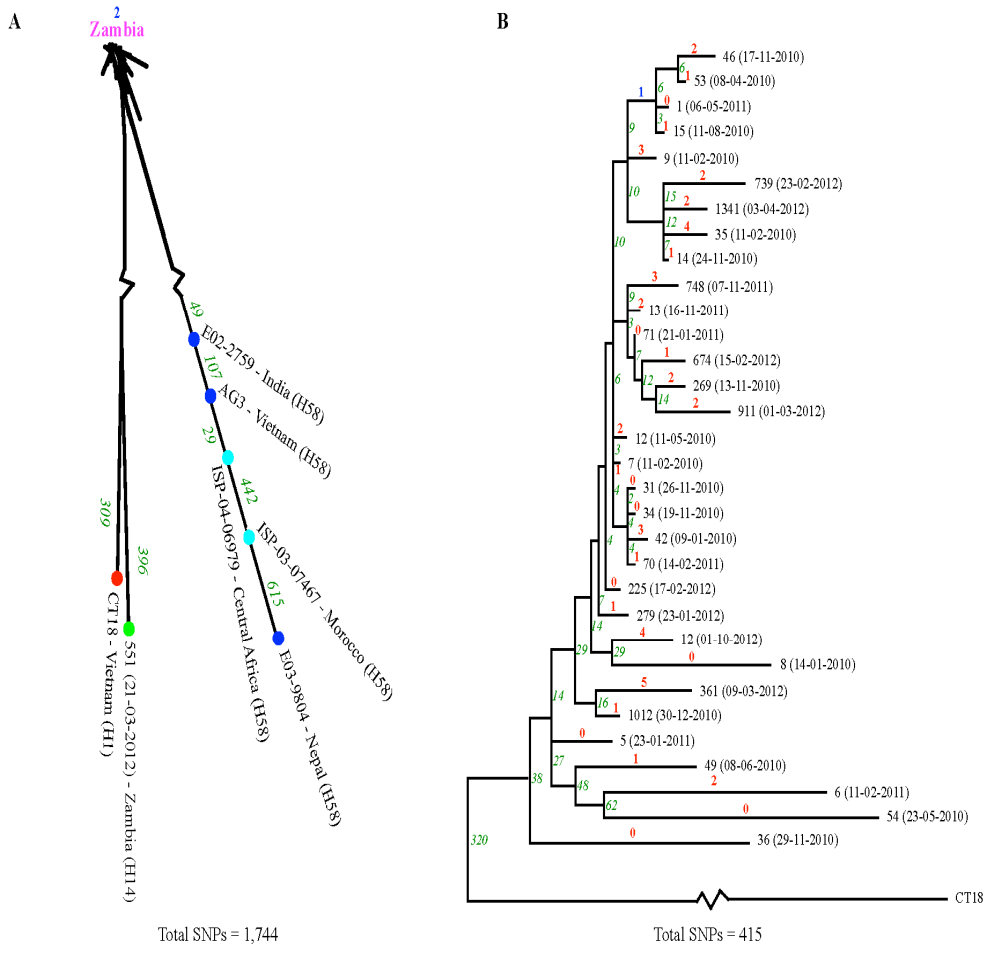

Figure 3.4. Phylogenetic reconstruction of the evolutionary relationships among the *Salmonella* serovar Typhi genomes.Numbers marked: in red indicate autapomorphic SNPs (for Figure B), in blue indicate synapomorphic SNPs, and green indicate the total SNP difference between isolates. In Figure A, the genomes belonging to H58B* from Zambia are marked in pink.

The tree revealed 1,744 high quality whole genome SNPs. Two synapomorphic (clade specific) SNPs were detected among the Zambian genomes (excluding the genome of pan susceptible strain) defining the new minor variant, H58B of haplotype H58B (Figure 3.4A).Figure 3.4A showed that the closest neighbour to the 32 Zambian *S.* Typhi haplotype H58B variant were ISP-04-06979 from Central Africa and E02-2759 from India separated by 29 and 49 SNPs, respectively. In comparison, the genome of the Zambian pan-susceptible strain; #551 of H14 was more distantly

related than the Indian and Central African genomes separated by 396 SNPs to the remaining 32 S. Typhi isolates of haplotype H58B variant (Figure 3.4A).

The SNP tree (Figure 3.4B) containing only outbreak genomes belonging to haplotype H58B variant and the reference genome; CT18 of haplotype H1 excluding the pan susceptible isolate contained an overall 415 high quality whole genome SNPs including the two SNPs defining H58B. SNPs were relatively frequent among the 32 *S*. Typhi isolates of haplotype H58B variant separating individual isolates from the nearest neighbour with 2 to 62 SNPs pairwise separation. The phylogenetic analysis provided evidence for a high clonal diversity among the WGST population, with a large substructure (subclades) that displays clear differentiations. There was no obvious clustering related to time (year) within the *S.* Typhi phylogenetic groups. However, one of the monophyletic subclades contained the four isolates: #14, #35 #1341, and #739. All these four isolates showed a variation in their antimicrobial resistance genes compared to the other isolates.

Chapter 4

Discussion

4.1 Discussion

There is a high prevalence of infections caused by *Salmonella,* especially in developing countries where sanitation is poor and water supply is inadequate (Effa and Bukirwa, 2008). However, this has been complicated by the emergence of multiple-drug-resistant (MDR) *Salmonella* strains, which are resistant to chloramphenicol, ampicillin, and cotrimoxazole, and has led to a change in treatment options (Crump *et al.*, 2008; Dimitrov *et al.*, 2010). MDR strains of *S*. Typhi have been reported from all parts of the world (Effa and Bukirwa 2008, Holt *et al.,* 2011, Tatavarthy *et al.,* 2012, Hendriksen *et al.,* 2013). Molecular epidemiology of human bacterial pathogens provides valuable information for understanding the reservoir, pathogenicity and control of these bacteria (Singh *et al.,* 2006).

Data presented in this study demonstratethe occurrence of both typhoidal and non-typhoidal *Salmonella* in the outbreak. The majority of the cases were caused by *S.* Typhi, followed NTSand *S.* Paratyphi B. Most enteric fever cases were caused by *S.* Typhi followed by*S.* Paratyphi B. Several other studies have also reported that *S.* Typhi is the major cause of enteric fever (Gaind *et al.,* 2006;Baker*et al.,* 2010). Studies carried out in India and Bangladesh demonstrated *S.* Typhi infections were more prevalent than those caused by *S.* Paratyphi (INSARG, 2012; Afroz *et al.,* 2013). This could be attributed to the fact that *S.* Typhi is the most virulent serovar among the enteric fever serovars due to the presence of the Vi capsular polysaccharide that possessesimmuno-modulatory properties, potentially further dampeningthe immune response (Baker *et al.,* 2010).Reports have also shown that

the second major cause of enteric fever is *S.* Paratyphi A (Gaind *et al.,* 2006; Maskey*et al.,* 2006; Baker *et al.,* 2010; Verma*et al.,* 2010; INSARG, 2012) whilst *S.* Paratyphi B and C are relatively uncommon (Baker *et al.,* 2010). However, this study revealed that the second major cause of enteric fever in the outbreak was *S.* Paratyphi B. The isolation of *S.* Paratyphi B in this study could be due to regional differences andhas highlighted the emergence of *S.* Paratyphi B as one of the predominant serovar in 2010-2012 outbreaks in Zambia.This finding correlates with other studies which revealed increased incidence of *S*. Paratyphi B in France (Desenclos *et al.,* 1996), Canada (Stratton *et al.,* 2001), Italy (Mammina *et al.,* 2002) andGermany (Miko *et al.,* 2002) and indicated that this serovar could be of importance in the near future.Our findings, along with those from other studies, indicate that enteric fever is a major public health problem in resource limited countries such as Zambia. Hence, there is an urgent need for effective epidemiological surveillance as a basis for the development of rational control strategies.

