Application of molecular epidemiological methods to investigate strains of *Salmonella enterica* serovar Enteritidis in South Africa

Munyadziwa Muvhali
(Student number: 385400)

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Advisory committee

Supervisor 1

Doctor Anthony M. Smith
BSc (Hons), PhD

Supervisor 2

Doctor Karen H. Keddy
BSc (Med), MBBCh, DTM & H, MMed, FC Path

Department

Centre for Enteric Diseases at the National Institute for Communicable Diseases (NICD), AND Department of Clinical Microbiology and Infectious Diseases, School of Pathology, Faculty of Health Sciences, University of the Witwatersrand.

DECLARATION

The experimental work described in this dissertation was conducted under the supervision of Dr Anthony M. Smith and Dr Karen H. Keddy in the Centre for Enteric Diseases (CED), National Institute for Communicable Diseases (NICD), National Health Laboratory Service (NHLS).

I **Munyadziwa Muvhali, student number 385400** declare that this dissertation is my own, unaided work. It is being submitted for the fulfillment for the degree of Master of Science in Medicine to the Faculty of Health Sciences, University of Witwatersrand, Johannesburg. It has not been submitted before for any degree or examination in any other University.

Munyadziwa Muvhali

Day --------31th---------- of ---------May----------2017

DEDICATION

To my Grandmother,

Esther Rambelani Muvhali

To my Aunt,

Mariam Mashudu Muvhali

To my Mother,

Evangeline Sharon Muvhali

To my Father,

Henry Masindi Muvhali

To my Fiancée,

Louie Lubisi

To my Fur Baby

Dexter

PRESENTATIONS

Muvhali, M. Molecular epidemiology of *Salmonella* Enteritidis in South Africa: MLVA applications to one health. Presented at the NICD Scientific Research Forum, Johannesburg, South Africa, 30 September 2015.
(Oral presentation)

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ABSTRACT

In South Africa, *Salmonella* Enteritidis has become a significant pathogen and the numbers of cases reported to the Centre for Enteric Diseases (CED) have increased. Pulsed-field gel electrophoresis (PFGE) is a primary for molecular subtyping of *Salmonella.* However, this technique has poor discrimination for serotypes with high homogeneity such as *Salmonella* Enteritidis. Multi-locus variable-number tandem-repeats analysis (MLVA) has shown higher discriminatory power for *Salmonella* Enteritidis compared to PFGE. In this study, MLVA was used to investigate the molecular epidemiology and relatedness of human *Salmonella* Enteritidis strains from Gauteng and Western Cape, South Africa. Furthermore, MLVA was also used to investigate the relatedness of human and non-human *Salmonella* Enteritidis strains. MLVA included analysis of five VNTR loci, with varying degrees of diversity. A total of 1221 human isolates and 43 non-human isolates were included in the study. Eighty-six MLVA profiles were obtained; MLVA profiles 7, 21, 22 and 28 were the predominant MLVA profiles. MLVA profile 28 was the most common MLVA profile amongst both the human and non-human isolates. Isolates had low prevalence of antimicrobial resistance, however sulfamethoxazole resistance was notable amongst both the human (348; 29%) and non-human (10; 23%) isolates. During the study period, seven *Salmonella* Enteritidis outbreaks were investigated from six provinces and isolates from each individual outbreak showed an identical MLVA profile. MLVA was shown to be a successful molecular subtyping tool for *Salmonella* Enteritidis, for both surveillance purposes and outbreak investigations. *Salmonella* Enteritidis strains circulating within the human and non-human population were clonal. The study emphasizes the need for the one health approach, in order to curb the spread of *Salmonella* Enteritidis in South Africa.

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TABLE OF CONTENTS

LIST OF FIGURES

Chapter 1

Chapter 2

Chapter 3

LIST OF TABLES

Chapter 3

NOMENCLATURE

( )	Open bracket close bracket / parenthesis
[ ]	Open square bracket close square bracket
<	Less than
=	Equal to
>	Greater than
≤	Less than or equals to
≥	Greater than or equal to
°	Degree
°C	Degree Celsius
~	Approximately
µl	Microliter
µm	Micromolar
ARC-OVI	Agricultural Research Council-Onderstepoort Veterinary Institute
bp	Base pair
CDC	Centers for Disease Control and Prevention
CED	Centre for Enteric Diseases
CLSI	Clinical and Laboratory Standards Institute guidelines
D	Simpson's index of Diversity
DNA	Deoxyribonucleic acid
EDTA	Disodium ethylenediaminetetra-acetic acid

et al.	And others
EU	European Union
FAO	Food and Agriculture Organization of the United Nations
GERMS-SA	Group for Enteric, Respiratory and Meningeal Surveillance in South Africa
H_2S	Hydrogen sulphide
I bp	Intermediate break points
i.e.	That is/ such as
iNTS	Invasive non-typhoidal *Salmonella*
IQR	Interquartile range
kb	Kilobases
KIA	Kliger iron media
LPS	Lipopolysaccharide
M	Molar
MAC	MacConkey agar
MHA	Mueller Hinton agar
MIC	Minimum inhibitory concentration
ml	Milliliter
MLST	Multi-locus sequence typing
MLVA	Multi-locus variable-number tandem-repeats analysis
mM	Millimolar
MST	Minimum spanning tree
n	Total number

NICD	National Institute for Communicable Diseases
NTS	Non-typhoidal *Salmonella*
PCR	Polymerase chain reaction
PFGE	Pulsed-field gel electrophoresis
pH	Percentage Hydrogen
PHE	Public Health England
PT4	Phage type 4
PT8	Phage type 8
RAPD	Random amplified polymorphic DNA
R bp	Resistant break points
REP	Repetitive extragenic palindromic
RNA	Ribonucleic acid
rpm	Revolutions per minute
rRNA	Ribosomal RNA
SA	South Africa
S bp	Susceptible break points
SNPs	Single nucleotide polymorphisms
TE	Tris-EDTA
USA	United States of America
VNTR(s)	Variable number tandem repeat (s)
WGS	Whole genome sequencing
WHO	World Health Organization
XLD	Xylose-Lysine-Desoxycholate

CHAPTER 1: LITERATURE REVIEW

1.1 Introduction

Salmonella is a major cause of morbidity and mortality in children under the age of five in most developing countries worldwide (Kirk *et al*., 2015). The global human health impact of nontyphoidal *Salmonella* is high, with an estimated 93.8 million illnesses, of which 80.3 million are reported to be foodborne related, and 155,000 deaths each year (Majowicz *et al*., 2010). Human illness caused by *Salmonella enterica* serovar Enteritidis (*Salmonella* Enteritidis) has drastically increased worldwide and by the 1980's *Salmonella* Enteritidis had replaced *Salmonella* Typhimurium as the primary cause of salmonellosis globally (Rodrigue *et al*., 1990; Bern *et al*., 1992; Kosek *et al*., 2003). In Africa, the burden of *Salmonella* Enteritidis has not been established. However, it is estimated that *Salmonella* Enteritidis accounts for 33.1% of the total invasive non-typhoidal *Salmonella* (iNTS) infections (Ao *et al*., 2015).

Since 2011, *Salmonella* Enteritidis has overtaken *Salmonella* Typhimurium as the most commonly reported *Salmonella* serotype to the Centre for Enteric Diseases (CED) at the National Institute for Communicable Diseases (NICD) in South Africa. Since then, the numbers of *Salmonella* Enteritidis cases have continued to increase (GERMS - SA Annual Report, 2012).

Despite global efforts to curb its spread *Salmonella* Enteritidis infections persist, causing an on-going challenge to the global health system.

1.2 History

The genus *Salmonella* was accidentally discovered by Theobald Smith, during his quest to identify the causative agent of hog cholera in 1885. Although it was later discovered that a virus was the actual causative agent of hog cholera, the discovery

of *Salmonella* was of major importance and significance to microbiology (Meštrović, 2015).

1.3 Bacterial structure and characterization

Salmonella is a rod-shaped Gram-negative bacterium, belonging to the *Enterobacteriaceae* family. This facultative anaerobic, peritrichous flagella (flagella around entire surface) containing microorganism is highly motile and non-spore forming, with a diameter of about 0.7 µm to 1.5 µm and length between 2 µm and 5 µm (Richard, 2008).

Salmonella has 3 different types of antigens (O somatic, H flagellar and Vi capsular antigens) and the ability for these antigens to agglutinate with serum antibodies, enables them to be used serologically in the identification of over 2500 *Salmonella* serotypes (Gianella, 1996; Dutta *et al*., 2012).

Salmonella is divided into two species: *Salmonella bongori* and *Salmonella enterica*, with the former being further classified into six subspecies (*enterica*, *salamae*, *arizonae*, *diarizonae*, *houtenae*, and *indica*) and a large number of serovars. *Salmonella enterica* subspecies 1 (*enterica*) consists of the most common O-antigen serogroups (A, B, C1, C2, D and E), which harbour strains that make up 99% of all *Salmonella* infections (Achtman *et al*., 2012).

Salmonella enterica subspecies 1 can be divided into two groups; typhoidal and nontyphoidal *Salmonella* (NTS) serovars. NTS serovars such as *Salmonella* Typhimurium and *Salmonella* Enteritidis are the most common cause of salmonellosis globally (Tennant *et al*., 2016). These serovars commonly cause disease in both humans and animals (zoonotic) and illness is usually a mild, self-limiting gastrointestinal disease. Typhoidal *Salmonella* serovars such as *Salmonella* Typhi and *Salmonella* Paratyphi A are adapted to human infection and therefore are not commonly found in other animals (Uzzau *et al*., 2000; Gal-Mor *et al*., 2014). Serovars present in the other subspecies (*salamae*, *arizonae*, *diarizonae*, *houtenae*,

and *indica*) are commonly found in cold-blooded animals and the environment; they are rarely isolated from humans (Uzzau *et al*., 2000).

1.4 Epidemiology of *Salmonella* Enteritidis

1.4.1 Global prevalence

Salmonella has been well documented as the leading bacterial cause of acute gastroenteritis globally (Majowicz *et al*., 2010). Gastroenteritis is a major cause of morbidity and mortality worldwide, both in children <5 years old and in the general population as a whole. Despite these reports, there is still a lack of data describing the precise global impact of *Salmonella* infections to human health (Kirk *et al*., 2015).

With an estimated 2.8 billion cases of diarrhoeal illness worldwide, *Salmonella* infections represent ~3% of these illnesses (Majowicz *et al*., 2010). Thus, indicating the importance of this pathogen to human health (Scallan *et al*., 2005).

In a web-based surveillance conducted by Galanis *et al*., (2006) from years 2002-2003, *Salmonella* Enteritidis was the most common serotype reported from human isolates globally. In 2002, it accounted for 65% of all isolates, followed by *Salmonella* Typhimurium (12%) and *Salmonella* Newport (4%) respectively. In animals, *Salmonella* Enteritidis was the third most common serotype accounting for 9% of the isolates preceded by *Salmonella* Typhimurium and *Salmonella* Heidelberg (Galanis *et al*., 2006).

1.4.2 Prevalence in developed countries

In most developed countries, the numbers of *Salmonella* infection cases continue to increase and *Salmonella* Enteritidis has become the most commonly isolated *Salmonella* serotype in these countries (Ao *et al*., 2015).

In the United States of America (USA), the annual incidence of *Salmonella* Enteritidis infection remained relatively steady from 1996-2003, with an incidence rate of 1.9/100,000 population. However in 2008, the incidence rate steadily increased to a maximum of 2.8/100,000 population, which was representative of a 44% increase since the period 1996-1999 (Chai *et al*., 2012). During the years 2004 to 2009, 6777 *Salmonella* Enteritidis infections had been reported in the USA, the most affected age group was the <4 years old, with an incidence rate of 4.7/100,000 to 6.9/100,000 population (Chai *et al*., 2012). In 2011, the Centers for Disease Control and Prevention (CDC) reported *Salmonella* Enteritidis as the most common serotype accounting for 22%, followed by *Salmonella* Newport (14%), and *Salmonella* Typhimurium (13%).

In Canada, over 10 000 laboratory confirmed cases of *Salmonella* Enteritidis were reported between the years 2003-2009. The incidence rate was shown to have increased from 2.16/100,000 population in 2003 to 5.79/100,000 population in 2009 (63% increase). Of all reported cases of *Salmonella*, the proportion of *Salmonella* Enteritidis isolates rose from 12.7% in 2003 to 32.1% in 2009 (Nesbitt *et al*., 2012).

Salmonella Enteritidis is the most common *Salmonella* serovar and a major cause of outbreaks in Europe (European Food Safety Authority, 2014). Surveillance data collected from 23 European countries between the years 2006-2007 showed that *Salmonella* Enteritidis was the most common serovar isolated from humans (European Food Safety Authority, 2009). Between the years 2007 to 2013, 328 537 *Salmonella* Enteritidis cases were reported to the European Surveillance System (TESSy) by 27 countries, with Germany and the Czech Republic together accounting for 52% of all cases. The age group most affected were those <15 years of age, accounting for 43% (*n* = 139,090). In 2012, *Salmonella* Enteritidis accounted for 179 outbreaks in Europe, this making up 37% of all *Salmonella* outbreaks that year (European Food Safety Authority, 2014).

Asia has one of the highest incidences of *Salmonella* infection cases in the world, with 32-cases/100,000 population in high income areas of the Asia Pacific region to 3,600/100,000 population in Southeast Asia (McKeown *et al*., 2012). In Asia, *Salmonella* Enteritidis has emerged as the most common human isolated *Salmonella*

serotype in countries like Japan, the Republic of Korea and Thailand (Ng *et al*, 1997; Galanis *et al*., 2006). In Singapore, *Salmonella* Enteritidis accounted for 62.2% of the human non-typhoidal salmonellosis in 2007 (Ministry of Health Singapore, 2007).

1.4.3 Prevalence in Africa

In developed countries most *Salmonella* infections are often associated with gastroenteritis and have case-fatality rates of <1% (Varma *et al*., 2005; Feasey *et al*., 2012). However in Africa, NTS is a major concern because of the Human Immunodeficiency Virus (HIV) and malaria epidemic (Graham *et al*., 2000a; Galanakis *et al*., 2007). People with such illnesses are immune-compromised and research has shown that NTS is one of the most common causes for hospital admissions in such immune compromised people (Graham *et al*., 2000a; Graham, 2002). Despite the increased prevalence of HIV and malaria, most African countries are unable to provide clean water and proper sanitation to the communities. Such limitations play a major role in the continual increase of NTS morbidity and mortality, particularly in young children (Berkley *et al*., 2005; Enwere *et al*., 2006; Bessong *et al*., 2009).

In Sub-Saharan Africa, NTS is among the top three most common pathogens responsible for bacterial bloodstream infections in both adults and young children (Shaw *et al*., 2008; Sigauque *et al*., 2009). Young children under three years of age as well as adults infected with HIV carry most of the burden of invasive disease and mortality within these two groups is high (Morpeth *et al*., 2009). Furthermore, several studies have described the association of NTS infection and malaria infection, particularly amongst young children in malaria endemic countries (Morpeth *et al*., 2009; Takem *et al*., 2014). Studies conducted in Sub-Saharan Africa have shown that there seems to be an increased risk of invasive NTS amongst children with malaria and mortality associated with co-infection seems higher than that associated with malaria alone (Takem *et al*., 2014). Sadly, majority of these studies do not further characterise the NTS to serotype level.

Due to limited data from most African countries, the total burden of *Salmonella* Enteritidis in Africa has not been established. However, few studies have described

Salmonella Enteritidis as one of the major causes of iNTS in Africa (Feasey *et al*., 2012). A study by Ao *et al*. (2015) estimated that *Salmonella* Enteritidis accounts for 33.1% of the total burden of iNTS in Africa. In South Africa, between the years 2003 and 2004, *Salmonella* Enteritidis accounted for 10% of the iNTS cases (Feasey *et al*., 2010). However, poor reporting and poor surveillance in most African countries make estimation of burden of disease a challenge.

1.4.4 Prevalence in South Africa

In South Africa, the CED at the NICD initiated laboratory-based surveillance of enteric bacteria for public health importance in 2003 and this was primarily in response to the HIV epidemic in the country. During this time, the predominant *Salmonella* serotypes were *Salmonella* Typhimurium and *Salmonella* Isangi (Feasey *et al*., 2010; Feasey *et al*, 2012). The introduction of highly active antiretroviral therapy (HAART) in 2004, showed a gradual decline in invasive salmonellosis, more especially in those serotypes that were associated with HIV infection such as *Salmonella* Typhimurium, whose association with HIV in Africa has been extensively described (Keddy *et al*., 2009). However since 2011, *Salmonella* Enteritidis has become a more important pathogen and the numbers of cases reported to the CED have increased (Figure 1.1). This increase is still unexplained and it is independent of the HIV epidemic in the country (GERMS - SA Annual Report, 2012).

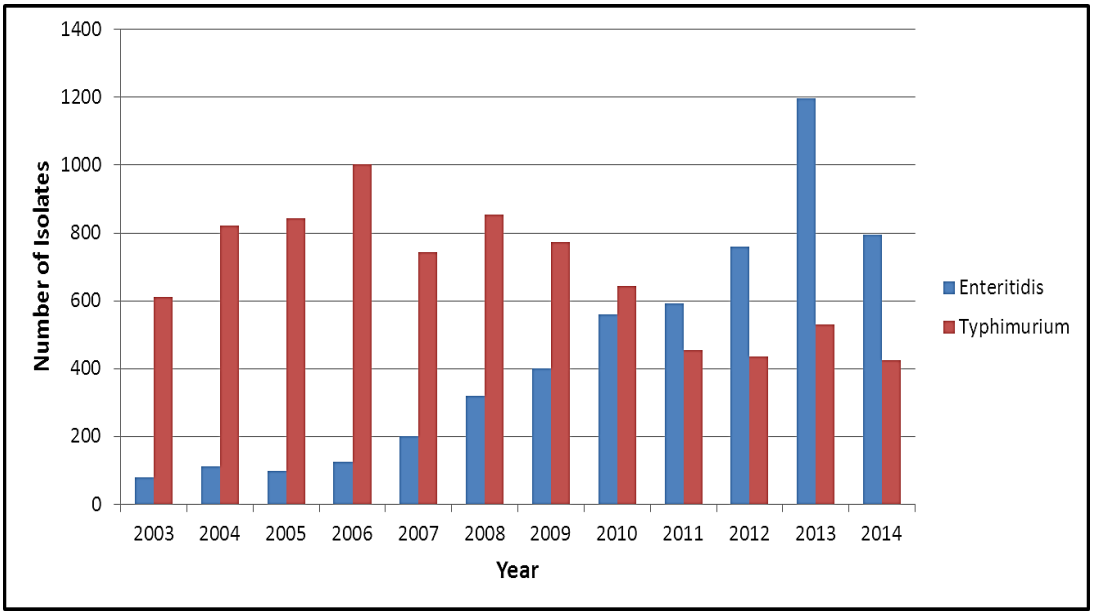


Figure 1.1. Bar graph illustrating the gradual increase of *Salmonella* Enteritidis isolates received at the CED from the year 2003.

1.4.5 Risk factors for *Salmonella* infection

1.4.5.1 Age as a risk factor

Globally, *Salmonella* incidence rates amongst the different age groups produces a bimodal distribution, with the first peak observed in children (<5 years) and the second observed in the elderly age group (WHO and FAO, 2002). However in Africa, a different bimodal distribution is described; this bimodal distribution has its initial peak in the <5 age group and the second peak occurring in the adult age groups (Ao *et al*., 2015).

Plausible risk factors amongst the different age groups include the lack of a fully developed immune system (young children), poor hygiene, a weakened immune system due to diseases and age-related decreased immune function (WHO and FAO, 2002).

1.4.5.2 Sex as a risk factor

Generally, *Salmonella* infections seem to infect both males and females equally. However, factors such as lifestyle (behavioural) may make another gender more prone to infection the other. Thus, sex as a risk factor for *Salmonella* infection remains a highly debatable subject (WHO and FAO, 2002).

1.4.6 Surveillance

Improvements are required on the estimation of the burden disease for pathogens such as *Salmonella*. These improvements would ensure that countries effectively design appropriate public health goals and allocate adequate resources to reduce disease burden (Senior, 2009).

Although laboratory-based surveillance provides useful trend information, it however does not give a true reflection of what is occurring in society, in terms of disease burden (underestimates disease burden) (Flint *et al*., 2005). In order to be certain that a laboratory-based surveillance system is an effective type of surveillance, every

ill person that seeks medical attention would have to submit a specimen. The laboratory would have to test for the pathogen and report a positive result and public health authorities would have to ascertain the laboratory-confirmed infection (Wheeler *et al*., 1999; Hall *et al*., 2005). However, even with such a system in place, not all countries are able to carry out such surveillance and compile such extensive data due to the financial constraints (laboratory based surveillances can be expensive); therefore making global estimates of disease burden difficult to calculate (Majowicz *et al*., 2010). Nonetheless, it is evident that *Salmonella* infections are a global challenge and despite limited data on the precise global incidence rate, small surveillance programmes from many parts of the world show the need for continual monitoring of *Salmonella* infections (Scallan *et al*., 2005; Majowicz *et al*., 2010; Kirk *et al*., 2015).

1.5 Transmission

Humans are the main reservoirs for typhoidal *Salmonella*. However, farm animals (chicken, cows, pigs and sheep) are the main reservoirs for human NTS infections. Other reservoirs include wild and domestic animals, as wells as reptiles (Mangni and Arvntikis, 2010).

Poultry and poultry products have been extensively described as the main source of *Salmonella* Enteritidis infection. In countries with increased occurrences of *Salmonella* outbreaks, it has been reported that 50-90% of all poultry related cases have been infected by *Salmonella* Enteritidis. Furthermore, in most outbreak investigations, eggs have been extensively described as the main reservoir for *Salmonella* Enteritidis infection in the poultry farming industry (Andino and Hanning, 2015).

Egg contamination by *Salmonella* serotypes has always been a major problem and this has been mainly attributed to the use of eggs with outer-shell contamination (environmental contamination factors) (Gianella, 1996; Mangni and Arvntikis, 2010). However, unlike most other *Salmonella* serotypes, *Salmonella* Enteritidis is able to

pass through the hard egg exterior and infect the egg internally. The pathogenic mechanisms involved in this occurrence are complex and are currently not fully understood. Furthermore, it seems that *Salmonella* Enteritidis has gained mechanisms that enable it to survive and grow in the internal egg contents more efficiently compared to the other serotypes (Gantois *et al*., 2009). *Salmonella* Enteritidis has the ability to infect an egg through the transovarian route and during intestinal carriage. This allows *Salmonella* Enteritidis to infect both the internal and external parts of the egg, and when eggs are stored at room temperature the number of bacterial cells in the egg increases and hence increasing the infectivity (Gantois *et al*., 2009).

Other modes of *Salmonella* Enteritidis infection include the consumption of vegetables, products such as peanut butter and milk. Other rare methods of transmission include drinking contaminated water and person-person spread (Gianella, 1996; Mangni and Arvntikis, 2010).

Although *Salmonella* Enteritidis infections are common in most populations globally, human asymptomatic carriage and spread is not common. However, asymptomatic carriage by chickens, birds and other animals such as mice has been reported (Davies and Wray, 1995; Hoelzer *et al*., 2011).

