

**UNIVERSITY OF GHANA
COLLEGE OF BASIC AND APPLIED SCIENCES**

**MOLECULAR SUBTYPING AND GENE EXPRESSION ANALYSIS OF DRUG
RESISTANT INVASIVE NON-TYPHOIDAL *SALMONELLA***

BY

**RITA OWUSU ADJEI
(10419727)**

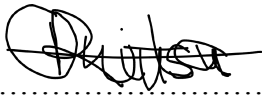
**A THESIS SUBMITTED TO THE SCHOOL OF GRADUATE STUDIES IN PARTIAL
FULFILMENT FOR THE REQUIREMENT OF THE AWARD OF DEGREE OF
MASTER OF PHILOSOPHY IN MOLECULAR CELL BIOLOGY OF INFECTIOUS
DISEASES**

DEPARTMENT OF BIOCHEMISTRY, CELL AND MOLECULAR BIOLOGY

JULY 2019

DECLARATION

I, Rita Owusu Adjei, hereby declare that the study was carried out at the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, under the combined supervision of Dr. Samuel Duodu and Dr. Lydia Mosi. I also declare that information derived from published and unpublished works of others has been duly acknowledged. I certainly certify that the thesis has not been presented elsewhere for the award of any degree.



Date... 21/10/2020 ..

RITA OWUSU ADJEI

(Student)



Date... 21/10/2020 ..

DR. SAMUEL DUODU

(Principal Supervisor)



22nd October 2020
Date.....

DR. LYDIA MOSI

(co-Supervisor)

DEDICATION

I dedicate this work to my family and friends. They made me reach this far.

ACKNOWLEDGMENT

I would like to express my deepest appreciation to Dr. Samuel Duodu. Without his guidance and assistance, this work would not have been possible. I express my appreciation to Dr. Lydia Mosi for her support and assistance to the success of this work.

I am grateful to Dr. Patrick Arthur for his immense contribution to my academic work. I thank Francis Krampah, Ethel Blessie and Michael Yakass for their valuable time and suggestions. I appreciate the efforts of Felix Ansah, Jonathan Suurbaar and members of Infectious Disease Research Laboratory (IDRL) especially by providing laboratory space for my molecular experiments. I thank Barbara Mensah, Samuel Asenso, Michelle Buckman, Evelyn Baaba Quansah, and my all colleagues for their extreme support to my academic career. I also thank Vivian Wellington and Genevieve Nuamah for their assistance and support during my project.

I thank all lecturers and staff members at the Department of Biochemistry, Cell and Molecular Biology for equipping me with the necessary skills and knowledge for a successful career. I am especially thankful to colleagues at Kumasi Centre for Collaborative Research for providing me with archive isolates for my project work.

My outmost gratitude goes to Professor Gordon Awandare who financially supported my project through World Bank Africa Centre for Excellence grant (ACE02-WACCBIP-Awandare). I deeply appreciate the full scholarship award I obtained as Brilliant and Needy student funded by WACCBIP-World Bank ACE. This award went a long way to provide for my financial needs throughout my master's program.

TABLE OF CONTENTS

DECLARATION	i
DEDICATION	ii
ACKNOWLEDGMENT.....	iii
TABLE OF CONTENTS.....	iv
LIST OF FIGURES	vii
LIST OF TABLES.....	viii
LIST OF ABBREVIATION	ix
ABSTRACT.....	xi
CHAPTER ONE	1
1.0 INTRODUCTION	1
1.1 Background	1
1.2 Problem Statement and Justification	5
1.2.1 Problem Statement.....	5
1.2.2 Justification.....	5
1.3 Hypotheses	6
1.4 Aim	6
1.5 Objectives	6
1.5.1 To determine the frequency of ST313 in clinical isolates	6
1.5.2 To characterize the resistance mechanisms of ST313 multidrug resistant clones.....	7
CHAPTER TWO	8
2.0 LITERATURE REVIEW	8
2.1 <i>Salmonella</i> classification	8
2.1.1 Systems of <i>Salmonella</i> Classification.....	8
2.1.2 Nomenclature.....	9
2.2 Major <i>Salmonella</i> serovars	10
2.2.1 Typhoidal <i>Salmonella</i> serovars	10
2.2.2 Non-typhoidal <i>Salmonella</i> serovars.....	11
2.2.3 Invasive Non-Typhoidal <i>Salmonella</i> Infections	12
2.3 Clonal Diversity and Spread invasive <i>Salmonella</i>	13
2.4 Non-Typhoidal <i>Salmonella</i> (NTS) Ecology, Pathogenesis, and Immune Response	14
2.4.1 Ecology	14

2.4.2 Infective dose and incubation period.....	15
2.4.3 Colonization, invasion, and immune response mechanisms	15
2.5 Laboratory identification	18
2.5.1 Bacterial Culture.....	18
2.5.2 Serotyping.....	18
2.5.3 Biochemical Tests.....	19
2.5.4 Phage Typing.....	19
2.5.5 Pulse-Field Gel Electrophoresis (PFGE).....	20
2.5.6 Quantitative Polymerase Chain Reaction	20
2.5.7 Multi-Locus Sequence Typing (MLST).....	21
2.5.8 Whole Genomic Sequencing	21
2.6 Antimicrobial agents and resistance.....	22
2.6.1 Mode of action and mechanism of resistance.....	22
2.6.2 Epidemiology of resistance in sub-Saharan Africa	23
2.6.3 Methods for Studying <i>Salmonella</i> antibiotic resistance mechanisms	24
CHAPTER THREE	26
3.0 Methodology	26
3.1 Characteristics of <i>Salmonella</i> Isolates	26
3.1.1 Sample size	26
3.1.2 Study Design.....	26
3.1.3 Bacterial isolates	27
3.1.4 Culturing conditions and morphological examination	27
3.2 Susceptibility Testing	28
3.3 DNA Extraction	29
3.4 <i>Salmonella</i> Genotyping	30
3.4.1 Amplification of virulence gene <i>st313-td</i> by polymerase chain reaction (PCR).....	30
3.4.2 Multi-Locus Sequence Typing	30
3.5 Antimicrobial Resistance Markers Amplification	31
3.6 Minimum Inhibitory Concentration Determination and Ciprofloxacin Resistance Induction	32
3.7 Induction Assay	33
3.8 Growth Comparison of Ciprofloxacin Resistant Isolates.....	33
3.9 PCR Amplification of Ciprofloxacin Resistance Genes.....	34

3.10 Gene Expression Analysis by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)	36
3.10.1 RNA Extraction	36
3.10.2 Gene Expression Analysis by RT-qPCR.....	37
3.10.3 DNA Sequence Analysis of Resistance target genes.....	38
3.11 Statistical Analyses	38
CHAPTER FOUR.....	39
4.0 RESULTS	39
4.1 Characteristics of <i>Salmonella</i> Isolates	39
4.2 Antimicrobial Susceptibility Profile	39
4.3 Detection of Antimicrobial Resistance Gene Markers	43
4.4 <i>Salmonella</i> genotyping by MLST	45
4.4.1 Detection of Virulent trait <i>st313-td</i> gene.....	45
4.4.2 Comparison of <i>Salmonella</i> Sequence Types and Resistance Phenotypes.....	45
4.5 Characterization of ST313 Resistance to Ciprofloxacin	48
4.5.1 MIC determination and growth patterns of ciprofloxacin resistant isolates.....	48
4.5.2 Expression analysis of genes related to ciprofloxacin resistance	50
4.5.3 DNA Sequence Analysis of Resistance Target Gene	53
CHAPTER FIVE	54
5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION	54
5.1 Discussion	54
5.2 Conclusion	60
5.3 Limitations and Recommendations	60
REFERENCES	62
APPENDIX.....	81

LIST OF FIGURES

Figure 1: <i>Salmonella</i> invasion and colonization.....	17
Figure 2: Representative 96-well plate set up for broth microdilution assay	32
Figure 3: Representative agar plate of antimicrobial susceptibility testing.....	40
Figure 4: Antimicrobial susceptibility profile.....	41
Figure 5: Distribution of antimicrobial resistance gene markers among <i>S. enterica</i> isolates.....	44
Figure 6: Comparison of growth patterns among <i>Salmonella</i> ST313 isolates.....	49
Figure 7: Normalized log ₁₀ transformed mRNA transcript levels of target genes in <i>Salmonella</i> isolates.....	51
Figure 8: Agarose gel representation of PCR amplicons.....	52
Figure 9: Sample Visualization of SNPs.	523

LIST OF TABLES

Table 1: Primers for Ciprofloxacin Resistance Targeted Genes.....	35
Table 2: Source, Serovars, and Drug Resistance Profile of <i>Salmonella enterica</i> Isolates.....	42
Table 3: Summary of st313-td gene amplification and Multi-Locus Sequence Typing.....	46
Table 4: Phenotypic and Genotypic Characterization of <i>S. Dublin</i>	46
Table 5: Relationship between <i>S. Typhimurium</i> Genotype, Resistance Profile and Invasive Virulence Trait	47
Table 6: Summary of Minimum Inhibitory Concentration and Resistance Characteristics	48
Table 7: Ciprofloxacin Concentrations used for RNA Expression.....	511

LIST OF ABBREVIATION

AFI	Acute Febrile Illness
AST	Antimicrobial Susceptibility Testing
ATCC	American Type Culture Collection
BHI	Brain heart Infusion
CDC	Centre for Disease Control
CLED	Cysteine-Lactose and Electrolyte Deficient
CLSI	Clinical and Laboratory Standards Institute
Ct	Cycle Threshold
DT	Definitive Phage Type
GI	Genome Island
IL	Interleukins
KCCR	Kumasi Centre for Collaborative Research
LB	Luria Bertani
LPS	Lipopolysaccharide
MDR	Multi-Drug Resistant
MHA	Mueller Hinton Agar
MHB	Mueller Hinton Broth

MIC	Minimum Inhibitory Concentration
MLST	Multi-Locus Sequence Typing
NCBI	National Center for Biotechnology Information
NCCLS	National Committee for Clinical Laboratory Standards
NTS	Non-Typhoidal <i>Salmonella</i>
OD	Optical Density
PAMPs	Pathogen Associated Molecular Patterns
PFGE	Pulse Field Gel Electrophoresis
PRR	Pathogen Recognition Receptor
PT	Phage Type
QRDR	Quinolone Resistant Determining Region
RT-qPCR	Reverse Transcription-quantitative Polymerase Chain Reaction
SCV	Salmonella Containing Vacuole
SPI	<i>Salmonella</i> Pathogenicity Island
ST	Sequence Type
T3SS	Type Three Secretory Systems
WACCBIP	West Africa Centre for Cell Biology of Infectious Pathogens
WHO	World Health Organization

ABSTRACT

Invasive Non-Typhoidal *Salmonella* infections have become widespread in sub-Saharan Africa. Predominant hyper-virulent clone *S. Typhimurium* ST313 is found to be associated with bloodstream invasion and multidrug resistance. Despite its importance, limited information is available on the circulating genotypes causing invasive non-typhoidal salmonellosis in Ghana.

A total of fifty-one NTS isolates from pediatric blood samples was used for this study. The resistance profiles of isolates were assessed against 8 classes of antibiotics by disc diffusion method followed by PCR detection of resistance markers. Molecular detection of the presence of a virulence associated gene, *st313-td*, was used as plausible test to select isolates for Multi-Locus Sequence Typing (MLST) analysis. Additionally, the mechanism of ciprofloxacin resistance was explored by monitoring changes in expression levels of important targeted genes including *gyrA*, *acrA*, *ydhJ*, *emrD*, and *soxR* under drug pressure using reverse transcription quantitative PCR.

Antibiotic susceptibility testing results showed all isolates were completely susceptible to imipenem, ceftriaxone and ceftazidime. In most cases, multidrug resistance (including reduced susceptibility to fluoroquinolones) were frequently recorded among *S. Typhimurium* isolates (63%, n=32/51). On the contrary, nine out of ten *S. Dublin* and the five unknown serovars were susceptible to all antibiotics used for screening. The virulence gene *st313-td* was detected in only 27% (n=14/51) isolates. Eight (8) other isolates showing varying resistance phenotypes were selected in addition to the 14 positive isolates for typing and analyses by MLST. Out of twenty-two (22) selected isolates, 82% (n=18), 4% (n=1) and 14% (n=3) were *S. Typhimurium* ST313, *S. Typhimurium* ST19, and *S. Dublin* ST 10 respectively. Most importantly, it was observed that about 83% (n=15/18) of *S. Typhimurium* ST313 clones were multidrug resistant (MDR).

Likewise, ten selected isolates for PCR analyses tested positive for the resistance markers; *CatA1* (30%), *StrA* (90%), *Bla_{Tem}* (50%), *TetA* (30%) & *TetB* (10%), *Sul1* (80%) & *Sul2* (100%) and *aadA* (70%). The normalized transformed mRNA transcript levels showed significant variations in gene expression of *gyrA*, *acrA*, *ydhJ*, *emrD* and *soxR* among susceptible, intermediate and laboratory induced resistant isolates. Further, sequence data analysis detected a single nucleotide polymorphism at position 324 resulting in a mutation (Phe → Asp) in the *gyrA* of clinical intermediate isolate, although there were no significant mutations in *acrA*, *ydhJ*, *emrD* and *soxR* genes among the three test isolates.

From the results, high frequencies of multidrug resistant invasive NTS pathogens carrying plasmid-borne resistance gene markers were found. Also, reduced susceptibility to fluoroquinolones and cephalosporin were observed which calls for increased surveillance and management of antimicrobial resistance to guide treatment. Detection of a SNP in *gyrA* gene and variations in mRNA levels suggest possible genetic and transcriptional regulation mechanisms employed by *Salmonella* pathogen to withstand drug stress. Therefore, further investigations are required to understand these mechanisms which will assist in developing alternative therapeutic interventions.

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background

Salmonellosis remains a significant disease problem to humans and livestock. Over 500million cases of illness due to *Salmonella* infections are reported annually and about 40% are cases found in children (Majowicz et al., 2010; Kariuki et al., 2015). Usually patients suffering from salmonellosis present with clinical symptoms such as fever, malaise, diarrhea, nausea and sometimes accompanied with vomiting (Chen et al., 2013). These conditions are expected to resolve within some few days but can progress and become complicated in patients with suppressed immunity. The disease incidence is highest during the peak of rainfall in the tropics, where pathogen burden and transmission is expected to be high (Guiraud et al., 2017). Zoonotic transmission and fecal oral transmission are reported as the major routes of infections in humans (MacLennan & Levine, 2013).

S. enterica is one of the major causative agents of salmonellosis, which belongs to the family of Gram negative facultative anaerobes called Enterobacteriaceae (Giannella, 1996). They are largely associated with acute gastrointestinal infections in humans (MacLennan & Levine, 2013; Labi, et al., 2014; Ashton, et al., 2017). These species of organisms are further divided into 6 subspecies and about 2600 serovars (McClelland et al., 2001; Issenhuth-Jeanjean et al., 2014). Generally, non-typhoidal *Salmonella* (NTS) serovars include all the serotypes except those that cause typhoid fever like *Salmonella* Typhi and *Salmonella* Paratyphi (Feasey et al., 2012). *S. Typhimurium* and *S. Enteritidis* are the most predominant NTS serovars globally associated with human salmonellosis (Gordon et al., 2008; Langridge et al., 2009). Unlike *S. Typhi* and *S. Paratyphi* which

are human host restricted and known for their systemic infections, most NTS serovars cause self-limiting infections in a broad range of hosts including farm animals (Chen et al., 2013).

In sub-Saharan Africa, some strains of NTS species have evolved into invasive forms, representing one of the major causes of bloodstream infections especially in malnourished children below age five, HIV-AIDS and severe malaria patients (MacLennan & Levine, 2013; Kariuki, et al., 2015). *S. Typhimurium* is the most predominant pathogenic serovar causing invasive NTS among children and immunocompromised individuals (Kingsley et al., 2009). Previous studies based on sub-genomic analysis identified less variability among NTS pathogens. However, recent reports have shown that these *S. Typhimurium* variants in sub Saharan Africa belong to a distinct genotype known by multi-locus sequence typing as ST313 (Kingsley et al., 2009; Okoro et al., 2012; MacLennan & Levine, 2013; Moon et al., 2015; Okoro et al., 2015). This genotype is known for its hyper-virulence activities and host specificity. Like *S. Typhi*, a whole genome analysis of invasive NTS serovars showed evidence of significant genome degradation and accumulation of non-coding genes which has markedly contributed to their human host adaptation (Kingsley et al., 2009). The serovars possess repertoire of virulence genes which has enabled them to cause systemic infections (Kingsley et al., 2009). For example, the gene *st313-td* is a virulence trait found on the genome island of bloodstream *S. Typhimurium* ST313 and *S. Dublin* pathogens which plays a significant role in host invasion mechanisms (Leekitcharoenphon et al., 2013; Herrero-Fresno et al., 2014). Molecular phylogenetic studies suggest that the ST313 clones evolved into two lineages over five decades ago during the emergence of HIV pandemics (Okoro et al., 2015). The sequence type ST313 was previously thought to be confined in sub Saharan Africa until a recent study reported its emergence in patients in the United Kingdom (Ashton et al., 2017).

Several hospital-based studies in Ghana have reported that invasive salmonellosis represented majority of bloodstream infection cases with the most predominant pathogens being *S. Typhimurium* and *S. Enteritidis* (Labi et al., 2014; Eibach et al., 2016; Andoh et al., 2017). In addition to causing high mortality and morbidity in sub-Saharan Africa, the resistance of iNTS pathogens to multiple antimicrobial agents is of growing concern (Mandomando et al., 2010; Kariuki et al., 2015; Eibach et al., 2016; Andoh et al., 2017). Strains expressing multidrug resistance (MDR) phenotype to sulphamethoxazole-trimethoprim (SXT), ampicillin, and chloramphenicol have been recorded in most sub-Saharan African countries thus limiting the value of these agents for management of invasive NTS (Kariuki, et al., 2015). Prolonged exposure to certain antibiotics has contributed to positive selection for resistance strains under drug pressure (Gordon, 2008). This has also been found to be one of the driving forces behind bacterial pathogen evolution (Kingsley et al., 2009; Okoro et al., 2012; Okoro et al., 2015).

Ciprofloxacin is a bactericidal antibiotic belonging to the group of first-generation fluoroquinolones produced in the 1980s. This broad-spectrum drug has proven active against several bacterial infections since its production until reports of resistance emerged in the early 2000s (Chattaway et al., 2016). Ciprofloxacin is still one of the most prescribed antibiotics for treatment of recalcitrant Gram negative/positive bacteria (Redgrave et al., 2014; Chattaway et al., 2016; Eibach et al., 2016). The drug's mode of action is by inhibiting bacterial DNA replication through its interaction with DNA gyrase or Topoisomerase IV enzymes which belong to the Topoisomerase Type II (Redgrave et al., 2014; Casper et al., 2017). The primary target in most Gram negative bacteria, such as *Salmonella*, is the DNA gyrase which is an enzyme that regulates negative supercoiling of the chromosomal DNA during replication (Casper et al., 2017). Mechanisms like point mutations in target genes resulting in conformational changes in enzymes,

horizontal acquisition of plasmids mediating resistance, and upregulation of efflux pumps to reduce drug accumulation in bacterial cells have been proposed by other studies to contribute to resistance (Giraud et al., 2000; Redgrave et al., 2014). Indeed, fluoroquinolone resistance has been found to involve several factors and not one could be associated with complete resistance (Redgrave et al., 2014). Therefore, it is important to study the resistance mechanisms employed by the hyper-virulent *Salmonella* strains against ciprofloxacin, a clinically relevant antibiotic administered during treatment of bacterial bloodstream infections.

1.2 Problem Statement and Justification

1.2.1 Problem Statement

ST313 is a hyper-virulent clone found in many countries in sub-Saharan Africa. Invasive NTS pathogens are the most frequently isolated blood-borne pathogens in Ghana (Nielsen et al, 2012; Labi et al., 2014; Cruz Espinoza et al., 2016). There has been reports of emerging invasive NTS bacteria with reduced susceptibility to fluoroquinolones which has become the first-line drug of choice for treatment of bacteremic infections in Ghana (Eibach et al., 2016; Andoh et al., 2017). This may compromise disease treatment and increase economic burden.

1.2.2 Justification

It is important as a country to identify the circulating genotypes to aid disease surveillance, management, and future epidemiological studies. The *st313-td* gene may be present in invasive NTS clinical isolates and therefore could be used as a preliminary screening method for identifying virulent clones.

Although, there are some reports on the resistance mechanisms of *Salmonella* serovars at the genomic (Kingsley et al., 2009; Hayden et al., 2016; Kagambèga et al., 2018) and transcriptomic levels (Li et al., 2017), these studies attempted to elucidate the mechanisms by inducing resistance in these pathogens against antimicrobial agents. A study by Eibach and his group on febrile Ghanaian children with invasive *Salmonella* infections found that some of the *Salmonella* isolates showed reduced ciprofloxacin susceptibility by employing resistance mechanisms such as mutated drug targets (particularly gyrase) and/or through the action of efflux pumps (Eibach et al., 2016). However, the contributions of these mechanisms were not clearly demonstrated. This suggests that other genes or pathways may be involved in the resistance of *Salmonella* to ciprofloxacin which

could be investigated using a gene expression approach. No study has yet reported on the transcriptomic analysis of clinical *S. Typhimurium* ST313 isolates with multidrug resistance phenotype.

Therefore, this current study was to identify predominant clones among invasive NTS serovars Typhimurium and Dublin and to carry out gene expression analysis on identified multidrug resistant hyper-virulent *S. Typhimurium* clone.

1.3 Hypotheses

- i. The genotype ST313 is a predominant circulating clone among Ghanaian febrile children with NTS bloodstream infections
- ii. The ST313 clones are hyper-virulent clones associated with high frequency of reduced ciprofloxacin susceptibility
- iii. Molecular analyses will identify possible resistance mechanisms in hyper-virulent ST313 clones.

1.4 Aim

To screen and elucidate the possible mechanisms of multidrug resistance in invasive NTS ST313 clones.

1.5 Objectives

1.5.1 To determine the frequency of ST313 in clinical isolates

- a) determine the antibiotic susceptibility profiles of invasive NTS serovars
- b) identify drug resistance molecular markers
- c) screen for the presence of *st313-td* in invasive NTS serovars

- d) identify the clones of invasive NTS serovars by multi-locus sequence typing

1.5.2 To characterize the resistance mechanisms of ST313 multidrug resistant clones

- a) Determine the Minimum Inhibitory Concentrations of Ciprofloxacin against selected *S. Typhimurium* isolates
- b) Induce resistance to ciprofloxacin in *S. Typhimurium* sensitive isolate
- c) Compare the expression of genes regulating resistance to ciprofloxacin by Reverse Transcription-quantitative PCR

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 *Salmonella* classification

2.1.1 Systems of *Salmonella* Classification

Salmonella belongs to a family of Gram negative, rod shaped bacteria called Enterobacteriaceae (Crum-Cianflone, 2008). The genus *Salmonella* is of clinical importance as members are generally implicated in diverse disease pathogenesis in humans and animals (Su & Chiu, 2007). *Salmonella* was first discovered in 1884 by D. E. Salmon as *Salmonella choleraesuis* from the intestinal tracts of pigs (Smith (1894), cited in Su & Chiu, 2007). Since then, the nomenclature system of the bacteria has evolved over several years and it is expected to change in the future (Brenner et al., 2000; Su & Chiu, 2007). Nomenclature of the bacteria presents a better way to understanding the organism's taxonomy (Tindall et al., 2005).