NTS were also isolated in this study and these were mainly isolated from blood and stool samples. Other studies in Sub-Saharan Africa have shown that NTS are mainly isolated from blood (Wadhwa *et al.,* 2007: Arora *et al.,* 2009). Several other studies in sub-Saharan Africa have indicated that there is an increased burden of non-typhoidal *Salmonella* (Gordon *et al.,* 2008; Sánchez-Vargas *et al.,* 2011; Wain *et al.,* 2013; Herrero-Fresno *et al.,* 2014). This has been attributed to the rise in HIV infections, malaria, malnutrition and poor sanitation, among others which make these individuals susceptible to NTS infections(Gordon *et al.,* 2008; Sánchez-Vargas *et al.,* 2011; Tadesse *et al.,* 2014). However, in this study, it was not possible to link our

findings toHIV infections, malaria or malnutrition due to lack of access to clinical information.

Several studies have reported that *S.* Senftenberg is a cause of serious human infections and is common in poultry (L'Ecuyer *et al.,* 1996; Kay *et al.,* 2007; Pezzoli *et al.,* 2007; Boumart *et al.,* 2012). Some studies have associated the source of infection with infant formula, mussels and vegetables (Rushdy *et al.,* 1998; Martinez-Urtaza *et al.,* 2004; Berger *et al.,* 2009; Pezzoli *et al.,* 2007, 2008; Veldman *et al.,* 2010). The probable source of the infection in one of the cases in this study could have been vegetables as one of the patients indicated that he had consumed vegetables before being taken ill. To the best of our knowledge, this is the first study to report this serovar in Zambia.

The use of antimicrobial drugs in food animals has resulted in the development of antimicrobial resistance (Begum *et al.,* 2010), through mutation and acquisition of resistance encoding genes (White *et al.,* 2001; Fluit, 2005). For many decades, antibiotics such as chloramphenicol, ampicillin, and cotrimoxazole were used as first line drugs for treating enteric fever (Holt *et al.*, 2011;Tatavarthy*et al.*, 2012; Hendriksen *et al.,* 2013). However, the emergence of MDR strains resistant to these antibiotics have led to changes in the treatment options of typhoid fever, leading to clinicians opting for second line drugs such as flouroquinolones (ciprofloxacin ofloxacin, and perfloxacin) and third generation cephalosporins (ceftriaxone, cefotaxime and cefixime) and azithromycin for treating MDR typhoid fever cases (Al-Sanouri *et al.*, 2008; Effa and Bukirwa 2008; Holt *et al.,* 2011).

In this study approximately 98.6% of *S.* Typhi and 100% of *S.* Paratyphi B isolates were resistant to ampicillin, cotrimoxazole, streptomycin, sulfamethoxazole and trimethoprim, while 85.9% of *S.* Typhi and 100% *S.* Paratyphi Bwere resistant to chloramphenicol. These findings are consistent with other studies in which it was noted that there was an increase in a number of *Salmonella* isolates being resistant to ampicillin, chloramphenicol and cotrimoxazole (Holt *et al.*, 2011;Tatavarthy*et al.*, 2012; Hendriksen *et al.,* 2013). A Study on the Malawi-Mozambique border reported findings similar to our study in which 100% of *S.* Typhi were resistant to ampicillin, chloramphenicol and sulfamethoxazole-trimethoprim (Lutterloh *et al.,* 2012) A study in Uganda showed that 76% of *S.* Typhi were resistant to ampicillin, streptomycin, sulfisoxazole, tetracycline, and cotrimoxazole, but were susceptible to chloramphenicol (Neil *et al.,* 2012) compared with 98.6% ampicillin and cotrimoxazole and 85.9%chloramphenicol resistance reported in this study. *S.* Typhi was only completely susceptible to ceftaxime, gentamicin, andspectinomycin.

The high susceptibility rates to flouroquinolones are important as these antibiotics act as alternatives in the treatment of resistant cases (Akinyemi *et al.,* 2005; Bayram *et al.,* 2011). However, high level resistance to quinolones and flouroquinolones, including single mutations in the QRDR of *gyrA* gene in *S.* Typhi have been reported from India (Zaki *et al.,* 2011; Holt *et al.,* 2011; 2012), Turkey (Bayram *et al.,* 2011), Egypt (Al-sanouri *et al.,* 2008) and Canada (Demczuk *et al.,* 2010). In this study flouroquinolone resistance was comparatively lower. Reduced resistance to ciprofloxacin was found comparatively lower as compared to 81% in the USA (Sjölund-Karlsson *et al.,* 2011), 100% in Turkey (Bayram *et al.,* 2011) and 15.4% in the Democratic Republic of Congo (Lunguya *et al.,* 2012). Nalidixic acid resistance

was also found to be low in this study compared to other studies (Lunguya *et al.,* 2012; Rai *et al.,* 2012; Tatavarthy *et al.,* 2012).This is probably because nalidixic acid is rarely prescribed.Additionally, thisstudy also demonstrated that 2/33(6.1%) of the isolates were nalidixic acid resistant, due to single mutations in the *gyrA* gene at codons Ser83 and Asp87. To the best of our knowledge this is the first time this phenotype has been reported in Zambia. The sensitivity of the isolates to quinolones and cephalosporins suggests that this group of antibiotics could be used for the treatment of enteric fever cases.

The increasing cephalosporin resistance in *Salmonella enterica* isolates in other parts of the world necessitates the search for alternative therapies for enteric fever in countries with a high disease burden. The World Health Organisation (WHO) recommends the use of azithromycin in uncomplicated typhoid fever cases (Effa and Bukirwa, 2008). However, this study demonstrated considerable resistanceto azithromycin (21.1%) in the *S.* Typhi isolates and this was similar to a study in India in which 33.6% of the *Salmonella* isolates were found to be resistant to this drug (Rai *et al.*, 2012). Therefore, the use of azithromycin or other antimicrobial drugs for the treatment of *Salmonella* infections should be carefully monitored in order to avoid the development of drug resistant strains.