1.6 Clinical features

The most common manifestation of *Salmonella* Enteritidis is the gastroenteritis syndrome. This involves moderate fever, nausea, diarrhoea and variable abdominal discomfort (Mangni and Arvntikis, 2010). Symptoms occur within 6-48 hours after ingestion of the contaminated food or water. The extent of the diarrhoea is variable, from a few loose stools to cholerae–like water diarrhoea, to the less common bloody diarrhoea (Gorbach *et al*., 2004). These symptoms usually resolve within in 3-7 days; however severe illness tends to occur in neonates, the elderly and immune compromised individuals, and chronic carriers are rare (occurring in less than 1% of the infected) (Dutta *et al*., 2012).

Most *Salmonella* infections result in mild-to-moderate gastroenteritis that usually resolves without treatment. However, some lead to severe invasive infections such as bacteraemia and meningitis. Invasive *Salmonella* may invade the body causing infections in the bloodstream, tissues such as muscle, fat and those that surround the brain and spinal cord. Clinical presentation of iNTS infection typically includes febrile systemic illness which resembles enteric fever. Diarrhoea is often absent in iNTS cases and the other clinical features observed are often diverse and non-specific (Feasey et al., 2012).

Invasive *Salmonella* infections can be life threatening and therefore hospitalization and proper treatment is required. iNTS is commonly known to occur in infants (<1 year of age), the elderly and those immune-compromised (e.g. HIV infected persons and cancer patients). In African countries with a high HIV prevalence, NTS is the most common bacteria isolated from blood cultures of hospital admitted febrile adults (Vugia *et al*., 2004; Gordon *et al*., 2008). iNTS bacteraemia in Africa is reported to have a fatality rate between 38%-47% and a recurrence rate of 43% amongst HIV infected individuals (Graham *et al*., 2000b; Galanakis *et al*., 2007).

In developed countries, invasive *Salmonella* Enteritidis is less common; however in Africa, *Salmonella* Enteritidis is one of the most commonly iNTS serotypes (Galanakis *et al*., 2007; Gordon *et al*., 2008). This may be attributed to certain virulence strains found in this serotype and its commonality within most African countries, thus making infection by this serotype a serious concern to public health (Vugia *et al*., 2004; Galanakis *et al*., 2007; Gordon *et al*., 2008).

1.7 Pathogenesis

In order for *Salmonella* to be pathogenic, it needs to possess a variety of virulence factors. These include (1) the ability to invade the host cells, (2) a fully equipped lipopolysaccharide (LPS) coat, (3) it must be able to replicate intracellularly and (4) it must contain a toxin or toxins (Gianella, 1996).

Salmonella enters the human digestive system through the consumption of *Salmonella*-contaminated food, water or environmental sources (such as person-person transmission). In the stomach, the bacterium survives the low acidic environment through the use of an adaptive acid tolerance response. The *Salmonella* bacterium then passes onto the small intestine using its peritrichous flagella to move and swim chemotactically towards the mucosal surface. The *Salmonella* fimbriae then adhere to the intestinal epithelium using receptors present on the epithelium. After epithelial entry, *Salmonella* multiplies intracellularly and spreads to the mesenteric lymph nodes and to the rest of the body via the systemic circulation. The bacterial cells are then taken up by the reticuloendothelial cells, which limit and control the bacterial spread. However, depending on the *Salmonella* serotype and the ability of the host immune defense system to fight off the infection, some organisms may infect the spleen, gallbladder, liver, bones, meninges, and other organs. Fortunately, most *Salmonella* serotypes are quickly destroyed in the extra-intestinal sites and most common human *Salmonella* infections remain confined to the intestine (Giannella, 1996; Younus, 2008; Elzouki *et al*., 2012).

In the case of gastroenteritis, after colonizing the lower intestine (ileum and cecum), *Salmonella* then invades the mucosal cell thus leading to acute inflammation (caused by release of cytokines by the epithelial cells) (Giannella, 1996). This inflammation leads to activation of the adenylate cyclase, increased production of fluids and the release of fluids to the intestinal lumen thus resulting in diarrhoea (Younus, 2008).

1.8 Diagnosis and laboratory identification

Salmonella is mainly isolated from a diarrhoeal stool. However, it can be isolated from blood culture and cerebral spinal fluid in cases of invasive *Salmonella* infection (Gillespie *et al*., 2006).

Culture is still the gold standard in the identification and diagnosis of *Salmonella*. These conventional microbiological methods take up to five days, from isolation to confirmation. Newer and more rapid methods have been have been developed to produce results more quickly, particularly for food and environmental samples. These

include immunology based assays such as, enzyme-linked immunosorbent assay (ELISA), latex agglutination assay, immunoffusion assay and immunochromatography (dipstick) assays. However, there is limited reporting of their use on human samples (Lee *et al*., 2015).

Molecular-based characterization methods used for identification of *Salmonella* include polymerase chain reaction (PCR) and deoxyribonucleic acid (DNA) probe hybridization assay. However, the DNA probe hybridization assay positive result still requires confirmation using culture based methods (Lee *et al*., 2015).

1.8.1 Culture methods

Laboratory diagnosis of *Salmonella* mainly relies on culture. The tests and media used to identify *Salmonella* take advantage of the unique aspects in salmonella's physiology or biochemistry, in order to differentiate it from the other genera within the *Enterobacteriaceae* family. *Salmonella* is a facultative anaerobe, oxidase-negative, catalase-positive and Gram-negative rod. Most *Salmonella* strains are motile and they ferment glucose thus producing acid and gas (Siegrist, 2009).

Many plating media are available for the differentiation and identification of *Salmonella*. They provide varying levels of selectivity; low selectivity media include MacConkey agar (MAC) and eosin methylene blue agar. Moderate selective media include Xylose-Lysine-Desoxycholate (XLD) agar, desoxycholate citrate agar, *Salmonella-Shigella* agar and Hektoen enteric (HE) agar. Highly selective media such as bismuth sulphite agar, XLD and HE have H_2S (hydrogen sulphide) indicator systems, which helps in the identification of lactose fermenting *Salmonella* (Murray *et al*., 2007; Siegrist, 2009). Selective plating media are used alongside screening media such as the Kliger iron media (KIA) or Triple sugar iron agar. The KIA media contains sucrose, ferric ammonium citrate and an indicator. It takes advantage of salmonella's ability to ferment glucose and not sucrose or lactose. In KIA media, *Salmonella* produces colonies with a black centre due to its ability to produce H_2S. However KIA media test must be used alongside indole and urease tests (*Salmonella* species do not produce indole or hydrolyse urease) and together these results can

be used to identify *Salmonella* as the causative agent of disease (Gillespie *et al*., 2006; Murray *et al*., 2007).

1.8.2 Molecular characterization

1.8.2.1 PCR

PCR is a commonly used molecular diagnostic technique. This technique involves the amplification of a single copy or a few copies of a segment of DNA, thereby producing thousands to millions of copies of a particular DNA sequence within two to three hours. PCR relies on thermal cycling conditions, which consist of cycles of repeated heating and cooling of the reaction, which enables the DNA to melt and allow enzymatic replication of the DNA (Chamberlain *et al*., 1988). The amplified DNA can then be analysed using gel-based systems (the traditional method of analysis).

PCR can amplify two or more genes simultaneously in the same reaction (multiplex PCR). This reduces cost (reduces the number of PCR reaction runs, thus fewer reagents used), and preparation time, and it uses less template DNA (Imen *et al*., 2012). Multiplex PCR is therefore being used to identify the *Salmonella* genus and the most common serovars (Porwollik *et al*., 2004; Porwollik *et al*., 2005). With the introduction of real-time PCR, identification of pathogens has become even quicker and less laborious. Several studies have been published on the use of multiplex real-time PCR for detection of *Salmonella* species and *Salmonella* Enteritidis (Malorny *et al*., 2007; Lee *et al*., 2009). One such published study, done by O'Regan *et al*., (2008) targeted the *ttRSBCA* gene for detection of *Salmonella* species and the *sdf* gene for the detection of *Salmonella* Enteritidis.

1.9 *Salmonella* subtyping

Subtyping methods (phenotypic and genotypic) enable differentiation of bacterial isolates beyond the genus level (i.e. species and subspecies level). Bacterial

subtyping methods enable us to detect and track foodborne outbreaks. Furthermore, they allow us to better understand the population genetics, epidemiology, and ecology of different foodborne pathogens (Wiedmann, 2002).

1.9.1 Phenotypic subtyping

Although culturing is used to identify *Salmonella*, identification to *Salmonella* species level is required. Phenotypic subtyping methods such as serotyping and phage typing have been used for many years. However, these methods are time and labour intensive and may have variable discrimination (Wiedmann, 2002).

1.9.1.1 Serotyping

Serotyping is considered the gold standard for phenotypic subtyping of *Salmonella*. *Salmonella* serotyping involves specific agglutination reactions between absorbed antisera and specific epitopes present on the lipopolysaccharide (LPS) (O antigen, encoded by the *rfb* genes) or flagella (H phase 1 and 2 antigens, encoded by the *fliB* and *fliC* genes) of the *Salmonella* bacteria. These antigens (46 O and 85 H antigens) are used in the differentiation of over 2500 *Salmonella* serotypes (Dwarkin and Alkow, 2006). *Salmonella* Enteritidis falls under the O:9 (D1) serogroup, with the antigenic formula 1,9,12:g,m:-. Furthermore, *Salmonella* Enteritidis lacks a phase 2 H antigen.

The O antigen is situated on the outer membrane of the bacterial cell known as the LPS. The LPS is an essential element responsible for maintaining the integrity of Gram-negative bacteria. The LPS also plays a major role in the interaction of the bacterium with the host and thus resulting in dramatic pathophysiological effect on the host's immune system. The LPS is one of the elements that gave rise to serotyping due to its high immunogenicity with the antibodies that are produced with specificity to the LPS polysaccharide glycosyl epitopes (O antigen). The O antigen is the most immunodominant region of the LPS; its structure is very variable amongst *Salmonella* strains (Cabello *et al*., 1993; Dwarkin and Alkow, 2006).

Salmonella H antigens are encoded by one of two genes, namely the *FliC* and *FliB*. The *FliC* gene is responsible for the expression of the phase 1 H antigens, whilst the *FliB* gene expresses the phase 2 H antigens. The *FliC* gene is situated in one of the operons responsible for flagella biosynthesis and it is present in all *Salmonella*. The *FliB* gene is situated in the part of the genome that is unique to *Salmonella enterica* and it is present in 4 of the 6 *Salmonella enterica* subspecies (*enterica*, *salamae*, *diarizonae* and *indica*). These two genes are coordinately regulated through a phase variation mechanism, so that only one flagella antigen type can be expressed at a time in a single bacterium. However, in other instances, certain serotypes can express both flagellin types simultaneously (diphasic), whilst other serotypes contain only one flagellar antigen type (monophasic). Sequence alignment has shown that the 5' and 3' ends of both the *FliC* and *FliB* genes are highly conserved, with the central regions of the sequences showing high variability between immunologically distinct antigen types and this is presumed to be the basis of antigenic differences of flagellar antigens (McQuiston *et al*., 2010).

However, negative agglutination can also occur due to the presence of a new serotype or/an unusual serotype and sometimes due to the presence of a capsular antigen (Vi antigen), which is present in only 3 *Salmonella* serovars: *Salmonella* Typhi, *Salmonella* Paratyphi C and *Salmonella* Dublin (Chart *et al*., 2000). In other instances, *Salmonella* organisms can be found in the non-specific phase and therefore variation can be induced by cultivating the isolate in semi-solid agar containing antisera against phase H 2, which will then select for phase H 1 (Gianella, 1996; Gillespie *et al*., 2006).

It is these unique *Salmonella* characteristic traits that lead to the development of the Kauffman-White-Le Minor scheme (Grimont and Weill, 2007), for serotyping of *Salmonella*. This scheme is currently used worldwide to ensure uniformity within the *Salmonella* nomenclature, which is essential for appropriate communication amongst scientists and health officials around the world (Gianella, 1996; Murray *et al*., 2007).

1.9.1.2 Phage typing

Phage typing is a non-molecular technique used to categorise certain *Salmonella* serotypes to a particular group based on their susceptibility to lysis by certain types of bacteriophage (virus that infects bacteria and replicates within it). Many *Salmonella* serotype strains differ in their susceptibility to lysis by different bacteriophages; this led to the development of a typing scheme based on reactivity to a panel of bacteriophage. In phage typing, *Salmonella* strains are exposed to a specific set of typing phages and the lytic pattern produced allows the assignment of the strain to a particular phage type.

Phage typing has been used in the typing of a number of serotypes including *Salmonella* Typhimurium, *Salmonella* Enteritidis and *Salmonella* Typhi (Threlfall and Frost, 1990; Threlfall, 2000). The technique is commonly used for surveillance and subtyping of *Salmonella*, and provides results easily and rapidly (within 24 hours). However, it is performed in few laboratories due to its requirement for standardized phage panels. Furthermore, phage typing could have less discrimination capacity for serotypes such as *Salmonella* Enteritidis, which has phage type 4 (PT4) as the most common infectious strain. This phage type is said to account for 75% of all *Salmonella* Enteritidis isolated during outbreaks (Hickman-Brenner *et al*., 1991; European Food Safety Authority, 2007). Despite its potential drawbacks, phage typing is one of the most commonly used techniques to subtype *Salmonella* Enteritidis (Cho *et al*., 2007).

1.9.2 Molecular subtyping

Molecular subtyping techniques address epidemiologic problems that cannot be approached or that would be more labour intensive, expensive, and time consuming to address by conventional non-molecular techniques (Foxman and Riley, 2001). Currently molecular subtyping techniques are based on restriction enzyme digestion, nucleic acid amplification or nucleotide sequencing techniques (Imen *et al*., 2012). Some of the most commonly used molecular techniques for subtyping of *Salmonella* include plasmid profiling, pulsed-field gel electrophoresis (PFGE), ribotyping, PCR,

multi-locus sequence typing (MLST), multi-locus variable-number tandem-repeats analysis (MLVA), and whole-genome sequencing (WGS) analysis.

1.9.2.1 Plasmid profiling

Plasmid profile analysis is one of the original DNA-based subtyping schemes. Plasmids are important because, most of them contain genes that encode for virulence and antimicrobial resistance. Plasmid profiling has proven to be useful because it has been shown that regardless of same serotype association, the plasmid content can differ based on the profile (the number and molecular size of the plasmid) obtained. The presence of different plasmid profiles in the same serotype indicates that lateral transfer through the gaining or loosing of plasmids occurs. Plasmid profiling is used for *Salmonella* because the plasmids found in this microorganism have been shown to differ in size 2-200 kilobases (kb) and have different functions (Rychlik *et al*., 2006). The method is based on isolation of the plasmids from the bacterial cell. The plasmids are then run on an agarose gel, which is then stained with ethidium bromide solution and viewed under ultraviolet (UV)-light (Helmuth *et al*., 1985).

Like many other techniques, plasmid profiling has limitations; plasmids can be rapidly acquired or lost. Furthermore, single major plasmids are now endemic within many *Salmonella* serotypes, and such was seen with *Salmonella* Enteritidis were 88% of the isolates in a particular outbreak in Maryland USA were found to all contain one main 36-*Mda* plasmid (Morris *et al*., 1992). Thus, the technique is most effective if the serotype of interest carries multiple plasmids with different sizes (Mendoza *et al*., 1999).

1.9.2.2 Pulsed-field gel electrophoresis (PFGE)

PFGE is currently the primary technique for subtyping of *Salmonella*. PulseNet International (an international molecular subtyping network) uses standardized PFGE protocols for various bacterial pathogens, and this allows for effective inter-laboratory comparison and sharing of PFGE data amongst the PulseNet network (Swaminathan *et al*., 2001; Ribot *et al*., 2006). Through its use on the PulseNet network, PFGE has

had a major impact on pathogen subtyping, surveillance and outbreak investigation (Ribot *et al*., 2006).

PFGE involves the cutting of bacterial DNA with rare-cutting restriction endonucleases and running the DNA on a special electrophoresis unit, which uses pulsed currents that change polarity at certain intervals. These pulsed currents enable separation of large fragments of DNA (up to 12 000 kb), therefore producing strain specific patterns (Peters *et al*., 2007).

A number of restriction endonucleases are specified for PFGE use. However *XbaI*, *SpeI* and *NotI* are the most commonly used restriction endonucleases for *Salmonella* subtyping. The discriminatory power of this technique can further be enhanced by the comparison of strain patterns produced from multiple enzymes, since it can reveal new subtypes (Liebisch & Schwarz, 1996). The success of such an approach was illustrated when 60 *Salmonella* Enteritidis isolates were cut by *Xbal* and they produced 28 different patterns and the same isolates produced 26 different patterns when cut with *SpeI*. Furthermore, when the patterns generated by the two restriction enzymes were combined, 32 different PFGE types were identified (Ridley *et al*., 1998).

PFGE is a highly reproducible technique and the introduction of computerized gel-based data collection and analysis has allowed for better standardization and comparison of patterns between laboratories (Swaminathan *et al*., 2001).

Despite the fact that PFGE is the primary technique for molecular subtyping of *Salmonella*, it is however not always successful. Other serotypes (mainly those with certain distinct phage types) are genetically homogeneous and therefore molecular subtyping techniques such as PFGE are unable to discriminate outbreak from non-outbreak strains, as seen with strains of *Salmonella* Enteritidis (Hopkins *et al*., 2011). This was observed during a Canadian-wide outbreak of gastroenteritis, wherein *Salmonella* Enteritidis strain DT8 was isolated. The *Salmonella* Enteritidis DT8 strain was evaluated using PFGE; nonetheless successful discrimination was only attained through the combination of intensive epidemiological, phenotypic and genotypic methods (Ahmed *et al*., 2000).

Additionally, PFGE is laborious and time consuming (takes minimum 3 days to complete), it requires expensive specialised equipment (which may not be affordable for developing countries) and it also requires experienced personnel (experienced staff may be scarce in developing countries and budget for training may be limited) (Herschleb *et al*., 2007).

1.9.2.3 Ribotyping

Ribotyping is a technique used to fingerprint ribosomal ribonucleic acid (rRNA) coding sequences. The technique involves the running of endonuclease-digested DNA through an agarose gel and then transferring the DNA fragments onto a membrane, which enables the DNA fragments to be hybridized to a probe that recognizes the *16SrRNA* and *23SrRNA* (Millemann *et al*., 1995).

Several copies of the rRNA operon exist in the *Salmonella* chromosome. The rRNA genes in this operon are very homologous. However, the interfering sequences tend to differ in their length and nucleotide composition (Mendoza *et al*., 1999; Imen *et al*., 2012). Ribotyping has been shown to successfully subtype isolates that fall within some of the most common serotypes and phage types (Landeras *et al*., 1996). Lin *et al*., (1996) identified 7 different ribotypes from 17 *Salmonella* Enteritidis phage type 8 (PT8) isolates whose chromosomal DNA was digested with *SphI*.

1.9.2.4 PCR-based methodologies

A number of PCR-based subtyping methodologies have been described and these include random amplified polymorphic DNA (RAPD) assay and repetitive extragenic palindromic (REP)-PCR (Olive and Bean, 1999).

The RAPD assay, also referred to as arbitrary primed PCR, is based on the use of short random sequence primers (~9-10 bases long) that hybridize to chromosomal DNA sequences at low annealing temperature, in order to be used to initiate amplification of regions of the bacterial genome. If two RAPD primers anneal within few kb of one another, a PCR product with a molecular length corresponding to the

distance between the two primers is produced. The number and location of these random primers tend to vary for different bacterial strains. Thus following separation of the PCR products by agarose gel electrophoresis, a pattern of bands which are characteristic (theoretically characteristic) to the particular bacterial strain are produced (Olive and Bean, 1999).

REP-PCR involves the fingerprinting of a bacterial genome by examining strain-specific patterns obtained from PCR amplification of repetitive DNA elements present within the bacterial genome. This assay consists of two main sets of repetitive elements (repetitive extragenic palindromic elements and enterobacterial repetitive intergenic consensus sequences), used for the typing process. The enterobacterial repetitive intergenic consensus sequences were primarily defined based on sequence data obtained from *Salmonella* Typhimurium and *E. coli*. This has enabled REP-PCR to generate unambiguous DNA fingerprints for differentiation of eubacterial species and strains (Olive and Bean, 1999).

Both assays have been successfully applied in the subtyping of *Salmonella* Enteritidis. Furthermore, advancement in their technologies have allowed for reduced preparation times and quicker results (Olive and Bean, 1999).

1.9.2.5 Multi-locus sequence typing (MLST)

MLST is a molecular typing technique that compares DNA sequences from internal regions of housekeeping or virulence genes and/or rRNA sequences, which tend to differ due to mutations or recombination events (Maiden *et al*., 1998).

The nucleotide differences found in each of the genes are combined and used to determine the type of strain (Yan *et al*., 2003). MLST produces data that is similar to that obtained by multi-locus enzyme electrophoresis, but in greater detail. This is because MLST has the ability to analyse individual nucleotide changes, rather than assessing the overall change in charge and expression of the enzyme in question (Maiden *et al*., 1998).

MLST is a useful technique for long-term epidemiological studies or phylogenetic analysis. Its potential use in *Salmonella* subtyping was observed from its ability to characterize over 200 salmonella's using sequences from the different housekeeping genes (Kotetishvili *et al*., 2002). These results were compared to those of PFGE and serotype analysis. It was shown that MLST was able to differentiate the strains better. This technique shows great potential in the accurate sharing of information between laboratories. Nonetheless MLST is still expensive and may be unaffordable in developing countries (Imen *et al*., 2012).

1.9.2.6 Multi-locus variable-number tandem-repeats analysis (MLVA)

MLVA is a subtyping method that uses naturally occurring variation in the number of tandem repeated DNA sequences found in the genome of most bacterial species. It is this polymorphism that occurs in repeat loci regions that makes MLVA a well discriminative (good typing) method (Kramer *et al*., 2010; Koninklijke Nederlandse Vereniging voor Microbiologie, 2014).

Tandem repeats are made up of two or more identical or nearly identical short DNA sequences that are not combined with any intervening DNA sequence. These tandem repeats are the result of errors made by the DNA polymerase, which incorrectly copies these segments by a mechanism called slipped strand mispairing. During replication, DNA polymerase stumbles in certain regions of the genome, therefore resulting in some DNA regions being duplicated or deleted. This DNA polymerase error can occur numerous times and may cause some regions to be multiplied several times and the size of these tandem repeat units can range from 3 to 100 base pair. The stuttering results in variation in the number of repeats, hence the name "variable number tandem repeat" (VNTR) (Kramer *et al*., 2010).

There are two different types of mechanisms by which variation in the number of tandem repeats can be generated. The first mechanism is replication slippage (also known as backward replication slippage), which occurs when the DNA polymerase copies the template DNA and then stutters in areas where tandem repeats are located, resulting in increased numbers of repeats (Figure 1.2). The second

mechanism is the forward slippage, which occurs if a tandem repeat region contains a large repeat sequence and during replication the single stranded genomic DNA forms a loop in the repeat region. The DNA polymerase then mistakenly skips this looped region and thus producing a replicated strand that has a smaller tandem repeat sequence (Figure 1.3) (Koninklijke Nederlandse Vereniging voor Microbiologie, 2014).