The Judicial Commission of the International Committee on the Systematics of Prokaryotes is among the organizations that approve the taxonomic classification and nomenclature system of *Salmonella* (Shelobolina et al., 2004). The antigenic formula, also called White-Kauffman-Le Minor Scheme, is one of the recent methods used for classifying *Salmonella* serovars which is based on their antisera and biochemical reactivity of (Issenhuth-Jeanjean et al., 2014). This scheme is updated yearly or biennially by WHO Collaborating Centre for Reference and Research on *Salmonella* (Su & Chiu, 2005). According to Issenhuth-Jeanjean et al (2014), the 9th edition of the scheme was issued in 2007 with reports of supplement updates in 2010 and 2014. Moreover, Center for Disease Control has adapted the WHO Collaborating Centre nomenclature system in classifying *Salmonella* (Su & Chiu, 2005).

2.1.2 Nomenclature

Presently, the genus *Salmonella* is divided into two major species, *enterica* and *bongori* (Popoff & Le Minor, 1997; Su & Chiu, 2007). Although a newly characterized species was identified and accepted by the Judicial Commission as *subterranea* (Shelobolina et al., 2004; Su & Chiu, 2005), such claim has been refuted by WHO Collaborating Center (Issenhuth-Jeanjean et al., 2014). *Salmonella enterica* constitutes the largest bacterial species under the genus. This group is further divided into six subspecies denoted by Roman numerals (or by names): subspecies I (*enterica*), subspecies II (*salamae*), subspecies IIIa (*arizonae*), subspecies IIIb (*diarizonae*) subspecies IV (*houstenae*), subspecies IV (*indica*) (Su & Chiu, 2007; Brenner et al., 2000; Popoff & Le Minor, 1997). Classification based on serology further categorize the subspecies into variants; popularly accepted by the Judicial Commission as serovars (Su & Chiu, 2007). Currently, there are about 2659 serovars identified under the species according to White-Kauffman-Le Minor scheme (Issenhuth-Jeanjean et al., 2014).

Before 1966, most *Salmonella* serovars were named based on their geographic origin, host, or pathogenic characteristics of the first isolated strain (Grimont & Weill, 2007; Su & Chiu, 2007). The names of clinically important serovars were, however, officially retained in 2005 even after the amendment of *Salmonella* nomenclature (Su & Chiu, 2007). A typical example is *Salmonella enterica* subspecies *enterica* serovar Typhimurium or simply *Salmonella* Typhimurium. Unnamed and newly discovered serovars after 1966 adopted the name given by their antigenic formula. In this case, the subspecies name is written in Roman numeral and is followed by their antigenic factors starting with O antigens (somatic) then H antigens (phase I, and if present, phase II) (flagellar), all of which are separated by a colon sign. Example is *Salmonella* serovar II 39:z₁₀:z₆ (Su & Chiu, 2007).

2.2 Major *Salmonella* serovars

Salmonella enterica belongs to a group of pathogenic bacteria often associated with mild to severe diarrheal or enteric diseases. The serovars may be grouped into two major types: typhoidal and non-typhoidal *Salmonellae*. The typhoidal *Salmonella* pathogens and therefore cause typhoid fever in healthy human hosts (Ashton et al., 2017). Their host restricted nature is suggested to be due to evolutionary development to become systemic after a protracted period (Ashton et al., 2017). Also, there is limited information to prove their survivability in other non-human hosts. Unlike typhoidal *Salmonella*, NTS consists of a large number of *Salmonella enterica* serovars mainly associated with gastroenteritis. They have a wide host range and easily adaptable to the environment.

2.2.1 Typhoidal *Salmonella* serovars

Typhoid disease arises when an individual is infected with either *S. Typhi* or *S. Paratyphi A* and *B* (Zand et al., 2003). There are about 22 million typhoidal cases reported each year and over 200,000 result in death (Crump et al., 2004). Healthy individuals in endemic countries in Asia and sub-Saharan Africa may suffer from the disease burden (Feasey et al., 2012). Consumption of fecal contaminated food and water is considered as one of the commonest routes of infection. Once ingested, the bacteria disseminate into the bloodstream (Groß et al., 2011). Typhoid fever cases are predominant in Asian countries although there have been increasing incidence rates in Africa over the years (Chau et al., 2007; Mweu & English, 2008; Groß et al., 2011; Kariuki et al., 2015). A study in Ghana estimated adjusted incidence rate of the disease based on setting and age as 100 cases per 100,000 person-years of observation with high proportion observed in rural children (Cruz Espinoza et al., 2016). Other studies have reported similar incidence rates in sub-Saharan Africa (Mweu & English, 2008). Generally, the incidence rate in Africa is 10 to 100 cases per

100,000 person-years of observation (Mweu & English, 2008). However, current report showed varied proportions ranging from 0 to 400 cases per 100,000 person-years of observation in some countries (Marks et al., 2017).

Studies have also shown marked increase in the proportion of drug resistant typhoid pathogens over the years (Groß et al., 2011). There have been several reports of *S. Typhi* resistance to 3rd and 4th generation antibiotics including cephalosporins and cepheids, carbapenems and fluoroquinolones (Kariuki et al., 2015). Increased proportion of *S. Typhi* MDR isolates ranging from 63% to 80% has been reported in Asia (Chau et al., 2007). Ghana Health Service recommends administration of cephalosporins and fluoroquinolones as well as resuscitation of loss fluids in severe cases (Andoh et al., 2017). Although there are WHO recommended typhoid vaccines, these are not widely being introduced especially in resource limited areas (Kariuki et al., 2015).

2.2.2 Non-typhoidal *Salmonella* serovars

Non-typhoidal *Salmonella* is known to cause gastrointestinal infection in both humans and animals. The Infection often lead to self-limited diarrhea in immune competent individuals. However, the disease conditions in sub-Saharan Africa tend to have fatal outcomes particularly in immune suppressed individuals with adjusted incidence rate of about 200 cases/100,000 person-years and case fatality rate ranging between 10 and 20% (Feasey et al., 2012; Marks et al., 2017; Uche et al., 2017). Some serovars classified under this group tend to be host adapted. For example, *S. Gallinarum* and *S. Dublin* are most frequently isolated from poultry and cattle respectively (Langridge et al., 2015).

Many studies in sub-Saharan Africa have reported high prevalence of *S. Enteritidis* and *S. Typhimurium* in NTS infections among other NTS serovars in humans (Guiraud et al., 2017; Uche

et al., 2017). In the USA, *S. Typhimurium* is implicated as the predominant pathogen causing foodborne diseases (Hendriksen et al., 2011). Similar case is observed in Europe, Asia and Southern America with the proportion of *S. Typhimurium* and *S. Enteritidis* proportions ranging from 17% to 45% (Hendriksen et al., 2011).

2.2.3 Invasive Non-Typhoidal *Salmonella* Infections

Bacterial bloodstream infection is considered as one of the most fatal health conditions especially among Children (Berkley et al., 2005; Groß et al., 2011; Feasey et al., 2012; Nielsen et al., 2012; Laupland & Church, 2014; Cruz Espinoza et al., 2016; Obeng-Nkrumah et al., 2016). Many studies have shown that the bacterial pathogens frequently isolated from blood include *Staphylococcus aureus*, *Streptococcus pneumoniae*, *Salmonella enterica* and *Escherichia coli* (Groß et al., 2011; Nielsen et al., 2012; Laupland & Church, 2014).

Invasive NTS pathogens have been implicated in majority of bloodstream infections in sub-Saharan Africa. A study by Nielsen et al (2012) showed that the annual cumulative incidence rate of invasive NTS bloodstream infections was 25.2 incidences per 1000 cases among rural Ghanaian children. The authors also reported the proportions of invasive NTS, *Staphylococcus aureus*, *Streptococcus pneumoniae* and *S. Typhi* as 53.3%, 13.2%, 9.1% and 7% respectively out of a total of 238 bloodstream pathogens identified (Nielsen et al., 2012). Another study in a tertiary hospital in Ghana reported invasive NTS (64%, n=115) as the most predominant cause of *Salmonella* bloodstream infections (Labi et al., 2014). Invasive NTS, among other bacterial pathogens, have been implicated in high mortality rate among cancer patients and neonates in Ghana (Obeng-Nkrumah et al, 2015; Labi et al., 2016). Similar patterns have been observed according to other studies in sub-Saharan Africa (Lunguya et al., 2013; Mandomando et al., 2015; Marks et al., 2017). For example, a study in Burkina Faso described about 56% of 118 systemic pathogens as iNTS

and high proportions were found in infants (Guiraud et al., 2017). In Malawi, neonates were found to be at a higher risk of suffering from bacteremia predominantly caused by invasive NTS and *Streptococcus pneumoniae* infections (Milledge et al., 2005). A study from Mozambique also reported high prevalence of invasive NTS and Pneumonia among children suffering from community-acquired bacterial bloodstream infections (Sigauque et al., 2009).

Several factors have been found to contribute to iNTS infections. These factors may be categorized as host and non-host. Mostly, host factors include age, malnutrition, severe malaria, pneumonia, meningitis, and HIV/AIDS coinfections (Feasey et al., 2012; MacLennan & Levine, 2013). On the other hand, the major non-host factor may be provision of effective healthcare services which may be restrained as a result of large endemic population, limited resources for effective diagnosis, and also the emergence of multidrug resistant bacteria (Kariuki et al., 2015).

2.3 Clonal Diversity and Spread invasive *Salmonella*

Salmonella has a clonal population structure displaying relatively low recombination and high recombination rates between and within subspecies respectively. Despite their close genetic relatedness, the disease phenotype and epidemiology can vary substantially between *Salmonella* subtypes. About 10% of *S. enterica* genome is made up of transposons, insertion sequences and plasmids which encode genes contributing to their serovar characteristics and clonal diversity (McClelland et al., 2001). In the early years of molecular sub-typing, studies based on Pulse Field Gel Electrophoresis and PCR amplification were not discriminatory enough to determine phylogenetic diversity among clones (Kingsley et al., 2009). Thus, application of sequence based MLST analysis has become widespread (Kagambèga et al., 2008). According to Kingsley et al (2009), there are about 20 distinct sequence types of *S. Typhimurium* available in the MLST

database for *S. enterica*. The authors reported in their study that about 31 (100%) and 13 (65%) of *S. Typhimurium* isolates from Malawi and Kenya respectively belonged to ST313 sequence type. They also discovered one Kenyan isolate with genotype ST394 which shared similar genetic identity to ST313. Six out of the twenty Kenyan isolates were ST19, a globally distributed clone which causes gastroenteritis. *S. Typhimurium* genotypes LT2, SL1344 and DT104 were the ubiquitous ST19 *Salmonella* serovars (Kingsley et al., 2009). On the other hand, DT56 and D23580 were of ST313 genotype (Kingsley et al., 2009). Unlike NTS, *Salmonella* Typhi is predominantly found in Asia and the most frequently isolated genotype is CT18 (McClelland et al., 2001). A virulent strain of *S. Typhi*, H58 Haplotype, has become the most predominant typhoidal pathogen in Asia (Kariuki et al., 2015).

2.4 Non-Typhoidal *Salmonella* (NTS) Ecology, Pathogenesis, and Immune Response

2.4.1 Ecology

Non-Typhoidal *Salmonella* is a ubiquitous bacterium known to survive in biotic and abiotic conditions (Crum-Cianflone, 2008). In the environment, they are found in various water sources, plant and animal food products and animal feed (Hendriksen et al., 2011; Olobatoke, 2017). In living host, these pathogens are often isolated from the gastrointestinal tract (Olobatoke, 2017). According to Olobake (2017), one of the commonest routes of transmission occurs during handling of animal products. During slaughter and dressing process, the pathogen passes through the intestines of infected animal into the feces which may contaminate food product (Crum-Cianflone, 2008; Olobatoke, 2017). In addition, crop farming in soil and water reservoirs may lead to contamination of produce particularly fruits and vegetables (Hanning et al., 2009).

2.4.2 Infective dose and incubation period

NTS infection, in most cases, may not result in disease symptoms in healthy adults. Studies have shown that infective dose for NTS infection is about 10^6 cfu/ml (Crum-Cianflone, 2008). Contrarily, typhoidal *Salmonella* infections cause disease at a lower infective dose of about 10^3 cfu/ml (Blaser & Newman, 1982).

In general, the number of bacterial cells needed to cause salmonellosis largely depends on several factors including *Salmonella* strain type and the individual's health status (Blaser & Newman, 1982). Direct human contact has been implicated in the transmission of NTS infection (Crum-Cianflone, 2008). This could be due to unsanitary conditions and unhygienic lifestyles adopted by some people. Asymptomatic individuals may serve as major source of transmission among endemic population (Kariuki et al., 2015). The incubation period for salmonellosis is between 16 hours to 20 hours which is followed by a prodromal stage where patients suffer from diarrhea (Glynn & Palmer, 1992). Diarrhea, fever, and vomiting are examples of disease symptoms that may last for about 4-7 days and in severe cases, may result in bloodstream infections.

2.4.3 Colonization, invasion, and immune response mechanisms

Salmonella pathogen is found to be an intracellular facultative bacterium (de Jong et al., 2012). The pathogen may invade phagocytic and non-phagocytic host cells (Figure 2). After ingestion, *Salmonella* traverses the gastro-intestines where it attaches itself to the epithelial cells after passing through the mucosal walls (Cooke et al., 2007). The bacteria may invade M cells and enterocytes in the intestinal epithelia as well as dendritic cells (de Jong et al., 2012). As the bacteria transmigrate, some become phagocytosed by stationed macrophages and dendritic cells. Within the phagocytes, the bacteria form a protective vacuole called Salmonella Containing Vacuole

within which they grow and multiply (Santos et al., 2002; Olobatoke, 2017). While inside the vacuole, the pathogen secretes several effector proteins into the cytosol of the phagocytic cells via the type three secretory system-1 (TTSS3-1) which modulate the activity of host cell cytoskeleton to enhance pathogen survival (Knodler et al., 2010; de Jong et al., 2012).

In an *in vivo* study, Reis et al (2003) used the ileal ligated loop model in cattle to demonstrate the enteric pathogenesis of *S. Typhimurium* infections. The authors showed that the bacteria may invade enterocytes or M cells found in the follicle-associated epithelium of the Peyer's Patches. Within an hour of infection, *S. Typhimurium* pathogens rapidly invaded the intestinal tissues by first penetrating the apical region to the basal side of the epithelia. The bacteria then migrate to the lamina propria, a connective tissue beneath the epithelia, within 5 hours of infection (Reis et al., 2003). During this period, about 3 hours post-infection, innate immune cells, particularly neutrophils, lying underneath the epithelia sense and become activated by the presence of the pathogens (Santos et al., 2002; Reis et al., 2003).

Immune response is triggered by the interaction between the immune cell pattern-recognition receptors (PRRs) such as Toll-Like Receptors (TLRs) and pathogen-associated molecular patterns (PAMPs) such as LPS, resulting in the stimulation and activation of neutrophils and macrophages (de Jong et al., 2012). There is also increased production of pro-inflammatory cytokines particularly interleukins (e.g. IL-1 β , IL6, & IL17, TNF) which lead to further recruitment of several immune cells to the site (Santos et al., 2002; de Jong et al., 2012).

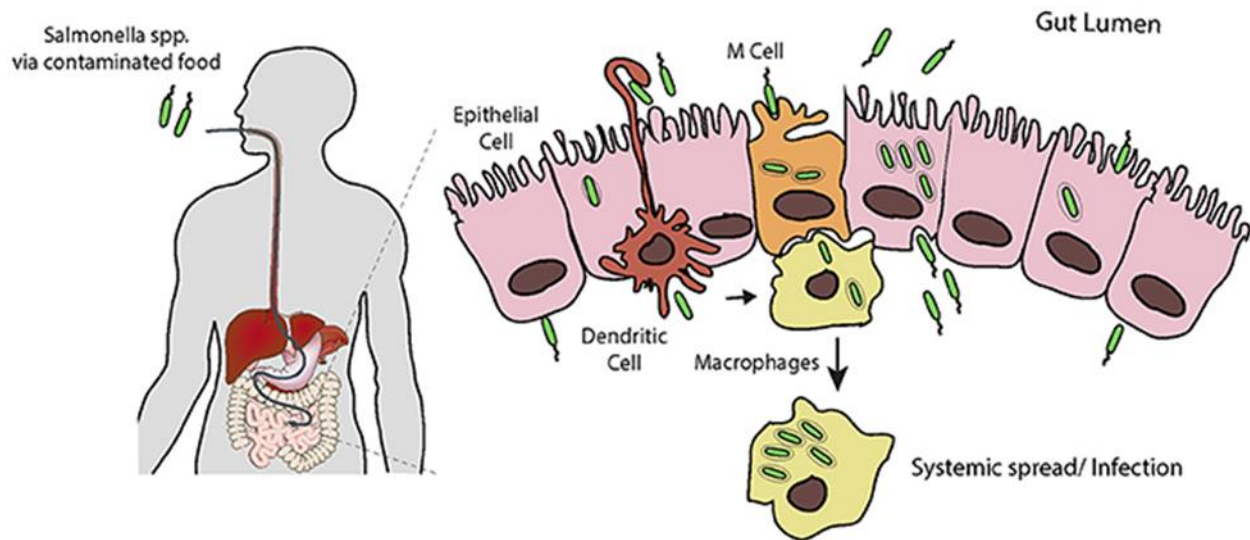


Figure 1: *Salmonella* invasion and colonization (adapted from Hume et al., 2017)

This action overwhelms the body leading to high levels of chloride ion secretion by epithelial cells into the intestinal lumen. This is eventually accompanied by osmotic influx of sodium ions and water resulting into severe diarrhea and dehydration in patients (Zhang et al., 2003).

NTS is often associated with systemic infections in immunocompromised patients (Gordon, 2008). Studies in murine models have shown that T3SS-2 mediates *Salmonella* bloodstream dissemination and survival in phagocytes (de Jong et al., 2012). Other studies have proposed several mechanisms leading to systemic infections in immunosuppressed individuals particularly HIV-AIDS patients. Some evidences suggest that bloodstream infections occur due to deficiency in immune responses. Defective cytokine production (e.g. IL-12, IL-23 and IL-17) may enhance NTS translocation to distant sites such as the mesenteric tissues, the lymph nodes, liver, gall bladder, and the spleen (Gordon, 2008; Raffatellu et al., 2008; de Jong et al., 2012). Also, MacLennan et al (2010) found that over-stimulation of Immunoglobulin G against lipopolysaccharide component of NTS in HIV-infected patients enabled the pathogen to escape killing by humoral immune response.

2.5 Laboratory identification

Most NTS infections may lead to mild or severe disease symptoms. For effective treatment, it is important to ascertain the presence of *Salmonella* as the etiological agent of the disease (Iddrisu, 2014). This could be done by effective diagnostic and classification methods (Laupland & Church, 2014). In addition, several laboratory-based methods of identification have assisted in disease surveillance and control (Hendriksen et al., 2011). The identification methods described below focuses on both clinical and reference laboratory investigations of *S. enterica*.

2.5.1 Bacterial Culture

Microbiological examination by culturing methods is considered as the gold standard for phenotypic identification of causative organisms (Iddrisu, 2014). In the case of diarrheal disease, stool culture may lead to identification of viable *Salmonella* pathogens. Blood culture is a mandatory diagnostic method for confirming bacteremic infections (Laupland & Church, 2014). Selective enrichment media have been used to distinguish between *Salmonella* serovars (Pierce et al., 2012). In most cases, bacterial culturing procedures are followed by other basic identification methods, particularly microscopy. Some drawbacks may include low sensitivity and low specificity. Insufficient specimen collection may preclude the isolation of positive pathogens (Laupland & Church, 2014). Another limitation as a result of contamination may lead to false positive results. Lastly, the procedure has slow turn-around time which means clinicians may obtain results after about 2-7 days.

2.5.2 Serotyping

An agglutination test is used to differentiate between *Salmonella* serovars based on immunological reactivity in the presence of polyvalent antisera (Slauch et al., 1995). During bloodstream infections, antibodies are produced against lipopolysaccharides (LPS), a constituent of the outer

membrane of Gram-negative bacteria (Henderson et al., 1996; Tuin et al., 2006). The LPS consists of Lipid A, O antigen, and a polysaccharide core. The endotoxic part of LPS is Lipid A which is highly conserved and is made up of two phosphate groups attached to glucosamine moieties (Tuin et al., 2006). Mostly, the immunogenic part is the O antigen. In some cases, like *S. Typhi*, the polysaccharide core produces Vi antigen which agglutinates in the presence of anti-Vi serum (Tuin et al., 2006). The Kauffman-White Scheme of Classification is based on the reaction of *Salmonella* to the various antisera therefore giving rise to diverse serovars (Slauch et al., 1995). Some major limitations include cross-reactivity of the test leading to false positive results and insufficient discrimination between sporadic infections and chronic cases.

2.5.3 Biochemical Tests

This is an assay carried out to identify bacteria based on their biochemical reactions and enzyme activity. A combination of several assays is used to determine different species and strains of *Salmonella* (Hemraj et al., 2013). For example, *Salmonella enterica* is a Gram-negative facultative anaerobe, with negative oxidase or urease activity, negative indole producers and non-lactose fermenters and it shows motility due to the possession of flagella (Hemraj et al., 2013). Using these and other bacterial characteristics, scientists could distinguish between species and strains even to the level of serovars. Nevertheless, these biochemical tests have limitations in their low discriminatory power within strains and relies on several methods for distinction (Wattiau et al., 2011).

2.5.4 Phage Typing

This is a phenotypic screening of *Salmonella* based on the type of bacteriophage it harbors (Baggesen et al., 2010). The bacterium is transformed with a lysogenic or lytic phage which lyse the cell after incubation to form clear zones on an agar plate. Previously, scientists used this method

for classifying of bacterial strains within the same species during outbreaks (Baggesen et al., 2010). *Salmonella* bacteria especially *S. Typhimurium* and *S. Enteritidis* are classified as definitive phage types (DT) and Phage Types (PT) respectively (Andoh et al., 2017). In most cases, the typing patterns should conform to phage types defined by a reference laboratory for it to be recognized (Cooke et al., 2007; Baggesen et al., 2010; Andoh et al., 2017). The procedure has low specificity.

2.5.5 Pulse-Field Gel Electrophoresis (PFGE)

The PFGE method is based on restriction digest of bacterial genome and their electrophoretic mobility in agarose gel using alternating currents (Liu, 2006). PFGE results in 8-25 large DNA fragment patterns of about 40-600kb (Liu, 2006). This method has sufficiently high discriminatory power and has been used in many laboratories for identifying bacterial strains. The procedure is often laborious and there is lack of comparable data across various laboratories (Lui et al., 2011). Nonetheless, many scientists consider PFGE as the gold standard for molecular typing of bacteria in epidemiological studies (Liu, 2006).

A similar molecular typing method based on restriction digest analysis is ribotyping. Ribotyping uses ribosomal DNA probes to detect smaller fragment sizes (1-30kb) of the genomic DNA (Liu, 2006). This method is reproducible and has high discriminatory power (Liu, 2006). However, it faces limitations like that of PFGE.

2.5.6 Quantitative Polymerase Chain Reaction

Amplification of specific gene sequences enables easy detection and discrimination between bacterial species and strains. This is a rapid and sensitive procedure which is often used as molecular tool for identifying pathogens in the laboratory. Most studies employing PCR method amplify the 16S ribosomal RNA gene (*16SrRNA*) for bacterial identification (Mollet et al., 1997).

The amplification of RNA polymerase B subunit (*rpoB*) has also been used for phylogenetic analyses to discriminate between *Salmonella* species (Mollet et al., 1997). In general, the PCR primers may tend to have low binding specificity and probes used in the amplification of target gene sequence may be equivocal.