This study also demonstrated that NTS were resistant to most of the antimicrobial drugs and were susceptible to cefotaxime, apramycin, flofernicol and ceftiour. However, apramycin, flofernicol and ceftiour are only approved for animal use(Spapen *et al.*, 2011).The two *S.* Senftenberg isolates were both found to be extremely resistant to almost all the antibiotics tested, including nalidixic acid but

were susceptible to apramycin, cefoxitin, colistin, imipenem, meropenem, and tigecycline. However, colistin is difficult to administer and has renal toxicity (Spapen *et al.*, 2011). Cefoxitin is grouped with extended-spectrum cephalosporins and may not have any effect as a result of the isolates already being resistant to broad-spectrum cephalosporins (CLSI, 2012), while the cost of carbapenems is very high (Hawkey and Livermore, 2012; Hendriksen *et al.,* 2013). Treatment with tigecycline may be effective toward NTS, but clinical trials need to be conducted to further investigate the full potential of its use for human treatment of infections caused by multidrug-resistant NTS. Both isolates were also found to be producers of extended spectrum β-lactamases (ESBLs), which is in line with an Indian study that demonstrated that *S.* Senftenberg was resistant to nalidixic acid and extended spectrum β-lactamase (ESBLs) (Rai *et al.*, 2012).

Studies conducted in Kenya, India, and the United States have reported an emergence of MDR strains among *Salmonella* isolates (Kariuki *et al.*, 2010; Kubota *et al.*, 2010; Tatavarthy *et al.*, 2012). This study also demonstrated the occurrence of MDR strains of *S.* Typhi, *S.* Paratyphi B, *S.* Senftenberg and other NTS which were resistant to all traditional first line drugs tested: ampicillin, chloramphenicol and cotrimoxazole. The commonest resistance pattern observed in this study was ampicillin-chloramphenicol-sulfamethoxazole-streptomycin-trimethoprimcotrimoxazole for *S.* Typhi and ampicillin-chloramphenicol-spectinomycin-sulfamethoxazole-streptomycin-trimethoprim-cotrimoxazole for *S.* Paratyphi B. A similar study by Demczuk and colleagues (2010), revealed 26 resistance patterns with the commonest patterns being nalidixic acid-resistant and ampicillin-chloramphenicol-nalidixic acid-streptomycin-sulfisoxazole-cotrimoxazole

in *S*. Typhi compared to this to this study. This study indicated that most of the *Salmonella* isolates were resistant to five or more antibiotics andthere was variability in the resistant patterns of NTS.The MDR detection rate was 85.9% for *S*. Typhi, 100% for *S.* Paratyphi B, 100% for *S.* Senftenberg 100% and 55.6% for the other NTS. Similar findings of MDR strains of *S.* Typhi and NTS have been reported by various groups. In Pakistan 69% of *S.* Typhi isolated from blood were MDR (Mirza*et al.,* 1996), in Vietnam 89.9% of isolates obtained between 1998 and 2002 were MDR (Le *et al.,*2004), whereas in Turkey 100% were MDR (Bayram *et al.,* 2011). However, resistance was considerably lower in Tajikistan and in the Democratic Republic of Congo where 27% and 30.3% of isolates, respectively, were MDR (Mermin*et al.,* 1999; Lunguya *et al.,* 2012). A study done in New Delhi, India, showed that there was a gradual rise in the number of MDR strains of *S.* Typhi over a 7-year period from 34% in 1999 to 58% in 2002, and rose to 66% in 2005, along with rising resistance to all the other drugs (Kumar *et al.,* 2008).

Despite, this wide spread of MDR *S.* Typhi strains observed in different parts of the worldand this study, other studies in different parts of India have shown a decrease in drug resistance in these strains to ampicillin, chloramphenicol and cotrimoxazole (Hosoglu *et al.,* 2003; Raza *et al.,* 2012). Another Indian study showed increased sensitivity to chloramphenicol (95.3%) observed in all the isolates, thereby showing a reversal of the susceptibility pattern and favouring the reuse of chloramphenicol (Rai *et al.*, 2012).

In this study antimicrobial drug resistance could be attributed to the overuse of these drugs and also the use of cotrimoxazole in HIV/AIDS patients as prophylaxis for

opportunistic infections prevention (Chintu *et al.,* 2004; Moodley *et al.,* 2013). The other reason, could be that laws and regulations are not strict and not followed on the sale and purchase of antimicrobial drugs in Zambia (Pandey and Sharma, 1994, Hang'ombe, 1999). This observation has been noted in India and has attributed the increase of MDR *S.* Typhi and other antibiotics to the widespread availability and uncontrolled use of antibiotics in the tropics and Indian subcontinent, leading to selective pressure on a large bacterial population of endemic *Salmonella* species (Rai *et al.,* 2012). Therefore, these practices together with the horizontal gene transfer have contributed to the emergence of *Salmonella* resistant to common antimicrobial drugs used for therapy. To the best of our knowledge this is the first enteric fever study to report MDR strains of *S.* Typhi, *S.* Paratyphi B and *S.* Senftenberg in Zambia. It indicates emerging problems of MDR *Salmonella* strains in Zambia and has provided baseline data necessary in formulating guidelines for monitoring of MDRs.

Whole Genome sequencing of a selected number of *S.* Typhi isolates revealed that all but one pan-susceptible isolate, harboured drug resistance genes. This may suggest possible transfer of drug resistance genes to other bacteria in the environment. Some isolates of *S.* Typhi and *S.* Paratyphi B were found to harbour Integron class Isequences containing dihydrofolate reductase (*dfrA7*) gene. A study inthe United States also demonstrated presence of this gene, which confers resistance to trimethoprim.In Zambia trimethoprim is given as a prophylactic drug especially in HIV infected individuals and may be ineffective due the presence of this drug resistance gene.

Previous studies have indicated that *S*. Typhi is a monomorphic bacterium and its sequence diversity is limited (Kidgell *et al.,* 2002; Roumagnac *et al.,* 2006; Holt *et al.,* 2010). The generation of a rooted phylogenetic tree based upon rareSNPs has permitted a greater understanding and definitionof the global population of *S*. Typhi, and provided apotential method for tracking the pathogen in anendemic setting (Roumagnac*et al.,* 2006). The high resolution SNP analysis of the Zambia outbreak isolates, in relation to the non-outbreak strains, demonstrated that the Zambian *S.* Typhi belonged to a monophyletic group and were closely related to the previously sequenced non-outbreak strains,ISP-04-06979 from Central Africaand E02-2759 from India. This clustering affirms the notion that *S.* Typhi is monomorphic and sequence diversity is limited (Kidgell *et al.,* 2002; Holt *et al.,* 2010). This close relatedness between our isolates in this study and those from Central Africa and India may suggest that these isolates have a common ancestor. The dominant strain in this study was the H58B haplotype minor variantand this contradicts the findings of studies carried out in Vietnam, Kenya and other endemic regions (Roumagnac *et al.,* 2006; Holt *et al.,* 2010; Kariuki *et al.,* 2010) where it was shown that the H58 haplotype was the main strain. Our findings also contradicts the notion that the H58 strain is a dominant clone that spreads easily within and betweenhuman populations andis the common cause of MDR typhoid fever in endemic areas (Holt *et al.,* 2010; Kariuki *et al.,* 2010).