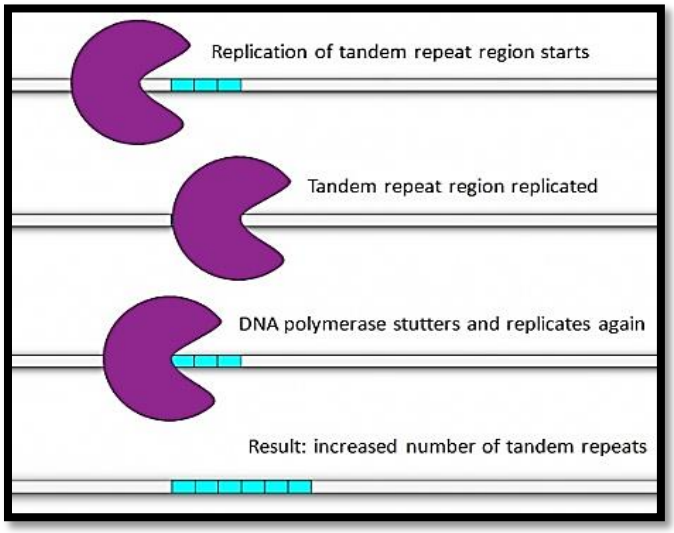


Figure 1.2. Mechanism of replication slippage (Koninklijke Nederlandse Vereniging voor Microbiologie, 2014).

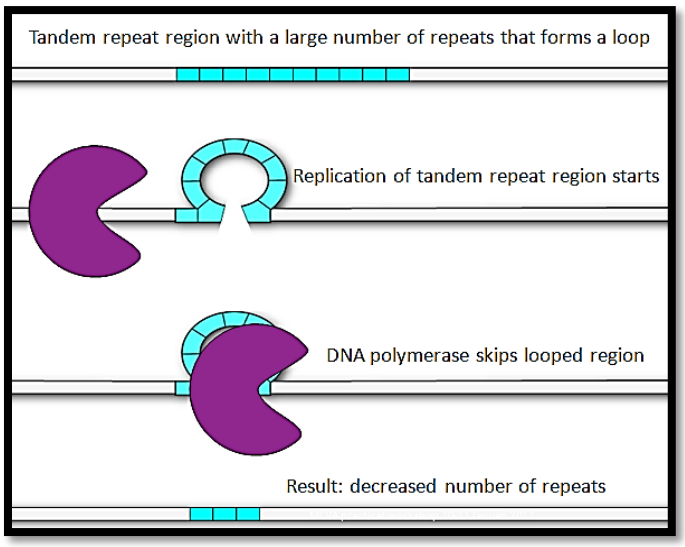


Figure 1.3. Mechanism of forward slippage (Koninklijke Nederlandse Vereniging voor Microbiologie, 2014).

The MLVA method involves performing a PCR to amplify the repeat regions in the bacterial genome. The size of the PCR product is then analysed using a multicolour capillary gel electrophoresis or by sequence analysis. The PCR product sizes are then used to determine the number of repeats in each region. Therefore, by combining the size differences from several repeat loci regions, a multi-digit, strain specific code (profile) can be acquired and these profiles can therefore be used for cluster analysis. The introduction of DNA sequencers in product sizing has made MLVA a much reliable method and the numerical aspect of the data makes it suitable for inter-laboratory exchange (Kramer *et al*., 2010).

MLVA's performance (reproducibility and epidemiological relevance) is highly dependent on the stability of its target sequence. Some VNTR's however are unstable and can therefore lead to strain separation during an outbreak, thus confounding the actual epidemiology. In other occasions extremely unstable VNTR's can even undergo change during routine laboratory sub-culturing and therefore affect the reproducibility of the MLVA method. Another possible MLVA drawback is that, since the primers used to amplify the VNTR's are designed based on currently known whole genome sequences, it cannot be possible to successfully amplify all VNTR's from all strains in the same species, therefore making typeability a challenge. For example an insertion within a VNTR region would change the VNTR size, thus confounding the VNTR size analysis. This signifies the importance of VNTR selection and primer design in developing an epidemiologically relevant MLVA scheme (Wiedmann and Zhang, 2011).

Although MLVA is a fragment-based method, its use of consistent molecular markers, PCR and capillary electrophoresis produces a more phylogenetically significant and less-ambiguous product, thus making it more advantageous in comparison to other fragment-based subtyping methods. MLVA's discriminative properties were observed by Boxrud *et al*., (2007), where they evaluated MLVA, phage typing and PFGE for the subtyping of *Salmonella* Enteritidis. Their discriminatory indexes were 0.965, 0.850 and 0.839 respectively, thus demonstrating MLVA's potential as a reliable and fast subtyping method for *Salmonella* Enteritidis.

To date, a number of MLVA schemes for *Salmonella* Enteritidis have been published (Boxrud *et al*., 2007; Cho *et al*., 2007; Cho *et al*., 2008; Malorny *et al*., 2008; Ross and Heuzenroeder, 2009). However, a difference in the MLVA setups and data interpretation makes comparison of data between laboratories a challenge. Another challenge with MLVA is that there are limited published data available describing the stability of loci regions and the speed at which tandem repeats evolve, and if such evolution can occur during an outbreak caused by a single ancestral isolate. Such concerns may threaten the use of MLVA for *Salmonella* Enteritidis outbreak detection unless specific guidelines are developed for performing MLVA and a common procedure is reached on how to interpret the MLVA data, as seen in MLVA for *Salmonella* Typhimurium (Larsson *et al*., 2009; Hopkins *et al*., 2011).

Hopkins *et al*., (2011) emphasized the standardization of MLVA for *Salmonella* Enteritidis by implementing guidelines for the MLVA protocol, result analysis and interpretation. The use of this protocol in laboratories around the world will ensure preservation of this technique for *Salmonella* Enteritidis subtyping.

In their study, Hopkins *et al*., (2011) identified 71 different MLVA profiles from 298 *Salmonella* Enteritidis isolates, thereby showing MLVA to be a promising subtyping method for this *Salmonella* serotype. Furthermore, MLVA was able to subtype isolates that belonged to the same phage type and in most cases isolates from different phage types clustered together. A similar finding was previously observed by Malorny *et al*., (2008) and Cho *et al*., (2010).

1.9.2.7 Whole-genome sequencing (WGS) analysis

Despite the fact that good clinical results are obtained from the serological and molecular methodologies mentioned above, the techniques however give limited information about the pathogenic organism. WGS analysis is changing this. The technique has greatly improved since its inception, with the reduction in process time as well as the move of WGS into high-throughput next generation sequencing technology, with comparatively simple benchtop technology and efficient library preparation. Furthermore, the technology is becoming cheaper and more user-friendly.

WGS involves the determination of the complete DNA sequence of an organism's genome at a single time, thus attaining all information regarding its genetic make-up. This gives more information beyond identification level (Hasman *et al*., 2014).

For genetically monomorphic bacteria such as *Salmonella* Enteritidis, *Salmonella* Montevideo, *Staphylococcus aureus* and many others, the current typing methods are not adequate for outbreak detection, trace backs, and identification of transmission routes. The use of WGS analysis on these organisms has shown better discrimination compared to other molecular typing methods (Den Bakker *et al*., 2014).

Numerous studies describing WGS analysis for *Salmonella* Enteritidis have been published. A study by Feasey *et al*. (2016) analysed *Salmonella* Enteritidis isolates from 45 countries (from six continents). They found evidence of three clades (grouping of organisms with a common ancestor); the global epidemic clade and two African clades (West African clade and Central/Eastern African clade). The study was able to link the global epidemic clade with foodborne outbreaks associated with chicken eggs in Europe. The West African clade and Central/Eastern African clade were shown to be related; however they were phylogenetically and geographically distinct from one another. The *Salmonella* Enteritidis strains in these two clades were also different from those in the industrialized world. These strains showed evidence of changing host adaptation, different virulence determinates and multidrug resistance. Furthermore, these strains had possible links to epidemics of bloodstream infection in at least three countries in Sub-Saharan Africa. Interestingly, the study also showed that the South African *Salmonella* Enteritidis strains were associated with the global epidemic clade.

Currently PFGE is the primary technique for typing *Salmonella* species; however PFGE has limited discriminatory power for *Salmonella* Enteritidis strains and clusters, and although MLVA improves discrimination of disease clusters, it still assigns 30% of isolates to a single MLVA type (Den Bakker *et al*., 2014).

A study by Den Bakker *et al*. (2014) comparing WGS to PGFE and MLVA, demonstrated that whole-genome cluster analysis of *Salmonella* Enteritidis showed

vast improvement on the detection of clusters of common PFGE types, as well as improving outbreak resolution. Furthermore, comparison of WGS analysis to MLVA gave corresponding results (multiple types) and PFGE had given a single type, which yielded no useful molecular clustering information (Den Bakker *et al*., 2014).

Despite well-published success on the typing of various bacterial organisms, the full potential of WGS has not been fully explored. Currently, majority of the WGS analysis is based on single nucleotide variants or single nucleotide polymorphisms (SNPs) that have been identified from comparisons to an already present reference genome sequence. Consequently, analysis is thus dependent on the quality of sequencing, genome assembly, as well as the quality and selection of the reference genome. SNPs comparative analysis excludes a significant proportion of phylogenetic data and some bioinformaticians have even suggested conducting phylogenetic analyses based on all loci in a genome, rather than solely analysing SNPs (Bertels *et al*., 2014). However, such analysis would require large computer resources and time (Kwong *et al*., 2015). Although the cost of WGS has significantly decreased, it is still unaffordable for most African countries. However, in South Africa, WGS is being explored in research facilities.

1.10 Treatment and management

Infection by NTS such as *Salmonella* Enteritidis tends to be mild and resolve easily without any treatment by antimicrobials. Antimicrobial treatment of uncomplicated salmonellosis is contra-indicated because it tends to extend the carrier state (D'Aoust, 1991; Chiu and Su, 2014). However, in order to prevent dehydration and electrolyte imbalances of the uncomplicated cases, replacement fluids and electrolytes are administered to the patient (Chiu and Su, 2014).

Antimicrobial treatment is administered in severe and complicated cases such as in patients with prolonged fever and those that are immune-compromised (such as infants, the elderly, HIV infected, cancer patients etc.). This is to prevent further morbidity or mortality amongst these high-risk groups (Alcaine *et al*., 2007). The first

line of treatment often includes fluoroquinolones like ciprofloxacin or a third generation cephalosporin *β*-lactam such as ceftriaxone, penicillins such as ampicillin and folic acid pathway inhibitors (sulfonamides). However, children and pregnant women are treated with *β*-lactams, because fluoroquinolones interfere with cartilage formation (Barceloux, 2012; Chiu and Su, 2014).

In healthy patients, antimicrobials are often prescribed and administered for 5 days in order to limit faecal carriage that may occur with prolonged use of antimicrobials (Hohmann, 2001). In patients with systematic disease, antimicrobials are administered for a longer period of time (3-4 weeks), to ensure effective drug penetration and treatment as well as to prevent recurrence (Mangni and Arvntikis, 2010). In circumstances where infection is caused by first line drug-resistant *Salmonella*; second line drugs such as aminoglycosides, sulfonamides like cotrimoxazole are therefore used (Guerrant *et al*., 2001; Chiu and Su, 2014). Treatment of multidrug-resistant *Salmonella* infections includes carbapenems such as imipenem or meropenem that are administered intravenously (Alanis, 2005; Huehn *et al*., 2010; Chiu and Su, 2014).

1.11 Antimicrobial resistance

Antimicrobials play an important role in the controlling of bacterial infections, reducing morbidity and in preventing mortality. It is estimated that antimicrobials increase life expectancy by 20 years. These compounds have not only saved human life, but have also saved life-stock in the agricultural industry (Alanis, 2005; National Department of Health, 2015). However the extensive use of these substances has resulted in bacterial resistance, thus threatening to reverse the life-saving power of these drugs. It is estimated that 25000 patients die in Europe each year from resistant bacterial infections and in South-East Asia 1 child dies every five minutes from a resistant bacterial infection (National Department of Health, 2015).

Antimicrobial resistance is a serious problem, which requires immediate global attention, because certain microorganisms have become extremely resistant to all

the existing antimicrobials; such events have been mainly described in Gram-negative rods such as *Escherichia coli* (*E. coli), Salmonella* species, *Klebsiella* species and *Acinetobacter* species (Hughes, 2011).

On the other hand, the production of new antimicrobials has become slow; few antimicrobials have been produced in the past decade (Hughes, 2011). Several powerful compounds, which are active against Gram-positive cocci, have been produced in the last few years. However, this has not been the case with Gram-negative bacteria and there is almost no new drugs that can be anticipated to be effective against multidrug-resistant Gram-negative rods in the near future (Carlet *et al*., 2012).

1.11.1 Drivers of antimicrobial resistance

The most important drivers of antimicrobial resistance include the amount of antimicrobials used, dependence on broad-spectrum antimicrobials and acquisition of hospital acquired infections (National Department of Health, 2015).

It is estimated that 50% of all antimicrobials prescribed to humans are unnecessary, since in many cases there is no infection or the infection is not caused by a bacterium or the antimicrobials are prescribed for an unnecessary expended period of time. It is also estimated that 80% of all antimicrobials used globally are used in agriculture and animal health for prevention, treatment and growth promotion (National Department of Health, 2015). Broad-spectrum antimicrobials have activity against a wide range of different bacteria and this may lead to the selection of a greater range of resistant populations compared to narrow-spectrum antimicrobials. The rising levels of bacterial resistance in hospitals and community settings, increases the need for use of empirical antimicrobials with a broader spectrum of activity. Therefore, escalating the selection of resistant bacteria within the human population (National Department of Health, 2015). Hospitalized patients are at high risk of developing multidrug-resistant bacterial infections, because they are often immune-compromised and poor hygiene practices by health care professionals may leave them vulnerable to such infections, mostly during the performance of invasive procedures (National Department of Health, 2015).

1.11.2 Mechanisms of antimicrobial resistance

Resistance to antimicrobials was detected in the target pathogens, a few years into their use as therapeutic agents in humans. Selective pressure created by their extensive use was the driving force in the emergence of genetically encoded resistance, which could be transferred to the offspring bacterium and in other events resistance, could be caused by horizontal gene transfer to even distant bacterial species (Linton, 1977; National Department of Health, 2015).

Mechanisms of antimicrobial resistance mainly fall into three categories: (1) inactivation of the antimicrobial agent, (2) efflux or alterations in permeability or transport of the antimicrobial agent, and (3) modification or replacement of the antimicrobial target site (McDermott *et al*., 2003; Walsh, 2003; Boerlin and Reid-Smith, 2008; Foley and Lynne, 2008).

Antimicrobial resistance is encoded genetically and can vary from mutations that are found in the chromosomal DNA, to horizontally acquired resistance genes that are carried by mobile genetic elements such as plasmids. Both point mutations and horizontally acquired genes can encode for all the three mechanisms of resistance.

Point mutations that occur in the promoter or operator coding regions can lead to overexpression of the endogenous genes such as those that encode for antimicrobial inactivating enzymes like the β-lactamase *AmpC* gene (Siu *et al*., 2003). Point mutations that occur in genes encoding for antimicrobial target regions can result in a target site that is resistant to the antimicrobial activity. Such a mutation was seen in the *gyrase* gene, whose mutation led to the expression of a fluoroquinolone-resistant *gyrase* enzyme (Eaves *et al*., 2004; Hopkins *et al*., 2005).

Non-chromosomal resistance genes encoded on plasmids, integrons, phages and transposons can be transferred horizontally through transformation, transduction or conjugation. These exogenous DNA's include genes which encode for enzymes that inactivate antimicrobials (e.g. β-lactamases that cleave the four membered ring in β-lactams), genes which encode for efflux systems (which expels the drug out of the bacterial cell) and genes that encode for an altered form of the enzyme that is

targeted for by the antimicrobial agent (Boerlin and Reid-Smith, 2008; Ajiboye *et al*., 2009; Carattoli, 2009).

1.11.3 Resistance to antimicrobials used for *Salmonella* treatment

Although most *Salmonella* cases are mild and require no treatment, complicated cases require antimicrobials and antimicrobial resistance often leads to treatment failures and potential death of patients by pathogens that were once treatable. *Salmonella* is already a huge burden globally and resistant *Salmonella* strains threaten to cause a global pandemic, leading to the loss of millions of lives (D'Aoust, 1991). Antimicrobials used for the treatment of complicated *Salmonella* infections include penicillins, fluoroquinolones, cephalosporins, tetracyclines, sulfonamides and macrolides to name a few (Parry and Threlfall, 2008; Barceloux, 2012).

Ampicillin is one of the most commonly used extended-spectrum penicillin antimicrobial worldwide (Root *et al*., 1999). Ampicillin is used in the treatment of complicated *Salmonella* Enteritidis cases. However, due to changes in susceptibility, it is no longer the drug of choice unless culture and sensitivity results indicate susceptibility (Barceloux, 2012). Although human ampicillin-resistant *Salmonella* Enteritidis isolate cases exist, there have been limited reports in literature. However, ampicillin resistance has been widely reported in other NTS serovars such as *Salmonella* Typhimurium (Mølbak *et al*., 1999; Pignato *et al*., 2010).

Fluoroquinolones are a family of synthetic antimicrobial agents, whose mode of activity involves the inhibition of topoisomerases (Hooper, 2001; Lorian, 2005). Fluoroquinolones such as ciprofloxacin are used in treatment of *Salmonella* infections. There is limited data on ciprofloxacin resistance by *Salmonella* Enteritidis; however Cheung *et al*. (2005) reported on plasmid mediated ciprofloxacin resistance of *Salmonella* Enteritidis in Hong Kong. Several studies have also described reduced susceptibility and intermediate resistance of *Salmonella* Enteritidis to ciprofloxacin (Threlfall, 2002; Eibach *et al*., 2016).

Ceftriaxone is an extended-spectrum cephalosporin, generally used to treat severe *Salmonella* infections and are the main drug of choice when treating patients for

whom fluoroquinolones are contra-indicated. Cephalosporin resistance amongst human *Salmonella* isolates is still low. Nonetheless, there is a continual increase of cephalosporin resistance in *Salmonella* isolates from animals, thus threatening food safety and human health (Goetez, 2012). Bacterial pathogens resistant to extended-spectrum cephalosporins such as ceftriaxone carry a plasmid-mediated *AmpC*-like beta-lactamase enzyme (encoded by the *blaCMY* gene), which hydrolyses cephalosporins. *Salmonella* isolates carrying the *blaCMY* gene have been isolated from bovine, porcine, human, and foods sources (Gray *et al*., 2004).

Tetracyclines are broad-spectrum antimicrobials, which are also used in the treatment of *Salmonella* infections. Tetracyclines mode of action is the inhibition of protein synthesis at the ribosome. Several different *tet* genes have been described to confer resistance to tetracyclines in *Salmonella* species. These genes have been detected on chromosomes of different *Salmonella enterica* serotypes including *Salmonella* Enteritidis, *Salmonella* Typhimurium, *Salmonella* Hadar, *Salmonella* Saintpaul, and *Salmonella* Choleraesuis (Pezzella *et al*., 2004).

Sulfamethoxazole and trimethoprim are one of the most common sulfonamides used in the treatment of infections caused by enteric pathogens such are *E. coli*, *Shigella* and *Salmonella*. Sulfamethoxazole is often used in combination with trimethoprim in a 5:1 ratio, with the trade name cotrimoxazole (Hamer and Gill, 2008; Sibanda *et al*., 2011). Sulfamethoxazole and trimethoprim (cotrimoxazole) are used as prophylactic drugs in HIV/Aids infected patients, as a means to prevent life threatening opportunistic infections in this risk group. This extensive use of cotrimoxazole within the HIV/Aids infected population is based on studies that were conducted in Africa showing reduced HIV-associated mortality and morbidity in sulfamethoxazole and cotrimoxazole users (Sibanda *et al*., 2011). Since the introduction of antiretroviral drugs, the prophylactic use of cotrimoxazole in developed countries has been greatly reduced. However, most African countries continue with this use of cotrimoxazole. Therefore, it is highly plausible that sulfonamide resistance may be attributed to the extensive use of these drugs in most African settings. Furthermore, the use of sulfonamides in the agricultural industry for animal prophylaxis might be playing a major role in their resistance (McEwen and Fedorka-Cray, 2002; Swartz, 2002;

Hamer and Gill, 2008; Morpeth *et al*., 2009). Resistance genes are often located on plasmids (Chiu and Su, 2014).

Macrolides are one of the most well established classes of antimicrobials used today. One of the most commonly used macrolides is azithromycin; this antimicrobial has been shown to have varying levels of activity against the family *Enterobacteriaceae*. However, it is effective against *Salmonella* and *Shigella* species (Parnham *et al*., 2002; Gaynor and Mankin, 2003). The high level of activity, safety, as well as their use as a penicillin alternative for allergic patients, has made macrolides a popular drug of choice. Such has led to the extensive use of these drugs and therefore aiding to the emergence and spread of resistant bacterial strains (Weisblum, 1995). Azithromycin resistance has not been reported in *Salmonella* Enteritidis.

1.12 Prevention

Prevention of salmonellosis is closely linked with food safety and therefore stringent regulations have to be emphasized in the food industry by government departments such as health and agriculture (FAO and WHO, 2002). The issue of foodborne disease prevention is very complex and complicated, because it involves several stages in food production such as processing, storage and transportation. Poultry, poultry products and meat products are the most commonly reported sources of *Salmonella* infection, therefore emphasis is required to ensure correct food safety and appropriate hygienic precautions are carried out (Mangni and Arvntikis, 2010).

Government departments cannot prevent foodborne diseases alone. The public has to be well informed about the risks involved in consumption of certain food products and also measures they can take to prevent infection. Another important aspect in the prevention of foodborne enteric infections relates to measures that have to be taken to ensure that restaurants conform to safety regulations when storing and preparing food. Regular monitoring (questionnaire based) of personnel who might be harbouring an enteric infection or not following proper hygienic procedures should be emphasized. Furthermore, hygienic practices should also be emphasized in

hospitals, children's day cares and in private homes. This is because food might have been safe during selection in supermarkets, but may become unsafe by the time it's consumed (Mangni and Arvntikis, 2010).

1.13 Vaccines

With food as the major route of transmission, reduction of *Salmonella* Enteritidis infections is a major global challenge and vaccination of food animals and/or humans may play a role in the fight against this pathogen. Animal vaccination would reduce the number of contaminated food animals and therefore reduce human infections caused by consumption of contaminated food. However, the theoretical efficacy of vaccines that prevent animal infection by *Salmonella* is uncertain. The main challenge is that most *Salmonella* serotypes, which colonise the animal species and are then passed on to humans, are actually part of the normal flora of these animals. Therefore the design of any vaccine to inhibit infection by "normal flora" is a challenging task (Chiu and Su, 2014). However, there are a number of live attenuated *Salmonella* vaccines licensed for use in poultry, swine and cattle industry (Chiu and Su, 2014).

1.14 *Salmonella* Enteritidis in food animals

Salmonella Enteritidis zoonotic capabilities enable it to infect both humans and animals successfully. Food animals have become a pathway for human infection by *Salmonella* Enteritidis, making this *Salmonella* serovar a pathogen of global concern (Mangni and Arvntikis, 2010; Gal-Mor *et al*., 2014).