2.5.7 Multi-Locus Sequence Typing (MLST)

MLST is considered as one of the indispensable molecular tools for epidemiological studies (Ranjbar et al., 2017). Bacterial characterization is based on the amplification and sequencing of conserved genes with sufficient polymorphisms between nucleotide sequences for allelic discrimination (Foley et al., 2009). Sequenced allele fragments of about 400-500bp are assigned allelic profiles and then submitted to a central database to generate bacterial sequence types (ST) (Foley et al., 2009). For *Salmonella*, the following seven housekeeping genes are often sequenced for comparative analyses: *thrA* (aspartokinase+homoserine dehydrogenase), *purE* (phosphoribosylaminoimidazole carboxylase), *sucA* (alpha ketoglutarate dehydrogenase), *hisD* (histidinol dehydrogenase), *aroC* (chorismate synthase), *hemD* (uroporphyrinogen III cosynthase), *dnaN* (DNA polymerase III beta subunit) (Lui et al., 2011; Ranjbar et al., 2017). According to Liu et al (2011), *hisD* showed the highest discriminating nucleotide sequences among the other targets in *Salmonella* isolates. In another study, *sucA* gene has been shown to distinguish between ST313 and ST19 (Krauland et al., 2009). MLST is important for genetic population studies especially due to its reproducibility. However, this tool may be less discriminatory among certain bacterial strains (Fakhr et al., 2005) and therefore rely on the combination of other identification tests.

2.5.8 Whole Genomic Sequencing

Complete genome sequencing is considered as the ideal molecular tool for characterizing bacteria (Philips et al., 2016). Sequencing techniques such as Sanger, Illumina, Pyrosequencing, Ion

Torrent and Nanopore Technology may be used to generate sequence data. With this method of analysis, data of MLST, resistance and other virulence traits could be obtained (Okoro et al., 2012; Kagambèga et al., 2018). This has sufficiently high discriminatory power. Due to the vast sequence data generated, gene annotations may become difficult and time consuming. Also, the cost involved in this procedure serve as one of the limitations.

2.6 Antimicrobial agents and resistance

2.6.1 Mode of action and mechanism of resistance

Classes of antimicrobials used for effective treatment of NTS disease include Fluoroquinolones, Carbapenems, Aminoglycosides, Macrolides, and Third Generation Cephalosporins (Su et al., 2004; Ando et al., 2017). Most of these classes target inhibition of protein synthesis or cell wall formation. For example, Aminoglycosides and Macrolides inhibit peptide elongation whereas Cephalosporins and Carbapenems act against bacterial cell wall formation of bacterial.

Fluoroquinolones have become widely used antibiotics in Ghana (Groß et al., 2011). This drug is bactericidal, and its mode of action is by inhibiting bacterial replication (Casper et al., 2016). Primarily, the compound interacts and inactivates DNA gyrase in Gram negative bacteria or Topoisomerase IV in Gram positive bacteria (Casper et al., 2016). The enzyme is a tetramer which consists of dimer subunits A and B (Casper et al., 2016). This enzyme complex releases tension in the bacterial chromosome during replication by negative supercoiling (Sutera et al., 2013; Casper et al., 2016).

Polymorphisms due to single base-pair change at designated sequence sites may alter the conformation of the enzyme and reduce affinity for drug resulting in resistance (Sutera et al.,

2013). These sites are known as quinolone resistance-determining regions (QRDRs) (Sutera et al., 2013). Point mutations in the GyrA QRDR codons 83 (Ser to Phe, Ser to Tyr, or Ser to Ala) and 87 (Asp to Tyr, Asp to Gly, or Asp to Asn) were found to confer significant level of resistance to fluoroquinolones (Giraud et al., 2000; Rahmani et al., 2013; Eibach et al., 2016). In addition, Single nucleotide polymorphisms at QRDR codons 463 to 466 of GyrB were also associated with resistance (Sutera et al., 2013; Eibach et al., 2016). Further, mutations in the ParC gene codon 80 (Ser to Iso, or Ser to Arg) of Topoisomerase IV may confer some level of resistance to fluoroquinolones, however, this form of mutation is rarely found in *Salmonella* (Giraud et al., 2000; Su et al., 2004; Rahmani et al., 2013; Eibach et al., 2016). Horizontal transfer of quinolone resistance plasmids (qnr) are also found to play major role in resistance to quinolones and fluoroquinolones.

The efflux of drug to reduce its accumulation in bacterial cell is another mechanism of decreasing the effect of the drug. Thus, active efflux is an important bacterial resistance mechanism to fluoroquinolone (Giraud et al., 2000; Caspar et al., 2017). Giraud et al (2000) suggested that increased expression of efflux mediated proteins like AcrAB coupled with reduced membrane permeability may be the initial resistance mechanism employed by the bacteria prior to GyrA mutations.

2.6.2 Epidemiology of resistance in sub-Saharan Africa

Treatment failure as a result of antimicrobial resistance poses a heavy economic burden aside increased morbidity and mortality rates (Bernabe et al., 2017). There have been vast reports of moderate to high prevalence of antimicrobial resistant pathogens in sub-Saharan Africa (Bernabe et al., 2017). Resistance to first line drugs particularly Chloramphenicol, Amino-penicillin and Sulphamethoxazole-Trimethoprim has driven the use of advanced antimicrobials such as

fluoroquinolones, carbapenems and third generation cephalosporins (Su et al., 2004; Al kraiem et al., 2018). A review by Bernabe et al reported the rate of resistance to the first line drugs in sub-Saharan Africa ranged from 50-70% (Bernabe et al., 2017). They also observed moderate levels of resistance to Gentamicin (38%) and Amoxicillin-Clavulanic Acid (42%), 3rd generation Cephalosporins (17%) and Fluoroquinolones (12%) among Gram negative bloodstream pathogens. Most importantly, *Salmonella enterica* isolates showed no resistance to Ceftriaxone (Bernabe et al., 2017). Molecular analysis of resistance among multidrug resistant *Salmonella* isolates have shown high proportions of plasmid mediated resistance genes *CatA1*, *Sul 1*, *Sul 2*, *Tet A*, *Tet B*, *Bla_{Tem}*, *aadA*, *dfrA* (Orman et al., 2002; Kagambèga et al 2018).

2.6.3 Methods for Studying *Salmonella* antibiotic resistance mechanisms

Mostly, antibiotic resistance is studied using phenotypic and molecular based techniques. Antimicrobial susceptibility testing using disc diffusion method is considered as the gold standard for assessing resistance among *Salmonella* pathogens (Mandomando et al., 2010). The results are interpreted using standard breakpoints according to Clinical Laboratory Standards Institute (CLSI) or European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines. With this method, several antibiotics could be screened simultaneously on an agar plate. The drawback is that, the minimum concentration to inhibit bacterial growth may not be determined. Also, persistent isolates may be missed during the analysis.

Another method is based on determination of the minimum inhibitory concentration (MIC) of an antimicrobial agent (Wiegand et al., 2008). This could be done by using agar diffusion or broth dilution assay. Test organisms may be exposed to progressively increasing concentrations of a given antimicrobial agent to determine the concentration at which the drug inhibits bacterial

growth. This method may be laborious and prone to error. An alternative way of carrying out MIC test is by using commercially available E-Test kit which may also be costly.

PCR detection of plasmid-borne resistance genes could serve as a good platform to monitor antimicrobial resistance (Orman et al., 2002). Resistance genes borne on plasmids and transposons are associated with major spread of drug resistance among bacteria across the globe.

Sequencing of genes particularly chromosomal encoded resistance genes to identify synonymous mutations could be used to study resistance (Okoro et al., 2015; Kagambèga et al., 2018).

Additionally, expression profiling of bacterial genes including efflux pump and metabolic genes using RT-qPCR and RNA sequencing has been employed in the study of antibiotic resistance mechanisms in *Salmonella* (Li et al., 2017).

CHAPTER THREE

3.0 Methodology

3.1 Characteristics of *Salmonella* Isolates

3.1.1 Sample size

The sample size for the study was calculated according to the method reviewed by Charan & Biswas (2013). Briefly, the formula was given as:

Sample size = $(Z_{1-\alpha/2})^2 P (1-P) / d^2$ where $Z_{1-\alpha/2}$ represents the standard normal variate (if the level of significance, α is 5%, then the value of Z is 1.96), d is the absolute error which is decided by the researcher and P is the expected proportion in the population and is usually based on previous study.

Using the formula above, the value of $\alpha = 0.05$, giving $Z_{1-\alpha/2}$ value as 1.96. The absolute error was 0.05. A study published by Labi et al (2014) showed that the prevalence of invasive NTS infected patients among those presenting with bacteremia at Korle Bu Teaching Hospital – a tertiary hospital in Ghana was 4%. From this, the expected proportion of patients suffering from iNTS infections was 0.04. Thus, the expected sample size was calculated as follows: Sample Size = $1.96^2 \times 0.04 \times (1-0.04) / 0.05^2 = 59$.

3.1.2 Study Design

This was a laboratory-based retrospective study conducted at Department of Biochemistry, Cell and Molecular Biology, University of Ghana. Archive *S. Typhimurium* and *S. Dublin* isolates were obtained from Kumasi Centre for Collaborative Research in Tropical Medicine (KCCR) at Kwame Nkrumah University of Science and Technology. These bacterial isolates were previously isolated

from blood samples taken from pediatric febrile patients between September 2007 and September 2012. Other presumptive *Salmonella enterica* isolates included in the study were obtained from the Acute Febrile Illness (AFI) project, an on-going study at the Department of Biochemistry, Cell and Molecular Biology/West Africa Centre for Cell Biology of Infectious Pathogens (WACCBIP), University of Ghana.

3.1.3 Bacterial isolates

Pure invasive NTS archive isolates from KCCR (n=46) were aerobically revived and grown on Cystine-lactose-electrolyte-deficient agar (C.L.E.D, Oxoid) at 37°C for 24 hours. The cultures were then transported within few hours in a media plate sealed with paraffin and stored in a zipper storage bag kept in a leakage-free box. The samples were immediately brought to the Department of Biochemistry, Cell and Molecular Biology, University of Ghana, microbiology lab where they were kept at 4°C to arrest their growth or stored at -20°C in 20% glycerol (v/v). Prior, *S. enterica* isolates from the AFI study (n=5) were kept under -20°C storage condition. A total 51 bacterial isolates (*S. Typhimurium* n=36, *S. Dublin* n=10, Unknown serovars n=5) were available for the study.

3.1.4 Culturing conditions and morphological examination

The isolates were sub-cultured on chocolate agar and incubated at room temperature for 24 hours. The cultures were routinely grown using Luria-Bertani (LB) media (Sigma-Aldrich) or chocolate agar. In the case of antimicrobial susceptibility testing, Mueller Hinton Agar 2 (Merck, Germany) was used. Gram's staining was carried out to confirm the phenotypic characteristics of isolates as previously described (Grainger et al., 2001).

3.2 Susceptibility Testing

The antibiotic susceptibility profiles of each isolate were assessed using disc diffusion method and by following CLSI M100 recommendations. The experimental procedure was carried using NCCLS manual for antimicrobial susceptibility testing. First, bacterial cells were cultured on LB agar at 37°C overnight. Single colonies from the overnight cultures were suspended in sterile 0.9% saline (w/v) and adjusted to 0.5McFarland standard (BBL, Becton Dickson). A sterile cotton-tipped swab stick was then dipped into the suspension. Excess liquid was removed from the swab by pressing against the side of the tube. The agar plate was inoculated by swabbing the surface starting from the top to cover entire plate. Streaking was done in a forward and backward manner and then rotating the plate to approximately 60° to repeat the procedure. The isolates were screened against the following antimicrobials: Ampicillin (10µg), Ceftazidime (30µg), Cefuroxime (30µg), Ceftriaxone (30µg), Ciprofloxacin (5µg), Levofloxacin (5µg), Sulphamethoxazole-Trimethoprim (300µg), Gentamicin (10µg), Imipenem (10µg), Chloramphenicol (30µg), and Tetracycline (30µg) (Oxoid). These agents for AST were chosen by first comparing and then selecting frequently used antimicrobial agents in recent NTS research publications from Ghana (Donkor et al., 2012; Labi et al., 2014; Eibach et al., 2016; Andoh et al., 2017). The AST cultured plates were incubated at 25°C for 16-18hours after which the zones of inhibition were measured in millimeters using a ruler. The breakpoints were interpreted according to CLSI M100S standards (2014) and using the reference strain *E. coli* ATCC 25922® as control organism.

3.3 DNA Extraction

Single cell colony was cultured in LB broth (Sigma-Aldrich, UK) by incubating at 37°C while shaking at 220rpm overnight. Cultured cells were harvested, and genomic DNA were extracted with Quick-DNA Plus Isolation Kit (Zymo Research). The procedure for extraction were carried out by following the manufacturer's instructions, although, with few modifications. Briefly, a volume of 1000µL of each sample culture was harvested by centrifugation. Excess media was washed off by resuspending pellets in 500µL Phosphate Buffered Saline (PBS) and centrifuging at $500 \times g$ for 2 minutes. The Pellets were finally resuspended in 200µL PBS, followed by addition of 200µL Biofluid and Cell Buffer® (Zymo Research) and 20µL Proteinase K (20mg/mL). After vortexing for 10-15 seconds, the mixture was incubated at 55°C for 15minutes. In addition, a bead-beating step was introduced by homogenizing the cells with sterile inert beads (Zymo Research) using the Disruptor Genie for 5-10 minutes. An equal volume of Genomic Binding Buffer® (Zymo Research) was added to the mixture, vortexed for 10-15 seconds, then transferred into a column placed in collection tube (Zymo Research). The column was washed with 400µL Pre-Wash Buffer®. Following this step was additional washing using 700µL and then 200µL gDNA Wash Buffer® by centrifugation ($12,000 \times g$ for 1 minute). Finally, the Spin Columns were transferred to a sterile micro-centrifuge tube where 50µL DNA Eluting Buffer® preheated at 70°C was added. This was incubated at room temperature for 5 minutes before centrifuging at maximum speed ($16,000 \times g$ for 1 minute) to elute the DNA. The quantity and purity of the isolated DNA samples were analyzed using Nanodrop™ Spectrophotometer (ThermoFisher Scientific, USA).

3.4 *Salmonella* Genotyping

3.4.1 Amplification of virulence gene *st313-td* by polymerase chain reaction (PCR)

All conventional PCR reaction components were assembled using NEB OneTaq[®] 2X Master Mix with Standard Buffer protocol. The mixture consisted of 1X Master Mix buffer with Taq Polymerase, 0.2µM of each primer set and 1-5µL genomic DNA as template in a total reaction volume of 20µL. The PCR primers were obtained from previous study (Herrero-Fresno et al, 2014). The reaction set up and conditions for *st313-td* gene was carried out according to the experimental conditions used by Herrero-Fresno et al with few modifications. First, elongation temperature was set based on the Taq Polymerase used. In addition, the number of reaction cycles were increased from 30 cycles to 33 cycles. The primer sequences specific for the gene *st313-td* is given as follows: forward 5'-GGTGATGTAGAAAGACGAA-3' and reverse 5'-GGAAGAGTAGGGAAAAGAA-3' (synthesized by Inqaba Biotec, SA) with expected amplicon size of about 1029bp. The thermocycling conditions using ProFlex[®] PCR System (Thermo Fisher Scientific, USA) were set as follows: 94°C for 5 minutes as initial denaturation step, then amplification step- 94°C for 1minute, 55°C for 1 minute, 68°C for 1 minute for 33 cycles, and final extension for 10min at 68°C. The amplicons were analyzed using gel electrophoresis by resolving in 1.5% agarose prepared in 1X TAE buffer and stained with EZ-Vision[®] in gel-solution fluorescent dye. The gel was visualized using Amersham Imager 600 and the estimated band size of the amplicons were determined by comparing to the Quick-Load[®] Purple 100bp DNA Ladder (New England BioLabs Inc).

3.4.2 Multi-Locus Sequence Typing

Twelve *S. Typhimurium* and Two *S. Dublin* DNA samples showing presence of *st313-td* gene and 8 other isolates (seven *S. Typhimurium* and one *S. Dublin*) with varying antibiotic susceptibility

patterns were selected for MLST analyses (ID Genomics, Seattle, USA). The MLST targeted 7 housekeeping genes in the bacterial genome as described in a previous study (Moon et al., 2015). The target genes were *aroC*, *dnaN*, *hemD*, *hisD*, *purE*, *sucA*, and *thrA*.

3.5 Antimicrobial Resistance Markers Amplification

The PCR reaction was carried out based on the conditions previously described above by using NEB OneTaq[®] 2X Master Mix with Standard Buffer protocol. The annealing temperatures used for the reactions were based on the primers obtained for the specific markers of resistance.

3.6 Minimum Inhibitory Concentration Determination and Ciprofloxacin Resistance Induction

The MICs of Ciprofloxacin were determined by broth microdilution assay according to the Wiegand et al, (2008) protocol. Two *S. Typhimurium* ST313 isolates 113552 and 113821, which showed complete susceptibility and MDR phenotypes respectively, were selected. The reference strain *E. coli* ATCC 25922® was used as control organism. Isolates were cultured overnight on Mueller Hinton Agar (MHA) by incubating at 37°C. Ciprofloxacin serial dilutions in cation-adjusted Mueller Hinton Broth (MHB 2) were freshly prepared. Bacterial cells were adjusted to 0.5McFarland's Standard before exposing to the antibiotic. The experiment was repeated in three biological replicates and two technical replicates. MICs were measured with a microplate reader (Varioskan LUX) after overnight incubation at 37°C with shaking. A typical 96-well plate set up was as shown below:

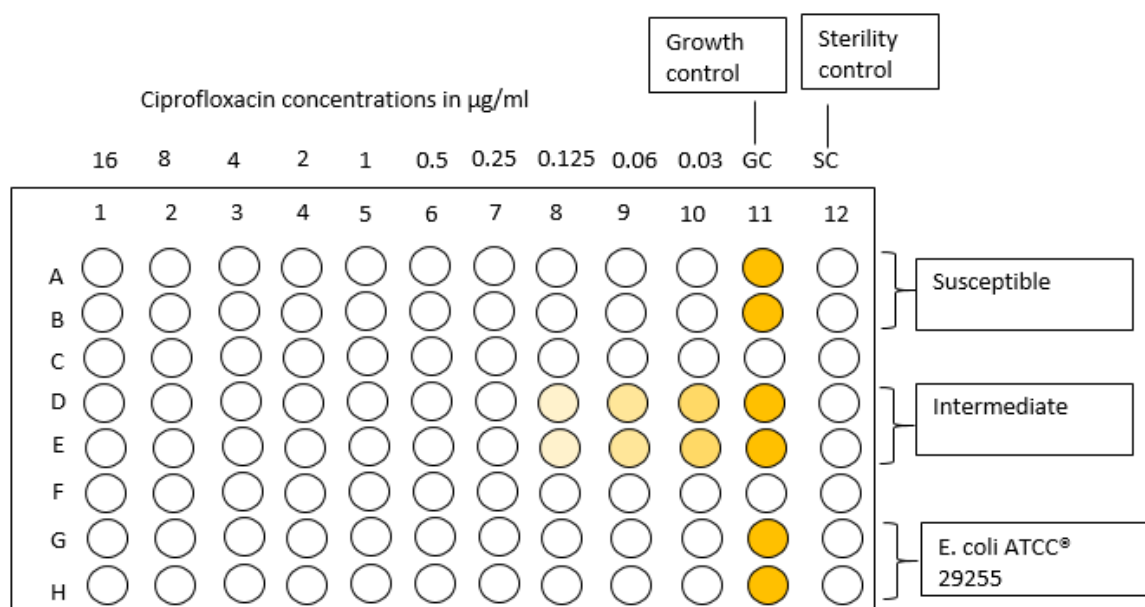


Figure 2: Representative 96-well plate set up for broth microdilution assay

3.7 Induction Assay

Following the MIC results, the susceptible isolate (113552) was cultured on Mueller-Hinton agar (MHA) overnight. These susceptible cells, after adjusting to 0.5 McFarland's Standard, were exposed to increasing concentration of ciprofloxacin starting with the concentration of half the established MIC (0.015µg/mL) (Appendix Table 3). The test, growth control and sterility control cultures were incubated overnight at 37°C while shaking. In addition, MHA culture plates with or without ciprofloxacin were prepared. The optical density (OD) at 600nm was measured and cell viability on MHA plates was assessed before proceeding to the next higher concentration. The procedure was repeated until resistant strains with elevated ciprofloxacin MIC began to show delayed growth (in this case, at 4µg/mL). Selected resistant bacteria were cultured in antibiotic-free MHB for five consecutive days to check for stability.

3.8 Growth Comparison of Ciprofloxacin Resistant Isolates

The three induced resistant (113552_Res1, 113552_Res2, & 113552_Res4) isolates and the susceptible parental (113552) isolates were grown on LB agar overnight. The growth patterns were determined by culturing individual isolate in LB broth starting with an initial OD of 0.02. The OD of the cultures were measured after every hour for 14 hours and then after 24 hours of incubation to plot a growth curve. MICs of the resistant and the susceptible isolates were determined as previously described. All set ups were carried out in two technical replicates. The significant differences in growth behavior between the induced resistant and parental isolates were analyzed using Mann-Whitney Test at a two-tailed p-value of 0.05.

3.9 PCR Amplification of Ciprofloxacin Resistance Genes

The genomic DNA of induced resistant (113552_Res4), susceptible (113552) and clinically intermediate resistant (113821) isolates were extracted using Quick-DNA Plus Isolation Kit (Zymo Research) as previously described. The listed primers for PCR (Table 1) were designed using the Primer3Plus Bioinformatics program (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi>). These designed primers were purchased from Inqaba Biotech Company, SA. PCR reaction conditions were as previously described except for the annealing temperature which was set at 60°C for all primers. Amplicons were resolved on 1.5% agarose gel stained with Ethidium Bromide. The PCR products are sent for Sanger sequencing. Upon receipt, sequenced data will undergo quality control checks by assessing the size of distribution using Agilent 2200 Tapestation (~ 250-1000bp). Further, sequence data will be edited using CLC Genomics Workbench software (CLC bio, Denmark) to remove adapter sequences as previously described (Philips et al., 2016). Genetic variations based on nucleotide polymorphisms will be determined by mapping FastQ files of the three isolates to the reference genome of *S. Typhimurium* LT2 (Accession number: NC_003197) at National Centre for Biotechnology Information (NCBI) database.