Overall, the clonal diversity observed in this study, linked with the neutral stabilizing selection and the differences of genomic deletion among the genomes led to the conclusion that multiple clones and lineages of *S.* Typhi H58B variable have been responsible for the *Salmonella*outbreak in Zambia due to either environmental

changes and poor sanitation or a high influx of infected people from other regions. This hypothesis is also supported by the epidemiological data from the Ministry of Health that typhoid fever is endemic and that the outbreak had been ongoing for several years with minimum intervention and control programs (MOH, 2012).

4.2 Conclusion

The study demonstrated that the 2010-2012 salmonellosis outbreak was both typhoidal and non-typhoidal in nature. The serovars responsible for the outbreak were *S.* Typhi, *S.* Paratyphi B, *S.* Senftenberg and other NTS. The majority of the cases were caused by *S.* Typhi. To our knowledge this was the first study to report *S.* Senftenberg in Zambia.

The majority of the *Salmonella* isolates were MDR strains, with a small number of the isolates being classified as nalidixic acid-resistant. Amongst the NTS *S.* Senftenberg was extremely resistant to most antibiotics tested and were also ESBLs producers. The isolatesharboured drug resistance genes towards six classes of antimicrobial drugs: trimethoprims, sulphonamides, phenicols, β-lactams, aminoglcosides and flouroquinolones. These isolates may have the potential to transmit these genes horizontally to other bacteria in environment.

The *S.* Typhi in this study exhibited genetic diversity, indicating that the isolates in the outbreak were distantly related. This suggests that multiple clones and lineages were responsible for the outbreak.The isolates also showed common ancestry to those from Central Africa and India, suggesting movement of people, food animals and products to and from these regions. The dominant strain was *S.* Typhi H58B

minor variant as opposed to the H58 strain which is dominant in East Africa and some parts of Asia (Holt *et al.,* 2010; Kariuki *et al.,* 2010).

4.3 Limitations of the Study

It was not possible to link our findings with clinical information due to lackof access to clinicaldata and incomplete patient records. Identification of all the NTS isolates in this study was not possible owing to limited resources. Molecular typing of the *Salmonella* isolates was mainly focused on *S.* Typhi and not for the other serovars isolateddue to inadequate resources. This may not reflect the characteristics of all the isolates in the present study.In addition, results in this study do not give a true reflection of the molecular characteristics of *S.* Typhi or other *Salmonella* in Zambia since the study was only limited to a few outbreak strains.

4.4Future Directions

It would be of interest to identify the NTS to species level so as to give an insight of the common circulating serovars of NTS in Zambia.This can be achieved by DNA sequencing of the Internal Transcribed Spacer (ITS) region in the NTS. This would help in providing useful information on the common NTS serovars in Zambia.

There is also a need to perform molecular typing on*S.* Paratyphi B serovars and NTS by Pulsed-Field Gel Electrophoresis (PFGE) or by whole genome sequencing.This will provide information on the diversity of these organisms, their source and the basis for identifying means of control of these global pathogens.Furthermore, studies on the distribution of *Salmonella enterica* serovars and molecular epidemiologyfrom other parts of Zambia should be performed to give an accurate picture of the strains

circulating in the country. These studies should include the collection of clinical and epidemiological information so as to give an insight of who the most vulnerable groups are as well as the common risk factor in our region.

It is also evident in this study that there is a presence of typhoidal, non-typhoidal *Salmonella* and MDR strains of *Salmonella*inZambia**.** Hence, an effective national *Salmonella* surveillance system should be set up for monitoring and control of *Salmonella* as well as control of antimicrobial resistance by the Ministry of Health (MOH) and Ministry of Mother, Child and Community Development. Furthermore, these ministries should improve laboratory capacity and this could be achieved through collaboration with WHO Global *Salmonella* Surveillance programs. Clinicians should also ensure that antibiotic susceptibility testing is done before antibiotic administration.The MOH and Ministry of Mother, Child and Community Developmentshould enhance outbreak surveillance to prevent outbreaks and further dissemination of MDR strains in Zambia.

Finally, information, education and communication through awareness campaigns and notifications, as and when necessary, should be organized and incorporated in the routine process by the Public Health Department at the MOH and Ministry of Mother, Child and Community Development and should also emphasise on educating the food business entrepreneurs and people involved in the food business as well as educating people on the dangers of antimicrobial drug resistance.

7.0 References

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Appendices

Appendix I: Ethics Approval Letter

THE UNIVERSITY OF ZAMBIA

BIOMEDICAL RESEARCH ETHICS COMMITTEE

Telephone: 260-1-256067
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Ridgeway Campus
P.O. Box 50110
Lusaka, Zambia

12th October, 2012.

Your Ref: 013-09-12.

Ms Annie Kalonda,
School of Medicine,
Department of Biomedical Sciences,
PO Box 50110,
Lusaka.

Dear Ms Kalonda,

RE: SUBMITTED RESEARCH PROPOSAL: **"MOLECULAR CHARACTERIZATION OF SALMONELLA ENTERICA SEROVAR TYPHI STRAINS ISOLATED AT THE UNIVERSITY TEACHING HOSPITAL FROM 2010 TO 2012"**

Your application for a waiver of ethics review for the protocol **"Molecular Characterization of Salmonella Enterica Serovar Typhi Strains Isolated at the University Teaching Hospital From 2010 to 2012"** was reviewed. The waiver is hereby granted in accordance with the University of Zambia Biomedical Research Ethics Committee procedure on granting waiver of ethics review.

CONDITIONS:

- The waiver is based strictly on your submitted proposal. Should there be need for you to modify or make changes to the proposal you will need to seek clearance from the University of Zambia Biomedical Research Ethics Committee.
- This waiver does not release you from any other applicable obligations in ensuring confidentiality.
- If you need any clarifications please consult this office.
- **Ensure that a final copy of the results is submitted to this Committee.**

Yours sincerely,

Dr. J.C Munthali
CHAIRPERSON

Date of approval: 12 October, 2012 **Date of expiry:** 11 October, 2013

Appendix II:A Representative DNA Sequence of Class I integron

Isolate #: 161

gi|312183497|gb|HQ132376.1|*Salmonella enterica subsp. enterica* serovar Typhi class I integron dihydrofolate reductase type 7 (dhfr7) gene, complete cds.
Length = 768, Score = 1358 (706), Expect = 0.0, Gaps = 1/714 (0%), Strand = Plus/Plus

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Query  1    GATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGC  60
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  43   GATGTTATGGAGCAGCAACGATGTTACGCAGCAGGGCAGTCGCCCTAAAACAAAGTTAGC  102