Salmonella Enteritidis is one of the most common *Salmonella* serovars isolated from food animals and food products. In farm animals, this pathogen is commonly isolated from poultry and chicken eggs. Although it can also be isolated from other farm animals such as swine, cattle and sheep; *Salmonella* Enteritidis infections in these

animals are considered less important compared to poultry infections (Kidanermariam *et al*., 2010; Andino and Hanning, 2015).

It has been widely reported that the incidence of *Salmonella* infections in various species of farm animals is closely linked to the husbandry methods used in the farms. It has also been reported that intensive farming methods are favourable to the spread of infections, thus leading to an increase in clinical disease. It is due to such potential disease risks that countries like Sweden have more stringent rules to control foodborne pathogens such as *Salmonella* (Commission of the European Communities, 2000; Kidanermariam *et al*., 2010). In South Africa, *Salmonella* Enteritidis is a notable disease and measures have been taken by the agricultural department to eradicate it. However, it remains a huge burden to both the local and the global farming industry (Kidanermariam *et al*., 2010).

1.14.1 Animal vaccination

A number of *Salmonella* vaccines are effective for use in poultry, swine and cattle (Chiu and Su, 2014). The emphasis placed in vaccination of food animals indicates the importance of contaminant free food, thus eliminating human exposure to these foodborne pathogens. *Salmonella* Enteritidis is the most prevalent *Salmonella* serotype in the poultry industry and therefore vaccinating chickens against this serotype is essential to ensuring food safety (Alvarado, 2011). In South Africa both live and inactivated *Salmonella* Enteritidis vaccines are licensed for use in efforts to reduce contamination of chickens, eggs and chicken products (DAFF and ARC-OVI, 2000).

1.14.2 Animal farming and antimicrobial resistance

Animals, like humans, acquire bacterial infections that need to be treated through antimicrobial therapy and the veterinary requirements for the treatment of these bacterial infections with antimicrobials is similar to those in human medicine (Acar and Röstel, 2001; South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs, *et al*., 2007). However, in agriculture the use of antimicrobials is more complex, more especially in food-

producing animals as it includes treatment, prophylaxis, metaphylaxis and growth promotion. This kind of animal treatment regime is essential in order to maintain a sustainable and economically feasible animal industry (Acar and Röstel, 2001; South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs, *et al*., 2007).

However, with the increasing prevalence of antimicrobial-resistant bacteria, there is considerable debate on the use of antibiotics in agricultural animals, more especially on those raised for human consumption. The main concern is inappropriate use of antibiotics in food animals, which could pose a potential threat to human health. This is because antimicrobial-resistant microorganisms propagate in these food animals and could easily enter the food supply, therefore spreading to humans. In 2011, the World Health Organisation (WHO) reported that the use of fluoroquinolones in food animals had resulted in the development of ciprofloxacin-resistant *Salmonella*, *Campylobacter* and *E. coli*, which have caused human infections that have proven difficult to treat (Teuber, 2001; WHO, 2011; Landers *et al*., 2012).

Modern day farming often involves intensively managed livestock operations, therefore increasing the potential for rapid dissemination of infectious agents from animals to humans. This is mainly due to the close proximity in which animals live in (feeding through same food channel etc.). Food animals grown in such environments usually require assertive infection management strategies, which often involves the use of antimicrobial therapy. Although the main goal is to prevent infections in animals, thus providing safe meat to humans, the use and administration of antimicrobials in the agricultural sector in different parts of the world is concerning. Many different classes of antimicrobials are used in the food animal industry, some of which are also used in human medicine (e.g. penicillins, cephalosporins, sulfonamides etc.). Microorganisms that are resistant to these classes of antimicrobials have been isolated from human clinical samples and such resistance has been extensively described in literature (Teuber, 2001; Landers *et al*., 2012).

In 2006, the European Union (EU) banned the use of antimicrobial feed additives (AFA) as growth promoters in livestock animals. A study done by Aarestrup (2012) in Denmark proved that the elimination of antimicrobials from animal feed had no

impact on their meat produce, since Denmark remains the world's largest exporter of pork. However, such strategies have not been implemented in South Africa (Henton *et al*., 2011; Eagar *et al*., 2012).

In South Africa, all main classes and types of antimicrobials are authorized for animal use and these antimicrobials include those used for growth promotion, most of which have been banned from inclusion as food additives in the EU (Henton *et al*., 2011). Approximately 234 antimicrobials have been registered for use in food animals and 64 (27%) in-feed antimicrobials are registered as stock remedies used for treatment, prophylaxis or growth promotion (Eagar *et al*., 2012). The most commonly sold class of antimicrobials included macrolides, tetracyclines, sulfonamides and penicillins, all of which are used in human treatment.

In most instances, a small concentration of antimicrobials are used in animal feed over long extended periods of time and such a practice can accelerate the emergence of antimicrobial-resistant microorganisms in food animals; which in turn may lead to human infection with these resistant microorganisms (Eagar *et al*., 2012). However, if the quality of industrial farming is improved there will be no need for such extensive use of antimicrobials to prevent and combat disease. The farming industry needs to improve on hygiene and reduce overcrowding in order to prevent rapid spread of disease and consequently reduce the need for prophylactic therapy (Garces, 2002; Moyane *et al*., 2013).

1.15 Study objectives

Salmonella Enteritidis is an important pathogen globally and has become a public health concern in epidemic areas, particularly in developing countries such as South Africa. Poor surveillance in most African countries makes it difficult to ensure proper estimates of disease burden and hence the true burden of salmonellosis caused by *Salmonella* Enteritidis has not been established.

The exact factors responsible for the *Salmonella* Enteritidis epidemic are still globally unclear (Duchet-Suchaux *et al*., 1995; Yim *et al*., 2010). Therefore, efficient surveillance structures have to be put in place to prevent massive outbreaks, which may take lengthy periods of time to control. Molecular epidemiology is necessary to identify the strains of importance that are common in the population. This aids in the assignment of proper preventative measures. In the case of zoonotic pathogens such as *Salmonella* Enteritidis, comparison of molecular data gathered from human and non-human (such as animal and environmental) isolates may help answer questions of transmission and the source of antimicrobial resistance spread (Gruner *et al*., 1994; Teuber, 2001; Ranjbar *et al*., 2014).

To date, no comprehensive molecular epidemiological studies of *Salmonella* Enteritidis strains in South Africa have been carried out. Therefore, the aim of our study is to investigate the molecular epidemiology and relatedness of *Salmonella* Enteritidis strains from humans in Gauteng and Western Cape, South Africa using MLVA; secondly to investigate the relatedness of human and non-human *Salmonella* Enteritidis strains in South Africa, using MLVA. Furthermore, we aim to investigate the antimicrobial susceptibility profiles of the *Salmonella* Enteritidis isolates, in order to estimate the burden of antimicrobial-resistant *Salmonella* Enteritidis isolates in circulation.

The gradual increase in the prevalence of *Salmonella* Enteritidis in South Africa over recent years emphasizes the need to conduct a molecular epidemiological study. This would essentially contribute to monitoring the evolving epidemiological patterns of the pathogen. Similarly, this study will allow us to identify and distinguish outbreaks from clusters of temporally and geographically proximate cases.

Lastly, we aim to analyse *Salmonella* Enteritidis outbreaks that occurred during the years 2013-2015, in order to ascertain MLVA's ability to identify outbreak isolates and group them into one cluster (MLVA profile).

CHAPTER 2: MATERIALS AND METHODS

2.1 Bacterial isolates

2.1.1 Human isolates

The CED serves as a reference centre for human enteric pathogens. The CED participates in the Group for Enteric, Respiratory and Meningeal Surveillance in South Africa (GERMS-SA) national laboratory-based surveillance. Microbiology laboratories across the country voluntarily submit isolates to the CED, for conformation and further characterization (National Institute for Communicable Diseases, 2016). The human isolates included in this study were obtained from the CED, through the GERMS-SA laboratory-based surveillance. The isolates were isolated from various body sites (Appendix A). Each isolate received at the CED was assigned an identification number (TCD number) and a unique database reference number. The TCD number and unique database reference number were used for identification of the patient. The patient details and demographic information obtained from the sender (laboratory) form were entered into the CED Microsoft Access 2010 (Microsoft Corporation, Redmond, USA) database. After identification/characterization of the isolates, the results were entered on the CED Microsoft Access database, using the TCD number and unique database reference number to identify the patient to whom the isolate/s belong.

At the onset of the study it was anticipated that due to resource constraints, mainly time and finances, that only a certain number (approximately 1220) of *Salmonella* Enteritidis isolates would be processed and included for the purpose of this study. Gauteng and the Western Cape provinces submit the highest number of *Salmonella* Enteritidis isolates for surveillance purposes to the CED annually. Therefore, to eliminate selection bias of isolates and to ensure randomness of selection, all *Salmonella* Enteritidis isolates from the year 2013-2015, present in the CED Access database from the Western Cape and Gauteng Province were identified and exported onto a Microsoft Excel 2010 (Microsoft Corporation, Redmond, USA)

spread-sheet. Random numbers were assigned to these isolates, using the RAND function in Microsoft Excel. The isolates were then selected by sorting them in ascending order of random number and selecting the top 1220 isolates. This process yielded one duplicate random number and for this reason, this isolate was also included, resulting is a total of 1221 (832 from Gauteng and 389 from the Western Cape) randomly selected study isolates.

2.1.2 Non-human isolates

A total of 43 non-human isolates from the years 2014-2015 were included in the study. Isolates were obtained from the Agricultural Research Council - Onderstepoort Veterinary Institute (ARC-OVI) (*n* = 27), University of Pretoria (*n* = 7), Deltamune (*n* = 3), private suppliers (*n* = 4) and the Western Cape Veterinary Laboratory (*n* = 2). The isolates were from animals (*n* = 20), animal environments (*n* = 2) and some were of unknown origin (*n* = 21). Upon arrival at the CED, the non-human isolates were cultured on MAC agar [Diagnostic Media Products, Johannesburg, South Africa (SA)] and 5% sheep blood agar (Diagnostic Media Products, Johannesburg, SA) and incubated overnight at 37˚C. Following overnight incubation, the plates were observed for purity. Culture on 5% sheep blood agar was then harvested using a sterile loop and re-suspended in a 10% skimmed-milk (Diagnostic Media Products, Johannesburg, SA) suspension and stored at -70°C.

2.1.3 Outbreak isolates

During the years 2013-2015, seven *Salmonella* Enteritidis outbreaks were reported to the CED. These outbreaks occurred within six provinces (Gauteng, KwaZulu-Natal, Mpumalanga, Limpopo, Free State and Eastern Cape) in South Africa. A total of 39 isolates (one goat meat isolate and 38 human isolates) were received from these outbreaks. The isolates were cultured on MAC agar (Diagnostic Media Products, Johannesburg, SA) and 5% sheep blood agar (Diagnostic Media Products, Johannesburg, SA) and incubated at 37˚C overnight. Following overnight incubation, the plates were observed for purity. Culture on 5% sheep blood agar was then

harvested using a sterile loop and re-suspended in a 10% skimmed-milk (Diagnostic Media Products, Johannesburg, SA) suspension and stored at -70°C.

2.2 Culturing of the bacterial isolates

Isolates were obtained from the CED -70˚C storage freezer. A sterile metal loop (heated then cooled) was used to scrap a loop-full of the top part of the frozen bacterial culture/skimmed-milk mixture. The contents were plated out on 5% sheep blood agar and incubated at 37˚C overnight.

2.3 Phenotypic and molecular characterization

2.3.1 Serotyping

Human isolates obtained from 2013-2014 were identified using serotyping. *Salmonella* isolates received at the CED were plated out on 5% sheep blood agar and MAC agar. The plates were incubated overnight at 37˚C and the culture was observed for purity the following day.

The Kauffman-White-Le Minor scheme (Grimont and Weill, 2007) was used in the serotyping of *Salmonella* to determine the antigenic properties, which are the O (somatic) antigens, H (flagellar) antigens and Vi (capsular) antigens. For determination of the O phase, a single colony was inoculated onto a tryptose slope (Diagnostic Media Products, Johannesburg, SA). The tryptose slope was incubated overnight at 37˚C. Following incubation, 1 ml of normal saline was added to the tryptose slope and the mixture was vortexed gently to achieve homogeneity. A loop-full of bacterial suspension was placed on to a clean microscope slide and a drop of OMA polyvalent (BioRad, Paris, France) was added onto the microscope slide bacterial suspension. The microscope slide was tilted back and forth for 30-60 seconds (sec), while viewing under good light against a dark background, with the aid of an eyepiece. Positive agglutination would be observed for *Salmonella*

positivity. For identification of *Salmonella* Enteritidis, monovalents 1, 9 and 12 (Statens Serum Institute, København, Denmark; Davies Diagnostics, Randburg, SA; Bioweb, Randburg, SA) would show positive agglutination.

For determination of the H phase, swarm agar was autoclaved for 10 minutes (min) and cooled to 52˚C using a water bath. A 20 ml aliquot of cooled swarm agar was added into a sterile plate and allowed to solidify before use. A loop-full of culture was inoculated by touching the centre surface of the swarm agar. The swarm agar plate was incubated in an upright position overnight at 37˚C. Following incubation, 2-3 drops of H polyvalents antisera (Statens Serum Institute, København, Denmark; Davies Diagnostics, Randburg, SA; BioRad, Paris, France; Bioweb, SA) was added onto a clean glass slide. Using a sterile loop, a small amount of growth was selected from the edge of the swarm agar and a suspension was prepared using the relevant polyvalent H antisera. The mix was tilted back and forth for 30-60 sec while viewing under good light against a dark background, with the aid of an eyepiece. If agglutination was observed, serotyping was repeated using the appropriate monovalents. For *Salmonella* Enteritidis, H phase 1 monovalents (g, m) would show positive agglutination and H phase 2 would have no agglutination. Each test isolate was performed against a negative control; containing sterile saline. This was to ensure that no auto-agglutination was occurring during serotyping.

2.3.2 Molecular identification (real-time PCR)

2.3.2.1 Crude DNA template extraction in preparation for PCR

Real-time PCR was used for the identification of *Salmonella* Enteritidis isolates (human and non-human) from the year 2015. Four-hundred microliters of autoclaved TE buffer (Appendix B) was aliquoted into 1.5 ml tubes. Using a sterile loop, bacterial culture (loop-full) was harvested from a 5% sheep blood agar plate and was re-suspended in the 1.5 ml tubes containing TE buffer. The suspension was vortexed for 10 sec and placed on a heating block at 95˚C for 25 min. After incubation, the tube was vortexed for 10 sec and the solution was centrifuged at 13200 rpm for 3 min to pellet the cellular debris. A 20 µl aliquot of the supernatant was transferred into a 1.5 ml tube with 80 µl of autoclaved TE buffer. The solution was vortexed for 5 sec

and centrifuged (13200 rpm) for 10 sec to collect the contents at the bottom of the tube. The DNA extracts were stored at -20˚C.

2.3.2.2 Real-time PCR for *Salmonella* identification

A multiplex real-time PCR was performed using previously described primers (Malorny *et al*., 2004; O'Regan *et al*., 2008; Park *et al*., 2008), that were used to amplify genes specific for *Salmonella* species (*ttRSBCA* gene), *Salmonella* Enteritidis (*sdf* gene) and *Salmonella* Typhimurium (*STM4497* gene) respectively (Appendix C). Each PCR reaction contained a known positive control as well as a non-template control. The primer/probe re-suspension, primer probe mix preparation, as well as PCR reaction setup are described in Appendix C. The reagents were then added into a 96-well reaction plate (BioRad, Hercules, USA) and the PCR reaction was ran on a BioRad CFX 96 real-time system (BioRad, Jurong, Singapore). The cycling conditions included 1 cycle at 50°C for 2 min, followed by 95°C for 2 min (1 cycle), 95°C for 15 sec and 60°C for 1 min (25 cycles). Results were viewed after completion of the real-time PCR run. A *Salmonella* Enteritidis positive result contained two sigmoidal curves, with *Salmonella* species in blue and *Salmonella* Enteritidis in grey as depicted in Figure 2.1. For a confident call on a positive result, the sigmoidal curves should also be accompanied by a Ct value within the range of 15-25.

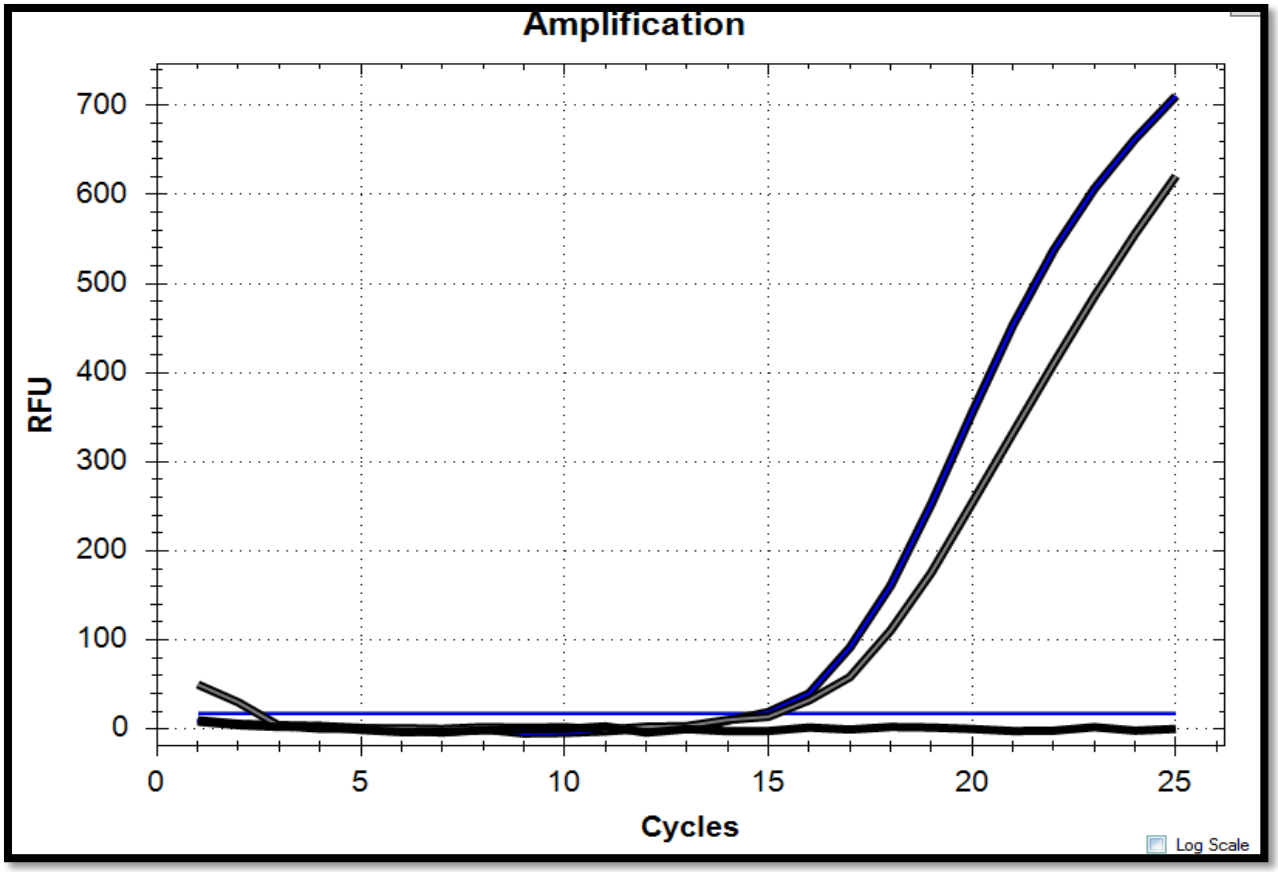


Figure 2.1 Real-time PCR amplification curve of a *Salmonella* species (blue) and *Salmonella* Enteritidis (grey) positive result.

2.4 MLVA

2.4.1 PCR

The MLVA technique used in this study was as described by Hopkins *et al*., (2011). A multiplex PCR was performed to amplify the five VNTR loci: SENTR7_SENTR5_SENTR6_SENTR4_SE-3 (Appendix D), using previously extracted DNA (refer to section 2.3.2.1). The primer mixes, as well as the PCR reaction setup are described in Appendix D. The PCR reaction was then performed in a DNA thermal cycler (Applied Biosystems, Singapore), with the following run conditions; 1 cycle of denaturation at 95°C for 15 min, followed by 35 cycles involving an initial step at 94°C for 1 min, annealing at 55°C for 90 sec, polymerization at 72°C for 90 sec and final cycle of polymerization at 72°C for 10 min.

2.4.2 Processing of PCR products

The PCR products were vortexed for 3 sec at medium speed and 2 µl of the PCR product was diluted in 198 µl of autoclaved deionised water. A master mix of GeneScan 600 LIZ Standard v2.0 (Applied Biosystems, Foster City, USA) and Hi-Di formamide (Life Technologies, London, United Kingdom) was prepared by adding 0.2 µl and 12 µl of each solution respectively into a 1.5 µl amber tube. A 1 µl aliquot of the PCR product dilution was added onto the GeneScan 600 LIZ Standard v2.0 and Hi-Di formamide mix. The suspension was vortexed (5 sec), centrifuged (5 sec) at 13 200 rpm and incubated at 95°C for 3 min. This was followed by immediate incubation on ice for 1 min. Each suspension was subsequently transferred onto a 96-well barcoded reaction plate (Life Technologies, Beijing, China), sealed and centrifuged to remove any air bubbles.

2.4.3 Analysis of PCR products using capillary electrophoresis

The Applied Biosystems 3500 Genetic Analyzer (Applied Biosystems, Tokyo, Japan) was used for capillary electrophoresis analysis of the PCR products. The plate content (sample) information was assigned to each well and the fragment analysis assay was selected as the run method.

2.4.4 MLVA data analysis using GeneMapper software

After capillary electrophoresis analysis, the GeneMapper version 4.1 software (Applied Biosystems, Foster City, USA) was used for visual analysis of the sample fragment sizes. The software identified each VNTR locus by its distinctive colour (SENTR7-blue, SENTR5-blue, SENTR6-black, SENTR4-green and SE-3-green). The PCR products (DNA fragments) were automatically sized via comparison to the internal size standard. The DNA fragments were automatically allocated to length bins and the alleles were assigned based on the bin fragments sizes. The sample VNTR allele size pattern (SENTR7_SENTR5_SENTR6_SENTR4_SE-3) could then be determined. Successful MLVA for *Salmonella* Enteritidis strains would produce five peaks and validity of test results was assured through the analysis of the

Salmonella Enteritidis reference strain (positive control, TCD736744), with a particular expected VNTR allele size pattern (123_292_184_112_306).

2.5 BioNumerics analysis of MLVA profiles

The VNTR allele size patterns obtained from MLVA analysis were captured into the BioNumerics version 6.5 software (Applied Maths, Sint-Martens-Latem, Belgium) as character values. The VNTR allele size numbers were used to assign MLVA profile numbers. A single VNTR locus difference between isolates resulted in a new MLVA profile being defined (e.g. 123, 268, 184, 112, 318_ MLVA profile 1 and 123, 262, 184, 112, 318_ MLVA profile 2). To compare differences in the VNTR allele size patterns of the isolates, a dendrogram was constructed by the UPGMA method, using the categorical coefficient with a 0 tolerance. This dendrogram was used to construct a minimum spanning tree (MST), using the MST categorical coefficient.