Table 1: Primers for Ciprofloxacin Resistance Targeted Genes

No.	Gene	DNA Primer sequence 5’-3’	Gene Annotation	Ref
1.	<i>GyrA</i>	F- AGTACGTGGGCGAATGTGTT	DNA Gyrase subunit A	This study
		R- AGGATGGTGTTCATGGGTGTT		
2.	<i>AcrA</i>	F- AAACGGAACCACTGCAAATC	Acridine Efflux Pump	This study
		R- TTTCAATCCGTCAGTCACCA		
3.	<i>YdhJ</i>	F- CGCGTGGTAAATATGTCGTT	Multidrug Resistance Efflux Pump	This study
		R- GTCACTTTCCACGCTTTGGT		
4.	<i>EmrD</i>	F- CCATTTATATTCCCGCCATC	Multidrug Resistance Protein D	This study
		R- AGACGTTCATCACGCCAAAC		
5.	<i>SoxR</i>	F- CGGGCGAGTATAATTCCTCAA	Redox-Sensing Transcriptional Activator of SoxS	This study
		R- CAGTCGCTACGCGATAAACA		
NB: F=Forward, R=Reverse				

3.10 Gene Expression Analysis by Reverse Transcription-quantitative Polymerase Chain Reaction (RT-qPCR)

3.10.1 RNA Extraction

Selected bacterial isolates (113552, 113821, & 113552_Res4) were grown on LB agar at 37°C overnight. Each isolate was sub-cultured in 10ml LB broth for 18-24hours. Next, 1ml of each culture was inoculated into 10ml Brain Heart Infusion (BHI) containing ciprofloxacin at half the MICs for the individual isolates (Table 6). However, the induced resistant isolate (113552_Res4) was exposed to the same ciprofloxacin concentration as the clinical intermediate (113821) to mimic that of the intermediate. The cultures were incubated at 37°C while shaking at 225rpm for 6-8 hours after which the cells were harvested. Two volumes of bacterial RNA Protect® (Qiagen, Germany) was added to one volume of bacterial culture by mixing and leaving on bench for about 5 minutes to preserve the integrity of the RNA. Total RNA from the cell pellets were extracted using ZymoResearch RNA isolation kit and by following the manufacturer's instructions. Briefly, cell pellets were resuspended in 400µl RNA Lysis Buffer, and then transferred into a Zymo-Spin™ filter in a collection tube. This was centrifuged at $16,000 \times g$ for 30seconds. One volume of absolute ethanol was added to the saved flow-through supernatant. The mixture was transferred into a Zymo-Spin™ IIICG Column and genomic DNA eliminated by DNase I Treatment. Following this step, about 400µl of RNA Prep Buffer was added to the column, centrifuged at $16,000 \times g$ for 30seconds, and then discarded flow-through. A volume of 700µl RNA Wash Buffer was added to the column and centrifuged at $16,000 \times g$ for 30 seconds. The flow-through was discarded. Lastly, 400µl RNA Wash Buffer was added to the column. Here, centrifugation was set at $16,000 \times g$ for 2 minutes to ensure complete removal of Wash Buffer. The RNA bound to the matrix of the spin column was subsequently washed and eluted in 50µl nuclease free water. The

integrity and purity of RNA samples collected in RNase free microcentrifuge tubes were assessed using Nanodrop™ Spectrophotometer (ThermoFisher Scientific, USA). The samples were stored at -20°C for further experiments.

3.10.2 Gene Expression Analysis by RT-qPCR

The expression levels of *GyrA*, *AcrA*, *YdhJ*, *EmrD* and *SoxR* genes in susceptible (113552), intermediate resistant (113821) and induced resistant (113552_Res4) isolates under drug stress (exposure to ciprofloxacin) were analyzed by RT-qPCR. The cDNA synthesis and amplification of gene transcripts were achieved by using Luna® Universal One-Step RT-qPCR kit (New England Biolabs). For a 20µL RT-qPCR reaction, 10µL of Luna Universal One-Step Reaction Mix (2X), 1µL of Luna WarmStart® Reverse Transcriptase Enzyme Mix (20X), 0.8µL of forward (10µM) primer, 0.8µL of reverse (10µM) primer, 2µL of template RNA (ranging between 100 and 200ng/µL), and 5.4µL nuclease-free water were used. The cycling conditions were as described below:

55°C for 10minutes as reverse transcription step, 95°C for 1 minute as initial denaturation step, and 40 cycles of denaturation and extension steps at 95°C for 10seconds and 60°C for 30seconds respectively.

The reaction was carried out in a 96-well microplate using the Quant Studio™ 5 Real Time PCR thermal system (ThermoFisher Scientific, USA). A negative no-template (distilled water) control was included in each run. The housekeeping gene 16SrRNA was used as the endogenous control. The mRNA transcript levels of targeted genes were determined as described previously (McGann et al., 2007; Duodu et al., 2010). The Ct values of the target genes and the endogenous control gene were transformed using the formula, E^{-Ct} , where E is the reaction efficiency and Ct is the

threshold cycle. The expression levels were normalized using the formula; $N = \text{transformed Ct (target)} / \text{transformed Ct (endogenous control)}$.

3.10.3 DNA Sequence Analysis of Resistance target genes

Amplified gene products were sequenced using Sanger sequencing platform. Sequence analysis was performed using Geneious bioinformatics software platform (<https://www.geneious.com>). Raw reads were edited and analyzed by aligning the nucleotide sequences of susceptible, intermediate, and resistant isolates to corresponding genes of reference strain *S. Typhimurium* LT2.

3.11 Statistical Analyses

Data generated from the study were computed using Microsoft Excel. Results of AST experimental replicates as well as data for resistance gene amplification were expressed as percentages. Further statistical analyses of data were exported into and analyzed using Graph Pad Prism 6 Software. Categorical data obtained for the sequence types and multidrug resistance were compared using Chi-Square/Fisher's Exact Tests. Continuous data from determination of growth pattern were analyzed as mean $\pm$ S.D. The significant differences were assessed using non-parametric using Mann Whitney Test. The mRNA transcript levels were analyzed using normalized $\log_{10}$ transformed transcript levels. Comparison of expression levels among samples were determined using 1-Way ANOVA and Tukey's multiple comparison test. All statistical analysis was set at a significant level of 5% (p value < 0.05) except for SNP analysis in which significant level was set at 0.1% (p -value < 0.001).

CHAPTER FOUR

4.0 RESULTS

4.1 Characteristics of *Salmonella* Isolates

All the fifty-one archived *Salmonella* isolates yielded growth on chocolate agar at 100% revival success rate. *Salmonella* appeared as small, round, smooth colonies with cream-to-grey color. Also, colonies did not show any discoloration of the media. All isolates were sampled from pediatric blood. Forty-six of them were serotyped based on White-Kauffmann-LeMinor scheme using agglutination tests (Eibach et al., 2016). Out of the 51 isolates, 70% (n=36) and 20% (n=10) belonged to *S. Typhimurium* and *S. Dublin* serovars respectively (Table 2). All bacterial isolates were identified as Gram negative rod-shaped. PCR detection of *Salmonella* specific *invA* gene tested positive for all isolates from AFI and KCCR.

4.2 Antimicrobial Susceptibility Profile

Susceptibility test was carried out to examine the phenotypic resistance patterns of *Salmonella* isolates (figure 3 and 4). The bacteria were tested against eight different classes of antibiotics and multidrug resistance was defined as resistance to three or more classes of drugs. The types of antibiotics were selected based on their frequent use in current NTS studies carried out in Ghana and also based on Ghana Health Service recommendation for the treatment of NTS disease in the country (Donkor et al., 2012; Labi et al., 2014; Eibach et al., 2016; Andoh et al., 2017). All isolates (100%) were completely susceptible to Imipenem, Ceftazidime and Ceftriaxone (figure 4). Reduced susceptibility to levofloxacin (n=6) and ciprofloxacin (n=3), gentamicin (n=1), tetracycline (n=1), amoxicillin-clavulanic acid (n=1) and cefuroxime (n=1) was also observed

among isolates (Figure 1). In general, resistance frequency of over 60% was recorded against chloramphenicol (n=32/51, 63%), amoxicillin (n=34/51, 67%), ampicillin (n=35/51, 69%), streptomycin (n=31/51, 61%) and Sulphamethoxazole-Trimethoprim (n=33/51, 65%) (Figure 1A). Only 2% of the isolates (n=1) were resistant to tetracycline or Amoxicillin-Clavulanic Acid (figure 4A). *S. Dublin* isolates were susceptible to almost all antibiotics used except for ampicillin and amoxicillin (figure 4C). High levels of resistance and reduced susceptibility were mostly observed among *S. Typhimurium* isolates (figure 4B). Overall, there were 32 MDR isolates (Table 2).

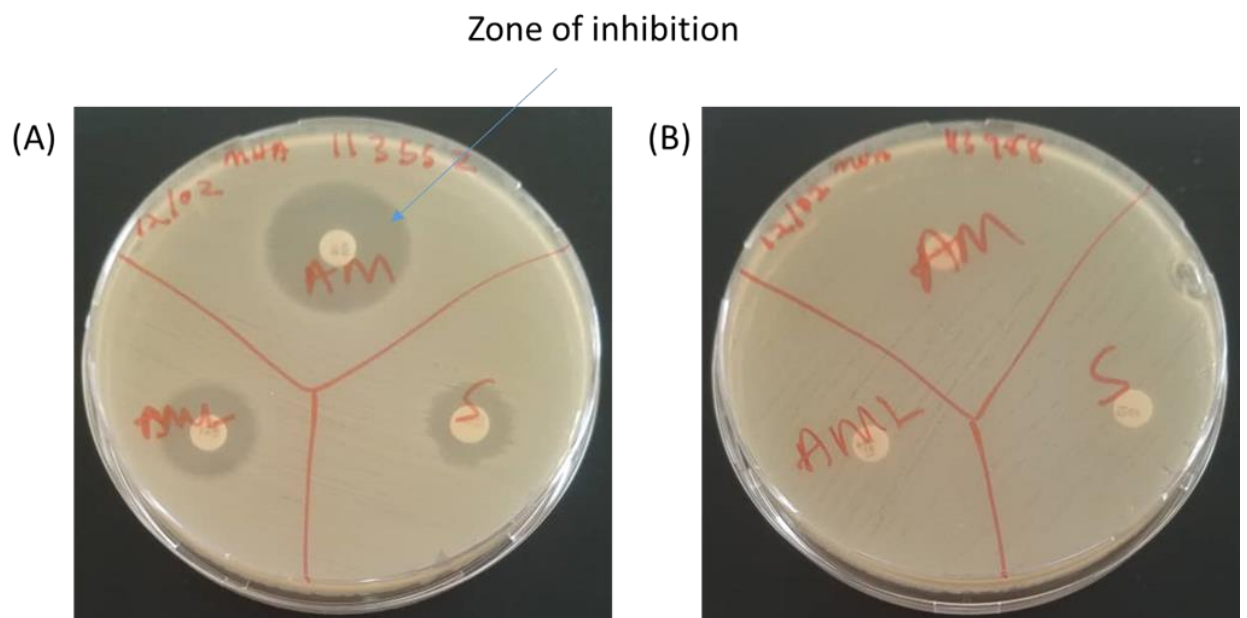


Figure 3: Representative agar plates of antimicrobial susceptibility testing using disc diffusion method.

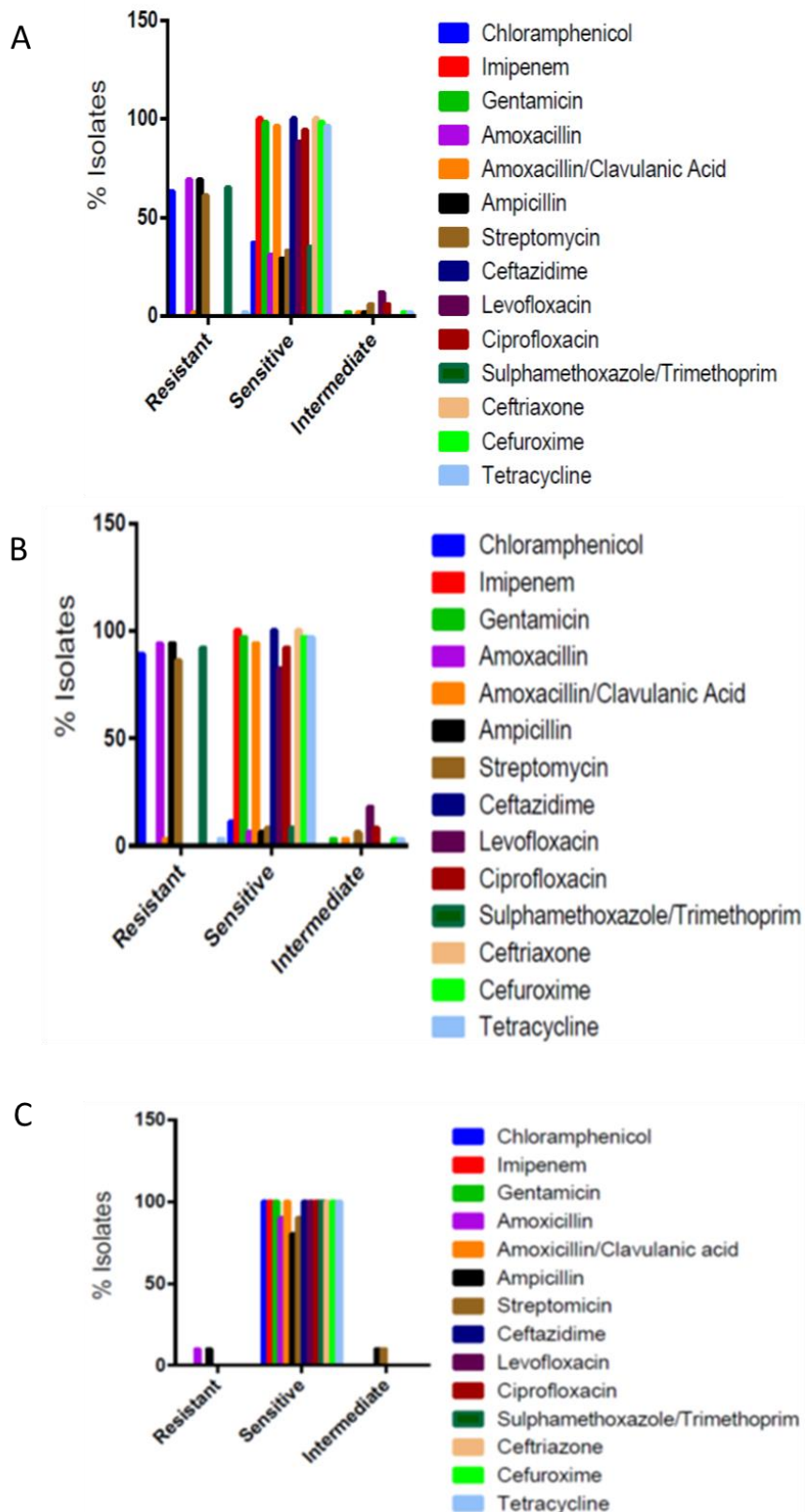


Figure 4: Antimicrobial susceptibility profile of (A) all *Salmonella enterica* (B) *Salmonella* Typhimurium and (C) *Salmonella* Dublin isolates.

Table 2: Source, Serovars, and Drug Resistance Profile of *Salmonella* enterica Isolates

Serovar	Source	Number of Isolates (%)	Susceptible to all antibiotics (N)	Resistant to ≥3 classes of antibiotics (N)
Typhimurium	Blood	36 (70%)	1	32
Dublin	Blood	10 (20%)	8	-
NTS	Blood	5 (10%)	5	-
Total		51 (100%)		
ABBREVIATION:				
N= number of isolates				

4.3 Detection of Antimicrobial Resistance Gene Markers

Following the phenotypic susceptibility testing, the molecular detection of plasmid-mediated resistance genes was carried out by PCR to confirm the resistance phenotype of the isolates. Ten isolates showing varying antimicrobial susceptibility profiles were selected for the molecular analysis. The targeted resistance genes, based on AST results, were plasmid-encoded and predominantly associated with corresponding drug resistance (Appendix Table 1). The genes were *CatA1*, *Bla_{Tem}*, *StrA*, *aadA*, *TetA & B*, *Sul1 & 2*, *aac (3) III-IV*, and *drfA14* encoding resistance to chloramphenicol, β -lactams, streptomycin, streptomycin, tetracycline, sulfamethoxazole, gentamicin, and trimethoprim respectively (figure 5). Three isolates (30%) carried *CatA1* gene (figure 5) although a total of 5 showed phenotypic resistance to Chloramphenicol. Interestingly, one of *CatA1*-carrying isolates was susceptible to chloramphenicol in the AST results (Appendix Table 2). The test was repeated to confirm the result. The presence of *Bla_{Tem}* gene was detected among five of the 7 isolates which showed phenotypic resistance to amino-penicillin drugs. About 90% of the isolates contained *StrA* gene including 2 isolates which were susceptible to streptomycin (Figure 5). Gene *aadA* was detected in 70% isolates (figure 5) and it was profoundly present in isolates showing streptomycin resistance. *TetA* gene was present in 30% of the isolates (figure 5) including one *S. Typhimurium* with no phenotypic resistance to tetracycline (Appendix Table 2). On the other hand, *Tet B* gene was detected in only one MDR *S. Typhimurium* isolate (10%) with phenotypic resistance to Tetracycline (figure 5). Ninety percent of *Sul1* and 100% of *Sul2* genes were detected (figure 5) including 3 other sulfamethoxazole susceptible isolates. However, there was no detection of *aac-III-IV* or *drfA14* genes which codes for Gentamicin and Trimethoprim resistance, respectively.

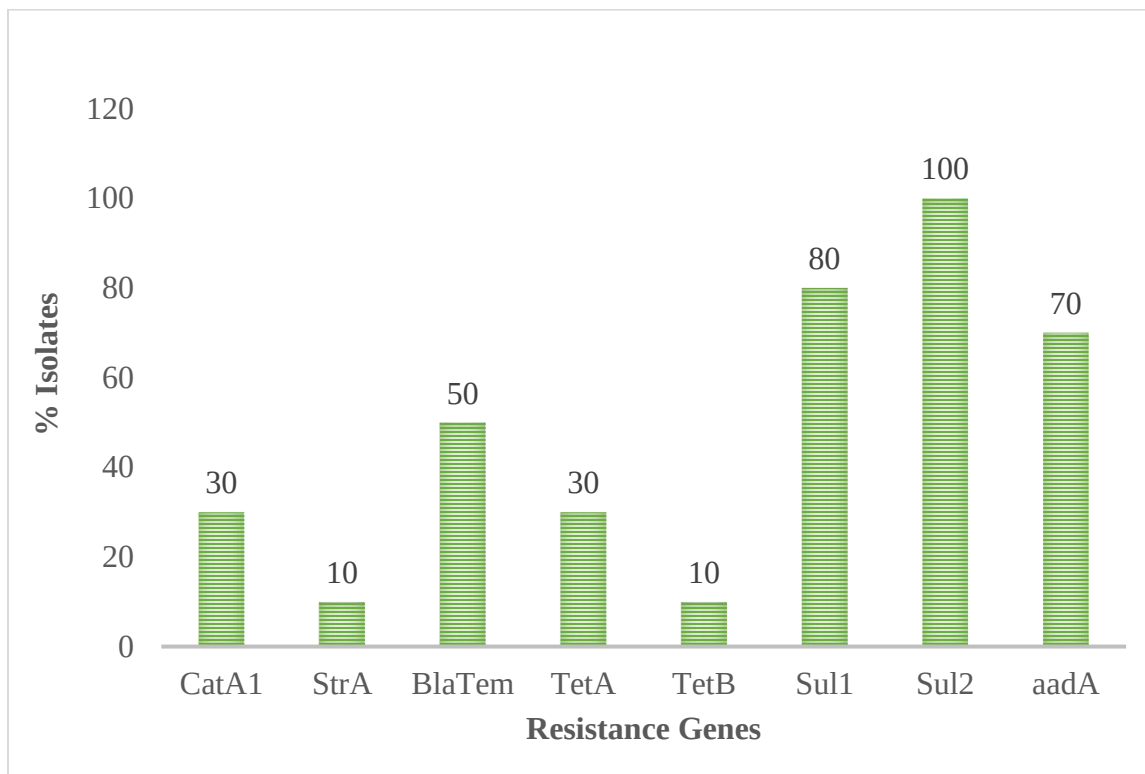


Figure 5: Distribution of antimicrobial resistance gene markers among *Salmonella enterica* isolates. Genes *CatA1*, *Bla_{Tem}*, *Tet A & B*, *Sul 1 & 2*, and *StrA* & *AadA* encode resistance to chloramphenicol, beta lactams, tetracycline, sulphamethoxazole and streptomycin respectively

4.4 *Salmonella* genotyping by MLST

4.4.1 Detection of Virulent trait *st313-td* gene

Amplification of *st313-td* gene was carried out as a preliminary investigation to identify possible virulent clones prior to selection for Multilocus Sequence Typing (MLST). Conventional PCR was performed for all fifty-one isolates. Out of this, only 14 (27%) of them carried the gene of which *S. Typhimurium* and *S. Dublin* isolates were 12 and 2 respectively (Table 3). Other *S. Typhimurium* (n=7) and *S. Dublin* (n=1) isolates showing varying resistance phenotypes, were selected in addition to the 14 positive isolates for typing and analyses (Table 3). *S. Typhimurium* isolates with sequence type ST313 represented 82% (n=18/22) of all the typed isolates whereas only 4% (n=1/22) was ST19 (Table 3). All *S. Dublin* isolates (14%, n=3/22) were of sequence type ST10 (Table 4).

4.4.2 Comparison of *Salmonella* Sequence Types and Resistance Phenotypes

The antimicrobial susceptibility results showed that *S. Dublin* isolates used in the study belong to sequence type 10 (ST10) and were susceptible to all the antimicrobial agents used (Table 4). On the other hand, a higher proportion of these isolates carried the *st313-td* gene (Table 4). Out of the 19 genotyped *S. Typhimurium* isolates, 15 (78%) of them were classified as MDRs (Table 5). The only ST19 isolate showed resistance to two classes of antibiotics (Table 5). Interestingly, one *S. Typhimurium* ST313 isolate was found to be susceptible to all the antibiotics (Table 5). By comparing the virulence trait with MDR phenotype among ST313 clones, a greater proportion (53%) carried the *st313-td* gene. This observation was shown to be statistically insignificant by using Fisher's Exact Test ($p > 0.05$)

Table 3: Summary of *st313-td* gene amplification and Multi-Locus Sequence Typing

Serovar	Presence of <i>st313-td</i> (n)	Absence of <i>st313-td</i> (n)	Total	Sequence Type (% of total)
<i>S. Typhimurium</i>	12	7	19	ST313 (82%) ST19 (4%)
<i>S. Dublin</i>	2	1	3	ST10 (14%)
Total	14	8	22	
Abbreviation:	n=number of isolates			

Table 4: Phenotypic and Genotypic Characterization of *S. Dublin*

Serovar	Isolate (%)	<i>st313-td</i> gene	Antimicrobial Sensitivity	Sequence Type
<i>S. Dublin</i>	67	Present	Susceptible to all antibiotics	ST10
	33	Absent	Susceptible to all antibiotics	ST10

Table 5: Relationship between *S. Typhimurium* Genotype, Resistance Profile and Virulence Trait

No.	<i>st313-td</i> gene	Sequence Types	Resistance Profile
1	present	ST313	MDR
2	present	ST313	MDR
3	present	ST313	MDR
4	present	ST313	Resistant to two classes of antibiotics
5	present	ST313	MDR
6	absent	ST313	susceptible to all antibiotics
7	present	ST313	MDR
8	present	ST313	MDR
9	absent	ST313	MDR
10	present	ST313	MDR
11	present	ST313	MDR
12	absent	ST19	Resistant to two classes of antibiotics
13	present	ST313	MDR
14	present	ST313	MDR
15	absent	ST313	MDR
16	absent	ST313	MDR
17	present	ST313	MDR
18	absent	ST313	MDR
19	absent	ST313	Resistant to two classes of antibiotics
MDR=multidrug resistant			

4.5 Characterization of ST313 Resistance to Ciprofloxacin

4.5.1 MIC determination and growth patterns of ciprofloxacin resistant isolates

Prior to induction of resistance, the MICs of Ciprofloxacin susceptible (113552) and intermediate (113821) isolates were determined as 0.015 µg/ml and 0.25 µg/ml respectively (Table 6). The resistant isolates (113552_Res1, 113552_Res2, and 113552_Res4) were obtained after exposing the susceptible ST313 isolate (113552) to increasing concentrations of ciprofloxacin as outlined in the methods. The MICs for 113552_Res1 and 113552_Res2, and 113552_Res4 were 1, 2, and 4 µg/ml respectively (Table 6).