Query  61   CATTACGGGGGTTGAATTGAAAATTTCATTGATTTCTGCAACGTCAGAAAATGGCGTAAT  120
            |||||||||||||||| ||||||||||||||||||||||||||||||||||||||||||||
Sbjct  103  CATTACGGGGGTTGAAT-GAAAATTTCATTGATTTCTGCAACGTCAGAAAATGGCGTAAT  161

Query  121  CGGTAATGGCCCTGATATCCCATGGTCAGCAAAAGGTGAGCAGTTACTCTTTAAAGCGCT  180
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  162  CGGTAATGGCCCTGATATCCCATGGTCAGCAAAAGGTGAGCAGTTACTCTTTAAAGCGCT  221

Query  181  CACATATAATCAGTGGCTCCTTGTTGGAAGGAAAACATTTGACTCTATGGGTGTTCTTCC  240
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  222  CACATATAATCAGTGGCTCCTTGTTGGAAGGAAAACATTTGACTCTATGGGTGTTCTTCC  281

Query  241  AAATCGAAAATATGCAGTAGTGTCGAGGAAAGGAATTTCAAGCTCAAATGAAAATGTATT  300
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  282  AAATCGAAAATATGCAGTAGTGTCGAGGAAAGGAATTTCAAGCTCAAATGAAAATGTATT  341

Query  301  AGTCTTTCCTTCAATAGAAATCGCTTTGCAAGAACTATCGAAAATTACAGATCATTTATA  360
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  342  AGTCTTTCCTTCAATAGAAATCGCTTTGCAAGAACTATCGAAAATTACAGATCATTTATA  401

Query  361  TGTCTCTGGTGGCGGTCAAATCTACAATAGTCTTATTGAAAAAGCAGATATAATTCATTT  420
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  402  TGTCTCTGGTGGCGGTCAAATCTACAATAGTCTTATTGAAAAAGCAGATATAATTCATTT  461

Query  421  GTCTACTGTTCACGTTGAGGTTGAAGGTGATATCAATTTTCCTAAAATTCCAGAGAATTT  480
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  462  GTCTACTGTTCACGTTGAGGTTGAAGGTGATATCAATTTTCCTAAAATTCCAGAGAATTT  521

Query  481  CAATTTGGTTTTTGAGCAGtttttttttGTCTAATATAAATTACACATATCAGATTTGGAA  540
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  522  CAATTTGGTTTTTGAGCAGTTTTTTTTTGTCTAATATAAATTACACATATCAGATTTGGAA  581

Query  541  AAAAGGCTAACAAGTCGTTCCAGCACCAGTCGCTGCGCTCCTTGGACAGTTTTTAAGTCG  600
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  582  AAAAGGCTAACAAGTCGTTCCAGCACCAGTCGCTGCGCTCCTTGGACAGTTTTTAAGTCG  641

Query  601  CGGTTTTATGGTTTTGCTGCGCAAAAGTATTCCATAAAACCACAACTTAAAAACTGCCGC  660
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  642  CGGTTTTATGGTTTTGCTGCGCAAAAGTATTCCATAAAACCACAACTTAAAAACTGCCGC  701