2.6 VNTR diversity measurement

Diversity and degree of polymorphism in each VNTR locus was measured using the Simpson's index of Diversity (*D*) and the 95% confidence intervals (CI's) was calculated using a free online tool available at the Public Health England (PHE) website (http://www.hpa-bioinformatics.org.uk/cgi-bin/DICI/DICI.pl).

2.7 Antimicrobial susceptibility

Antimicrobial susceptibility testing against ampicillin, azithromycin, ciprofloxacin, ceftriaxone, trimethoprim, tetracycline, sulfamethoxazole and cotrimoxazole was conducted using the Etest method. *Salmonella* Enteritidis isolates were sub-cultured on 5% sheep blood agar plates overnight at 37˚C. Mueller Hinton agar (MHA) (Diagnostic Media Products, Johannesburg, SA) and the Etest strips (BioMérieux, Paris, France) were placed at room temperature to thaw for 1 hour and 30 min

respectively. Using a sterile swab, 3-5 well-isolated colonies, from an overnight culture, were selected and transferred to a sterile saline tube to prepare a 0.5 MacFarland bacterial suspension. The turbidity of the bacterial suspension was thus determined using a Microscan Turbidity Meter (Dade Behring, Deerfield, USA). Subsequently, a sterile swab was immersed into the bacterial suspension and drained on the sides of the saline tube. Using a rotating plate holder (BioMérieux, Askim, Sweden), the MHA plates were swabbed in three directional patterns to obtain uniform growth. The plates were left to incubate at room temperature for 15 min. Two Etest strips facing opposite directions were placed in each plate, and the plates were then incubated overnight at 37˚C.

The minimum inhibitory concentration (MIC) of each antimicrobial was read based on whether the antimicrobial was bactericidal or bacteriostatic. Bactericidal antimicrobial MIC was read at the point of complete inhibition of growth and bacteriostatic antimicrobial MIC was read at 80% growth inhibition. Interpretation of the result as being susceptible, intermediate or resistant was based on the Clinical and Laboratory Standards Institute (CLSI) guidelines (CLSI, 2013; CLSI, 2014 and CLSI, 2015) of each antimicrobial. Control strains *E. coli* ATCC 25922 and *Klebsiella pneumoniae* ATCC700608 were cultured every two weeks and upon initial use of a new Etest batch to validate the antimicrobial susceptibility test and the Etests strips. Both control strains had to be susceptible to all tested antimicrobials, in order to deem the Etest and antimicrobial susceptibility test as valid.

2.8 Study data analysis

2.8.1 Human isolates

Demographic information (age, gender, province, town etc.) of the human *Salmonella* Enteritidis isolates was obtained from the CED Access database. The demographic information was exported onto a Microsoft Excel spread-sheet. The MLVA profiles and antimicrobial susceptibility results obtained were also exported to the Microsoft Excel spread-sheet. Construction of tables for the results section (Chapter 3) was done using Microsoft Excel. The tabled results were stratified by province (Gauteng

and Western Cape). Statistical analysis was performed using Epi Info 7 (CDC, Atlanta, USA) and testing for statistical significance was performed using Chi-squared and Fisher's Exact tests, from which a p-value of <0.05 was taken to be statistically significant.

The interpretation of the MST data was based on previously described interpretation (Noller *et al*., 2006). Analysis of the diversity and degree of polymorphism (statistical difference) in each locus was based on previously described interpretation of Simpson's diversity index data (Simpson, 1949).

2.8.2 Non-human isolates

Demographic information of the non-human *Salmonella* Enteritidis isolates was obtained from the sender. The information was transferred onto a Microsoft Excel spread-sheet. The MLVA profiles and antimicrobial susceptibility results obtained were exported to the Microsoft Excel spread-sheet and tables were constructed from the data.

CHAPTER 3: RESULTS

3.1 Human study population demographic information

3.1.1 Isolate distribution (2013-2015)

During the years of the study (2013-2015), random selection of isolates by Microsoft Excel 2010 selected more isolates from Gauteng compared to Western Cape. Of the total number of isolates selected from the Gauteng Province during the years 2013-2015, more isolates were selected in the year 2014, thus accounting for 38% (319/832) of the total Gauteng isolates. In Western Cape, more isolates were selected in the year 2013, accounting for 41% (159/389) of the total isolates from the province. Overall more isolates were selected in the year 2013 (*n* = 415) and the lowest number of isolates were selected in 2015 (*n* = 400). The overall p-value of the total number of isolates selected during the study years was p = 0.42 (Table 3.1).

3.1.2 Age distribution of patients

The median age of patients in Gauteng was 29 years (range, <1-95), with an interquartile range (IQR) of 4-47. The age group <1-4 years had the highest number of isolates, which accounted for 25% (*n* = 210) of the total isolates in this province. Age group 35-44 years had the second highest number of isolates, thus accounting for 13% (*n* = 110) of the total isolates in Gauteng. Furthermore, the third highest age groups in Gauteng were the 25-34 years (*n* = 102; 12%) and 5-14 years (*n* = 96; 12%) respectively. Age groups with the lowest number of isolates were the 55-64 years (6%; *n* = 48) and the 15-24 years (6%; *n* = 50) (Table 3.1).

The median age of patients in Western Cape was 31 years (range, <1-89), with an IQR of 5-50. Age group <1-4 years also accounted for a large number of isolates in Western Cape. This age group accounted for 26% (*n* = 102) of the total number of isolates in this province. Age group 35-44 years had the second highest number of isolates, thus accounting for 14% (*n* = 55) of the total isolates in the province. In

Western Cape, the third highest age groups were the 45-54 years (*n* = 46; 12%), ≥65 years (*n* = 46; 12%) and the 25-34 years (*n* = 45; 12%) respectively. Age groups with the lowest number of isolates were the 55-64 years (7%; *n* = 80) and the 15-24 years (6%; *n* = 69) (Table 3.1).

Overall the median age was 30 years, with an IQR of 4-48 years, and an age range of <1-95 years. The overall age p-value was not statistically significant at p = 0.1. Furthermore, age group <1-4 years had the highest number of isolates in the study (*n* = 312; 26%), followed by age group 35-44 years (*n* = 165; 14%) and age group 25-34 years (*n* = 147; 12%) respectively. The overall age distribution p-value was p = 0.91 (Table 3.1).

3.1.3 Sex (gender) distribution of patients

In Gauteng, females had a higher number of isolates (*n* = 414: 50%) than males (*n* = 404; 49%) and 14 (1%) isolates were of unknown sex. In Western Cape, males had higher numbers of isolates (*n* = 604; 50%) than females 49% (*n* = 601) and a total of 16 (1%) isolates were of unknown sex. The overall sex distribution p-value was not statistically significant at p = 0.83 (Table 3.1).

3.1.4 Site of isolation

The *Salmonella* Enteritidis isolates included in the study were isolated from various parts of the body (Appendix A). The isolates either caused an invasive (isolated from sterile site) or a non-invasive (isolated from non-sterile site) infection. A total of 274 (33%) invasive isolates and 558 (67%) non-invasive isolates were present amongst Gauteng isolates. In Western Cape, a total of 142 (37%) invasive isolates and 247 (63%) non-invasive isolates were selected. Overall invasive isolates accounted for 34% (*n* = 416) of the total isolates. The overall p-value of invasive isolates amongst the two provinces (Gauteng and Western Cape) was p = 0.22 (Table 3.1).

Table 3.1. Demographic information of the study population.

Characteristic	*GA (n=832) (%)	*WC (n=389) (%)	Overall (n=1221) (%)	ΔOverall p-value
Year				
2013	256 (31)	159 (41)	415 (34)	0.42
2014	319 (38)	87 (22)	406 (33)	
2015	257 (31)	143 (37)	400 (33)	
Age (years)				
Median (^IQR)	29 (4-47)	31 (5-50)	30 (4-48)	0.1
Range	<1-95	<1-89	<1-95	
Age category				
0-4	210 (25)	102 (26)	312 (26)	0.91
5-14	96 (12)	39 (10)	135 (10)	
15-24	50 (6)	19 (5)	69 (6)	
25-34	102 (12)	45 (12)	147 (12)	
35-44	110 (13)	55 (14)	165 (14)	
45-54	94 (11)	46 (12)	140 (11)	
55-64	48 (6)	32 (8)	80 (7)	
≥65	83 (10)	46 (12)	130 (10)	
Unknown	38 (5)	5 (1)	43 (4)	
Sex				
Female	414 (50)	187 (48)	601 (49)	0.83
Male	404 (49)	200 (51)	604 (50)	
Unknown	14 (1)	2 (1)	16 (1)	
Isolate type				
Invasive	274 (33)	142 (37)	416 (34)	0.22
Non-invasive	558 (67)	247 (63)	805 (66)	

* Province: GA – Gauteng; WC – Western Cape

Δ Statistics: Statistical data calculated using the Chi-squared and Fisher's Exact tests and a p-value <0.05 is statistically significant

^IQR – Interquartile range

3.1.5 Invasive isolates and age distribution

Age group <1-4 years, had the highest number of invasive isolates 86/416 (21%), followed by age groups 35-44 years (81/416; 19%), 45-44 years (61/416; 15%), 25-34 years (58/416; 14%) and >65 years (45/416; 11%) (Table 3.2). Age groups 5-14 years, 15-24 years, 55-64 years and the unknown age group contained less than 10% invasive isolates. They accounted for 4% (16/416), 5% (20/416), 8% (35/416) and 3% (14/416) of the total invasive isolates in the study respectively (Table 3.2).

Table 3.2. Number of invasive isolates per age group.

Age groups	Total isolates in age group	Total invasive isolates in age group*	% of invasive isolates in age group (*/416x100)
<1-4	312	86	21
5-14	135	16	4
15-24	69	20	5
25-34	147	58	14
35-44	165	81	19
45-54	140	61	15
55-64	80	35	8
>64	13	45	11
Unknown	43	14	3
Total	1221	416	100

3.2 MLVA profiles for human *Salmonella* Enteritidis isolates

3.2.1 MLVA profile distribution

MLVA was performed on a total of 1221 human *Salmonella* Enteritidis isolates; 832/1221 from Gauteng and 389/1221 isolates from the Western Cape. A total of five VNTR loci (SENTR7, SENTR5, SENTR6, SENTR4 and SE-3) were included in the study. These loci had size variations amongst them, which were used to determine strain variability within the isolates.

A total of 20/1221 (1.6%) isolates had no (0) amplification on one or two loci, with lack of amplification occurring on SENTR6 (13/1221; 1%), SE-3 (4/1221; 0.3%) and SENTR4 (3/1221; 0.2%). Furthermore, a total of 5/1221 isolates had a locus with two alleles. This was observed on SENTR7 (two isolates), SENTR5 (one isolate) and SENTR4 (two isolates). The electropherogram of each of the isolates were analysed and two peaks were observed in the one respective locus. From the two peaks, the peak with the highest frequency was selected as the result.

MLVA profiles were constructed based on single VNTR locus difference between isolates. A total of 84 MLVA profiles were determined from 1221 human isolates (Appendix D). Overall (2013-2015), MLVA profile 28 (661/1221; 54%) had the largest number of isolates, followed by MLVA profile 7 (135/1221; 11%), MLVA profile 22 (101/1221; 8%) and MLVA profile 21 (68/1221; 6%) respectively (Table 3.3; Table 3.4; Figure 3.1).

MLVA profile 42 (33/1221) and MLVA profile 41 (24/1221) accounted for 3% and 2% of the total isolates respectively (Table 3.3). A total of five MLVA profiles contained 6-20 isolates per MLVA profile, these MLVA profiles accounted for 3% of the total isolates. Furthermore, 73 MLVA profiles consisted of ≤5 isolates per MLVA profile and these MLVA profiles accounted for 13% of the total isolates (Figure 3.1). Collectively, these 80 MLVA profiles were grouped together and named ‘’other MLVA profiles’’ for analysis purposes (Table 3.4).

MLVA profiles with significant numbers of isolates (i.e. predominant MLVA profiles) (MLVA profiles 28, 7, 21 and 22) were present in both the Gauteng and Western Cape Province. MLVA profile 28 was the most common MLVA profile in both the Gauteng and Western Cape Province, accounting for 62% (513/832) and 38% (148/389) of the total isolates in the provinces respectively. Of the total number of isolates in MLVA profile 28, 78% (513/661) of the isolates were from Gauteng Province. MLVA profile 7 accounted for 6% (47/832) of the total isolates in Gauteng. In Western Cape, MLVA profile 7 accounted for 22% (87/389) of the total isolates. Furthermore, of the total number of isolates in MLVA profile 7, 64% (87/135) of the isolates were from the Western Cape Province. MLVA profiles 21 and 22 accounted for 6% (48/832) and 9% (78/832) of the total isolates in Gauteng respectively.

Similarly in the Western Cape, MLVA profiles 21 and 22 accounted for 5% (20/389) and 6% (23/389) of the total isolates respectively. Furthermore, MLVA profiles 21 and 22 contained more isolates from Gauteng, which accounted for 71% (48/68) and 77% (78/101) of the total isolates in these MLVA profiles respectively (Table 3.4; Figure 3.2).

Of the 80 smaller MLVA profiles (other MLVA profiles), 50 were present in only one province; 28 in Gauteng and 22 in Western Cape. Furthermore, 17/80 MLVA profiles contained more isolates from Western Cape, while 8/80 contained more isolates from Gauteng and 5/80 contained equal numbers of isolates in both provinces. Collectively, these MLVA profiles accounted for 17% (145/832) of the total isolates in Gauteng and 29% (111/389) of the total isolates in Western Cape (Table 3.4; Figure 3.2).

MLVA profiles 28, 21, and 22 were also present in outbreak investigated isolates from six provinces in South Africa; namely Gauteng, KwaZulu-Natal, Mpumalanga, Limpopo, Free State and Eastern Cape (refer to section 3.6). Isolates that did not have a known province were represented in white (only present in the non-human isolates; refer to section 3.4) (Figure 3.2).

Table 3.3. Example of MLVA profile numbers; MLVA profiles 7, 21, 22 and 28 are predominant.

SENTR7	SENTR5	SENTR6	SENTR4	SE-3	no: of human isolates with MLVA profile (*n* = 1221)	MLVA profile number (*n* = 84)
132	268	177	112	318	1	**1**
132	268	219	112	318	1	**2**
132	280	184	112	318	1	**3**
132	280	177	119	318	3	**4**
132	280	184	112	306	1	**5**
132	280	177	112	306	3	**6**
132	280	177	119	306	135	**7**
132	280	184	119	306	10	**8**
132	244	170	112	306	1	**9**
123	262	177	126	306	1	**10**
123	250	212	140	306	15	**11**
123	226	212	126	306	4	**12**
123	262	191	112	318	1	**18**
123	274	0	112	318	2	**19**
123	262	0	112	318	1	**20**
123	274	184	112	318	68	**21**
123	262	184	112	318	101	**22**
123	280	184	112	318	17	**23**
123	244	184	112	318	2	**24**
123	268	0	112	318	8	**27**
123	268	184	112	318	661	**28**
123	268	177	112	306	5	**29**
132	286	177	119	306	24	**41**
132	268	177	119	306	33	**42**

Table 3.4. Predominant MLVA profiles and other MLVA profiles present in the Gauteng and Western Cape Province during the years 2013-2015.

	***GA (*n* = 832)**					***WC (*n* = 389)**				
	Predominant MLVA profiles				Other MLVA profiles (%)	**Predominant MLVA profiles**				Other MLVA profiles (%)
Year	MLVA 7 (%)	MLVA 21 (%)	MLVA 22 (%)	MLVA 28 (%)		MLVA 7 (%)	MLVA 21 (%)	MLVA 22 (%)	MLVA 28 (%)	
2013 (*n* = 415)	30 (7)	8 (2)	11 (3)	136 (33)	71 (17)	54 (13)	13 (3)	2 (0.5)	44 (10.5)	46 (11)
2014 (*n* = 406)	16 (4)	24 (6)	23 (6)	218 (54)	38 (9)	19 (5)	4 (1)	2 (0.5)	45 (11)	17 (4)
2015 (*n* = 400)	2 (0.5)	16 (4)	44 (11)	159 (40)	36 (9)	14 (3)	3 (0.5)	19 (5)	59 (15)	48 (12)
Total (*n* = 1221)	48 (4)	48 (4)	78 (6)	513 (42)	145 (12)	87 (7)	20 (2)	23 (2)	148 (12)	111 (9)

*Province: GA – Gauteng; WC – Western Cape

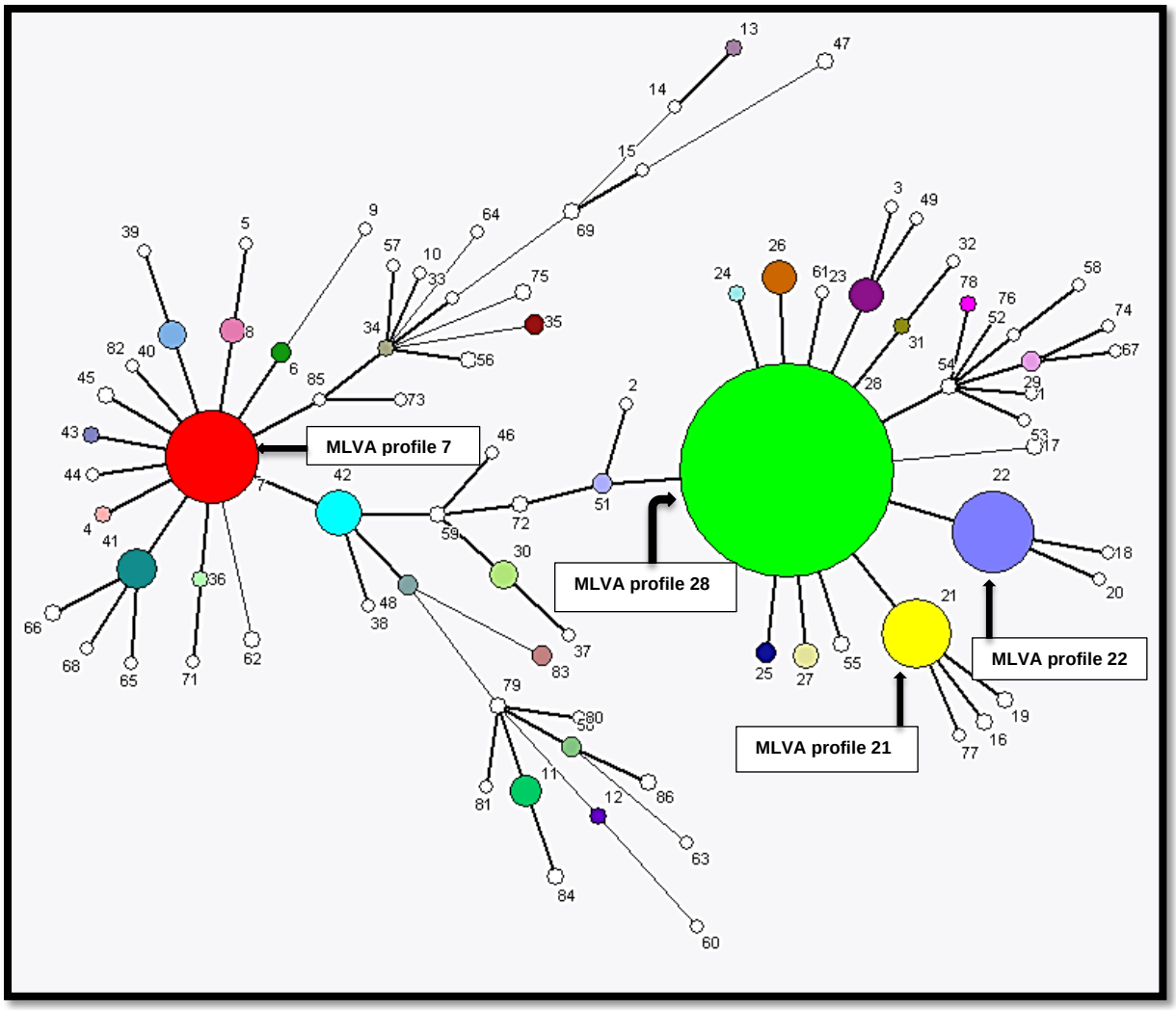


Figure 3.1. MLVA MST for *Salmonella* Enteritidis isolates from the years 2013-2015, drawn using the MLVA profile numbers.

The node (circle) represents the number of isolates in that MLVA profile. MLVA profiles are connected by branches and the thickness of the branch indicates how many VNTR loci differences are between the connected MLVA profiles (nodes). The thick solid lines connect nodes that differ by one VNTR allele and thin solid lines connect nodes that differ by two VNTR alleles. The distance between the MLVA profiles (nodes) represents the genetic divergence between two neighbouring MLVA profiles.

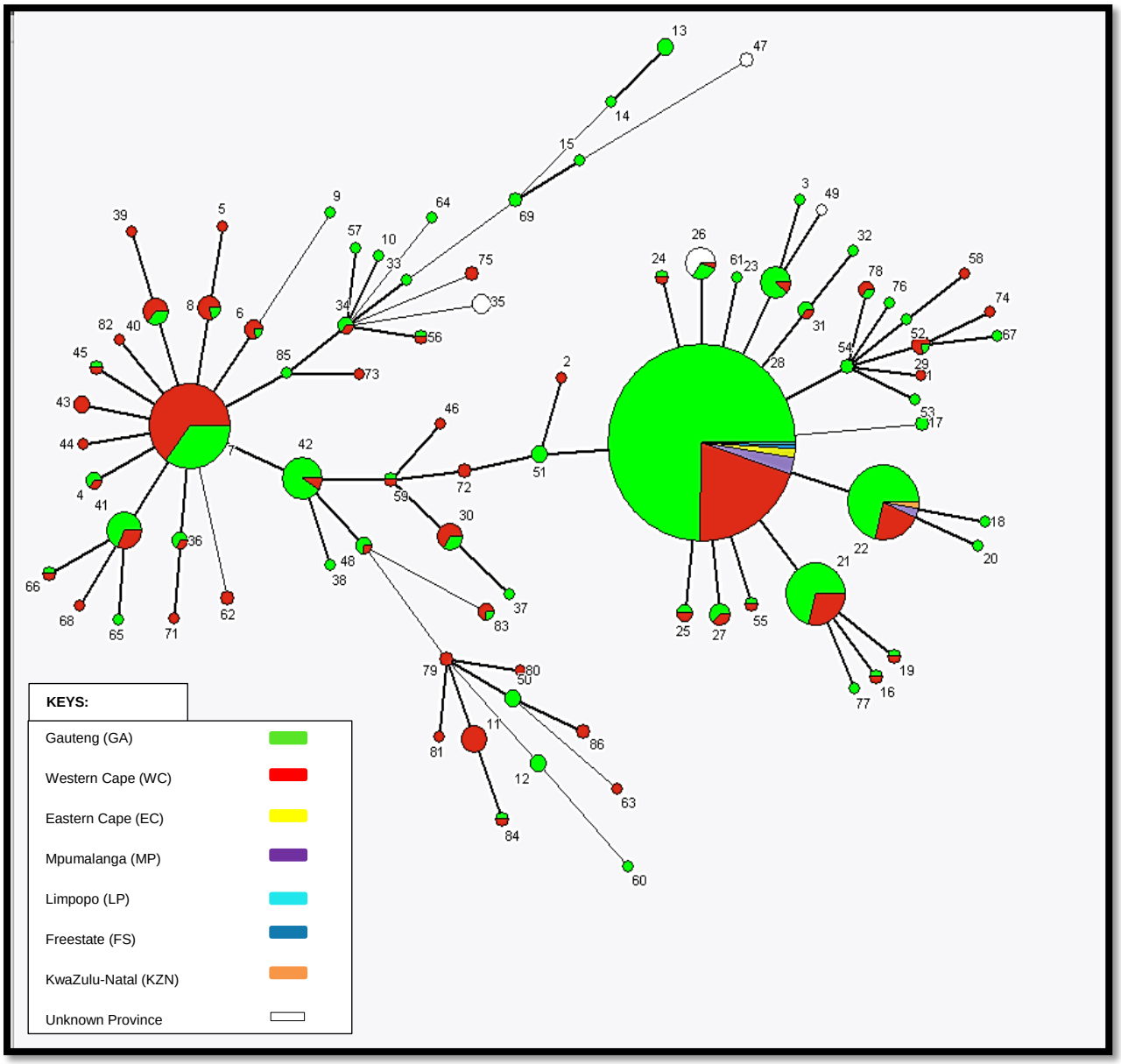


Figure 3.2. MLVA MST for *Salmonella* Enteritidis, indicating the provinces included on the CED BioNumerics database.