Table 6: Summary of Minimum Inhibitory Concentration and Resistance Characteristics

Sample	MIC (µg/ml)	Ciprofloxacin Susceptibility
113552	0.015	Susceptible
113821	0.25	Intermediate
113552_Res1	1	Resistant
113552_Res2	2	Resistant
113552_Res4	4	Resistant

To establish the effects of ciprofloxacin resistance on bacterial growth, the optical density (OD₆₀₀) of each bacterial culture including the control parental isolate (113552) was monitored at different time points (figure 6). There was no statistically significant difference between the resistant and susceptible isolates. However, 113552_Res4 isolate showed the highest exponential growth rate as compared to the other isolates (figure 6). This observation was contrary to that of 113552_Res1 which measured the lowest OD (figure 6). After 14 hours, the isolates seemed to have entered the stationary phase except for 113552_Res2 (figure 6).

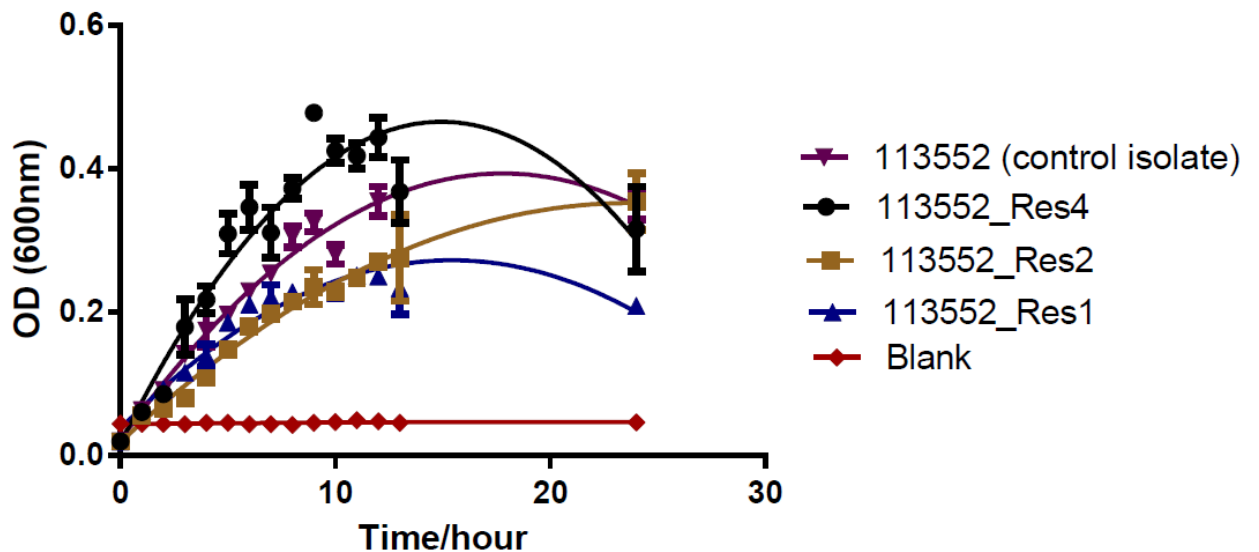


Figure 6: Comparison of growth patterns among *Salmonella* ST313 isolates. The graph shows the mean $\pm$ SD optical density (OD) measured for each isolate over time. The blank was LB broth and the control organism was 113552. Resistant isolates 113552_Res1, 113552_Res2, and 113552_Res4 were obtained after exposing the control organisms to 1ug/ml, 2ug/ml and 4ug/ml Ciprofloxacin concentrations respectively.

4.5.2 Expression analysis of genes related to ciprofloxacin resistance

To understand the mechanism of resistance, changes in mRNA transcript levels of selected genes (*gyrA*, *acrA*, *ydhJ*, *emrD*, and *soxR*) after exposure to ciprofloxacin were monitored by reverse transcription quantitative PCR (figure 7). These targeted genes are known to be regulated in responses to fluoroquinolones (Li et al., 2017). Amplification of the endogenous control *16SrRNA* gene resulted in low Ct values (between 4.9 and 6.9) corresponding to its high abundance in the cultured samples. Some differences in *16SrRNA* abundance were also observed between tested samples, which could be attributed to differential growth of *Salmonella* cells under varying ciprofloxacin exposure. To avoid any confounding effect of cell numbers on the quantitative analysis of mRNA transcript levels, the expression of each targeted gene was first log transformed and normalized to the *16S rRNA* reference control. The results showed significant variation in gene expression levels between the susceptible, intermediate, and resistant isolates (figure 7). The transcript levels were generally higher for susceptible than the resistant isolates. For most of the genes, significant differences in mRNA transcript levels were also observed between the intermediate and induced resistant isolates (figure 7). For the induced resistant isolate, there were no significant differences between the mRNA transcripts levels of the five genes notwithstanding *emrD* gene was the least expressed.

Table 7: Ciprofloxacin Concentrations used for RNA Expression

No.	Sample ID	Ciprofloxacin concentration used
1	susceptible	0.0075 µg/ml
2	intermediate	0.125 µg/ml
3	Resistant	0.125 µg/ml

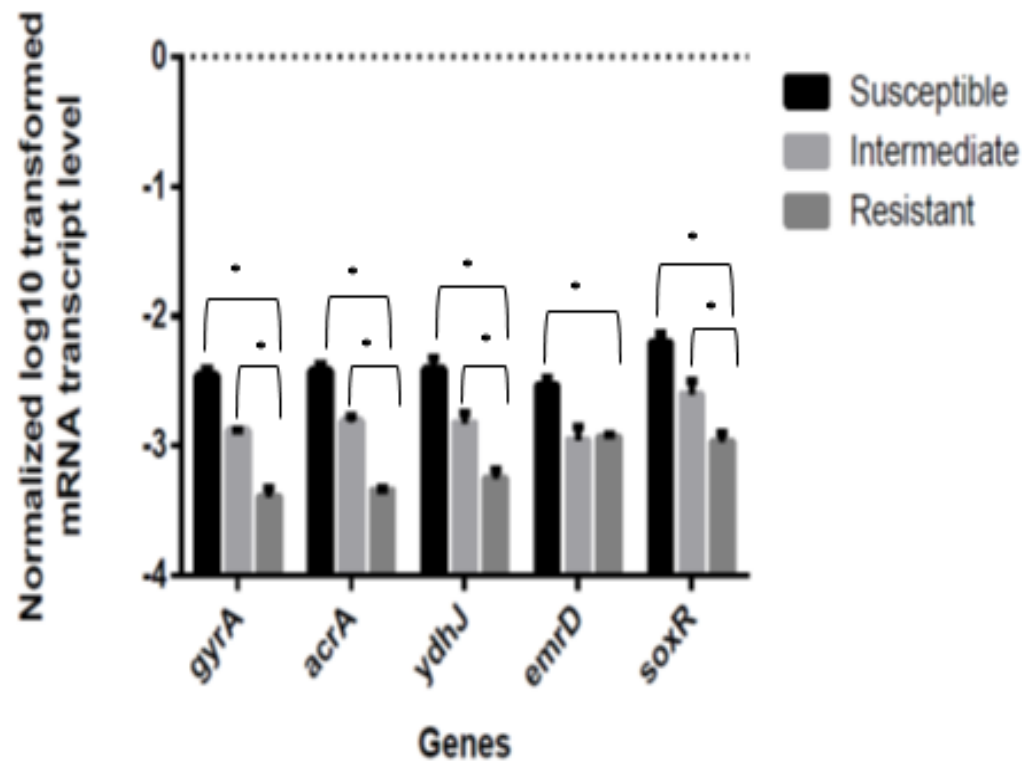


Figure 7: Normalized log₁₀ transformed mRNA transcript levels of target genes in *Salmonella* isolates. The expression levels of *gyrA*, *acrA*, *ydhJ*, *emrD* and *soxR* in susceptible, intermediate and resistant isolates after exposure to ciprofloxacin were determined by RT-qPCR analysis. The mRNA transcript levels were normalized to the endogenous control 16SrRNA. The data represent mean $\pm$ SD of three technical replicates and (*) is $p < 0.05$

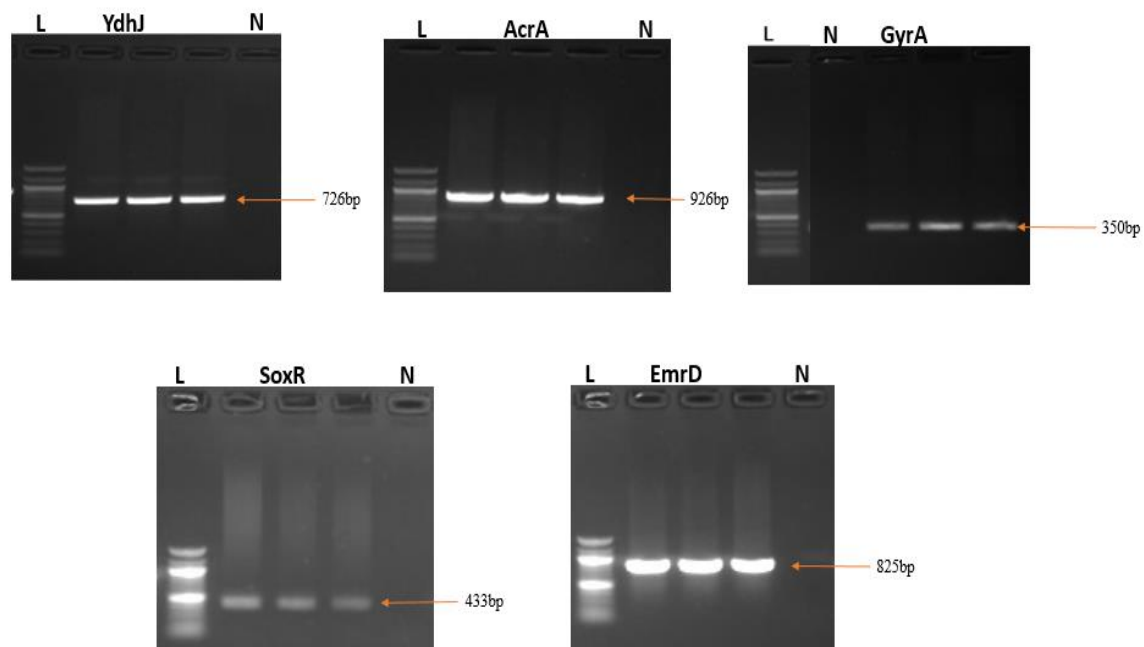


Figure 8: Agarose gel representation of PCR amplicons. PCR products of the following genes (from left to right) *YdhJ* (726bp), *AcrA* (926bp), and *GyrA* (350bp), *SoxR* (433bp) & *EmrD* (825bp) among resistant, intermediate and susceptible isolates. N was the negative no template control and L was 100bp DNA ladder

4.5.3 DNA Sequence Analysis of Resistance Target Gene

Although no changes in gene expression were detected in the resistant isolate, this does not preclude the generation of mutations in those genes. To explore this further, PCR amplification and sequencing of all five genes (*gyrA*, *acrA*, *ydhJ*, *emrD*, and *soxR*) were performed for the susceptible, intermediate and resistant isolates (figure 8). From the sequence analyses, a SNP was observed at codon 324 (C → A) leading to a change from phenylalanine to isoleucine (p-value, << 0.001) in the *gyrA* gene of the clinical intermediate resistant isolate (Figure 9) However, no statistically significant changes in SNPs were observed for *acrA*, *ydhJ*, *emrD*, and *soxR* genes among the three isolates (Appendix Table 5).

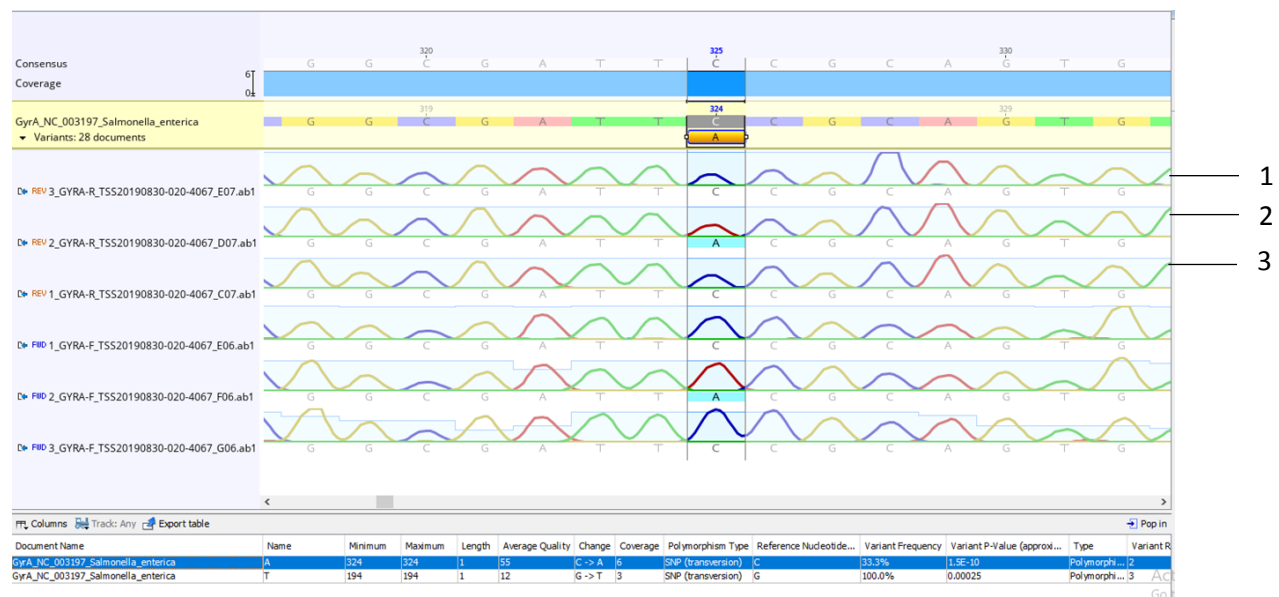


Fig 9: Sample visualization of SNPs. Nucleotide sequence of *gyrA* gene where 1, 2, and 3 represent susceptible, intermediate, and resistant respectively.

CHAPTER FIVE

5.0 DISCUSSION, CONCLUSION AND RECOMMENDATION

5.1 Discussion

Invasive NTS disease is considered as the one of the most important cause of morbidity and mortality in sub-Saharan Africa (Kariuki et al., 2015; Guiraud et al., 2017). There have been reports of high levels of resistance to ampicillin, sulphamethoxazole-trimethoprim and chloramphenicol among pathogens causing bacterial bloodstream infections in Africa (Bernabe et al., 2017). *S. Typhimurium* is one of the major serovars implicated in human invasive NTS infections (Kariuki et al., 2015; Bernabe et al., 2017). Moderate proportions of host adapted serovars such as *S. Dublin* which causes salmonellosis in cattle have also been found in humans (Andoh et al., 2017). This study was set to investigate the virulent genotypes of NTS and their importance in driving multidrug resistance in Ghana. Earlier reports in other countries in sSA have associated multidrug resistance to invasiveness (Gordon et al., 2008; Kariuki et al. 2015; Akullian et al., 2018). To determine the frequency of multi-drug resistant NTS pathogens causing bloodstream infections in febrile children, fifty-one (51) invasive NTS isolates of *S. Typhimurium* and *S. Dublin* were obtained for the study (Table 2). It was observed that high proportions of *S. Typhimurium* isolates displayed MDR phenotype. These pathogens were mostly resistant to chloramphenicol, sulphamethoxazole-trimethoprim, ampicillin, and amoxicillin. The results obtained in this study is supported by those conducted by Andoh et al (2017) who reported high prevalence of NTS isolates with resistance to the multiple antimicrobials described above. These drugs were previously used as first-line therapeutic agents for the treatment of bacterial infections in Ghana (Ando et al., 2017). However, 3rd generation cephalosporins and fluoroquinolones are currently prescribed as treatment drugs for salmonellosis (Kariuki et al., 2005; Ando et al., 2017). The authors also reported about

2-8% level of resistance to cephalosporins. In this current study, it was observed that one *S. Typhimurium* isolate showed reduced susceptibility to cefuroxime, a 2nd generation cephalosporin. Interestingly, all isolates were susceptible to ceftriaxone (figure 4). Several other studies have no reports of *Salmonella* isolates associated with ceftriaxone resistance (Schwarz et al., 2010; Lui et al., 2011; Bernabe et al., 2017). This suggests that third generation cephalosporins may serve as important drug choice when other third-line drugs are less effective in sub-Saharan Africa. Another important observation was the reduced susceptibility to fluoroquinolone, albeit in a low proportion of *S. Typhimurium* isolates (figure 4B). Recent reports have shown the emergence of fluoroquinolone non-susceptible invasive NTS pathogens in Ghana (Eibach et al., 2016). This report was consistent with the AST and MIC results obtained in this study (figure 4 and Table 6). Studies by Giraud et al (2000) observed that *Salmonella* pathogens showing resistance to fluoroquinolones were also resistant to non-related antimicrobials. It was evident from our analysis that all isolates with reduced ciprofloxacin susceptibility were also classified as MDR. Fluoroquinolone resistance has become of great concern since these drugs are widely being used for treatment of invasive salmonellosis (Kariuki et al., 2015). Generally, the AST have shown high prevalence of MDR NTS pathogens implicated in bloodstream infections.

Resistance genes borne on mobile genetic elements such as plasmids and integrons have been found to mediate the spread of resistance among *Salmonella* isolates (Orman et al., 2002; Krauland et al., 2009). Most studies have identified resistance genes including *qnr*, *bla_{Tem}*, *catA1*, *sul1*, *sul2*, *tetA*, *tetB*, *dfrA14*, *dfrA1*, *aac(3)IV*, *aadA*, and *strA* among others in MDR pathogenic bacteria (Bräu et al., 1984; Heuer et al., 2002; Orman et al., 2002; Leekitcharoenphon et al., 2013; Rahmani et al., 2013; Vuthy et al., 2017; Kagambèga et al., 2018). In this study, there were no detection of *aac(3)IV* or *dfrA14* encoding gentamicin or trimethoprim resistance respectively among the

isolates screened. However, *strA* and *tetB* genes, *bla_{Tem}*, *catA*, and *tetA* genes and *sul1*, *sul2*, and *aadA* genes were present in low, moderate, and high levels respectively (Figure 5). Some isolates carried resistance genes yet did not elicit phenotypic resistance to the antibiotics. This observation was similar to the results obtained by Kagambèga et al (2018) who revealed the presence of *tetB* gene among *Salmonella* whole genome sequences without corresponding phenotypic resistance to tetracycline. Generally, most of the isolates showed corresponding antibiotic resistance phenotype. This goes on to confirm the suggestions of Orman et al (2002) that drug resistance is often mediated by horizontal acquisition of resistance genes.

NTS pathogens were originally associated with local infections in the gastro-intestines. In sub-Saharan Africa, however, NTS has been implicated in bacteremia (Kariuki et al., 2015). To evolve into invasive forms, researchers have proposed that the hyper-virulent pathogen may possess repertoire of virulence genes which increases its pathogenicity (Herrero-Fresno et al., 2014). In a study conducted by Herrero-Fresno et al, whole genome comparison among *Salmonella* strains revealed the presence of a pathogenicity island in *S. Typhimurium* ST313 genome known as ST313-GI which encodes prophage proteins for replication, excision, and integration. This 18kb genome island (GI) is found to harbor a virulence gene, *st313-td*, which plays important role in bacterial bloodstream invasion. The authors found about 93% of 82 bloodstream *S. Typhimurium* and 100% of 50 *S. Dublin* isolates harbored the *st313-td* gene by PCR amplification. MLST analysis showed that this proportion of *S. Typhimurium* strains belonged to ST313 clade and originated from sub-Saharan Africa. It was noted that about all other *S. Typhimurium* isolates sampled from various sources aside human blood lacked the presence of the gene. Interestingly, a smaller fraction (about 6.8kb) of ST313-GI were present in *S. Dublin* chromosome. They therefore concluded that the complete GI responsible for increased pathogenicity was exclusively found in

S. Typhimurium ST313. In this present study, *st313-td* was detected in 14 out of 51 (27%) bloodstream NTS pathogens (Table 3). The MLST analysis showed 18 out of a total of 22 invasive NTS isolates were *S. Typhimurium* ST313, representing the most common genotype (Table 3). Additionally, two *S. Dublin* isolates carrying the *st313-td* belonged to ST10 genotype (Table 3 & 4). One ST19 isolate lacking the *st313-td* gene was also identified (Table 3 & 5). This study further sought to establish the virulence of ST313 genotypes by comparing the resistance phenotype with virulence trait. Although there was no statistically significant association, it was noted that a moderate level (about 58%) of *S. Typhimurium* ST313 isolates harboring the *st313-td* gene displayed MDR phenotype (Table 5). In addition, these pathogens carried several of the plasmid encoded antimicrobial resistance genes (Appendix Table 2). Importantly, increasing levels of resistance to third line drugs were observed among ST313 isolates. On the Other hand, all *S. Dublin* ST10 isolates were susceptible to all antibiotics (Table 4), and this was consistent with the findings of Herrero-Fresno et al (2014).

Few studies have investigated the expression of important genes regulating resistance to fluoroquinolones (Qin et al., 2018). Mostly, molecular investigation is carried by identifying nucleotide polymorphisms in the resistant strains (Chattaway et al., 2016). Such studies have limitation in the sense that certain genetic mutations may be non-synonymous. Thus, determining the impact of fluoroquinolones on gene expression of pathogenic bacteria may provide a step towards understanding their resistance mechanisms. The study therefore aimed to investigate possible mechanisms of resistance in a hyper-virulent *S. Typhimurium* ST313 clone by gene expression analysis. Firstly, laboratory induced ciprofloxacin resistant isolates were generated from susceptible clinical isolate *in vitro*. This was to reduce confounding factors such as host stress response adaptation which may influence the effects of ciprofloxacin on the bacteria. Analysis of

the growth behavior revealed there was no statistical difference between the susceptible and induced resistant isolates (figure 7). On the contrary, a study in *Haemophilus parasuis* showed faster growth rate in tildipirosin induced resistant strains (Li et al., 2017). The growth pattern observed in this present study was important since it established successful generation of isogenic resistant isolates which differed from the susceptible wildtype based on their response to ciprofloxacin. Variation in gene expression was investigated by comparing normalized mRNA transcript levels of *gyrA*, *acrA*, *ydhJ*, *emrD* and *soxR* among susceptible, intermediate, and induced resistant isolates (figure 7). These genes were selected based on a transcriptomic study of ciprofloxacin resistant *S. Typhimurium* reference strain ATCC 13311 (Li et al., 2017). In the current study, high levels of mRNA transcripts were found in the susceptible wildtype as compared to the intermediate and induced resistant isolates for all five genes (p value <0.05) (figure 7). This contradicted the findings of Li et al where the authors revealed increased expression of *gyrA*, *acrA*, *ydhJ* and *emrD* in *Salmonella* resistant isolates. High levels of expression in the susceptible isolate have been described by Erickson et al (2017) as adaptive resistance mechanism used by bacteria in transient stress response. In their study, the authors found increased expression of resistance genes among *E. coli* strains after exposure to antibiotic stress (Erickson et al., 2017). Therefore, it could be suggested that the observed relatively high expression in the NTS wildtype isolate may be due to adaptive measures mounted to withstand new environmental stress (that is exposure to ciprofloxacin) unlike the resistant or intermediate that may require higher dosage to induce similar level of response. Surprisingly, Li et al also found out by transcriptomic and RT-qPCR analyses that *soxR* gene expression was downregulated in induced resistant *Salmonella* strain. This present study reported low levels of *soxR* mRNA transcripts in resistant isolates indicating downregulation of the gene, which agrees with the data obtained by Li et al (2017).