Query  661  TGAACTCGGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAG  714
            ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct  702  TGAACTCGGCGTTAGATGCACTAAGCACATAATTGCTCACAGCCAAACTATCAG  755
```

Appendix III: Whole Genome Sequencing Supplementary Data

A.Full Genomic Information of Genomes Included in this Study

Isolate no.	Read length (bp)	Total Sequences	percent GC	Sequencing platform	N50 (bp)	Total contigs	Total length of sequence (bp)
1	95	10465710	51	Illumina (BGI)	25180	374	4744214
5	35-251	502445	51	Illumina MiSeq	59520	177	4729455
6	35-251	1211293	51	Illumina MiSeq	143433	88	4780503
7	95	5720046	51	Illumina (BGI)	24726	377	4744419
8	35-251	1161340	51	Illumina MiSeq	153429	85	4685827
9	95	11664325	51	Illumina (BGI)	25179	385	4745648
12	95	6091141	51	Illumina (BGI)	24206	390	4745025
13	95	5118271	51	Illumina (BGI)	23938	389	4746965
14	95	7236224	51	Illumina (BGI)	23676	404	4783218
15	95	6006275	51	Illumina (BGI)	24765	391	4742136
31	31-100	3046914	51	Illumina HiSeq	206184	78	4750645
34	35-100	3351745	51	Illumina HiSeq	204269	75	4750804
35	45-100	2580654	51	Illumina HiSeq	186675	84	4792846
36	35-251	1349664	51	Illumina MiSeq	144286	88	4732620
42	29-100	2582796	51	Illumina HiSeq	204269	74	4751179
46	49-100	1484940	51	Illumina HiSeq	221745	78	4751180
49	35-251	972455	51	Illumina MiSeq	143479	80	4737442
53	55-100	1762789	51	Illumina HiSeq	206193	74	4752104
54	35-251	1263152	51	Illumina MiSeq	154782	84	4741519
70	55-100	1492338	51	Illumina HiSeq	206120	68	4755515
71	43-100	1884095	51	Illumina HiSeq	204269	78	4750221
1012	35-251	1233724	51	Illumina MiSeq	28409	480	4699244
12	35-251	993271	51	Illumina MiSeq	2694182	58	4713610
1341	35-251	1392883	51	Illumina MiSeq	28980	363	4742770
225	35-251	1152319	51	Illumina MiSeq	35231	309	4718813
269	35-251	1088375	51	Illumina MiSeq	4612297	73	4746801
279	35-251	1022280	51	Illumina MiSeq	32411	317	4710498
361	35-251	881620	51	Illumina MiSeq	30284	359	4703371
551	35-251	1681977	51	Illumina MiSeq	2959711	260	4686183
674	35-251	1327234	51	Illumina MiSeq	42197	224	4716255
739	35-251	1241995	51	Illumina MiSeq	4671909	46	4785946
748	35-251	1394046	51	Illumina MiSeq	42747	264	4715900
911	35-251	1376523	51	Illumina MiSeq	3821004	351	4681861

Public genomes

Isolate ID	Country	Year	Haplotype	Genbank ID	Source	Total contigs	Total length of sequence (bp)
CT18	Vietnam	1993	H1	AL513382	NCBI	1	4809037
AG3	Vietnam	2004	H58	CAAY00000000	NCBI	7336	4751206
E02-2759	India	2002	H58	-	Sanger	1	4809037
ISP-04-06979	Central Africa	2004	H58	-	Sanger	1	4809037
E03-9804	Nepal	2003	H58	-	Sanger	1	4809037
ISP-03-07467	Morocco	2003	H58	-	Sanger	1	4809037

Download link

ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/typhi/solexa/E02-2759_solexa_genome.fastq

ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/typhi/solexa/ISP-04-06979_solexa_genome.fastq

ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/typhi/solexa/E03-9804_solexa_genome.fastq

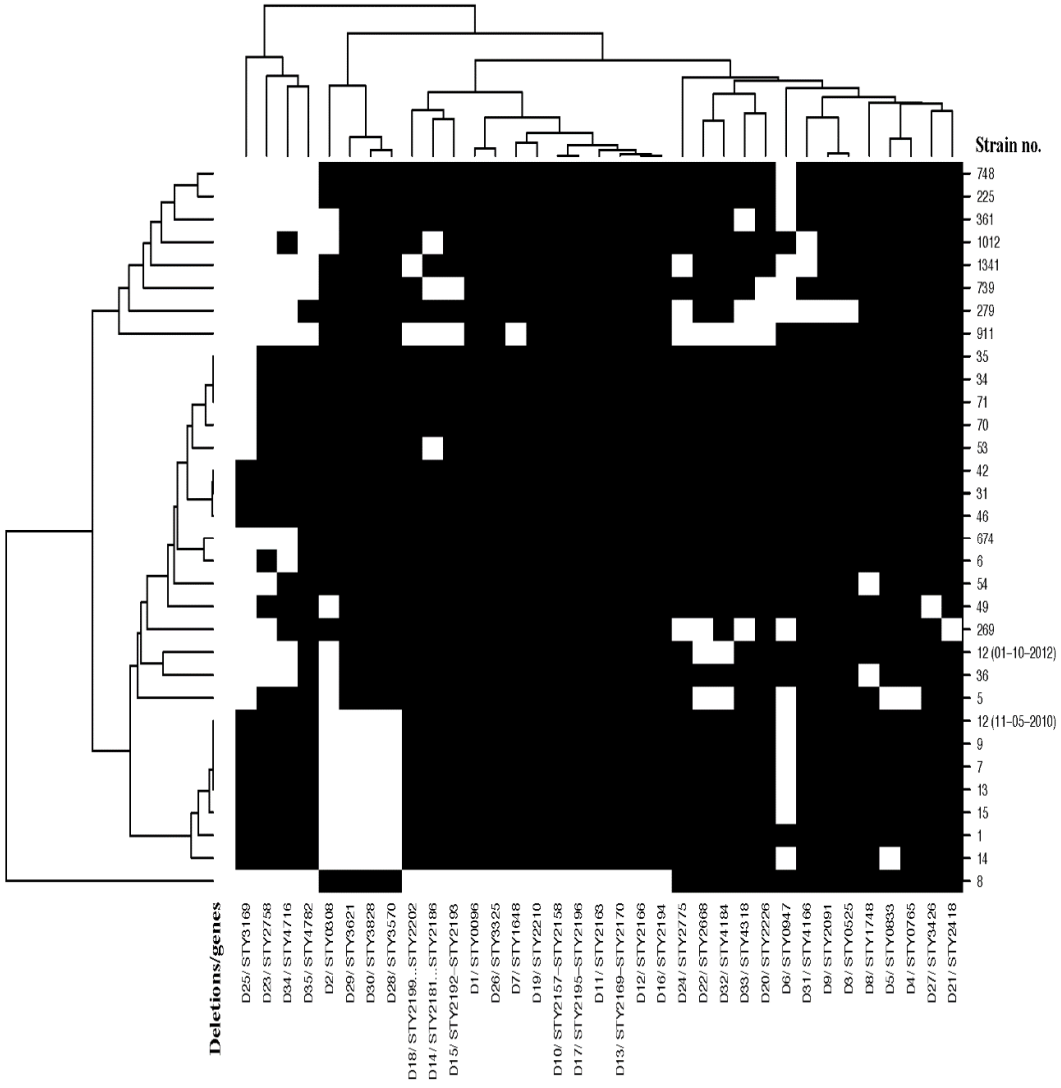
ftp://ftp.sanger.ac.uk/pub/pathogens/Salmonella/typhi/solexa/ISP-03-07467_solexa_genome.fastq

B.Representative SNP Positions of the 33 WGST Isolates.

pos	ref	alt	ref_codon	alt_codon	aa_change
12478	C	T	CAC	CAT	His
17187	G	A	GTG	GTA	Val
22623	T	C	TTG	CTG	Leu
32762	G	A	CTG	CTA	Leu
35122	T	C	-	-	-
40159	G	A	GGG	GAG	Gly11Glu
61892	T	C	TAC	CAC	Tyr520His
63831	C	T	ACG	ATG	Thr339Met
76422	A	G	AGC	GGC	Ser243Gly
80983	T	C	TAC	CAC	Tyr150His
89102	A	G	AAA	AGA	Lys201Arg
93158	A	G	TAG	TGG	*388Trp
115909	C	T	-	-	-
117735	T	C	-	-	-
124045	C	T	ATC	ATT	Ile
136763	T	C	TGC	CGC	Cys261Arg
158891	G	T	GCG	GCT	Ala
160293	T	C	TTG	CTG	Leu
163958	G	A	CTG	CTA	Leu
175120	C	T	CCT	CTT	Pro415Leu
202039	C	T	CTG	TTG	Leu
204478	C	T	CCG	CTG	Pro37Leu
214999	A	G	ACC	GCC	Thr286Ala
219989	C	T	GCA	GTA	Ala265Val
260848	T	C	TTG	CTG	Leu
313260	G	A	GCG	GCA	Ala
316812	C	G	ACT	AGT	Thr147Ser
354895	G	A	GTG	ATG	Val563Met
362463	A	T	CAC	CTC	His642Leu
381217	A	G	AAT	AGT	Asn748Ser
381514	G	A	CGG	CAG	Arg649Gln
387082	G	A	CGA	CAA	Arg33Gln
387595	C	T	ACC	ATC	Thr204Ile
397389	A	G	AAC	GAC	Asn171Asp
399478	T	C	CTT	CTC	Leu
401918	T	C	ACT	ACC	Thr
408989	G	A	CTG	CTA	Leu
413125	T	C	CTG	CCG	Leu452Pro
426870	A	G	AGG	GGG	Arg205Gly
429115	T	C	-	-	-
435831	C	T	GCG	GTG	Ala122Val
445543	A	G	GCA	GCG	Ala
448607	G	A	GGG	GGA	Gly
448862	A	G	CTA	CTG	Leu
461438	C	T	CGT	TGT	Arg4Cys

Synonymous SNPs = 141
Non-synonymous SNPs = 222
* = SNPs in stop codon
"-" = SNPs in intergenic region
dN/dS = 0.940

C.Genomic Deletions Detected in the *S.*Typhi genomes



Genomic deletions detected in the *Salmonella* serovar Typhi genomes from Zambia. Deletions (marked in black) are based on a 95% hit score. Affected genes are partially or entirely deleted.

Appendix IV: Media and Reagents

A. Media

MacConkey Agar (Oxoid Ltd, Basingstone, Hampshire, England)

50g of MacConkey agar base in 1litre distilled water

Autoclave at 121˚C for 15 minutes.

Xylose-Lysine Deoxycholate Agar (Oxoid Ltd, Basingstone, Hampshire, England)

53g in 1000ml of distilled water

Boil and dispense

Mueller Hinton Agar (Mast Diagnostics, Mast Group Ltd, Merseyside, UK)

38.0g in 1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Triple Iron Sugar (TSI) Agar (REMEL)

59.5g in 1000ml of distilled water

Autoclave at 121˚C for 15 minutes.

Simmons Citrate Agar (HiMedia Laboratories Pvt. Ltd. India)

24.2g in 1000ml distilled water

Autoclave at 121˚C for 15 minutes.

Lysine Iron Agar (HiMedia Laboratories Pvt. Ltd. India)

34.56g in 1000ml distilled water

Autoclave at 121˚C for 15 minutes.

Urease Agar (HiMedia Laboratories Pvt. Ltd. India)

24.01g of urea base in 950ml distilled water

Autoclave at 115˚C for 20 minutes.

Cool to 50˚C and add 50ml of sterile 40% urea

Agarose Gel (1.5%)

1.5% Agarose gel

Up to 100ml 1X TBE buffer

Skim milk, Tryptone, Glucose, Glycerol (STGG)

2g Skim milk powder

3g Tryptone soya broth

0.5g Glucose

10ml Glycerol

100ml distilled water

Autoclave at 121˚C for 15minutes

Store STGG frozen at -20°C or refrigerate until use.

B. Reagents

0.89% Sodium Chloride

0.89g Sodium chloride in 100ml distilled water

Autoclave.

McFarland standard 0.5

Barium chloride (1.175%) 0.05ml

Sulphuric acid (1%) 9.95ml