3.2.2 Simpson's diversity index for *Salmonella* Enteritidis VNTR loci

Simpson's diversity index for the five VNTR loci ranged from 0.525 to 0.876. The 95% confidence intervals (CI) ranged from 0.433 - 0.617 (lowest; SENTR7) to 0.844-0.908 (highest; SENTR5). The number of alleles found in each locus was also calculated from the Public Health England (PHE) website online tool. SENTR5 had the highest number of alleles (17), followed by SENTR6 (13) and SENTR4 (8)

respectively. SENTR7 and SE-3 had the least alleles with seven and four alleles each respectively (Table 3.5).

Table 3.5. Simpson's diversity index for the *Salmonella* Enteritidis five VNTR loci.

Locus	*Diversity index (*D*)	˚95% CI	ΔNumber of alleles (K)
SENTR7	0.525	0.433 - 0.617	7
SENTR5	0.876	0.844 - 0.908	17
SENTR6	0.799	0.745 - 0.853	13
SENTR4	0.743	0.686 - 0.800	8
SE3	0.539	0.463 - 0.615	4

*Diversity index (*D*): measures the variation at each locus based on the number of alleles found at that locus from the study population. Ranges from 0.0 (no diversity) to 1.0 (complete diversity).
˚Confidence interval (CI): Precision of Diversity Index at 95% upper and lower boundaries.
ΔK: Number of different alleles present at a locus in the study population (Simpson, 1949).

3.2.3 Invasive *Salmonella* Enteritidis isolates and MLVA profiles

Of the predominant MLVA profiles, MLVA profile 28 had the largest number of invasive isolates in both provinces (Gauteng and Western Cape). This MLVA profile accounted for 37% (153/274) and 13% (55/142) of the total invasive isolates in Gauteng and Western Cape respectively. MLVA profile 7 had the second highest number of invasive isolates in Western Cape (20%; 29/142), however it had the second lowest numbers of invasive isolates in Gauteng (7%; 19/274). MLVA profile 22 contained the second highest number of invasive isolates in Gauteng, thus accounting for 12% (33/274) of the total isolates in the province. Overall, MLVA profile 28 accounted for 50% (208/416) of the total number of invasive isolates in this study. MLVA profile 7 contained the second highest number of invasive isolates in the study (11.5%; 48/416) (Table 3.6).

The other MLVA profiles accounted for 24.5% (101/416) of the total invasive isolates. Their largest number of invasive isolates were found in Gauteng Province (53/274), were they accounted for 19% of the total invasive isolates in this province. However,

in Western Cape these MLVA profiles accounted for a large percentage of 34% (48/142) of the total invasive isolates in the province (Table 3.6).

Table 3.6. Invasive isolates present in predominant MLVA profiles and the other MLVA profiles from Gauteng and Western Cape.

	*GA invasive isolates (*n* = 274)					*WC invasive isolates (*n* = 142)				
	Predominant MLVA profiles				Other MLVA profiles (%)	Predominant MLVA profiles				Other MLVA profiles (%)
Year	MLVA 7 (%)	MLVA 21 (%)	MLVA 22 (%)	MLVA 28 (%)		MLVA 7 (%)	MLVA 21 (%)	MLVA 22 (%)	MLVA 28 (%)	
2013 (*n* = 128)	9 (7)	0	3 (2)	34 (26.5)	29 (23)	15 (12)	5 (4)	0	16 (13)	17 (12.5)
2014 (*n* = 149)	9 (6)	8 (5)	7 (5)	81 (54)	14 (10)	10 (7)	0	0	14 (9)	6 (4)
2015 (*n* = 139)	1 (1)	8 (6)	23 (16)	38 (27)	10 (7)	4 (3)	1 (1)	4 (3)	25 (18)	25 (18)
Total (*n* = 416)	19 (4.5)	16 (4)	33 (8)	153 (37)	53 (13)	29 (7)	6 (1)	4 (1)	55 (13)	48 (11.5)

* Province: GA – Gauteng; WC – Western Cape

3.3 Antimicrobial profiles of human *Salmonella* Enteritidis isolates

Antimicrobial resistance was tested on eight antimicrobials (ampicillin, trimethoprim, cotrimoxazole, sulfamethoxazole, ciprofloxacin, tetracycline, ceftriaxone and azithromycin) from six different classes of antimicrobials (penicillins, sulfonamides, fluoroquinolones, tetracyclines, cephalosporins and macrolides). All 1221 human isolates showed full susceptibility (100%) to azithromycin. Over 98% of isolates showed full susceptibility to antimicrobials ampicillin, cotrimoxazole, trimethoprim, tetracycline and ceftriaxone respectively (Table 3.7).

Low prevalence of intermediate resistance (≤2%) was observed in 2/8 antimicrobials (ampicillin and tetracycline). However, 9% (113/1221) of the isolates were intermediately resistant to ciprofloxacin and no intermediate resistance was observed for ceftriaxone. Of the 1221 isolates, 29% (348/1221) were resistant to sulfamethoxazole. Low prevalence of antimicrobial resistance (≤2%) was observed in 5/8 antimicrobials (ampicillin, cotrimoxazole, trimethoprim, tetracycline and ceftriaxone). No antimicrobial resistance was observed for ciprofloxacin (Table 3.7).

Table 3.7. Antimicrobial-susceptibility profiles of human *Salmonella* Enteritidis isolates (*n* = 1221).

Antimicrobials	***Susceptible (%)**	**[Δ]S bp (µg/ml)**	***Intermediate (%)**	**[Δ]I bp (µg/ml)**	***Resistant (%)**	**[Δ]R bp (µg/ml)**
Ampicillin	1201 (98.4)	≤8	2 (0.1)	16	18 (1.5)	≥32
Cotrimoxazole	1215 (99.5)	≤2	-	-	6 (0.5)	≥4
Trimethoprim	1215 (99.5)	≤8	-	-	6 (0.5)	≥16
Sulfamethoxazole	873 (71)	≤256	-	-	348 (29)	≥512
Ciprofloxacin	1108 (91)	≤0.06	113 (9)	0.12-0.5	0	≥1
Tetracycline	1195 (98)	≤4	15 (1)	8	11 (1)	≥16
Ceftriaxone	1216 (99.5)	≤1	0	2	5 (0.5)	≥4
Azithromycin	1221(100)	≤16	-	-	0	≥32

*****Antimicrobial patterns: Antimicrobial patterns observed in the human study population isolates and percentage (%) of isolates that are susceptible, intermediately resistant and fully resistant to the tested antimicrobials.
[Δ]Antimicrobial beak points: **S bp** - Susceptible break points; **I bp** - Intermediate Resistance break points; **R bp** - Resistant break points

3.3.1 MLVA profiles and antimicrobial resistance

MLVA profile 28 had the largest number of sulfamethoxazole-resistant isolates. This MLVA profile accounted for 58% (202/348) of the total sulfamethoxazole-resistant isolates. In the two provinces (Gauteng and Western Cape), MLVA profile 28 accounted for 65% (162/250) and 41% (40/98) of the total sulfamethoxazole-resistant isolates in each province respectively. MLVA profile 7 contained the second highest number of isolates with sulfamethoxazole resistance, amongst the predominant MLVA profiles. Overall, this MLVA profile accounted for 9% (30/ 348) of the total sulfamethoxazole-resistant isolates, with its highest number of sulfamethoxazole-resistant isolates present in Western Cape (*n* = 21). Predominant MLVA profiles 21 and 22 contained fewer numbers of isolates with sulfamethoxazole resistance. The other MLVA profiles had the second highest numbers of sulfamethoxazole-resistant isolates in the study, thus accounting for 20% (71/348) of the total sulfamethoxazole-resistant isolates. These MLVA profiles accounted for 16% (40/250) and 32% (31/98) of total sulfamethoxazole-resistant isolates in Gauteng and Western Cape respectively (Table 3.8).

MLVA profile 28 had the largest number of ciprofloxacin intermediate resistant isolates in both provinces (Gauteng and Western Cape). This MLVA profile accounted for 66% (61/92) and 38% (8/21) of the total ciprofloxacin intermediate resistant isolates in Gauteng and Western Cape respectively. Overall, MLVA profile 28 accounted for 61% (69/113) of the total ciprofloxacin intermediate resistant isolates in the study. The other predominant MLVA profiles (MLVA profile 7, 21 and 22) contained fewer ciprofloxacin intermediate resistant isolates compared to MLVA profile 28 and the other MLVA profiles. The other MLVA profiles contained the second largest ciprofloxacin intermediate resistant isolates. In Gauteng, these MLVA profiles accounted for 20% (18/92) of the total ciprofloxacin intermediate resistant isolates in the province. Similarly, in Western Cape the other MLVA profiles accounted for 28% (6/21) of the total ciprofloxacin intermediate resistant isolates. Overall, the other MLVA profiles accounted for 21% (24/113) of the total ciprofloxacin intermediate resistant isolates in the study (Table 3.8).

Table 3.8. Predominant MLVA profiles and other MLVA profiles with sulfamethoxazole resistance and ciprofloxacin intermediate resistance.

[Δ]Sulfamethoxazole resistance			
MLVA profiles	*GA (%)	*WC (%)	Total isolates (%)
MLVA 7	9 (4)	21 (21)	30 (9)
MLVA 21	16 (6)	3 (3)	19 (5.5)
MLVA 22	23 (9)	3 (3)	26 (7.5)
MLVA 28	162 (65)	40 (41)	202 (58)
Other MLVA profiles	40 (16)	31 (32)	71 (20)
[°]Ciprofloxacin intermediate resistance			
MLVA profiles	*GA (%)	*WC (%)	Total isolates (%)
MLVA 7	3 (3)	5 (24)	8 (7)
MLVA 21	7 (8)	1 (5)	8 (7)
MLVA 22	3 (3)	1 (5)	4 (3)
MLVA 28	61 (66)	8 (38)	69 (61)
Other MLVA profiles	18 (20)	6 (28)	24 (21)

*Province: *GA – Gauteng; *WC – Western Cape

[Δ]Sulfamethoxazole resistance: *GP (*n* = 250); * WC (*n* = 98); * Total isolates (*n* = 348)

[°]Ciprofloxacin intermediate resistance: *GA (*n* = 92); * WC (*n* = 21); * Total isolates (*n* = 113)

3.4 Non-human *Salmonella* Enteritidis isolates

3.4.1 Origin of non-human isolates

During the years 2014-2015, a total of 43 non-human isolates were obtained from various sources. Of the 43 non-human isolates, 51% (22/43) were of known origin [avian (*n* = 3), wild animal (*n* = 2), equine (*n* = 5), poultry (*n* = 10) and environmental (*n* = 2)], with various sources. Isolates of unknown origin and source accounted for 49% (21/43) of the total non-human isolates. Although the origin and source of these isolates could not be established, they are from establishments (companies) that only work on non-human isolates (Table 3.9).

Table 3.9. Non-human *Salmonella* Enteritidis isolates included in the study from the years 2014-2015.

Origin	**Source**	**Total isolates (*n* = 43)**
Avian	Unknown	3
Wild animal	Cheetah foetus	1
	Cheetah pyothorax	1
Equine	Horse faeces	3
	Unknown	2
Poultry	Chicken abdominal swab	1
	Chicken egg swab (internal)	1
	Chicken peritonitis	1
	Chicken faeces	1
	Unknown	6
Environmental	Chicken kraal dust	1
	Chicken kraal boot covers	1
Unknown	Unknown	21

3.4.2 MLVA profiles for non-human isolates

MLVA was performed on all 43 non-human isolates and ten MLVA profiles (MLVA profiles 21, 22, 26, 28, 35, 40, 41, 47, 48 and 49) were generated. MLVA profile 28 accounted for 35% (15/43) of the total non-human isolates. MLVA profile 26 was the second most common MLVA profile accounting for 28% (12/43) of the total isolates. Other MLVA profiles (MLVA 21, 22, 35, 40, 41, 47, 48 and 49) accounted for a

smaller percentage of isolates. These MLVA profiles accounted for 5%(2/43); 5% (2/43); 12% (5/43); 5%(2/43); 2%(1/43); 5%(2/43); 2%(1/43); 2%(1/43) of the total isolates respectively (Table 3.10).

Table 3.10. MLVA profiles obtained from the 43 non-human *Salmonella* Enteritidis isolates.

MLVA profiles	**Total isolates in MLVA profile (%)**
MLVA 21	2 (5)
MLVA 22	2 (5)
MLVA 26	12 (28)
MLVA 28	15 (35)
MLVA 35	5 (12)
MLVA 40	2 (5)
MLVA 41	1 (2)
MLVA 47	2 (5)
MLVA 48	1 (2)
MLVA 49	1 (2)

3.4.3 Antimicrobial profiles of non-human isolates

A total of eight antimicrobial agents (ampicillin, trimethoprim, cotrimoxazole, sulfamethoxazole, ciprofloxacin, tetracycline, ceftriaxone and azithromycin) were tested on the 43 non-human *Salmonella* Enteritidis isolates. All isolates were fully susceptible (100%) to 5/8 antimicrobials (ampicillin, trimethoprim, cotrimoxazole, tetracycline, and azithromycin). A 7% (3/43) and 5% (2/43) intermediate resistance was observed for ciprofloxacin and ceftriaxone respectively. Antimicrobial resistance amongst non-human isolates was observed for sulfamethoxazole; a total of 10/43 (23%) isolates were resistant to this antimicrobial (Table 3.11).

Table 3.11. Antimicrobial-susceptibility profiles of non-human *Salmonella* Enteritidis isolates.

Antimicrobials	***Susceptible (%)**	**[Δ]S bp (µg/ml)**	***Intermediate (%)**	**[Δ]I bp (µg/ml)**	***Resistant (%)**	**[Δ]R bp (µg/ml)**
Ampicillin	43 (100)	≤8	0	16	0	≥32
Cotrimoxazole	43 (100)	≤2	-	-	0	≥4
Trimethoprim	43 (100)	≤8	-	-	0	≥16
Sulfamethoxazole	33 (77)	≤256	-	-	10 (23)	≥512
Ciprofloxacin	40 (93)	≤0.06	3 (7)	0.12-0.5	0	≥1
Tetracycline	43 (100)	≤4	0	8	0	≥16
Ceftriaxone	41 (95)	≤1	2 (5)	2	0	≥4
Azithromycin	43 (100)	≤16	-	-	0	≥32

*****Antimicrobial patterns: Antimicrobial patterns observed in the non-human study population isolates and percentage (%) of isolates that are susceptible, intermediately resistant and fully resistant to the tested antimicrobials.
[Δ]Antimicrobial beak points: **S bp** - Susceptible break points; **I bp** - Intermediate Resistance break points; **R bp** - Resistant break points

3.5 Human and non-human *Salmonella* Enteritidis association

Eight of the ten MLVA profiles present in non-human isolates were also present in the human isolates. MLVA profiles 35 and 49 were only found in non-human isolates. MLVA profile 28 had the highest number of isolates in both human (*n* = 661) and non-human (*n* = 15) isolates. MLVA profile 26 contained more non-human isolates (*n* = 12) (Table 3.12; Appendix D).

Table 3.12. Non-human *Salmonella* Enteritidis isolates found in the same MLVA profiles as human *Salmonella* Enteritidis isolates.

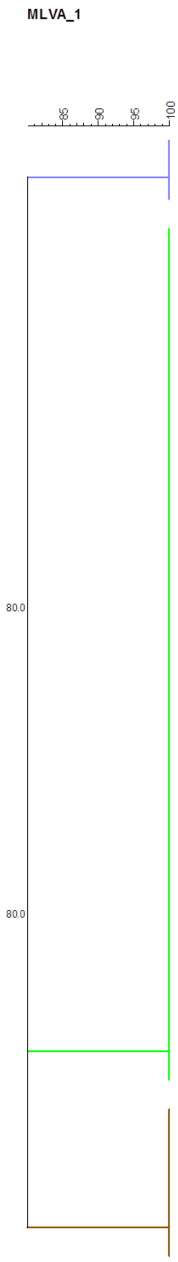
MLVA profiles	Total non-human isolates in MLVA profile	Total human isolates in MLVA profile
MLVA 21	2	68
MLVA 22	2	101
MLVA 26	12	6
MLVA 28	15	661
MLVA 40	2	9
MLVA 41	1	24
MLVA 47	2	1
MLVA 48	1	3

3.6 *Salmonella* Enteritidis outbreaks investigated during the study

During the study (2013-2015), seven *Salmonella* Enteritidis outbreaks were investigated from six provinces (Gauteng, KwaZulu-Natal, Mpumalanga, Limpopo, Free State and Eastern Cape) in South Africa. All provinces had one outbreak episode reported within the years except Mpumalanga, which had two outbreaks; one in November 2013 and one in July 2014. All the outbreaks were associated with food poisoning- details on each outbreak are summarized in Appendix E. A total of 39 isolates associated with the seven reported outbreaks were received at the CED; three (KwaZulu-Natal); 17 (Mpumalanga); three (Limpopo); three (Free State); 10

(Eastern Cape) and three (Gauteng). Serotyping was done on all the isolates and they were all confirmed to be *Salmonella* Enteritidis. MLVA was performed and dendrogram analysis of the results on BioNumerics showed that within each individual outbreak, isolates showed the identical MLVA profile (Figure 3.3).

Among all outbreaks, a total of three MLVA profiles were shown (MLVA profiles 21, 22 and 28). MLVA profile 21 was present in 3/39 isolates (7.7%) from Gauteng Province. MLVA profile 22 was present in 6/39 (15.4%) isolates; three isolates from the KwaZulu-Natal outbreak and three isolates from the 2013 Mpumalanga outbreak. MLVA profile 28 was the most predominant MLVA profile, accounting for 77% (30/39) of the overall outbreak isolates. This profile contained three isolates from Limpopo, 14 isolates from Mpumalanga (2014), three isolates from Free State and 10 isolates from Eastern Cape Province. A MST of the outbreak isolates is shown in Figure 3.2, with different colour keys for each province.



MLVA_1

SENTR7	SENTR5	SENTR6	SENTR4	SE3	Province	Date of Isolation	Outbreak	MLVA profile number
123	274	184	112	318	Gauteng	20/10/2015	Yes, foodborne	21
123	274	184	112	318	Gauteng	20/10/2015	Yes, foodborne	21
123	274	184	112	318	Gauteng	25/10/2015	Yes, foodborne	21
123	268	184	112	318	Limpopo	22/01/2014	Yes, foodborne	28
123	268	184	112	318	Limpopo	22/01/2014	Yes, foodborne	28
123	268	184	112	318	Limpopo	22/01/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Free State	24/11/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	25/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	25/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	27/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	27/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	25/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	30/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	26/07/2014	Yes, foodborne	28
123	268	184	112	318	Mpumalanga	08/08/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Eastern Cape	23/12/2014	Yes, foodborne	28
123	268	184	112	318	Free State	24/11/2014	Yes, foodborne	28
123	268	184	112	318	Free State	24/11/2014	Yes, foodborne	28
123	262	184	112	318	Kwa-Zulu Natal	29/05/2013	Yes, foodborne	22
123	262	184	112	318	Kwa-Zulu Natal	30/05/2013	Yes, foodborne	22
123	262	184	112	318	Kwa-Zulu Natal	29/05/2013	Yes, foodborne	22
123	262	184	112	318	Mpumalanga	11/11/2013	Yes, foodborne	22
123	262	184	112	318	Mpumalanga	11/11/2013	Yes, foodborne	22
123	262	184	112	318	Mpumalanga	11/11/2013	Yes, foodborne	22

Figure 3.3. Dendrogram of the *Salmonella* Enteritidis outbreak isolates from the years 2013-2015. MLVA profile 21 is highlighted in purple, MLVA profile 28 in green and MLVA profile 22 in bronze.

CHAPTER 4: DISCUSSION

4.1 Human *Salmonella* Enteritidis study population

4.1.1 Isolate distribution

The random selection of isolates from Microsoft Excel spread-sheet selected more isolates from Gauteng (*n* = 832) than Western Cape (*n* = 389). This was because, during the years 2013-2015, the CED received more isolates from Gauteng compared to Western Cape, and the reason for this is unknown.

However, despite variations in the number of isolates selected from the two provinces, the overall number of isolates selected throughout the three years (2013-2015) was closely similar. This was further supported by the p-value obtained (p = 0.42), which showed no statistical difference amongst the years.

4.1.2 Age distribution

Analysis of age distribution amongst the two provinces revealed numerous similarities from the age median (Gauteng, 29 years; Western Cape, 31 years), IQR (Gauteng, 4-47; Western Cape, 5-50) and age ranges (Gauteng, <1-95; Western Cape, <1-89). Overall, the study population median age was 30 years, with an IQR of 4-48 and an age range of <1-95. This suggested that the study population mainly consisted of adults (median), and that 50% of our study population falls between the ages of 4-48 years (IQR). An age range of <1-95 years, showed that *Salmonella* Enteritidis infections had affected all age groups, from the very young to the very old. This finding is consistent with global reports of *Salmonella* Enteritidis infections (WHO and FAO, 2002). The overall age distribution gave a p-value of p = 0.91, thus indicating that there was no statistical difference in age distribution amongst the two provinces. Despite this p-value, in-depth analysis of the age groups showed reportable variations.