In this work, resistant isolates were exposed to lower ciprofloxacin concentration (0.125µg/ml), far less than its determined MIC. Goh et al (2002) have previously suggested that bacterial transcription are greatly affected by the concentrations of the antimicrobial agents used for induction. The group further suggested that the effects of sub-inhibitory concentrations on transcription is independent of the inhibitory actions of the drugs. During transcription, the expression machinery employs various mechanisms to detect subtle levels of antimicrobial agents to produce the appropriate stress response by upregulation or downregulation of target genes (Goh et al, 2002).

Point mutations resulting in polymorphic differences in bacteria genome may contribute to antimicrobial resistance mechanisms. The fact that we do not observe changes at the transcript level does not rule out mutations in the target genes. Polymorphism arising from point mutation at the quinolone resistance determining region (QRDR) has served as important contributing factor to fluoroquinolone resistance (Chattaway et al., 2016). Here, we exploited the nucleotide sequence variations among susceptible, intermediate, and resistant *S. Typhimurium* isolates to identify SNPs involved in ciprofloxacin resistance. Notably, a SNP at position 324 in the *gyrA* gene of the clinical intermediate isolate was identified that resulted in transversion mutation C→A (p value << 0.001) and a subsequent amino acid change from Phe to Asp. According to several published reports, the most frequently observed point mutations in *gyrA* occur at QRDR codon ⁸³Ser to Phe/Tyr/Ala or ⁸⁷Asp to Tyr/Gly/Asn. Thus, the single *gyrA* mutation (³²⁴Phe to Asp) could be a novel one. Although, mutation in *gyrA* may have a more direct contribution to the resistance phenotype (Casper et al., 2017), further analysis is recommended to validate the synonymy of the mutations and/or its association with reduced ciprofloxacin susceptibility. On the other hand, no significant SNPs were found in *AcrA*, *SoxR*, *EmrD*, and *YdhJ* genes (*p-value* ≥0.001).

5.2 Conclusion

There is high preponderance of MDR *S. Typhimurium* ST313 among children suffering from fever according to the study. This situation is worsened by the observed reduced susceptibility to fluoroquinolones. Further investigation of possible ciprofloxacin resistance mechanisms by gene expression showed significant variations in mRNA transcript levels among susceptible, intermediate, and resistant *S. Typhimurium* isolates. Nonetheless, the activities of the drug at both genomic and transcriptional levels suggest the need for a whole genomic and transcriptomic approach to fully understand the interplay of fluoroquinolone resistance mechanisms in iNTS ST313.

This study has a practical public health implication as fluoroquinolones are still being used as drug of choice for treatment of salmonellosis, the knowledge of the endemic genotypes and their resistance mechanisms would provide a step towards developing effective therapeutic interventions.

5.3 Limitations and Recommendations

The study involved a retrospective approach and therefore could not completely reflect on the current disease situation and treatment results. The limited time available for fresh sample collection and for conducting the entire research necessitated the use of archived isolates. Nevertheless, the information obtained in the study could still be relevant for informed decision making on antibiotic resistance management as well as a premise for future NTS genotype research.

The expected proportion of patients suffering from iNTS was calculated based on a study at Korle Bu Teaching Hospital – a tertiary hospital in Ghana (Labi et al, 2014). There is limited epidemiological information on invasive non-typhoidal salmonellosis in Ghana. The few studies have suggested a wide prevalence rate ranging between 2.5% and 57% (Schwarz et al. 2010;

Nielsen et al., 2012; Labi et al. 2016. Opintan and Newman, 2017). This include studies from both rural and tertiary hospitals. The present study based its sample size calculation on Labi's study at a tertiary hospital with a prevalence rate of 4% because it is also a referral hospital where several clinical NTS cases are expected to be reported.

Data from MLST could not allow for the discovery of new emerging strains since the method involves the amplification of 7 housekeeping genes which rarely undergo molecular changes. Thus, whole genome sequences would be a recommended option since this approach provide vast genetic information including MLST data.

In the future, it is recommended that a prospective research approach should be carried with a larger sample size which would provide a true reflection of the present moment. With the fight against multidrug resistance, the development of vaccines should more promising solution to the problem. Also, avoiding sub-optimal concentrations and indiscriminate use or abuse of drugs against *Salmonella* infections is critical. Prescription of antimicrobial targeting specific causative organism, rather than relying on a broad-spectrum antimicrobial should be encouraged for the management of febrile illness. Antibiotics should only be prescribed by health professionals when necessary. We should improve on national surveillance for antimicrobial resistance.

REFERENCES

- Akullian, A., Montgomery, J. M., John-Stewart, G., Miller, S. I., Hayden, H. S., Radey, M. C., Hager, K. R., Verani, J. R., Ochieng, J. B., & Juma, J. (2018). Multi-drug resistant non-typhoidal Salmonella associated with invasive disease in western Kenya. *PLoS Neglected Tropical Diseases*, 12(1), e0006156.
- Al kraiem, A. A., Yang, G., Al kraiem, F., & Chen, T. (2018). Challenges associated with ceftriaxone resistance in Salmonella. *Frontiers in Life Science*, 11(1), 26–34.
- Andoh, L. A., Ahmed, S., Olsen, J. E., Obiri-Danso, K., Newman, M. J., Opintan, J. A., Barco, L., & Dalsgaard, A. (2017). Prevalence and characterization of Salmonella among humans in Ghana. *Tropical Medicine and Health*, 45(1), 3.
- Ashton, P. M., Nair, S., Peters, T., Bale, J., Powell, D. G., Painset, A., Tewolde, R., Jenkins, C., Dallman, T. J., & DePinna, E. M. (2015). Identification and typing of Salmonella for public health surveillance using whole genome sequencing. *PeerJ PrePrints*, 3, e1425v1.
- Baggesen, D. L., Sørensen, G., Nielsen, E., & Wegener, H. C. (2010). Phage typing of Salmonella Typhimurium—is it still a useful tool for surveillance and outbreak investigation? *Eurosurveillance*, 15(4), 19471.
- Berkley, J. A., Lowe, B. S., Mwangi, I., Williams, T., Bauni, E., Mwarumba, S., Ngetsa, C., Slack, M. P., Njenga, S., & Hart, C. A. (2005). Bacteremia among children admitted to a rural hospital in Kenya. *New England Journal of Medicine*, 352(1), 39–47.
- Bernabe, K. J., Langendorf, C., Ford, N., Ronat, J.-B., & Murphy, R. A. (2017). Antimicrobial resistance in West Africa: A systematic review and meta-analysis. *International Journal of Antimicrobial Agents*, 50(5), 629–639.
- Blaser, M. J., & Newman, L. S. (1982). A review of human salmonellosis: I. Infective dose. *Reviews of Infectious Diseases*, 4(6), 1096–1106.

- Boadu, E. A. (2014). *The use of antibiotics and the pattern of antimicrobial resistance at SDA Hospital, Kumasi, Ghana.*
- Bräu, B., Pilz, U., & Piepersberg, W. (1984). Genes for gentamicin-(3)-N-acetyltransferases III and IV. *Molecular and General Genetics MGG*, 193(1), 179–187.
- Brenner, F., Villar, R., Angulo, F., Tauxe, R., & Swaminathan, B. (2000). Salmonella nomenclature. *Journal of Clinical Microbiology*, 38(7), 2465–2467.
- Caspar, Y., Siebert, C., Suter, V., Villers, C., Aubry, A., Mayer, C., Maurin, M., & Renesto, P. (2017). Functional characterization of the DNA gyrase in fluoroquinolone-resistant mutants of *Francisella novicida*. *Antimicrobial Agents and Chemotherapy*, 61(4).
- Charan, J., & Biswas, T. (2013). How to calculate sample size for different study designs in medical research? *Indian Journal of Psychological Medicine*, 35(2), 121.
- Chattaway, M. A., Aboderin, A. O., Fashae, K., Okoro, C. K., Opintan, J. A., & Okeke, I. N. (2016). Fluoroquinolone-resistant enteric bacteria in sub-Saharan Africa: Clones, implications and research needs. *Frontiers in Microbiology*, 7, 558.
- Chau, T. T., Campbell, J. I., Galindo, C. M., Van Minh Hoang, N., Diep, T. S., Nga, T. T. T., Van Vinh Chau, N., Tuan, P. Q., Page, A. L., Ochiai, R. L., Schultsz, C., Wain, J., Bhutta, Z. A., Parry, C. M., Bhattacharya, S. K., Dutta, S., Agtini, M., Dong, B., Honghui, Y., ... Dolecek, C. (2007). Antimicrobial Drug Resistance of *Salmonella enterica* Seroovar Typhi in Asia and Molecular Mechanism of Reduced Susceptibility to the Fluoroquinolones. *Antimicrobial Agents and Chemotherapy*, 51(12), 4315.
<https://doi.org/10.1128/AAC.00294-07>

- Chen, H.-M., Wang, Y., Su, L.-H., & Chiu, C.-H. (2013). Nontyphoid Salmonella Infection: Microbiology, Clinical Features, and Antimicrobial Therapy. *Pediatrics & Neonatology*, 54(3), 147–152. <https://doi.org/10.1016/j.pedneo.2013.01.010>
- Cooke, F. J., Wain, J., Fookes, M., Ivens, A., Thomson, N., Brown, D. J., Threlfall, E. J., Gunn, G., Foster, G., & Dougan, G. (2007). Prophage Sequences Defining Hot Spots of Genome Variation in *Salmonella enterica* Serovar Typhimurium Can Be Used To Discriminate between Field Isolates. *Journal of Clinical Microbiology*, 45(8), 2590. <https://doi.org/10.1128/JCM.00729-07>
- Crum-Cianflone, N. F. (2008). Salmonellosis and the gastrointestinal tract: More than just peanut butter. *Current Gastroenterology Reports*, 10(4), 424–431. PubMed. <https://doi.org/10.1007/s11894-008-0079-7>
- Crump, J. A., Luby, S. P., & Mintz, E. D. (2004). The global burden of typhoid fever. *Bulletin of the World Health Organization*, 82, 346–353.
- Cruz Espinoza, L. M., Nichols, C., Adu-Sarkodie, Y., Al-Emran, H. M., Baker, S., Clemens, J. D., Dekker, D. M., Eibach, D., Krumkamp, R., Boahen, K., Im, J., Jaeger, A., von Kalckreuth, V., Pak, G. D., Panzner, U., Park, S. E., Park, J. K., Sarpong, N., Schütt-Gerowitt, H., ... May, J. (2016). Variations of Invasive Salmonella Infections by Population Size in Asante Akim North Municipal, Ghana. *Clinical Infectious Diseases*, 62(suppl_1), S17–S22. <https://doi.org/10.1093/cid/civ787>
- De Jong, H. K., Parry, C. M., van der Poll, T., & Wiersinga, W. J. (2012). Host–pathogen interaction in invasive salmonellosis. *PLOS Pathog*, 8(10), e1002933.
- Duodu, S., Holst-Jensen, A., Skjerdal, T., Cappellet, J.-M., Pilet, M.-F., & Loncarevic, S. (2010). Influence of storage temperature on gene expression and virulence potential of *Listeria*

- monocytogenes strains grown in a salmon matrix. *Food Microbiology*, 27(6), 795–801.
<https://doi.org/10.1016/j.fm.2010.04.012>
- Eibach, D., Al-Emran, H. M., Dekker, D. M., Krumkamp, R., Adu-Sarkodie, Y., Cruz Espinoza, L. M., Ehmen, C., Boahen, K., Heisig, P., Im, J., Jaeger, A., von Kalckreuth, V., Pak, G. D., Panzner, U., Park, S. E., Reinhardt, A., Sarpong, N., Schütt-Gerowitt, H., Wierzba, T. F., ... May, J. (2016). The Emergence of Reduced Ciprofloxacin Susceptibility in *Salmonella enterica* Causing Bloodstream Infections in Rural Ghana. *Clinical Infectious Diseases*, 62(suppl_1), S32–S36. <https://doi.org/10.1093/cid/civ757>
- Erickson, K. E., Otoupal, P. B., & Chatterjee, A. (2017). Transcriptome-Level Signatures in Gene Expression and Gene Expression Variability during Bacterial Adaptive Evolution. *MSphere*, 2(1), e00009-17. PubMed. <https://doi.org/10.1128/mSphere.00009-17>
- Fakhr, M. K., Nolan, L. K., & Logue, C. M. (2005). Multilocus Sequence Typing Lacks the Discriminatory Ability of Pulsed-Field Gel Electrophoresis for Typing *Salmonella enterica* Serovar Typhimurium. *Journal of Clinical Microbiology*, 43(5), 2215.
<https://doi.org/10.1128/JCM.43.5.2215-2219.2005>
- Feasey, N. A., Dougan, G., Kingsley, R. A., Heyderman, R. S., & Gordon, M. A. (2012). Invasive non-typhoidal salmonella disease: An emerging and neglected tropical disease in Africa. *The Lancet*, 379(9835), 2489–2499. [https://doi.org/10.1016/S0140-6736\(11\)61752-2](https://doi.org/10.1016/S0140-6736(11)61752-2)
- Foley, S. L., Lynne, A. M., & Nayak, R. (2009). Molecular typing methodologies for microbial source tracking and epidemiological investigations of Gram-negative bacterial foodborne pathogens. *Infection, Genetics and Evolution*, 9(4), 430–440.
<https://doi.org/10.1016/j.meegid.2009.03.004>

Giannella, R. (1996a). Salmonella. Chapter 21. *Medical Microbiology*. 4th Edn. Galveston, TX.

Giannella, R. (1996b). Salmonella, in medical microbiology, Baron S, Editor. *Galveston (TX)*:

University of Texas Medical Branch at Galveston.

<https://www.ncbi.nlm.nih.gov/books/NBK8435/>

Giraud, E., Cloeckert, A., Kerboeuf, D., & Chaslus-Dancla, E. (2000). Evidence for Active Efflux as the Primary Mechanism of Resistance to Ciprofloxacin in *Salmonella enterica* Serovar Typhimurium. *Antimicrobial Agents and Chemotherapy*, 44(5), 1223.

<https://doi.org/10.1128/AAC.44.5.1223-1228.2000>

Goh, E.-B., Yim, G., Tsui, W., McClure, J., Surette, M. G., & Davies, J. (2002). Transcriptional modulation of bacterial gene expression by subinhibitory concentrations of antibiotics. *Proceedings of the National Academy of Sciences*, 99(26), 17025.

<https://doi.org/10.1073/pnas.252607699>

Gordon, M. A. (2008). Salmonella infections in immunocompromised adults. *Journal of Infection*, 56(6), 413–422. <https://doi.org/10.1016/j.jinf.2008.03.012>

Gordon, M. A., Graham, S. M., Walsh, A. L., Wilson, L., Phiri, A., Molyneux, E., Zijlstra, E. E., Heyderman, R. S., Hart, C. A., & Molyneux, M. E. (2008). Epidemics of Invasive *Salmonella enterica* Serovar Enteritidis and *S. enterica* Serovar Typhimurium Infection Associated with Multidrug Resistance among Adults and Children in Malawi. *Clinical Infectious Diseases*, 46(7), 963–969. <https://doi.org/10.1086/529146>

Grainger, J., Hurst, J., & Burdass, D. (2001). *Basic practical microbiology: A manual* (4th ed.). The Society for General Microbiology.

<https://microbiologyonline.org/file/fa3f7a18b16eef6c8eb80069a17c4bcc.pdf>

- Grimont, P. A., & Weill, F.-X. (2007). Antigenic formulae of the *Salmonella* serovars. *WHO Collaborating Centre for Reference and Research on Salmonella*, 9, 1–166.
- Groß, U., Amuzu, S. K., de Ciman, R., Kassimova, I., Groß, L., Rabsch, W., Rosenberg, U., Schulze, M., Stich, A., & Zimmermann, O. (2011). Bacteremia and antimicrobial drug resistance over time, Ghana. *Emerging Infectious Diseases*, 17(10), 1879–1882. PubMed. <https://doi.org/10.3201/eid1710.110327>
- Guiraud, I., Post, A., Diallo, S. N., Lompo, P., Maltha, J., Thriemer, K., Tahita, C. M., Ley, B., Derra, K., Bottieau, E., Kazienga, A., Schurmans, C., Ravinetto, R., Rouamba, E., Van Griensven, J., Bertrand, S., Tinto, H., & Jacobs, J. (2017). Population-based incidence, seasonality and serotype distribution of invasive salmonellosis among children in Nanoro, rural Burkina Faso. *PLOS ONE*, 12(7), e0178577. <https://doi.org/10.1371/journal.pone.0178577>
- Hanning, I. B., Nutt, J. D., & Ricke, S. C. (2009). Salmonellosis Outbreaks in the United States Due to Fresh Produce: Sources and Potential Intervention Measures. *Foodborne Pathogens and Disease*, 6(6), 635–648. <https://doi.org/10.1089/fpd.2008.0232>
- Hayden, H. S., Matamouros, S., Hager, K. R., Brittnacher, M. J., Rohmer, L., Radey, M. C., Weiss, E. J., Kim, K. B., Jacobs, M. A., Sims-Day, E. H., Yue, M., Zaidi, M. B., Schifferli, D. M., Manning, S. D., Walson, J. L., & Miller, S. I. (2016). Genomic Analysis of *Salmonella enterica* Serovar Typhimurium Characterizes Strain Diversity for Recent U.S. Salmonellosis Cases and Identifies Mutations Linked to Loss of Fitness under Nitrosative and Oxidative Stress. *MBio*, 7(2), e00154-16. <https://doi.org/10.1128/mBio.00154-16>

- Hemraj, V., Diksha, S., & Avneet, G. (2013). A review on commonly used biochemical test for bacteria. *Innovare Journal of Life Sciences*, 1(1).
<https://innovareacademics.in/journals/index.php/ijls/article/view/30>
- Henderson, B., Poole, S., & Wilson, M. (1996). Bacterial modulins: A novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. *Microbiological Reviews*, 60(2), 316.
- Hendriksen, R. S., Vieira, A. R., Karlsmose, S., Lo Fo Wong, D. M. A., Jensen, A. B., Wegener, H. C., & Aarestrup, F. M. (2011). Global Monitoring of Salmonella Serovar Distribution from the World Health Organization Global Foodborne Infections Network Country Data Bank: Results of Quality Assured Laboratories from 2001 to 2007. *Foodborne Pathogens and Disease*, 8(8), 887–900. <https://doi.org/10.1089/fpd.2010.0787>
- Herrero-Fresno, A., Wallrodt, I., Leekitcharoenphon, P., Olsen, J. E., Aarestrup, F. M., & Hendriksen, R. S. (2014). The Role of the st313-td Gene in Virulence of Salmonella Typhimurium ST313. *PLOS ONE*, 9(1), e84566.
<https://doi.org/10.1371/journal.pone.0084566>
- Hume, P. J., Singh, V., Davidson, A. C., & Koronakis, V. (2017). Swiss Army Pathogen: The Salmonella Entry Toolkit. *Frontiers in Cellular and Infection Microbiology*, 7, 348.
<https://doi.org/10.3389/fcimb.2017.00348>
- Iddrisu, L. F. (2014). Aetiological agents and their antimicrobial susceptibility patterns in children under five years of age presenting with bloodstream infections at Kintampo Municipal Hospital. *University of Ghana, Legon*.
- Issenhuth-Jeanjean, S., Roggentin, P., Mikoleit, M., Guibourdenche, M., de Pinna, E., Nair, S., Fields, P. I., & Weill, F.-X. (2014). Supplement 2008–2010 (no. 48) to the White–

- Kauffmann–Le Minor scheme. *Research in Microbiology*, 165(7), 526–530.
<https://doi.org/10.1016/j.resmic.2014.07.004>
- Kagambèga, A., Lienemann, T., Frye, J. G., Barro, N., & Haukka, K. (2018). Whole genome sequencing of multidrug-resistant *Salmonella enterica* serovar Typhimurium isolated from humans and poultry in Burkina Faso. *Tropical Medicine and Health*, 46(1), 4.
<https://doi.org/10.1186/s41182-018-0086-9>
- Kariuki, S., Gordon, M. A., Feasey, N., & Parry, C. M. (2015). Antimicrobial resistance and management of invasive *Salmonella* disease. *Global Progress on Use of Vaccines for Invasive Salmonella Infections*, 33, C21–C29.
<https://doi.org/10.1016/j.vaccine.2015.03.102>
- Kingsley, R. A., Msefula, C. L., Thomson, N. R., Kariuki, S., Holt, K. E., Gordon, M. A., Harris, D., Clarke, L., Whitehead, S., Sangal, V., Marsh, K., Achtman, M., Molyneux, M. E., Cormican, M., Parkhill, J., MacLennan, C. A., Heyderman, R. S., & Dougan, G. (2009). Epidemic multiple drug resistant *Salmonella* Typhimurium causing invasive disease in sub-Saharan Africa have a distinct genotype. *Genome Research*, 19(12), 2279–2287.
 PubMed. <https://doi.org/10.1101/gr.091017.109>
- Knodler, L. A., Vallance, B. A., Celli, J., Winfree, S., Hansen, B., Montero, M., & Steele-Mortimer, O. (2010). Dissemination of invasive *Salmonella* via bacterial-induced extrusion of mucosal epithelia. *Proceedings of the National Academy of Sciences*, 107(41), 17733. <https://doi.org/10.1073/pnas.1006098107>
- Krauland, M. G., Marsh, J. W., Paterson, D. L., & Harrison, L. H. (2009). Integron-mediated multidrug resistance in a global collection of nontyphoidal *Salmonella enterica* isolates.

- Emerging Infectious Diseases*, 15(3), 388–396. PubMed.
<https://doi.org/10.3201/eid1503.081131>
- Labi, A.-K., Obeng-Nkrumah, N., Addison, N. O., & Donkor, E. S. (2014). Salmonella blood stream infections in a tertiary care setting in Ghana. *BMC Infectious Diseases*, 14(1), 3857. <https://doi.org/10.1186/s12879-014-0697-7>
- Labi, A.-K., Obeng-Nkrumah, N., Bjerrum, S., Enweronu-Laryea, C., & Newman, M. J. (2016). Neonatal bloodstream infections in a Ghanaian Tertiary Hospital: Are the current antibiotic recommendations adequate? *BMC Infectious Diseases*, 16(1), 598.
<https://doi.org/10.1186/s12879-016-1913-4>
- Langridge, G. C., Fookes, M., Connor, T. R., Feltwell, T., Feasey, N., Parsons, B. N., Seth-Smith, H. M. B., Barquist, L., Stedman, A., Humphrey, T., Wigley, P., Peters, S. E., Maskell, D. J., Corander, J., Chabalgoity, J. A., Barrow, P., Parkhill, J., Dougan, G., & Thomson, N. R. (2014). Patterns of genome evolution that have accompanied host adaptation in Salmonella. *Proceedings of the National Academy of Sciences*, 201416707. <https://doi.org/10.1073/pnas.1416707112>
- Langridge, G. C., Nair, S., & Wain, J. (2009). Nontyphoidal Salmonella serovars cause different degrees of invasive disease globally. *The Journal of Infectious Diseases*, 199(4), 602–603. PubMed. <https://doi.org/10.1086/596208>
- Laupland, K. B., & Church, D. L. (2014). Population-Based Epidemiology and Microbiology of Community-Onset Bloodstream Infections. *Clinical Microbiology Reviews*, 27(4), 647. <https://doi.org/10.1128/CMR.00002-14>
- Leekitcharoenphon, P., Friis, C., Zankari, E., Svendsen, C. A., Price, L. B., Rahmani, M., Herrero-Fresno, A., Fashae, K., Vandenberg, O., Aarestrup, F. M., & Hendriksen, R. S.