10X TBE Buffer

108g Tris

55g Boric acid

9.3g EDTA

Up to a 1000ml of distilled water.

TBE (x1)

1 volume of 10x TBE buffer

9 volumes distilled water

Ethidium bromide solution

10mg/ml in distilled water.

Stored in a dark bottle at $4^{o}C$

Appendix V: List of Publications and Presentations

The following article, based on this dissertation, has been published.

1. Hendriksen, R. S., Joensena, K. G., Lukwesa-Musyani, C., Kalondaa, A., Leekitcharoenphona, P., Nakazwe, R., Aarestrupa, F. M., Hasmana, H. and Mwansa, J. C. L. (2013). Extremely Drug-Resistant *Salmonella enterica* Serovar Senftenberg Infections in Patients in Zambia. *Journal Clinical Microbiology*, **51**:284-286.

The following manuscripts, based on this dissertation, have been prepared for publication:

1. Hendriksen, R. S., Leekitcharoenphon, P., Lukwesa-Musyani, C., Tambatamba, B., Mwaba, J., Kalonda, A., Nakazwe, R., Kwenda, G., Jensen, J. D., Kaas, R. S., Cavaco, L. M., Aarestrup, F. M., Hasman, H. and Mwansa, J. C. L. (2014). Genomic signature of multi-drug resistant *Salmonella* Typhi related to a massive outbreak in Zambia during 2010 - 2012. To be submitted to *PLoS Pathogens.*

2. Annie Kalonda, Geoffrey Kwenda, Chileshe Lukwesa-Musyani, Rene S. Hendriksen, Trevor Kaile, Clemence Marimo and James C.L Mwansa. (2014).Detection of Class I Integrons In Multidrug Resistant *Salmonella enterica* serovar Typhi and Paratyphi B Isolated at the University Teaching Hospital, Lusaka, Zambia. To be submitted to *Journal of Infection in Developing Countries.*

Part of this work has been presented at the following scientific meetings:

1. Kalonda, A., Kwenda, G., Lukwesa-Musyani, C. and Mwansa, J. C. L. (2014) Detection of Multidrug Resistant *Salmonella enterica* serovars isolated at the University Teaching Hospital, Lusaka. Oral presentation at the Seventh National HealthResearch Conference held on 14^{th}-16^{th} October, 2013 at Government Complex, Lusaka, ZambiaScientific Program.

2. Kalonda, A., Kwenda, G., Lukwesa-Musyani, C. and Mwansa, J. C. L. (2014) Molecular Characterisation of *Salmonella enterica* Serovars Isolated at the University Teaching Hospital. Oral and Poster presentation at the University of Zambia Graduate Forum held on the 7^{th} -11^{th} April, 2014.

JCM
Journals.ASM.org

Extremely Drug-Resistant *Salmonella enterica* Serovar Senftenberg Infections in Patients in Zambia

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Two cases of extremely drug-resistant *Salmonella enterica* serovar Senftenberg isolated from patients in Zambia were investigated by utilizing MIC determinations and whole-genome sequencing. The isolates were resistant to, and harbored genes toward, nine drug classes, including fluoroquinolones and extended-spectrum cephalosporins, contained two plasmid replicons, and differed by 93 single-nucleotide polymorphisms.

Infections with *Salmonella* that are resistant to multiple antimicrobials are associated with increased morbidity and mortality (1, 2), and the global emergence of such organisms is leaving clinicians with few, or no, treatment options (3). Recently, several studies have indicated the emergence and spread of multidrug-resistant *Salmonella* clones in Africa (4–7). Often, those clones have a different epidemiology than what is observed in developed countries, complicating control and prevention strategies (8). It is paramount to identify new multidrug-resistant clones as early as possible to hamper further dissemination (1).

Here, we describe two clinical cases of human salmonellosis in Zambia caused by extremely drug-resistant (EDR) *Salmonella enterica* serovar Senftenberg. The genomes of the isolates were sequenced to determine the multilocus sequence type (MLST) and to investigate the occurrence and genetic mechanisms of antimicrobial resistance, plasmid replicons, and genetic relatedness by single-nucleotide polymorphism (SNP) analysis.

On 18 January 2012, a 34-year-old male from Mazabuka, Zambia (72 km south of the capital, Lusaka), was admitted to the Mazabuka District Hospital. Based on medical examination, the patient was diagnosed with gastroenteritis and treated with ciprofloxacin and co trimoxazole. Two days later, the patient was discharged, with continuing treatment on cephalexin and co-trimoxazole, but was readmitted with epistaxis and occipital pulsatile headache and treated with adrenaline and vitamin K. The patient was discharged 6 days later and scheduled to be reviewed. On 6 February the patient was referred to the renal unit of the University Teaching Hospital (UTH) in Lusaka, as he was pale, dehydrated, afebrile, tachycardic, with a scaphoid abdomen, and later he also developed ureamic encephalopathy. This time, the patient was diagnosed with sepsis and chronic renal failure. Among other tests, which were all negative, a renal ultrasound was normal but the stool culture yielded *Salmonella* (isolate 588). Three days later, the patient was unable to eat and was fed through a nasogastric tube and intravenous fluids. The patient was transfused 4 days after admission, but no dialysis was initiated. The patient died the morning of 11 February 2012.

A second patient, a 30-year-old male from the Chibolya compound (2 km west of Lusaka and 74 km away from case 1), had spent most of his time in the compound. The patient was admitted to the UTH and diagnosed with gastroenteritis with tuberculosis (TB), after having been referred from a local clinic on 9 March 2012. Three months prior to admission the patient had been treated with antimicrobials due to sexually transmitted infections. Prior to admission on 9 March 2012, the patient complained of a headache, chills, fever, diarrhea, and general weakness. On 13 March, a stool sample was collected, and it yielded *Salmonella* (isolate 1028). The patient was reported to have consumed vegetables bought from the local market. Based on chest X ray, the patient was diagnosed with extrapulmonary TB and treated with rifampin, isoniazid, pyrazinamide, and ethambutol. On 16 March, the patient was also diagnosed with HIV and received emtricitabine, tenofovir, efavirenz, and co-trimoxazole.