In-depth analysis of the age groups in each province respectively, showed that age group <1-4 years had the highest number of isolates in both provinces (Gauteng, *n* = 210; Western Cape, *n* = 102), thus accounting for 25% (Gauteng) and 26% (Western Cape) of the total isolates in each province respectively. Overall, this age group accounted for the highest number of isolates in the study (26%; 312/1221). Similarly, the second highest age group in both provinces was the 35-44 years (Gauteng, *n* = 110; Western Cape, *n* = 55), which accounted for 13% (Gauteng) and 14% (Western Cape) of the total isolates in each province respectively. Overall, this age group also accounted for the second highest number of isolates in the study (13.5%; 165/1221).

The general global observation of *Salmonella* infections amongst the different age groups is that infection incidence rates peak in children and the elderly (bimodal distribution) (WHO and FAO, 2002). However in Africa, several studies have described a bimodal distribution, different to that seen in other parts of the world. This bimodal distribution has its initial peak in the <1-4 years age group (children) and the second peak occurring in the adult age groups (Ao *et al*., 2015).

Globally, the <1-4 years are the mostly commonly affected age group in terms of diarrhoeal disease and despite global efforts to reduce the spread, diarrhoea remains the 2nd leading cause of death amongst children under five years of age (Morpeth *et al*., 2009; Liu *et al*., 2012). This population age group is more susceptible to infection by *Salmonella* and other foodborne pathogens, because their immune system is not fully developed (Dropulic and Lederman, 2009).

In Africa, several studies have described the burden of NTS amongst young children under the age of five years (Gordon *et al*., 2008; Morpeth *et al*., 2009; Sigauque *et al*., 2009). In a study done by Mandomando *et al*. (2009) on invasive NTS amongst Mozambican children, *Salmonella* Enteritidis was the second most common *Salmonella* found in children (*Salmonella* Typhimurium most common) with an incidence rate of 28.9 cases/ 10^5 child-years and with an incidence rate of 108.6 cases/ 10^5 child-years in infants. This shows the severity of *Salmonella* Enteritidis infections amongst young children in Africa.

The burden of disease observed amongst the <1-4 year age group in this study correspond with the global observation, and highlights the need for continual improvement of strategies to reduce the burden of foodborne diseases, particularly amongst young children.

The second highest age group in the study was the 35-44 years (adults) and this trend is not common globally. This peak may be attributed to HIV infection; however, since the HIV status of the study population is unknown, we cannot say for certain. Nonetheless, previously published data from Africa has described the strong association between NTS infection and HIV infection, and this association has been noted to be strongest amongst the adult age group (Morpeth *et al*., 2009). Individuals with HIV are prone to infection and recurrent infections by enteric bacterial pathogens such as *Salmonella* Enteritidis (WHO and FAO, 2002; Feasey *et al*., 2016).

South Africa has a high prevalence of HIV and according to the Statistics South Africa 2015 mid-year population estimates, an estimated 6.19 million (6.19 million /54.96 million; 11.2%) South Africans are HIV infected (Statistics South Africa, 2015). Adults between the ages of 15-49 are the most affected, accounting for 16.6% of the total population in this age group.

In this study we also observed large numbers of isolates within age groups 25-34 years (147/1221; 12%) and 45-54 years (140/1221; 11%). These two age groups also contain a large portion of the HIV infected population, thus possibly supporting its (HIV) role in the prevalence of *Salmonella* Enteritidis amongst these age groups, as reported in most African literature (Gordon, 2009; Morpeth *et al*., 2009). This is further suggested by the overall IQR (4-48 years), which includes a large portion of the HIV infected population.

Children over the age of 5 years (5-14 years) were also significantly affected, thus accounting for 10% (*n* = 135) of the study population. This notable prevalence rate amongst the 5-14 years age group has been described in previous literature (Khanum *et al*., 2006). Possible causes of infection amongst the 5-14 years age group include; lack of access to clean drinking water and exposure to contaminated

food items that are available in open-air school cafeteria (Asghar *et al*., 2002; Khanum *et al*., 2006). However, there was a notable reduction in the number of cases observed in this age group compared to the <1-4 years age group. This reduction may be due to further development of immunity and the production of efficient antibodies necessary to disable the bacterium (MacLennan *et al*., 2008). This advanced immunity may also be responsible for the lower numbers of isolates (overall) amongst the 15-24 years age group.

Individuals in the >65 years age group also had a notable prevalence rate (130/1221; 10%), which has been extensively described in literature globally. Elderly individuals are immune compromised, this is due to age-related deterioration of their immune system and co-morbidities (such as heart disease, malignancies), which result in increased proneness to infection. (WHO and FAO, 2002; Lund and O'Brien, 2011; Chen *et al*., 2012).

Age group 55-64 years had lower numbers of isolates (*n* = 80; 7%) compared to the >65 years. This may be due to the fact that this age group falls outside the HIV infected population age bracket (lower HIV infection), and that these individuals may still have a considerably efficient immune system, compared to the >65 years.

Overall in this study, children (<1-4 years) and adults within the HIV infected population age groups (25-34 years, 35-44 years and 45-54 years) are at higher risk of *Salmonella* Enteritidis infection.

4.1.3 Gender distribution

Overall, the gender distribution in this study showed no statistical difference (p = 0.83) amongst the two sexes. Several studies with different views about NTS and gender association have been published. Some studies report an infection ratio of 1:1 for both male and female and others describe males being more susceptible to infection than women and vice versa (WHO and FAO, 2002). In this study the former is true; the number of males and females infected by *Salmonella* Enteritidis was relatively equal.

4.1.4 Invasive *Salmonella* Enteritidis isolates

The Gauteng Province contained the largest number of invasive isolates (*n* = 274; 33%), and this was expected since Gauteng had more isolates compared to the Western Cape. However, the Western Cape Province contained a larger percentage of invasive isolates (37%; *n* = 142). The cause of increased invasive isolates in Western Cape is unclear, and may not necessarily be a true reflection of invasive *Salmonella* Enteritidis prevalence in Western Cape. Furthermore, the overall p value (p = 0.22), indicates that there is no statistical significance amongst the two provinces invasive isolates.

The overall percentage of invasive isolates obtained in this study is 34% (416/1221). This percentage is highly notable and concerning since deaths from invasive *Salmonella* infections are common in Africa (Vugia *et al*., 2004; Feasey *et al*., 2012; Ao *et al*., 2015). Furthermore in Africa, invasive *Salmonella* Enteritidis is often associated with infants (<3 years) and immune-compromised people, such as those with HIV; which are the most vulnerable populations within society (Morpeth *et al*., 2009).

In this study we observed that young children (<1-4 years), the elderly and people within the adult age groups had a higher percentage of invasive disease (Table 3.2). Invasive disease amongst the young children and the elderly may be due to an under developed immune system (children) and an age related weakened immune system (elderly). Although we cannot definitively identify HIV infection as the contributing factor for invasive disease within the adult age groups (HIV status of the study population is unknown), such a phenomenon has been extensively described in previous literature.

Despite the correlation of our findings with previously published data, the exact cause of invasive disease amongst 34% of our human study population is unknown. However, the host's susceptibility to infection also plays an important role. This is

evident amongst the high risk groups, who have poor immune function, which may make them prone to invasive *Salmonella* Enteritidis infection (Feasey *et al*., 2012; Langridge *et al*., 2012 and Feasey *et al*., 2016).

On the other hand, the *Salmonella* Enteritidis isolates in this study were not analysed for any enhanced virulence traits, thus we cannot rule out increased virulence within these isolates. Further studies would need to be carried out to investigate the presence of such traits.

Furthermore, lack of access to clean water and other socioeconomic problems play an important role in increased exposure to *Salmonella* Enteritidis and the development of invasive disease. Therefore, emphasis needs to be put into providing clean water and sanitation, in order to reduce exposure to *Salmonella*, particularly amongst children, the elderly and immune-compromised individuals (Langridge *et al*., 2012).

4.2 MLVA

4.2.1 The *Salmonella* Enteritidis VNTR loci

A total of five VNTR loci (SENTR7, SENTR5, SENTR6, SENTR4 and SE-3) previously described by Malorny *et al*., (2008) and Hopkins *et al*., (2011) were used in the study. These loci were shown to have consistent sequence length and diversity. Malorny *et al*., (2008) first reported on these five VNTR loci, along with four other VNTR loci (SENTR1, SENTR2, SENTR3 and SE-7). In their study SENTR7, SENTR5, SENTR6, SENTR4 and SE-3 had a higher Nei's diversity index (diversity index similar to the Simpson's diversity index, refer to section 4.5) compared to the other four VNTR loci, thus allowing for better discrimination *Salmonella* Enteritidis strains.

4.2.2 Lack of amplification on VNTR loci

In this study, lack of amplification was noted on VNTR loci SENTR6 (*n* = 13), SENTR4 (*n* = 3) and SE-3 (*n* = 4), with SENTR6 having the most lack of amplification. The lack of amplification at a particular VNTR locus was logged as “0” in the MLVA profile and the “0” was used as a unique feature in the definition of MLVA profiles (Appendix D).

A similar event was observed by Haguenoer *et al*., (2011), who found that certain isolates within their study lacked amplification at a particular VNTR locus. The locus was part of a genomic island and the initial primer pair only targeted the border regions of the genomic island. Haguenoer *et al*., (2011) designed a second primer pair which targeted flanking regions beyond the borders of the genomic island. Failed amplification with the second primer pair confirmed the absence of this locus in some of the isolates. Although Haguenoer *et al*., (2011) confirmed the cause for lack of amplification in their isolates; lack of amplification is not only attributed to the absence of the VNTR locus, but it may also be due to modification of the region in which the primer binds (Haguenoer *et al*., 2011). However, in our study lack of amplification in some of the isolates cannot be confirmed as being due to the absence or modification of the VNTR locus, since no further investigation of these possibilities was performed.

4.2.3 Electropherogram with double peaks at a VNTR locus

Electropherogram analysis (GeneMapper analysis) of each MLVA PCR product, revealed five isolates with double peaks at a VNTR locus. This occurred in loci SENTR7 (*n* = 2); SENTR5 (*n* = 1) and SENTR4 (*n* = 2). The presence of double peaks at a locus may indicate that each bacterium in the culture has two copies of the locus in question. Alternatively, this may mean that each bacterium may have one copy of the locus, but that more than one strain is present in the culture population and so contributing to the multiple alleles sizes.

In order to verify the double peaks, the isolates were plated out for single colonies and two colonies from each plate were analysed individually by MLVA. The double peaks were reproducible on all the isolates. Observation of all the electropherograms generated from each of the isolates, revealed differences in the two peak's fluorescence intensities. One of the peaks consistently had higher fluorescence intensity compared to its counterpart. According to Nadon *et al*., (2013), the peak that consistently has higher fluorescence intensity is then taken as the main allele (true result) and the other peak should be ignored. The same analogy was used in this study, for all isolates with double peaks at a locus.

4.2.4 Association of bacterial divergence and MLVA profiles

Noller *et al*., (2006), defined isolates with no more than a single VNTR difference occurring between all loci as being of the same lineage, the same analogy was used in this study. In this study, 72 MLVA profiles were observed as belonging to the same lineage and 14 MLVA profiles showed close relation (two loci difference) to the former. The most predominant MLVA profiles (MLVA profile 28, 7, 22, and 21) belong to the same lineage and therefore are closely related to each other (Figure 3.1).

The distance between two MLVA profiles indicates the differences that have occurred between the two MLVA profiles (genetic divergence). Based on this, MLVA profile 28 is closely related to MLVA profile 21 and 22, thus indicating that little variation has occurred between these MLVA profiles (very similar). The distance between MLVA profile 28, 21 and 22 to MLVA profile 7 is large, thus indicating that major changes have occurred between them (high genetic divergence).

4.2.5 Association of environmental factors and MLVA profiles

According to Dawson (2012) diversification within a lineage often results from interactions between the intrinsic biological limitations of organisms and the extrinsic environmental factors. In order to better understand the evolution of resident

lineages within a particular environmental setting, the demographic patterns of the co-existing populations need to be analysed in context to the history of the region (Dawson, 2012). Many studies have described environmental variation as a common factor in the influence of bacterial lineage diversity (Avitia *et al*., 2014). Genetic divergence within a bacterial lineage that is attributed to environmental factors may have occurred in our study and this may be further supported by the dominance of certain MLVA profiles in specific provinces. MLVA profiles 28, 22 and 21 are closely related to one another and seem to be more predominant in the Gauteng province. Furthermore, the smaller MLVA profiles that are closely related to these three MLVA profiles are largely present in Gauteng than in Western Cape. Similarly, MLVA profile 7 is more predominant in Western Cape and the smaller MLVA profiles closely related to MLVA profile 7 are mainly present in Western Cape (Figure 3.2).

4.2.6 MLVA clusters

In studies done by Murphy *et al*., (2008) and Cho *et al*., (2008), arrangement of MLVA profiles into clusters was based on the number of VNTR loci differences between the MLVA profiles. They reported that MLVA profiles with three or more VNTR loci differences (nodes connected by dotted lines) were genetically distant and would therefore not fall into the same cluster as MLVA profiles with one-two VNTR loci differences. In our study, two VNTR loci differences were observed amongst the MLVA profiles, therefore only a single MLVA cluster was obtained in the study. This is an indication of clonality amongst *Salmonella* Enteritidis strains in the two provinces, which may indicate that the strains originate from a single *Salmonella* Enteritidis ancestor (Cho *et al*., 2008; Murphy *et al*., 2008). Such clonality may be the reason for such close relations amongst the MLVA profiles obtained in this study. A similar finding was observed in Iran, wherein six closely related MLVA profiles were identified in the study and the high clonality of the Iranian *Salmonella* Enteritidis isolates was deemed as the cause for the close relation amongst the MLVA profiles (Acton, 2013). An extensive phylogenetic analysis needs to be conducted on our *Salmonella* Enteritidis isolates to confirm that they share a common ancestor.

4.2.7 MLVA profile homogeneity

The human isolates included in this study were from only 2/9 provinces in South Africa. The Western Cape and Gauteng province are the most urbanised cities in the country, a large number of people from different provinces in the country, as well as different countries in Africa are found largely in these two provinces (Statistics South Africa, 2015). Therefore, these two provinces allow us to get a general view of the *Salmonella* Enteritidis strains circulating in the country. The high homogeneity of these isolates may be indicative of the stains circulating in the country. A study by Campioni *et al*. (2013) on the observation of the *Salmonella* Enteritidis strains circulating in Brazil, found that there was high genetic homogeneity in the *Salmonella* Enteritidis strains found in both humans and animals in Brazil. Due to such high strain homogeneity, they were able to identify the introduction of a new *Salmonella* Enteritidis strain, which was highly diverse compared to the native strains. Further analysis of the strain revealed that it originated from North America. A similar surveillance can be initiated in South Africa using MLVA and it can be useful in the identification of new strains associated with foreign travels and potential outbreaks caused by the newly introduced strains.

4.2.8 Discriminatory power of MLVA (Simpson's diversity index)

The Simpson's diversity index (*D*) calculates diversity within each locus and it also measures the probability that two epidemiologically unrelated isolates will be characterized as being different. This enables observation of VNTR polymorphism (Boxrud *et al*., 2007). A VNTR locus with a higher *D* value (value closer to one), shows that it is highly diverse and could be helpful in differentiating isolates from the same environment (geographical area), as well as give greater discrimination for epidemiologically unrelated strains. In our study SENTR4, SENTR5 and SENTR6 had *D* values between 0.7-0.9, and SENTR7 and SE3 had *D* values of 0.525 and 0.539 respectively. Therefore, SENTR4, SENTR5 and SENTR6 have greater polymorphism than SENTR7 and SE3. SENTR7 and SE3 are less variable and are the loci that define the MLVA profile. These loci (SENTR7 and SE3) serve as molecular clocks and are predominantly useful in phylogenetic analysis of isolates

that are more geographically dispersed (Boxrud *et al*., 2007; Haguenoer *et al*., 2011; Hopkins *et al*., 2011).

In our study, SENTR5 had the highest number of alleles (17 alleles), followed by SENTR6 with 13 alleles and SENTR 4 with eight alleles. SENTR7 and SE-3 had the least numbers of alleles, with seven and four respectively. These findings are comparable to those found by Malorny *et al*., (2008), whereby SENTR6 had the highest number of alleles (11 alleles) followed by SENTR5 (10 alleles) and SENTR4 with seven alleles. Malorny *et al*., (2008) also observed that SENTR7 and SE-3 had the least number of alleles, with three each.

Several studies have compared both MLVA and PFGE using Simpson's diversity index and most of these studies have shown that MLVA has higher discriminatory power than PFGE. Although we did not compare the two molecular techniques, literature has reported on MLVA's higher discriminatory power and the incorporation of VNTR loci with different Simpson's diversity indexes makes it possible to discriminate between closely related and distantly related *Salmonella* Enteritidis strains (Boxrud *et al*., 2007; Hopkins *et al*., 2011; Haguenoer *et al*., 2011).

4.2.9 MLVA profiles and invasive *Salmonella* Enteritidis isolates

The association of MLVA profiles and invasive isolates has not been reported in literature. This may be due to the fact that most countries do not use MLVA as a standard typing method. However, numerous studies have described the association of certain *Salmonella* Enteritidis phage types with invasive disease. *Salmonella* Enteritidis PT4 has been described as the most common and most virulent *Salmonella* Enteritidis phage type and studies have shown the association of this phage type with invasive disease in both humans and animals (Poppe *et al*., 1993; Indar-Harrinauth *et al*., 2001). The same could be possible using MLVA as a standard typing method.

MLVA profile 28 had the highest number of invasive isolates in the study (208/416). This is least surprising since it accounts for majority of the isolates in the study (*n* = 661). However, our data also showed that the other MLVA profiles contained large

numbers of invasive isolates (101/416). This is indicative of the fact that invasive isolates are not confined to one particular MLVA profile, but that even the smaller MLVA profiles can contain invasive isolates. More so, the possibility of increased virulence within these MLVA profiles cannot be disregarded. Further genotypic analysis of the invasive isolates present in these MLVA profiles is necessary.

In this study, we were able to show that not only one MLVA profile is associated with invasive *Salmonella* Enteritidis infection. However, our data does not indicate if there is any clinical significance on the association of MLVA profiles and invasive isolates. The establishment of such a trend may assist in the advancement of molecular epidemiological surveillance.

4.3 Antimicrobial susceptibility profiles of human *Salmonella* Enteritidis isolates

Over the past few decades, many reports have described the increase in resistance of NTS serotypes to medically important antimicrobial agents. Antimicrobial resistance in NTS serotypes is a global challenge, although the resistance rate varies amongst different serotypes and different antimicrobials. Numerous reports from different parts of the world have shown that *Salmonella* Enteritidis is still relatively susceptible to most antimicrobial agents compared to other serotypes (Su *et al*., 2004; CDC, 2013; Chiu and Su, 2014). However, isolated studies from various parts of the world have shown a major increase in resistance of *Salmonella* Enteritidis isolates (Morpeth *et al*., 2009; Jiayong *et al*., 2015).

In this study, 55.4% (677/1221) of the total human isolates were fully susceptible to all antimicrobials tested (section 3.3). Low prevalence of resistance was observed in 7/8 antimicrobials; however, high prevalence of resistance was observed for sulfamethoxazole, with 29% (*n* = 348) of the isolates having resistance to this antimicrobial. A similar finding was observed by Smith *et al*., (2014) in a study, which compared *Salmonella* Enteritidis isolates from humans and wild animals in South Africa. Of the 196 humans isolates included in their study, 43% were resistant to sulfamethoxazole.

There is limited data on sulfamethoxazole resistance in human *Salmonella* Enteritidis isolates. This is because sulfamethoxazole is generally used in combination with trimethoprim (cotrimoxazole) in the treatment of human infections. Many publications have described resistance of *Salmonella* isolates to cotrimoxazole, however in this study cotrimoxazole resistance was very minimal, with <1% of the isolates showing resistance to this drug. In most African settings, sulfamethoxazole (sometimes in combination with trimethoprim as cotrimoxazole) is used as a prophylactic drug in HIV/Aids patients as a means to prevent life threatening opportunistic infections. This use of sulfamethoxazole has been shown to reduce mortality and morbidity in people with HIV (Hamer and Gill, 2008). This prophylactic use of sulfonamides still continues today in South Africa and in many other parts of Africa (Sibanda *et al*., 2011; National Department of Health South Africa, 2015). Such continual exposure to antimicrobials has been shown to lead to the selection of resistant bacterial strains (Eagar *et al*., 2012; National Department of Health, 2015). Thus, it is possible that pathogens such as *Salmonella* Enteritidis may have gained resistance against sulfamethoxazole due to such constant exposure.

Another contributor of high sulfamethoxazole resistance may be its use in the agricultural sector. Several studies have described sulfamethoxazole's use in animal prophylaxis (Kumar *et al*., 2005; Eagar *et al*., 2012). Although we are unable to pin point the exact cause of increased sulfamethoxazole resistance in our isolates. The excessive use of sulfamethoxazole in the farming sector may be playing a role in the emergence of sulfamethoxazole-resistant isolates (refer to section 1.14.2 and section 4.5).

In this study, 9% (113/1221) of human isolates showed intermediate resistance to ciprofloxacin. Ciprofloxacin is an important antimicrobial used in the treatment of numerous bacterial infections including extra-intestinal *Salmonella* infections (Chiu and Su, 2014). Numerous studies from different parts of the world (including Africa), have described ciprofloxacin-resistant *Salmonella* Typhi isolates (Kariuki *et al*., 2010; Medalla *et al*., 2011; Rahman *et al*., 2014; Al-Emran *et al*., 2016). Such infections have led to clinical treatment failures with dire consequences. However, there is limited data on reduced susceptibility of NTS (such as *Salmonella* Enteritidis) to ciprofloxacin. A study by Eibach *et al*. (2016) in Ghana, reported on reduced

susceptibility of *Salmonella* Enteritidis isolates (10/19; 53%) to ciprofloxacin. Further genotypic analysis of the isolates revealed mutations in the *gyrA* gene, which confers low-level resistance to ciprofloxacin.

In Africa, there are limited reports on the amount of ciprofloxacin prescribed in health care facilities. Thus, we are unable to identify the exact cause of ciprofloxacin intermediate resistance amongst our isolates. However, in 2011 the WHO reported that the use of fluoroquinolones in the food animal industry was a major contributor to the emergence of ciprofloxacin-resistant *Salmonella*, *Campylobacter* and *E. coli*. This had resulted in human infections that had proven difficult to treat (Teuber, 2001; WHO, 2011; Landers *et al*., 2012); thus indicating the impact the food animal industry has on the emergence of antimicrobial resistance.

Although we cannot confirm the exact cause of ciprofloxacin intermediate resistance, it is however possible that the isolates may have acquired resistance genes through any of the previously described gene acquisition mechanisms (section 1.11.2). Further genotypic studies have to be done on our ciprofloxacin intermediate resistant isolates in order to conclude the presence or absence of resistance genes. On the other hand, we also recognize that limited data regarding the use of ciprofloxacin in African health care settings (public and private), may limit our understanding of the role ciprofloxacin use in these health care facilities plays in the emergence of antimicrobial resistance.

4.3.1 MLVA profiles and antimicrobial resistance

The highest sulfamethoxazole resistance was found amongst the Gauteng isolates. This was expected, due to the large number of isolates selected from Gauteng. Sulfamethoxazole resistance was present in both the predominant MLVA profiles and the other MLVA profiles, thus showing that different strains (found in various MLVA profiles) have mechanisms that confer for sulfamethoxazole resistance.