- (2013). Genomics of an emerging clone of *Salmonella* serovar Typhimurium ST313 from Nigeria and the Democratic Republic of Congo. *The Journal of Infection in Developing Countries*, 7(10). <https://doi.org/10.3855/jidc.3328>
- Lei, Z., Fu, S., Yang, B., Liu, Q., Ahmed, S., Xu, L., Xiong, J., Cao, J., & Qiu, Y. (2017). Comparative transcriptional profiling of tildipirosin-resistant and sensitive *Haemophilus parasuis*. *Scientific Reports*, 7(1), 7517. <https://doi.org/10.1038/s41598-017-07972-5>
- Li, L., Dai, X., Wang, Y., Yang, Y., Zhao, X., Wang, L., & Zeng, M. (2017). RNA-seq-based analysis of drug-resistant *Salmonella enterica* serovar Typhimurium selected in vivo and in vitro. *PLOS ONE*, 12(4), e0175234. <https://doi.org/10.1371/journal.pone.0175234>
- Liu, D. (2006). Identification, subtyping and virulence determination of *Listeria monocytogenes*, an important foodborne pathogen. *Journal of Medical Microbiology*, 55(6), 645–659.
- Liu, W., Liu, B., Zhu, X., Yu, S., & Shi, X. (2011). Diversity of *Salmonella* isolates using serotyping and multilocus sequence typing. *Food Microbiology*, 28(6), 1182–1189. <https://doi.org/10.1016/j.fm.2011.04.001>
- Lunguya, O., Lejon, V., Phoba, M.-F., Bertrand, S., Vanhoof, R., Glupczynski, Y., Verhaegen, J., Muyembe-Tamfum, J.-J., & Jacobs, J. (2013). Antimicrobial Resistance in Invasive Non-typhoid *Salmonella* from the Democratic Republic of the Congo: Emergence of Decreased Fluoroquinolone Susceptibility and Extended-spectrum Beta Lactamases. *PLOS Neglected Tropical Diseases*, 7(3), e2103. <https://doi.org/10.1371/journal.pntd.0002103>
- MacLennan, C. A., Gilchrist, J. J., Gordon, M. A., Cunningham, A. F., Cobbold, M., Goodall, M., Kingsley, R. A., van Oosterhout, J. J. G., Msefula, C. L., Mandala, W. L., Leyton, D. L., Marshall, J. L., Gondwe, E. N., Bobat, S., López-Macías, C., Doffinger, R.,

- Henderson, I. R., Zijlstra, E. E., Dougan, G., ... Molyneux, M. E. (2010). Dysregulated Humoral Immunity to Nontyphoidal Salmonella in HIV-Infected African Adults. *Science*, 328(5977), 508. <https://doi.org/10.1126/science.1180346>
- MacLennan, C. A., & Levine, M. M. (2013). Invasive nontyphoidal Salmonella disease in Africa: Current status. *Expert Review of Anti-Infective Therapy*, 11(5), 443–446.
- Majowicz, S. E., Musto, J., Scallan, E., Angulo, F. J., Kirk, M., O'Brien, S. J., Jones, T. F., Fazil, A., Hoekstra, R. M., & for the International Collaboration on Enteric Disease “Burden of Illness” Studies. (2010). The Global Burden of Nontyphoidal Salmonella Gastroenteritis. *Clinical Infectious Diseases*, 50(6), 882–889. <https://doi.org/10.1086/650733>
- Mandomando, I., Bassat, Q., Sigaúque, B., Massora, S., Quintó, L., Ácacio, S., Nhampossa, T., Vubil, D., Garrine, M., Macete, E., Aide, P., Saco, C., Herrera-León, S., Ruiz, J., Tennant, S. M., Menéndez, C., & Alonso, P. L. (2015). Invasive Salmonella Infections Among Children From Rural Mozambique, 2001–2014. *Clinical Infectious Diseases*, 61(suppl_4), S339–S345. <https://doi.org/10.1093/cid/civ712>
- Mandomando, I., Sigaúque, B., Morais, L., Espasa, M., Vallès, X., Sacarlal, J., Macete, E., Aide, P., Quintó, L., & Nhampossa, T. (2010). Antimicrobial drug resistance trends of bacteremia isolates in a rural hospital in southern Mozambique. *The American Journal of Tropical Medicine and Hygiene*, 83(1), 152–157.
- Marks, F., von Kalckreuth, V., Aaby, P., Adu-Sarkodie, Y., El Tayeb, M. A., Ali, M., Aseffa, A., Baker, S., Biggs, H. M., Bjerregaard-Andersen, M., Breiman, R. F., Campbell, J. I., Cosmas, L., Crump, J. A., Espinoza, L. M. C., Deerin, J. F., Dekker, D. M., Fields, B. S., Gasmelseed, N., ... Wierzbza, T. F. (2017). Incidence of invasive salmonella disease in

- sub-Saharan Africa: A multicentre population-based surveillance study. *The Lancet Global Health*, 5(3), e310–e323. [https://doi.org/10.1016/S2214-109X\(17\)30022-0](https://doi.org/10.1016/S2214-109X(17)30022-0)
- McClelland, M., Sanderson, K. E., Spieth, J., Clifton, S. W., Latreille, P., Courtney, L., Porwollik, S., Ali, J., Dante, M., Du, F., Hou, S., Layman, D., Leonard, S., Nguyen, C., Scott, K., Holmes, A., Grewal, N., Mulvaney, E., Ryan, E., ... Wilson, R. K. (2001). Complete genome sequence of *Salmonella enterica* serovar Typhimurium LT2. *Nature*, 413(6858), 852–856. <https://doi.org/10.1038/35101614>
- McGann, P., Wiedmann, M., & Boor, K. J. (2007). The alternative sigma factor sigma B and the virulence gene regulator PrfA both regulate transcription of *Listeria monocytogenes* internalins. *Applied and Environmental Microbiology*, 73(9), 2919–2930. PubMed. <https://doi.org/10.1128/AEM.02664-06>
- Milledge, J., Calis, J. C. J., Graham, S. M., Phiri, A., Wilson, L. K., Soko, D., Mbwini, M., Walsh, A. L., Rogerson, S. R., & Molyneux, E. M. (2005). Aetiology of neonatal sepsis in Blantyre, Malawi: 1996–2001. *Annals of Tropical Paediatrics*, 25(2), 101–110. <https://doi.org/10.1179/146532805X45692>
- Mollet, C., Drancourt, M., & Raoult, D. (1997). RpoB sequence analysis as a novel basis for bacterial identification. *Molecular Microbiology*, 26(5), 1005–1011. PubMed. <https://doi.org/10.1046/j.1365-2958.1997.6382009.x>
- Moon, T. D., Johnson, M., Foster, M. A., Silva, W. P., Buene, M., Valverde, E., Morais, L., Williams, J. V., Vermund, S. H., & Brentlinger, P. E. (2015). Identification of Invasive *Salmonella Enterica* Serovar Typhimurium ST313 in Ambulatory HIV-Infected Adults in Mozambique. *Journal of Global Infectious Diseases*, 7(4), 139–142. PubMed. <https://doi.org/10.4103/0974-777X.170496>

- Mweu, E., & English, M. (2008). Typhoid fever in children in Africa. *Tropical Medicine & International Health : TM & IH*, 13(4), 532–540. PubMed.
<https://doi.org/10.1111/j.1365-3156.2008.02031.x>
- Nielsen, M. V., Sarpong, N., Krumkamp, R., Dekker, D., Loag, W., Amemasor, S., Agyekum, A., Marks, F., Huenger, F., & Krefis, A. C. (2012). Incidence and characteristics of bacteremia among children in rural Ghana. *PloS One*, 7(9), e44063.
- Obeng-Nkrumah, N., Labi, A.-K., Acquah, M. E., & Donkor, E. S. (2015). Bloodstream infections in patients with malignancies: Implications for antibiotic treatment in a Ghanaian tertiary setting. *BMC Research Notes*, 8(1), 742.
<https://doi.org/10.1186/s13104-015-1701-z>
- Okoro, C. K., Barquist, L., Connor, T. R., Harris, S. R., Clare, S., Stevens, M. P., Arends, M. J., Hale, C., Kane, L., Pickard, D. J., Hill, J., Harcourt, K., Parkhill, J., Dougan, G., & Kingsley, R. A. (2015). Signatures of Adaptation in Human Invasive Salmonella Typhimurium ST313 Populations from Sub-Saharan Africa. *PLOS Neglected Tropical Diseases*, 9(3), e0003611. <https://doi.org/10.1371/journal.pntd.0003611>
- Okoro, C. K., Kingsley, R. A., Connor, T. R., Harris, S. R., Parry, C. M., Al-Mashhadani, M. N., Kariuki, S., Msefula, C. L., Gordon, M. A., de Pinna, E., Wain, J., Heyderman, R. S., Obaro, S., Alonso, P. L., Mandomando, I., MacLennan, C. A., Tapia, M. D., Levine, M. M., Tennant, S. M., ... Dougan, G. (2012). Intracontinental spread of human invasive Salmonella Typhimurium pathovariants in sub-Saharan Africa. *Nature Genetics*, 44(11), 1215–1221. PubMed. <https://doi.org/10.1038/ng.2423>
- Olobatoke, R. (2017). Public health burden of non-typhoidal Salmonella strains in sub-Saharan Africa. *International Research Journal of Public and Environmental Health*, 4, 112–119.

- Opintan, J. A., & Newman, M. J. (2017). Prevalence of antimicrobial resistant pathogens from blood cultures: Results from a laboratory based nationwide surveillance in Ghana. *Antimicrobial Resistance & Infection Control*, 6(1), 64.
- Orman, B. E., Piñeiro, S. A., Arduino, S., Galas, M., Melano, R., Caffer, M. I., Sordelli, D. O., & Centrón, D. (2002). Evolution of Multiresistance in Nontyphoid Salmonella Serovars from 1984 to 1998 in Argentina. *Antimicrobial Agents and Chemotherapy*, 46(12), 3963. <https://doi.org/10.1128/AAC.46.12.3963-3970.2002>
- Park, S. E., Pak, G. D., Aaby, P., Adu-Sarkodie, Y., Ali, M., Aseffa, A., Biggs, H. M., Bjerregaard-Andersen, M., Breiman, R. F., Crump, J. A., Cruz Espinoza, L. M., Eltayeb, M. A., Gasmelseed, N., Hertz, J. T., Im, J., Jaeger, A., Parfait Kabore, L., von Kalckreuth, V., Keddy, K. H., ... Marks, F. (2016). The Relationship Between Invasive Nontyphoidal Salmonella Disease, Other Bacterial Bloodstream Infections, and Malaria in Sub-Saharan Africa. *Clinical Infectious Diseases*, 62(suppl_1), S23–S31. <https://doi.org/10.1093/cid/civ893>
- Petti, C. A., Polage, C. R., Quinn, T. C., Ronald, A. R., & Sande, M. A. (2006). Laboratory Medicine in Africa: A Barrier to Effective Health Care. *Clinical Infectious Diseases*, 42(3), 377–382. <https://doi.org/10.1086/499363>
- Phillips, A., Sotomayor, C., Wang, Q., Holmes, N., Furlong, C., Ward, K., Howard, P., Octavia, S., Lan, R., & Sintchenko, V. (2016). Whole genome sequencing of Salmonella Typhimurium illuminates distinct outbreaks caused by an endemic multi-locus variable number tandem repeat analysis type in Australia, 2014. *BMC Microbiology*, 16(1), 211. <https://doi.org/10.1186/s12866-016-0831-3>

- Pierce, S. E., Bell, R. L., Hellberg, R. S., Cheng, C.-M., Chen, K.-S., Williams-Hill, D. M., Martin, W. B., & Allard, M. W. (2012). Detection and Identification of *Salmonella enterica*, *Escherichia coli*, and *Shigella* spp. Via PCR-electrospray ionization mass spectrometry: Isolate testing and analysis of food samples. *Applied and Environmental Microbiology*, 78(23), 8403–8411. PubMed. <https://doi.org/10.1128/AEM.02272-12>
- Popoff, M. Y., & Le Minor, L. (1997). *Antigenic formulas of the Salmonella serovars*.
- Qin, H., Lo, N. W.-S., Loo, J. F.-C., Lin, X., Yim, A. K.-Y., Tsui, S. K.-W., Lau, T. C.-K., Ip, M., & Chan, T.-F. (2018). Comparative transcriptomics of multidrug-resistant *Acinetobacter baumannii* in response to antibiotic treatments. *Scientific Reports*, 8(1), 3515–3515. PubMed. <https://doi.org/10.1038/s41598-018-21841-9>
- Raffatellu, M., Santos, R. L., Verhoeven, D. E., George, M. D., Wilson, R. P., Winter, S. E., Godinez, I., Sankaran, S., Paixao, T. A., Gordon, M. A., Kolls, J. K., Dandekar, S., & Bäumlér, A. J. (2008). Simian immunodeficiency virus–induced mucosal interleukin-17 deficiency promotes *Salmonella* dissemination from the gut. *Nature Medicine*, 14(4), 421–428. <https://doi.org/10.1038/nm1743>
- Rahmani, M., Peighambari, S. M., Svendsen, C. A., Cavaco, L. M., Agersø, Y., & Hendriksen, R. S. (2013). Molecular clonality and antimicrobial resistance in *Salmonella enterica* serovars Enteritidis and Infantis from broilers in three Northern regions of Iran. *BMC Veterinary Research*, 9(1), 66. <https://doi.org/10.1186/1746-6148-9-66>
- Ranjbar, R., Elhaghi, P., & Shokoohizadeh, L. (2017). Multilocus Sequence Typing of the Clinical Isolates of *Salmonella Enterica* Serovar Typhimurium in Tehran Hospitals. *Iranian Journal of Medical Sciences*, 42(5), 443–448. PubMed.

- Redgrave, L. S., Sutton, S. B., Webber, M. A., & Piddock, L. J. V. (2014). Fluoroquinolone resistance: Mechanisms, impact on bacteria, and role in evolutionary success. *Trends in Microbiology*, 22(8), 438–445. <https://doi.org/10.1016/j.tim.2014.04.007>
- Reis, B. P., Zhang, S., Tsolis, R. M., Bäumlér, A. J., Adams, L. G., & Santos, R. L. (2003). The attenuated sopB mutant of *Salmonella enterica* serovar Typhimurium has the same tissue distribution and host chemokine response as the wild type in bovine Peyer's patches. *Veterinary Microbiology*, 97(3), 269–277. <https://doi.org/10.1016/j.vetmic.2003.09.019>
- Santos, R., Zhang, S., Tsolis, R., Bäumlér, A., & Adams, L. (2002). Morphologic and molecular characterization of *Salmonella typhimurium* infection in neonatal calves. *Veterinary Pathology*, 39(2), 200–215. PubMed. <https://doi.org/10.1354/vp.39-2-200>
- Schwarz, N. G., Sarpong, N., Hüngrer, F., Marks, F., Acquah, S. E., Agyekum, A., Nkrumah, B., Loag, W., Hagen, R. M., Evans, J. A., Dekker, D., Fobil, J. N., Meyer, C. G., May, J., & Adu-Sarkodie, Y. (2010). Systemic bacteraemia in children presenting with clinical pneumonia and the impact of non-typhoid salmonella (NTS). *BMC Infectious Diseases*, 10, 319. PubMed. <https://doi.org/10.1186/1471-2334-10-319>
- Shelobolina, E. S., Sullivan, S. A., O'Neill, K. R., Nevin, K. P., & Lovley, D. R. (2004). Isolation, Characterization, and U(VI)-Reducing Potential of a Facultatively Anaerobic, Acid-Resistant Bacterium from Low-pH, Nitrate- and U(VI)-Contaminated Subsurface Sediment and Description of *Salmonella subterranea* sp. Nov. *Applied and Environmental Microbiology*, 70(5), 2959. <https://doi.org/10.1128/AEM.70.5.2959-2965.2004>
- Sigaúque, B., Roca, A., Mandomando, I., Morais, L., Quintó, L., Sacarlal, J., Macete, E., Nhampos, T., Machevo, S., Aide, P., Bassat, Q., Bardají, A., Nhalungo, D., Soriano-Gabarró, M., Flannery, B., Menendez, C., Levine, M. M., & Alonso, P. L. (2009).

- Community-Acquired Bacteremia Among Children Admitted to a Rural Hospital in Mozambique. *The Pediatric Infectious Disease Journal*, 28(2).
https://journals.lww.com/pidj/Fulltext/2009/02000/Community_Acquired_Bacteremia_Among_Children.6.aspx
- Slauch, J. M., Mahan, M. J., Michetti, P., Neutra, M. R., & Mekalanos, J. J. (1995). Acetylation (O-factor 5) affects the structural and immunological properties of *Salmonella typhimurium* lipopolysaccharide O antigen. *Infection and Immunity*, 63(2), 437–441.
 PubMed. <https://doi.org/10.1128/IAI.63.2.437-441.1995>
- Smith, T. (1894). The hog-cholera group of bacteria. *US Bur Anim Ind Bull*, 6, 6–40.
- Su, L., & Chiu, C. (2005). Current system of *Salmonella* nomenclature used by WHO, CDC and ASM. Retrieved on February, 26, 2016.
- Su, L.-H., & Chiu, C.-H. (2007). *Salmonella*: Clinical importance and evolution of nomenclature. *Chang Gung Medical Journal*, 30(3), 210–219. PubMed.
- Su, L.-H., Chiu, C.-H., Chu, C., & Ou, J. T. (2004). Antimicrobial Resistance in Nontyphoid *Salmonella* Serotypes: A Global Challenge. *Clinical Infectious Diseases*, 39(4), 546–551.
<https://doi.org/10.1086/422726>
- Sutera, V., Levert, M., Burmeister, W. P., Schneider, D., & Maurin, M. (2013). Evolution toward high-level fluoroquinolone resistance in *Francisella* species. *Journal of Antimicrobial Chemotherapy*, 69(1), 101–110. <https://doi.org/10.1093/jac/dkt321>
- Tindall, B., Grimont, P., Garrity, G., & Euzeby, J. (2005). Nomenclature and taxonomy of the genus *Salmonella*. *International Journal of Systematic and Evolutionary Microbiology*, 55(1), 521–524.

- Tuin, A., Huizinga-Van der Vlag, A., van Loenen-Weemaes, A.-M. M. A., Meijer, D. K. F., & Poelstra, K. (2006). On the role and fate of LPS-dephosphorylating activity in the rat liver. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, 290(2), G377–G385. <https://doi.org/10.1152/ajpgi.00147.2005>
- Uche, I. V., MacLennan, C. A., & Saul, A. (2017). A Systematic Review of the Incidence, Risk Factors and Case Fatality Rates of Invasive Nontyphoidal Salmonella (iNTS) Disease in Africa (1966 to 2014). *PLOS Neglected Tropical Diseases*, 11(1), e0005118. <https://doi.org/10.1371/journal.pntd.0005118>
- Vuthy, Y., Kruij Sun, L., Heng, S., Alexandra, K., & Awa, A.-K. (n.d.). Antibiotic susceptibility and molecular characterization of resistance genes among *Escherichia coli* and among *Salmonella* subsp. In chicken food chains. *Asian Pacific Journal of Tropical Biomedicine*.
- Wattiau, P., Boland, C., & Bertrand, S. (2011). Methodologies for *Salmonella enterica* subsp. Enterica subtyping: Gold standards and alternatives. *Applied and Environmental Microbiology*, 77(22), 7877–7885. PubMed. <https://doi.org/10.1128/AEM.05527-11>
- Wiegand, I., Hilpert, K., & Hancock, R. E. W. (2008). Agar and broth dilution methods to determine the minimal inhibitory concentration (MIC) of antimicrobial substances. *Nature Protocols*, 3(2), 163–175. <https://doi.org/10.1038/nprot.2007.521>
- Zand, J., Rountree, R., Rountree, B., & Walton, R. (2003). *Smart Medicine for a Healthier Child: A Practical A-to-Z Reference to Natural and Conventional Treatments for Infants and Children*. Penguin.
- Zhang, S., Kingsley, R. A., Santos, R. L., Andrews-Polymenis, H., Raffatellu, M., Figueiredo, J., Nunes, J., Tsolis, R. M., Adams, L. G., & Bäumler, A. J. (2003). Molecular pathogenesis

of *Salmonella enterica* serotype typhimurium-induced diarrhea. *Infection and Immunity*, 71(1), 1–12. PubMed. <https://doi.org/10.1128/iai.71.1.1-12.2003>

APPENDIX

Appendix Table 1: Primers for Antibiotic Resistance Genes

Antibiotic	Gene name	Sequence 5' - 3'	Fragment size (bp)	Annealing Temp (°C)	Author
Beta Lactam	<i>bla_{Tem}</i>	(F) ACCAATGCTTAATCAGTGAG	963	50	Vuthy et al., 2017
		(B) GCGGAACCCCTATTTG			
Sulfamethoxazole	<i>sul1</i>	(F) TGAGATCAGACGTATTGCGC	420	58	Rahmani et al., 2013
		(R) TTGAAGGTTTCGACAGCACGT			
Sulfamethoxazole	<i>sul2</i>	(F) GGCAGATGTGATCGACCTCG	405	58	Vuthy et al., 2017
		(R) TGCCGGGATCAAGGACAAG			
Chloramphenicol	<i>catA1</i>	(F) AATGTACCTATAACCAG	585	42	Leekitcharoenphon et al., 2013
		(R) TGCCTTAAAAAAATTAC			
Trimethoprim	<i>dfrA14</i>	(F) TGAGAACCTTGAAAGTATCATTG	483	55	Rahmani et al., 2013
		(R) ACCCTTTTCCAAATTTGATAG			
Tetracycline	<i>tetA</i>	(F) GCTACATCCTGCTTGCCTTC	210	58	Vuthy et al., 2017
		(R) CATAGATCGCCGTGAAGAGG			
Tetracycline	<i>tetB</i>	(F) TTGGTTAGGGGCAAGTTTTG	659	55	Leekitcharoenphon et al., 2013
		(R) GTAATGGGCCAATAACACCG			
Streptomycin	<i>strA</i>	(F) CCAATCGCAGATAGAAGGC	548	58	Vuthy et al., 2017
		(R) CTTGGTGATAACGGCAATTC			
Streptomycin	<i>aadA</i>	(F) GTGGATGGCGGCCTGAAGCC	528	58	Rahmani et al., 2013
		(R) AATGCCCAGTCGGCAGCG			
Gentamicin	<i>aac(3)III-IV</i>	(F) GACGACGAGCCGTTTCGAYCC	280	58	Bräu et al., 1984
		(R) CCATCAACTCGGCAAGATGSAG			

Appendix Table 2: Identification of Antibiotic Resistance Genes

No.	Sample ID	Serovar	Sequence Type (ST)	Antibiotic Susceptibility Pattern			Antibiotic resistance genes
				Susceptible	Intermediate	Resistant	
1	113378	S. Typhimurium	313	Chlo, Strep		Amx, Amp, SXT	Sul1, Sul2, StrA, aadA, CatA1, Bla _{Tem}
2	113552	S. Typhimurium	313	All drugs			Sul1, Sul2
3	113590	S. Typhimurium	313		Lev	Chlo, Amx, Amp, Strep, SXT	Sul1, Sul2, StrA, aadA, CatA1, Bla _{Tem}
4	113734	S. Typhimurium	313			Chlo, Amx, Amp, Strep, SXT	Sul1, Sul2, StrA, aadA, Bla _{Tem}
5	113821	S. Typhimurium	313		Lev, Cip	Chlo, Amx, Amp, Strep, SXT	Sul1, Sul2, StrA, Bla _{Tem} , aadA
6	114348	S. Typhimurium	313		Tet	Chlo, Amx, Amp, Strep, SXT	Sul1, Sul2, TetA, StrA, aadA
7	114361	S. Dublin	10	All drugs			Sul1, Sul2, StrA
8	114575	S. Typhimurium	19	Amp	Amox, Strep, Cefuroxime		Sul1, Sul2, TetA, StrA, aadA
9	115613	S. Dublin	-			Amx, Amp	Sul2, StrA
10	116208	S. Typhimurium	313			Chlo, Amx, Amp, Strep, SXT, Tet	Sul1, Sul2, Tet A, Tet B, StrA, CatA1, Bla _{Tem} , aadA

NB: The Antimicrobial Susceptibility Patterns above were mainly used as basis for selecting resistance genes for the PCR amplification. Amx= Amoxicillin, Amp= Ampicillin, Chlo= Chloramphenicol, Cip= Ciprofloxacin, Strep= Streptomycin, SXT= Sulphamethoxazole-Trimethoprim, Tet=Tetracycline.