The *Salmonella* isolates were shipped to the Technical University of Denmark (DTU) for further characterization. The isolates were serotyped, followed by MIC determinations as previously described, including the tigecycline MIC (9). Both isolates belonged to *Salmonella enterica* serovar Senftenberg and had an almost identical antimicrobial susceptibility pattern, conferring resistance to amoxicillin plus clavulanic acid (MIC, 16 μg/ml), ampicillin (MIC, ≥32 μg/ml), cefepime (MIC, ≥16 μg/ml), cefotaxime (MIC, ≥64 μg/ml), cefpodoxime (MIC, ≥32 μg/ml), ceftazidime (MIC, 128 μg/ml), ceftiofur (MIC, ≥8 μg/ml), ceftriaxzone (MIC, ≥128 μg/ml), chloramphenicol (MIC, ≥64 μg/ml), ciprofloxacin (MIC, ≥4 μg/ml), gentamicin (MIC, ≥16 μg/ml), nalidixan (MIC, ≥64 μg/ml), neomycin (MIC, ≥32 μg/ml), spectinomycin (MIC, ≥256 μg/ml), streptomycin (MIC, ≥128 μg/ml), sulfamethoxazole (MIC, ≥1,024 μg/ml), tetracycline (MIC, ≥32 μg/ml), and trimethoprim (MIC, ≥32 μg/ml). In addition, isolate 588 was also resistant to florfenicol (MIC, ≥64 μg/ml). The isolates were susceptible to apramycin, cefoxitin, colistin, imipenem, meropenem, and tigecycline.

However, one could question if those antimicrobials would be used for treatment, since florfenicol and apramycin are only approved for animal usage, colistin is difficult to administer and has

Received 20 August 2012 Returned for modification 25 September 2012
Accepted 12 October 2012
Published ahead of print 17 October 2012
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doi:10.1128/JCM.02227-12

TABLE 1 Distributions of antimicrobial resistance genes in *Salmonella* Senftenberg isolates from Zambia

Antimicrobial agent(s)	Resistance gene, class, and/or mutation in isolate ID no.:	
	588	1028
Aminoglycoside	*aac(6′)-IIc* *aac(6′)-Iy* *aph(3′)-Ic* *aac(6′) Ib cr* *strA* *strB*	*aac(6′)-IIc* *aac(6′)-Iy* *aadA2* *aac(6′) Ib cr* *strA* *strB*
Beta-lactam	bla_{TEM-1} $bla_{CTX-M-15}$ bla_{OXA-10}	bla_{TEM-1} $bla_{CTX-M-15}$ bla_{OXA-30}
Fluoroquinolone Low-level resistance High-level resistance	*aac(6′)-Ib-cr* *gyr*(A) (S-83-Y and D-87-G) *par*(C) (S-80-I)	*aac(6′)-Ib-cr* *gyr*(A) (S-83-Y and D-87-G) *par*(C) (S-80-I)
Macrolide-lincosamide-streptogramin	*ere*(A)	*ere*(A)
Phenicol	*catB3* *catA2* *cmlA1* *floR*	*catB3* *catA2*
Rifampin	*arr-2*	
Sulfonamide	*sul1* *sul2*	*sul1* *sul2*
Tetracycline	*tet*(A) *tet*(D)	*tet*(D)
Trimethoprim	*dfrA14* *dfrA18* *dfrA23*	*dfrA18*

renal toxicity (2), cefoxitin is grouped with extended-spectrum cephalosporins and would most likely not have any effect, due to the isolates already being resistant to broad-spectrum cephalosporins (10), and carbapenems are too expensive, considering that these patients were required to cover the hospital expenses themselves. Treatment with tigecycline may be effective toward nontyphoid *Salmonella*, but clinical trials need to be conducted to further investigate the full potential of its use for human treatment of infections caused by multidrug-resistant nontyphoid *Salmonella*.

The isolates were sequenced on the Ion Torrent PGM system (Life Technologies) following the manufacturer's protocols for 200-bp genomic DNA (gDNA) fragment library preparation (Ion Xpress Plus gDNA and Amplicon Library Preparation), template preparation (Ion OneTouch system), and sequencing (Ion PGM 200 sequencing kit). The sequence data were assembled and analyzed using the pipeline available on the Center for Genomic Epidemiology website (www.genomicepidemiology.org) (11, 12).

The isolates belonged to MLST ST14. The Resfinder tool detected the following resistance genes present either in both or in one of the isolates, as well as two mutations in *gyr*(A) and one mutation in *par*(C) responsible for high-level fluoroquinolone resistance (Table 1). Both isolates contained an incI I12 plasmid replicon, and isolate 588 contained an incA/C plasmid replicon.

The genetic relatedness of the two isolates was examined and identified 93 high quality SNPs (the informative SNPs were determined based on a minimum coverage of 20 times, base calling quality of 30, and a minimum distance of 10 bp between each SNP) between the two isolates, using the *S.* Senftenberg SS209 reference genome (Bio project number PRJEA82547) (13) and 530 and 521 SNPs between isolates 1028 and 588 and the reference genome. There are currently insufficient data on the nucleotide diversity between clonally related and unrelated *Salmonella* isolates to determine whether this was indicative of separate or clonally related strains. Whole-genome studies on *Salmonella* have indicated an accumulation rate of 1 to 2 SNPs per year (8). Thus, the 93 SNP differences observed here in combination with the differences in resistance profiles and genes may suggest that the isolates have an unrelated origin.

S. Senftenberg has also previously been reported as the cause of serious human infections (14–16). *S.* Senftenberg is well recognized as being common among poultry (17), but it has also been associated with infant formula (18), mussels (19), and vegetables (16, 20, 21). It is noteworthy that one of the patients claimed to have consumed vegetables prior to onset of symptoms. *S.* Senftenberg has the ability to adhere to plant leaves, perhaps contributing to infections in such cases (20). A similar case of one resistant *S.* Senftenberg isolate was recently reported for a traveler returning from Egypt, indicating the importance of this resistant serovar in Africa (22).

In conclusion, we have reported here cases from Zambia of extremely drug-resistant *S.* Senftenberg isolates that caused severe human infections and for which there were very few treatment options. We speculate that the clones are emerging and suggest that public health authorities become alert for any further dissemination.

ACKNOWLEDGMENTS

This work was funded by the Center for Genomic Epidemiology (www.genomicepidemiology.org) and by the World Health Organization Global Food-Borne Infections Network (www.who.int/gfn).

We also thank the Ministry of Health, Zambia, for permission to submit the isolates for study.

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