Appendix Table 3: Ciprofloxacin MIC Determination Results

	16ug/ml	8ug/ml	4ug/ml	2ug/ml	1ug/ml	0.5ug/ml	0.25ug/ml	0.125ug/ml	0.06ug/ml	0.03ug/ml	0.015ug/ml	0.0075ug/ml	Growth Control	Sterility Control	Antibiotic Susceptibility	
A	0.071	0.090	0.101	0.101	0.068	0.086	0.059	0.089	0.079	0.067	0.054	0.745	0.670	0.066	Susceptible	
B	0.083	0.064	0.059	0.085	0.088	0.079	0.057	0.057	0.063	0.080	0.034	0.532	0.734	0.067		
Average	0.077	0.077	0.080	0.093	0.078	0.083	0.058	0.073	0.071	0.074	0.044	0.639	0.702	0.067		
D	0.034	0.044	0.033	0.038	0.064	0.074	0.067	0.539	0.618	0.735	0.605	0.594	0.749	0.049	Intermediate	
E	0.032	0.033	0.042	0.033	0.058	0.063	0.065	0.542	0.674	0.776		0.744	0.652	0.051		
Average	0.033	0.039	0.038	0.036	0.061	0.069	0.066	0.541	0.646	0.756	0.605	0.669	0.701	0.050		
G	0.034	0.038	0.037	0.038	0.056	0.054	0.055	0.051	0.051	0.053	0.051	0.046	0.676	0.050	Reference	
H	0.034	0.034	0.035	0.041	0.050	0.051	0.051	0.053	0.051	0.048	0.046	0.046	0.747	0.053		
Average	0.034	0.036	0.036	0.040	0.053	0.053	0.053	0.052	0.051	0.051	0.049	0.046	0.712	0.052		

NB: the table above shows absorbance measured at 600nm and in duplicates

Appendix Table 4: Primers for RT-qPCR

Organism	Gene	Primer Sequence 5'-3'	Fragment size	Reference
<i>Salmonella</i> Typhimurium	16SrRNA	F: TGTAGCGGTGAAATGCGTAG	161bp	Li et al., 2017
		R: CAAGGGCACAACCTCCAAG		
<i>Salmonella</i> Typhimurium LT2	GyrA	F: AAGCCAACGAACGTATCACC	189bp	This study
		R: AAGTCAGGTCAACGCCAATC		
<i>Salmonella</i> Typhimurium ST313	SoxR	F: AGTTAGACCGACGTATTCAT	158bp	Li et al., 2017
		R: TTAATCATCTTCAAGCAGCC		
<i>Salmonella</i> Typhimurium ST313	EmrD	F: CCTCGTCGGCATGTCTATTT	133bp	Li et al., 2017
		R: GAGCGTTCTCGCCATTACTC		
<i>Salmonella</i> Typhimurium LT2	AcrA	F: TGATGCTCTCAGGCAGCTTA	152bp	This study
		R: ACGGTAAGCAACGGTACGAC		
<i>Salmonella</i> Typhimurium LT2	YdhJ	F: ATTATGCCAGCACCGGTAAG	161bp	This study
		R: GCCAACGTGACCCTGTAAC		

NB: all primers developed in this study have T_m of about 60°C

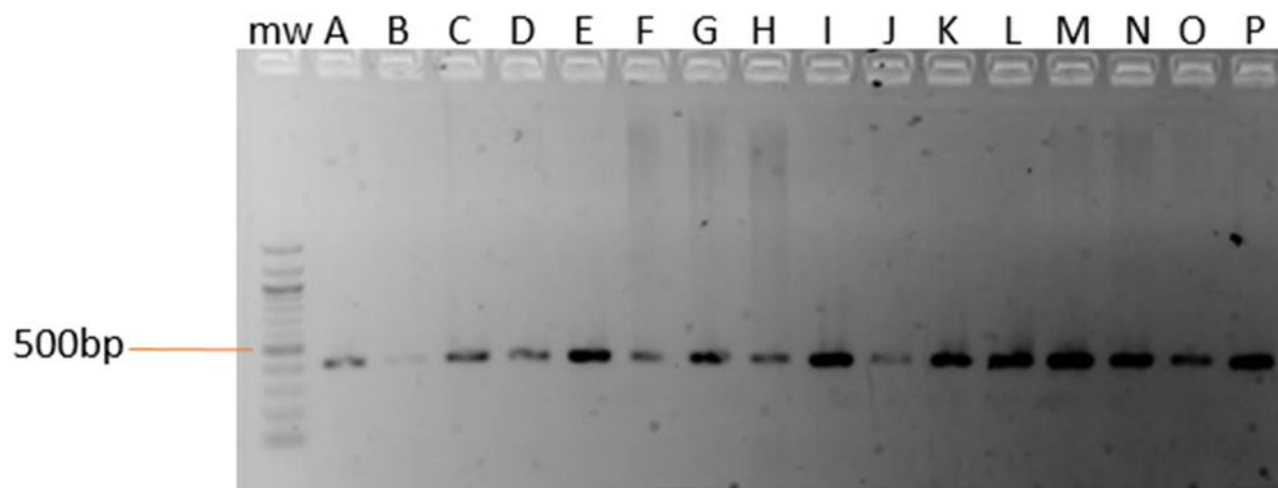


Fig 1. PCR detection of *sul1* and *sul2* resistance genes among eight (8) *Salmonella* isolates. From A to H is the amplification of *sul1* gene (fragment size= 420bp) and from I to P is *sul2* gene amplification (fragment size= 405). The samples were 113378 (A & I), 113552 (B & J), 113590 (C & K), 113734 (D & L), 113821 (E & M), 114348 (F & N), 114361 (G & O), 114575 (H & P). A 100bp molecular weight marker (mw) was used for 1.5% agarose gel electrophoresis.

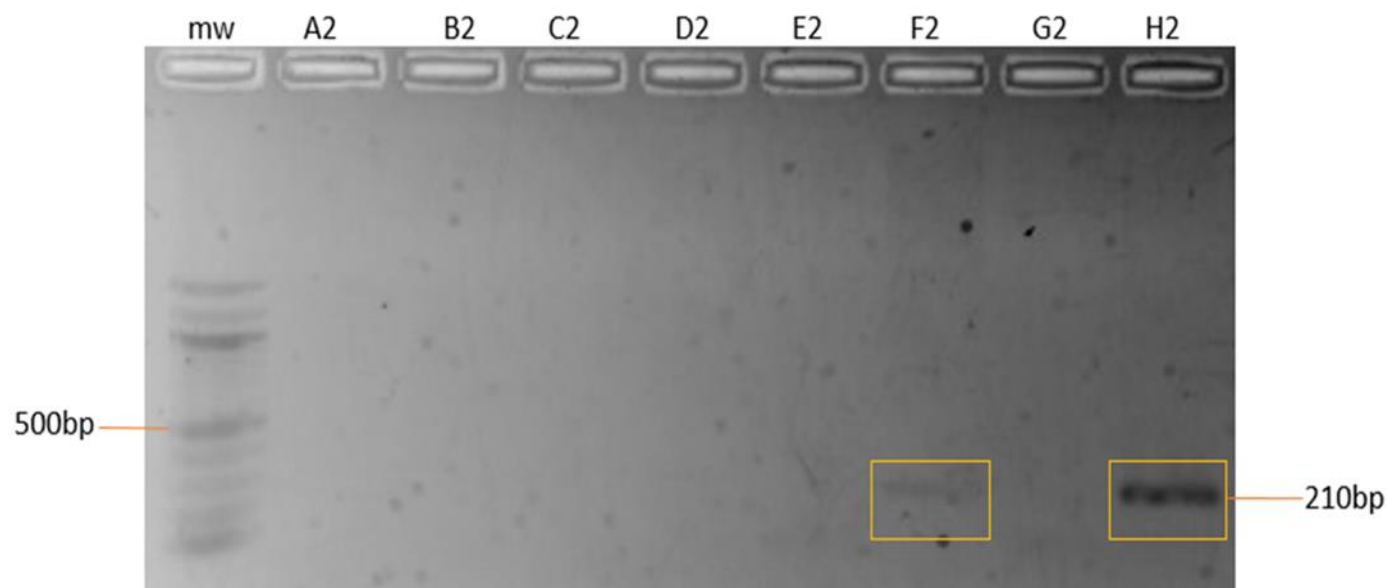


Fig 2: TetA gene PCR amplification for 8 *Salmonella* isolates. From Left to Right: 113378 (A2), 113552(B2), 113590(C2), 113734 (D2), 113821 (E2), 114348 (F2), 114361 (G2), 114575 (H2). The expected product size was 210bp

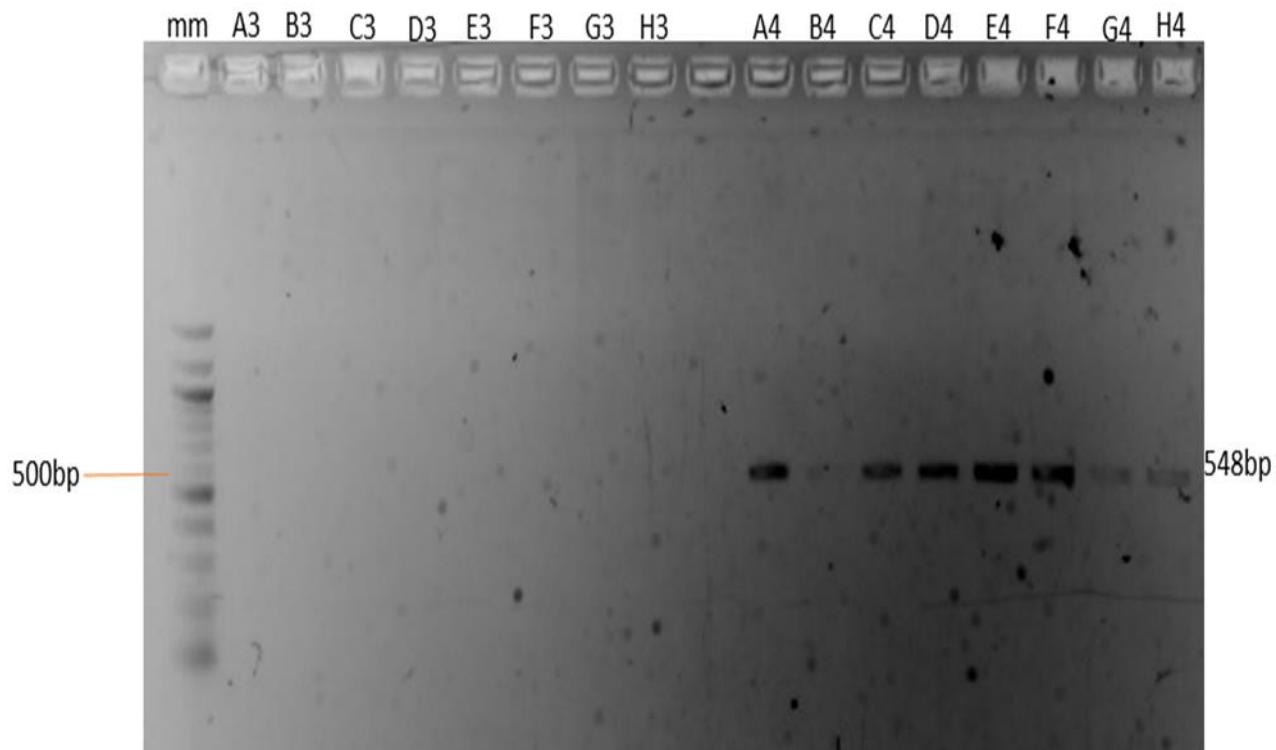


Fig 3: PCR amplification of *tetB* and *StrA* resistance genes. *TetB* and *StrA* are tetracycline and streptomycin resistance genes respectively. Among 8 *Salmonella* isolates, the presence of *TetB* gene was screened from Well A3 to H3 as well as *StrA* gene as shown in wells A4 to H4. The expected band size was 548bp. The amplicons were resolved on a 1.5% agarose gel and with 100bp molecular weight marker.

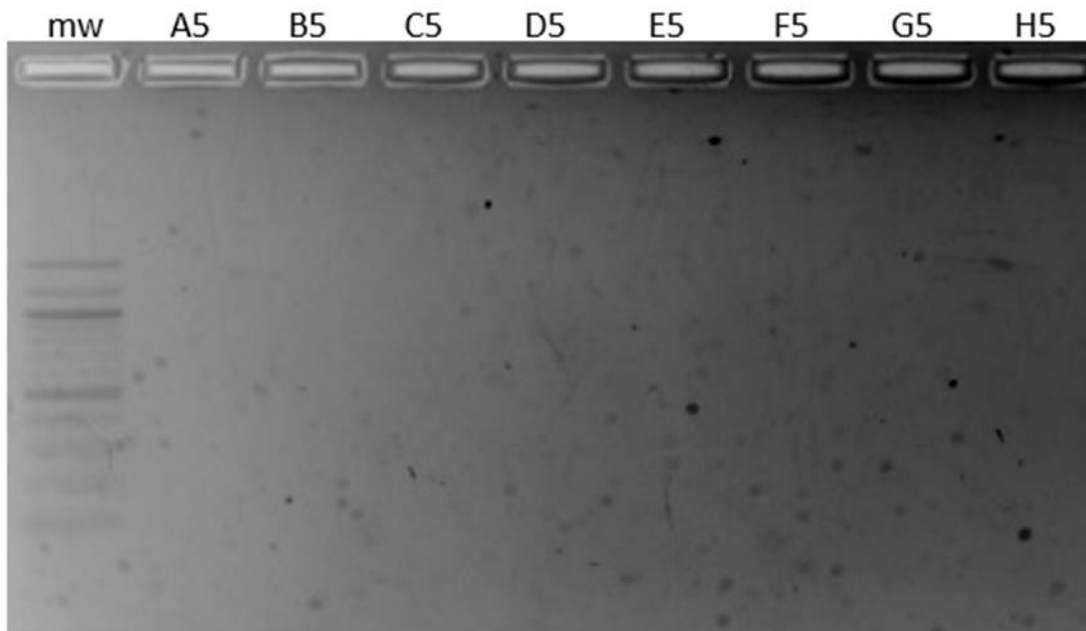


Fig 4: Screening for gentamicin resistance gene *aac* (3) III-IV by PCR amplification. The expected band size was 280bp

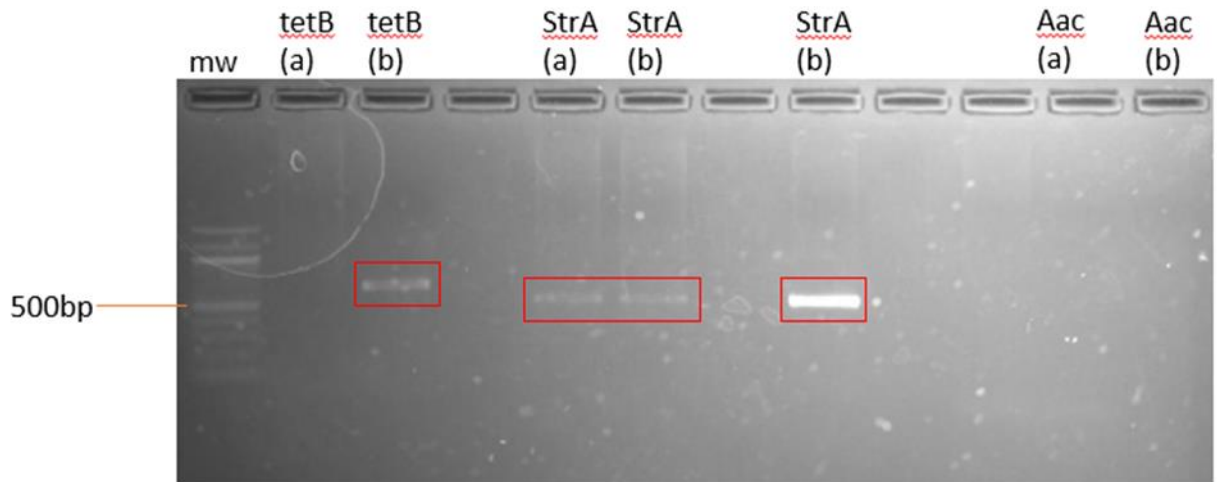


Fig : PCR amplification of *tetB*, *StrA* and *aac(3)III-IV* resistance genes in two *Salmonella* isolates 115613 (a) & 116208 (b) . The expected fragment size for *TetB* is 659bp, *StrA* is 548bp and *aac(3)III-IV* is 280bp. The amplicons were resolved on a 1.5% agarose gel and by using 100bp molecular weight marker (mw)

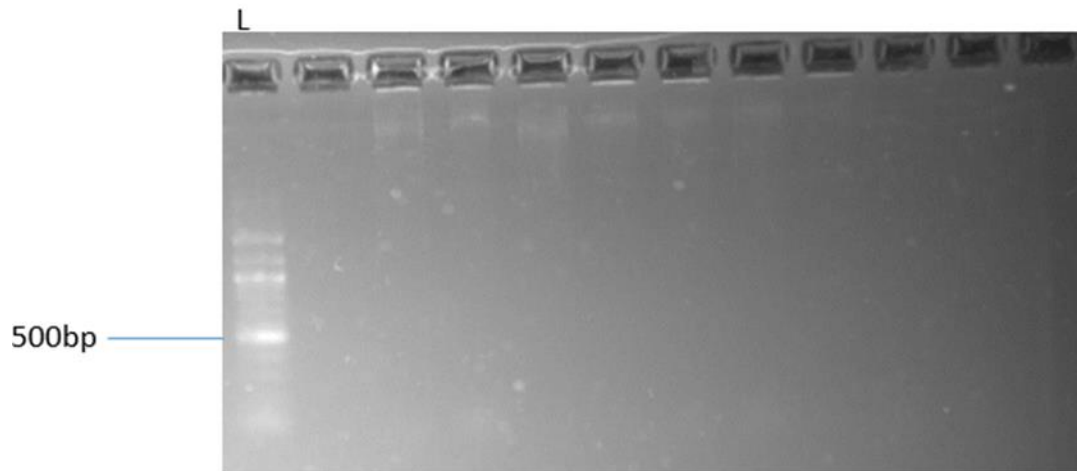


Fig 7: PCR detection of Trimethoprim resistance gene *dfrA14* among NTS isolates. The expected fragment size was 483bp. L was 100bp ladder and 1.5% agarose was used.

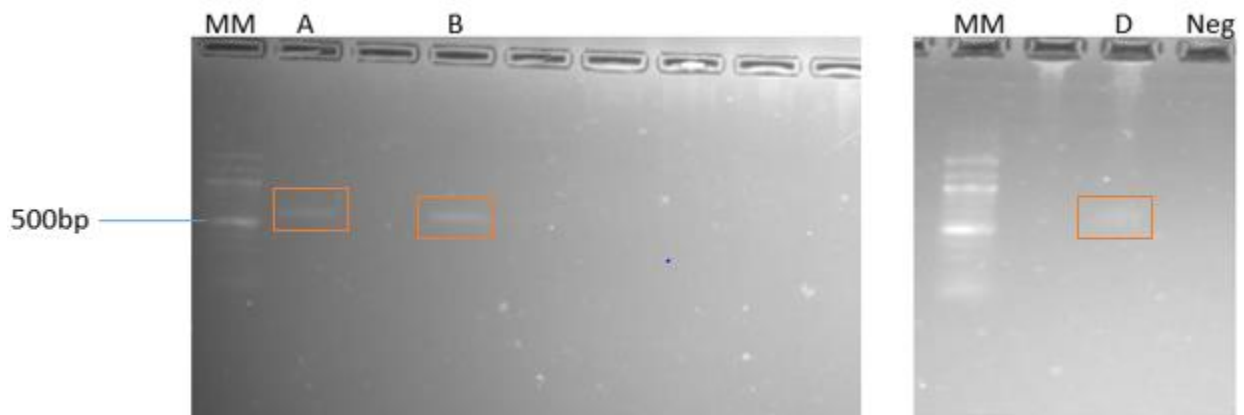


Fig 8: Chloramphenicol resistance gene *CatA1* amplification. MM= 100bp molecular weight marker, A= 113378, B= 113590, D= 116208 & Neg= negative control. The expected product size was 585bp

Appendix Table 5: Edit versions of SNPS

Sequence Name	Minimum	Maximum	Length	Average Quality	Change	Coverage	Polymorphism Type	Reference Frequency	Reference Nucleotide(s)	Strand-Bias >50 % P-value	Strand-Bias >65% P-value	Variant Frequency	Variant Nucleotide(s)	Variant P-Value (approximate)	Variant Raw Frequency
AcrA_NC_003197_Salmonella_enterica	242	242	1	14	G - > T	2	SNP (transversion)	50.00 %	G	1	1	50.00 %	T	0.078	1
EmrD_NC_003197_Salmonella_enterica	82	82	1	13	A - > T	1	SNP (transversion)		A	1	1	100.00 %	T	0.05	1
EmrD_NC_003197_Salmonella_enterica	85	85	1	14	C - > T	2	SNP (transition)		C	0.5	0.85	100.00 %	T	0.0025	2
GyrA_NC_003197_Salmonella_enterica	193	193	1	4	T - > A	3	SNP (transversion)		T	0.25	0.55	100.00 %	A	0.063	3
GyrA_NC_003197_Salmonella_enterica	194	194	1	12	G - > T	3	SNP (transversion)		G	0.25	0.55	100.00 %	T	0.00025	3
GyrA_NC_003197_Salmonella_enterica	324	324	1	55	C - > A	6	SNP (transversion)	66.70 %	C	1	1	33.30 %	A	1.50E-10	2
SoxR_NC_003197_Salmonella_enterica	27	27	1	9	T - > G	3	SNP (transversion)	33.30 %	T	0.5	0.85	66.70 %	G	0.067	2
SoxR_NC_003197_Salmonella_enterica	28	28	1	13	A - > T	3	SNP (transversion)	33.30 %	A	0.5	0.85	66.70 %	T	0.0073	2
SoxR_NC_003197_Salmonella_enterica	29	29	1	13	T - > A	3	SNP (transversion)	33.30 %	T	0.5	0.85	66.70 %	A	0.011	2
SoxR_NC_003197_Salmonella_enterica	30	30	1	20	A - > T	3	SNP (transversion)	66.70 %	A	1	1	33.30 %	T	0.03	1

SoxR_NC_003197_Salm onella_enterica	40 5	40 5	1	16	G - > A	4	SNP (transition)	75.00 %	G	1	1	25.00 %	A	0.097	1
SoxR_NC_003197_Salm onella_enterica	44 1	44 1	1	6	#N AM E?	2	Insertion			0.5	0.85	100.0 0%	G	0.063	2
YdhJ	49	49	1	10	(A) 5 - > (A) 4	3	Deletion (tandem repeat)		A	0.25	0.55	100.0 0%		0.001	3

NB: p-value<0.001