Of the predominant MLVA profiles, MLVA profile 28 had the highest number of sulfamethoxazole-resistant isolates in this study, thus accounting for 58% (202/348)

of the total sulfamethoxazole-resistant isolates. This is expected since majority of the isolates consist of this MLVA profile.

Further analysis of the MLVA profiles (MST analysis) seems to indicate that resistance mechanisms may have been acquired individually within the different MLVA profiles. This is because other MLVA profiles that are closely related to MLVA profiles with sulfamethoxazole-resistant isolates, show full susceptibility to sulfamethoxazole. This means resistance mechanisms could not have been passed down during strain diversity. Environmental pressures may have played a huge role in the acquisition of these resistant genes (Dawson, 2012). Furthermore, majority of the resistance mechanisms are found in non-chromosomal DNA, thus making it highly plausible that the isolates found in these MLVA profiles obtained them (resistance genes) after strain diversification (Hamer and Gill, 2008). However, further molecular investigation needs to be carried out in order to prove this hypothesis.

Similarly, higher ciprofloxacin intermediate resistance was observed amongst Gauteng isolates. MLVA profile 28 (61%; 69/113) had the highest number of isolates with ciprofloxacin intermediate resistance. Observation of the MST shows a picture similar to that observed in sulfamethoxazole-resistant isolates. Thus, emphasizing the role environmental pressures play in the acquisition of antimicrobial resistance mechanisms.

In this study, we observed that antimicrobial testing along with MLVA can potentially be used as a surveillance tool which may assist in identifying antimicrobial pattern changes in *Salmonella* Enteritidis isolates present within different MLVA profiles (i.e. acquisition of resistance genes that were not seen before in that MLVA profile). This is due to the fact that other MLVA profiles in the study contained isolates that were resistant to more than one antimicrobial. This may help in identifying *Salmonella* Enteritidis outbreak strains that belong to MLVA profiles with certain antimicrobial resistance patterns.

4.4 Non-human *Salmonella* Enteritidis isolates

A total of 43 non-human isolates from different origins (poultry, wild animal, equine, avian and environmental), collected from various parts of the country were included in the study. Disappointingly, 49% (21/43) of our non-human isolates were of unknown origin (and source) and lacked demographic data. The isolates were accepted at the CED and included in the study as non-human isolates due to the fact that they were obtained from known veterinary laboratories.

Obtaining isolates from private veterinary laboratories was difficult, because the laboratories have signed confidentiality agreements with their customers (usually food animal producers), which prevents them from disclosing any findings with any third party. In situations where isolates were obtained, some laboratories would withhold valuable information about the isolates such as their origin, collection date and place of collection (province and town), in fear of possible linking of data to their clients. Furthermore, laboratories may also be reluctant to give isolates or further details about the isolates, more especially in the case of *Salmonella* Enteritidis, since it is a controlled pathogen in South African agricultural sector. All these aspects resulted in very few non-human isolates being included in the study.

Despite the poor numbers received, the non-human isolates collected were from diverse origins such as agricultural animals, wild animals, domestic animals and animal environments. We hoped that this isolate diversity would enable us to observe *Salmonella* Enteritidis strains circulating in the non-human population and compare these strains to those found in humans. Isolates of poultry origin are of great importance to the study, because the association of human infection and poultry has been highly described in literature and eggs are still considered the main source of human *Salmonella* Enteritidis infection (Andino and Hanning, 2015). Furthermore, the South African government has a zero tolerance for *Salmonella* Enteritidis in poultry and thus theoretically there should not be any *Salmonella* Enteritidis infections in poultry (World poultry, 2016).

However in this study, we observed the presence of *Salmonella* Enteritidis in poultry. This is concerning because it increases the potential spread of this pathogen to

humans, therefore leading to potential outbreaks. This needs to be prevented, since recovery (economic and food security) from such events could be challenging and very costly to the country. Furthermore, in South Africa, chicken and chicken derivatives (e.g. eggs) are an important source of protein, since they are cheaper compared to other meat products. Thus, reduced control of *Salmonella* Enteritidis would have devastating effects on both consumers and food-producing companies; enhanced quality control would drive the price of poultry products up and make it unaffordable to minimum wage earners (Cogan and Humphrey, 2003; Hoelzer *et al*., 2011; Andino and Hanning, 2015; World poultry, 2016).

4.4.1 MLVA profiles of non-human *Salmonella* Enteritidis isolates

MLVA profile 28 represented the highest number of isolates; four poultry, one environmental, two cheetah and eight of unknown origin. MLVA profile 26 and 35 were the second and third most common MLVA profiles amongst the non-human isolates respectively. Isolates found in both MLVA profiles (MLVA profiles 26 and 35) lacked supporting demographic data and therefore were of unknown origin and source. MLVA profiles 22, 48, 40 and 41 contained isolates of poultry origin and MLVA profile 21 had isolates from poultry and equine origin. Collectively, isolates of poultry origin were present in 6/10 MLVA profiles (MLVA profiles 28, 22, 48, 40, 41 and 21). Due to lack of added demographic information, we cannot identify whether these isolates (poultry) originate for a single geographic region or not. However, the data indicates MLVA profile diversity amongst the poultry isolates (Cho e*t al*., 2007). In the case of MLVA profiles 28 and 21, the presence of isolates from various origins (poultry, equine and cheetah) indicates how widespread MLVA profiles can be, even crossing through different animal species. These events are somewhat not surprising since these interactions can easily occur in places where different animals live in close proximity to one another (i.e. farms). Furthermore, animals such as horses and cheetahs can also acquire the infection from the environment, wherein bird and poultry faeces may be present. The inclusion of environmental isolates in this study emphasizes the role the environment plays in the transmission of disease (Hoelzer *et al*., 2011).

Despite the fact that few wild and large domestic animal isolates were received for this study, we were still able to observe the presence of *Salmonella* Enteritidis amongst them. More isolates need to be collected, in order to identify the magnitude of *Salmonella* Enteritidis infection within these animal communities.

4.5 Non-human *Salmonella* Enteritidis antimicrobial susceptibility profiles

All non-human isolates showed full susceptibility to five of the antimicrobials (ampicillin, cotrimoxazole, trimethoprim, tetracycline, and azithromycin). Intermediate resistance was observed for ceftriaxone (5%; 2/43) and ciprofloxacin (7%; 3/43). Furthermore, 23% (10/43) of the isolates were resistant to sulfamethoxazole.

The number of non-human isolates received in this study was too small to reflect on possible antimicrobial patterns. However, we noted low prevalence of intermediate resistance for ceftriaxone and ciprofloxacin amongst the isolates received. The cause of this intermediate resistance is unknown and many factors may be involved. Ceftriaxone and ciprofloxacin are some of the most commonly used antimicrobials in the human health. The use of these antimicrobials in food animals for treatment of infections has been reported, yet their use in animal prophylaxis is less described as compared to the other cephalosporins and fluoroquinolones (Eagar *et al*., 2012; Landers *et al*., 2012; Moyane *et al*., 2013). Despite the small numbers of non-human isolates received, intermediate resistance to both ceftriaxone and ciprofloxacin is concerning. These intermediate resistant strains could potentially spread to humans, causing challenges in patient management (Tadesse, 2015).

In this study, sulfamethoxazole resistance amongst non-human isolates (23%; 10/43) was comparable to that found in human isolates (29%; 348/1221). However, it was not possible to establish any connection between the two. Nonetheless, several studies have shown evidence of obtaining the same antimicrobial-resistant strain in animal produce (e.g. meat and eggs) as that seen to cause clinical infection and subclinical colonization in humans (Landers *et al*., 2012).

The use of sulfonamides such as sulfamethoxazole in food animal agriculture for growth promotion and prophylaxis has been extensively described in literature from various parts of the world. In South Africa, many different classes of antimicrobials are used in the food animal industry; these include penicillins, cephalosporins and sulfonamides. Eagar *et al*., (2012), estimated the percentage volume of antimicrobials sold in South Africa for water medication between the years 2002-2004 and sulfonamides were the highest sold antimicrobials accounting for 95.40%, second by penicillins (1.80%) and quinolones (1.30%) respectively. Although we cannot conclude the main cause for resistance within the non-human isolates, supporting literature describes the excessive use of antimicrobials in the food animal industry and the role it (excessive use) plays in antimicrobial resistance.

Although sulfamethoxazole, ciprofloxacin and ceftriaxone are used in agriculture, we also acknowledge that fact that some of the non-agricultural isolates (equine and wild animals) included in this study had (either) intermediate resistance and/or antimicrobial resistance to these antimicrobials. Therefore, other factors are involved in the emergence of resistance amongst the non-human isolates. Furthermore, we do not know the extent at which antimicrobials are being used within the wild animal community. These areas need to be explored in order to try establish areas of concern and possible interventions of all possible drivers of antimicrobial resistance within the animal community. Indeed a larger number of non-human *Salmonella* Enteritidis isolates (from various origins) is necessary in order to achieve this.

4.6 Comparison and associations of human and non-human *Salmonella* Enteritidis isolates

The presence of certain MLVA profiles in both humans and non-humans may be indicative of transmission between these groups. This was further supported by the fact that, MLVA profiles 21, 22 and 28 (predominant amongst human isolates) were also present amongst non-human isolates. MLVA profile 28 was the most common MLVA profile in both groups (humans and non-humans). However, the reason for this predominance is unknown. MLVA profiles 35 and 49 were only present amongst non-human isolates. A similar finding was reported by Cho *et al*., (2008), wherein

certain MLVA profiles were only found in animals and not in humans (source specific MLVA profiles). However, a larger number of non-human isolates (with known origin and source) are necessary in order to confirm their source specific characteristic.

Comparison of MLVA profiles obtained in both human and non-human isolates showed that *Salmonella* Enteritidis strains circulating in both the humans and non-humans were clonal (i.e. fall under one cluster). However, the number of non-human isolates collected, along with the limited geographic data obtained made the association of human and non-human isolates challenging. Demographic information is vital in the accurate comparison of human and non-human (i.e. animal) infections and in the identification of possible transference of disease from animals to humans. Data from such studies assist in controlling the spread of zoonotic pathogens such as *Salmonella* Enteritidis in both humans and animals. This is crucial because in most animals, *Salmonella* Enteritidis causes asymptomatic infections, which can lead to sporadic outbreaks (Hoelzer *et al*., 2011). Furthermore, such studies also support the need for proper interventions on zoonotic pathogens (Mangni and Arvntikis, 2010; Kidanermariam *et al*., 2010).

4.7 Molecular investigation of outbreaks using MLVA

For many years, PFGE has been used as a primary method for molecular subtyping of salmonellae; it is still used as a tool for molecular epidemiological investigations and outbreak investigations - particularly still used by PulseNet International. However, *Salmonella* Enteritidis has limited heterogeneity (lacks genetic variation), thus making discrimination using PFGE challenging. The application of MLVA to a wide variety of bacterial species (including *Salmonella* species) has concluded that MLVA is more discriminatory compared to most other available molecular subtyping methods, including PFGE. Numerous studies have applied MLVA in the analysis of *Salmonella* Enteritidis in different parts of the world and many studies have applied it in outbreak investigations (Hopkins *et al*., 2011). However, many have remained doubtful about the stability of VNTR's during an outbreak. It is assumed that the VNTR's may evolve rapidly, thereby producing multiple MLVA profiles during an

outbreak. A study by Boxrud *et al*. (2007) analysed the stability of *Salmonella* Enteritidis VNTR's during an outbreak, they found that the MLVA profiles remained stable during the course of the outbreak and a similar finding was observed by Malorny *et al*., (2008).

In this study, we were able to successfully use MLVA as a molecular epidemiological tool for the investigation of outbreaks. MLVA was able to group all isolates from one outbreak into a single MLVA profile, therefore indicating the stability of the VNTR's in this study. MLVA profile 28 accounted for most of the outbreaks (5/7 outbreaks). This MLVA profile had caused outbreaks in four provinces (Mpumalanga, Limpopo, Free State and Eastern Cape). This showed that not only is MLVA profile 28 the most common MLVA profile in the study (predominantly found in both human and non-human isolates), but that it is also able to cause outbreaks throughout the country. This is however not surprising, since our data show that it could potentially be common within the population and thus the chances of it causing outbreaks throughout South Africa is highly plausible. Other predominant MLVA profiles in the study were also shown to cause outbreaks. MLVA profile 21 caused an outbreak in Gauteng, and MLVA profile 22 caused outbreaks in KwaZulu-Natal and Mpumalanga.

Observation of the MST suggests that MLVA profile 21, 22, and 28 are closely related, with a single VNTR locus difference between them (Figure 3.1). This may be indicative of shared pathogenicity characteristics between them, which enable them to effectively cause outbreaks compared to the other MLVA profiles. WGS analysis on these outbreak isolates is necessary in order to identify any shared pathogenicity characteristics. However, outbreaks caused by closely related strains (closely related MLVA profiles) have been reported. A study by Slinko *et al*. (2009) on an outbreak of *Salmonella* Typhimurium, found that outbreaks caused by the STm197 strain had produced several closely related MLVA profiles, which had caused outbreaks in many restaurants in the city of Brisbane for over two months.

Our findings emphasize the need for analysis of *Salmonella* Enteritidis isolates from different provinces in the country, in order to observe the circulating MLVA profiles in each province and their potential to cause outbreaks.

4.8 Limitations and future prospects

4.8.1 Limitations

The CED received fewer human isolates from the Western Cape Province in comparison to the Gauteng Province. Therefore, the random selection of isolates favoured the Gauteng Province (*n* = 832) more than the Western Cape Province (*n* = 389). This gave bias during study population analysis and made any comparison between the two provinces challenging. Furthermore, this may have limited our chances of obtaining more MLVA profiles from the Western Cape. However, statistical analysis did not reveal any significant differences between the two provinces and the MLVA profiles obtained in the study showed high clonality amongst isolates from both provinces.

Data obtained in this study could not be used to derive an incidence rate. This is because the study population was randomly selected from isolates that are obtained through the GERMS-SA surveillance programme. Such data does not give a true reflection of the burden of *Salmonella* Enteritidis in South Africa, since most people with less severe disease do not seek health care, and those who do often receive empiric therapy during consultation and a stool specimen is rarely taken. However, this aspect was not the primary focus of the study and thus it did not have an effect on the ability to identify *Salmonella* Enteritidis strain diversity in South Africa.

The non-human isolates received, as well as the demographic data that accompanied the isolates were extremely limited. Data was sometimes even completely non-existent. This was despite our continuous appeals and requests for isolates from the sending laboratories. This made linking of human and non-human *Salmonella* Enteritidis infection a difficult task.

In this study, reference strains were not used to normalize raw fragment data to actual fragment sizes. This was because no reference strains had been validated for

Salmonella Enteritidis MLVA at the time of the study. This made inter-laboratory comparison of the MLVA data impossible. However, Peters *et al*. (2017) published the first list of validated reference strains for *Salmonella* Enteritidis MLVA. This will be beneficial for future *Salmonella* Enteritidis MLVA studies.

4.8.2 Future prospects

Although clonality of *Salmonella* Enteritidis strains was presumed through analysis of the MST, further analysis of the MLVA profiles using WGS is necessary. This is because MLVA only looks at a small segment of the genome and mutations in any of the five VNTR's may affect its credibility. WGS analysis on the other hand analyses the pathogen's whole genome and it can identify strain diversity using other sequence variations such as SNPs. WGS analysis has become the ultimate tool, it is gradually becoming more accessible to all and will eventually replace all current subtyping methods.

Studies associating human and non-human (e.g. food animals) *Salmonella* Enteritidis infection are necessary within South Africa. Furthermore, studies with large numbers of non-human (i.e. animal) isolates are necessary to conclude definitive infection transference between humans and animals.

CHAPTER 5: CONCLUSIONS

Of the 1221 human *Salmonella* Enteritidis isolates (389 from Western Cape and 832 from Gauteng) were included in the study, a high number of cases was noted amongst the <1-4 years and 34-44 years age groups. The high number of isolates amongst the <1-4 years age group could be attributed to an immature immune system and the high number of isolates amongst the 34-44 years age group may be attributed to the HIV epidemic within the country. Invasive isolates accounted for 34% of the total isolates. We noted that invasive disease was prominent amongst the young children (<4 years), the elderly and the adult age groups (HIV infected population age groups). The main cause of invasive disease could not be established. However, poor immune function could be playing a role in susceptibility to invasive disease.

A total of 43 non-human isolates were included in the study. Such few non-human isolates were included in the study, because obtaining non-human isolates from veterinary laboratories was challenging. The veterinary laboratories were unwilling to provide isolates or further details about the isolates due to loyalty towards their customers. This led to the receiving of 21 (49%) isolates with absolutely no supporting data, including origin and source. However, the rest of the isolates were of avian, equine, poultry, environmental (animal environment) and wild animal origin.

This study represented the first ever-molecular subtyping analysis of large number of *Salmonella* Enteritidis isolates in any African country using MLVA. A total of 86 MLVA profiles were obtained from 1264 isolates (1221 human isolates and 43 non-human isolates). MLVA was able to successfully distinguish *Salmonella* Enteritidis strains that differed by one VNTR locus. MLVA also demonstrated the clonality of *Salmonella* Enteritidis strains circulating amongst the human and non-human isolates, because the MLVA profiles obtained in the study had a maximum of two VNTR loci differences. Therefore, a single cluster was obtained in the study.

Of the 86 MLVA profiles identified, four predominant MLVA profiles (MLVA profiles 7, 21, 22 and 28) were obtained amongst the human isolates. MLVA profile 28 was the most common MLVA profile throughout the study (2013-2015) and the exact cause for this was unknown. MLVA profiles 28, 21 and 22 were predominantly present in the Gauteng province and MLVA profile 7 was predominantly present in the Western Cape.

A total of 10 MLVA profiles were obtained from 43 non-human isolates. As found in the human isolates, MLVA profile 28 was the most predominant MLVA profile. Thus, indicating possible dominance within both populations. Isolates of poultry origin were present in a number of MLVA profiles (MLVA profiles 28, 22, 48, 40, 41 and 21). This suggests MLVA profile diversity amongst poultry isolates. However, more non-human isolates are necessary to identify the extent of *Salmonella* Enteritidis infection within the non-human population. Furthermore, 8/10 MLVA profiles were present in both human and non-human isolates. MLVA profiles 35 and 49 were only found amongst non-human isolates. However, more non-human isolates need to be analysed to conclude their source specificity.

A total of 348 (29%) human isolates were resistant to sulfamethoxazole. The cause of such resistance could not be established, however sulfamethoxazole resistance within the *Salmonella* Enteritidis isolates could be due to excessive (i.e. prophylactic) use. Furthermore, the excessive use of sulfamethoxazole in the agricultural sector could also be a key role player in sulfamethoxazole resistance. A similar finding was observed amongst the non-human isolates, with sulfamethoxazole resistance at 23% (n = 10), thus emphasizing that the excessive use of antimicrobials in the agricultural sector plays a role in the emergence of antimicrobial-resistant isolates.

During the years 2013-2015, seven *Salmonella* Enteritidis outbreaks were investigated from six provinces. Isolates from each individual outbreak showed an identical MLVA profile, thus showing VNTR stability. Three MLVA profiles were obtained (MLVA profiles 21, 22 and 28) and MLVA profile 28 accounted for most of the outbreaks (5/7 outbreaks), thus showing its ability to spread throughout the country. Observation of the MST showed that MLVA profile 21, 22, and 28 are

closely related, with a single VNTR locus difference between them. This may be indicative of shared pathogenicity mechanisms between them.

Finally, in this study MLVA has shown to be a successful molecular subtyping tool for *Salmonella* Enteritidis, for both surveillance purposes and outbreak investigations. MLVA is a relatively inexpensive, easy and consistent molecular typing method. In this study we showed that VNTR's can be stable during an outbreak, making MLVA a quicker way of discriminating outbreak strains from non-outbreak strains. We observed that *Salmonella* Enteritidis strains circulating within the Western Cape and Gauteng Province were very clonal and that such clonality may be an indication of the bigger picture occurring throughout the country. We also observed that *Salmonella* Enteritidis strains circulating in both the human and non-human populations were clonal, which may be indicative of active transmission between the two. The prevalence of antimicrobial resistance amongst all isolates was low although sulfamethoxazole resistance was notable. Emphasis needs to be placed in curbing the spread of *Salmonella* Enteritidis amongst the young, the immune-compromised individuals and the elderly, since they are more at risk of infection by invasive and antimicrobial-resistant *Salmonella* Enteritidis. This study also showed the need for more emphasis on the one health approach; both human and animal health sectors have to work together to curb the spread of zoonotic diseases within the country. In association with the current epidemiological surveillance programs, studies such as this can provide valuable information for the development of public health strategies to minimize or control the risk of outbreaks and epidemics by *Salmonella* Enteritidis.

REFERENCES

Aarestrup, F. (2012). Sustainable farming: Get pigs off antibiotics. *Nature*. 486: 465-466.

Acar, J. and Röstel, B. (2001). Antimicrobial resistance: An overview OIE. *Revue Scientifique et Technique*. 20 (3): 797–810.

Achtman, M., Wain, J., Weill, F., Nair, S., Zhou, Z., Sangal, V., Krauland, M.G., Hale, J.L., Harbottle, H., Uesbeck, A., Dougan, G., Harrison, L.H., Brisse, S. and the *S. enterica* MLST study group. (2012). Multilocus Sequence Typing as a Replacement for Serotyping in *Salmonella enterica. PLOS Pathogens*. 8 (6): e1002776. doi:10.1371/journal.ppat.1002776.

Acton, Q.A. (2013). *Advances in Salmonella research and treatment: 2013 Edition: Scholarly edition*. Atlanta, Georgia: Scholarly editions. pp 1-2.

Ahmed, R., Soule, G., Demczuk, W.H., Clark, C., Khakhria, R., Ratnam, S., Marshall, S., Ng, L.K., Woodward, D.L., Johnson, W.M. and Rodgers, F.G. (2000). Epidemiologic typing of *Salmonella enterica* serotype Enteritidis in a Canada-wide outbreak of gastroenteritis due to contaminated cheese. *Journal of Clinical Microbiology*. 38: 2403-2406.

Alanis, A.J. (2005). Resistance to antibiotics: are we in the post-antibiotic era? *Archives of Medical Research*. 36 (6): 697–705.

Alcaine, S.D., Warnick, L.D. and Wiedmann, M. (2007). Antimicrobial resistance in nontyphoidal *Salmonella*. *Journal of Food Protection*. 70 (3): 780–790.

Al-Emran, H.M., Eibach, D., Krumkamp, R., Ali, M., Baker, S., Biggs, H.M., Bjerregaard-Andersen, M., Breiman, R.F., Clemens, J.D., Crump, J.A., Cruz, Espinoza, L.M., Deerin, J., Dekker, D.M., Gassama, S.A., Hertz, J.T., Im, J.,

Ibrango, S., von Kalckreuth, V., Kabore, L.P., Konings, F., Løfberg, S.V., Meyer, C.G., Mintz, E.D., Montgomery, J.M., Olack, B., Pak, G.D., Panzner, U., Park, S.E., Razafindrabe, J.L., Rabezanahary, H., Rakotondrainiarivelo, J.P., Rakotozandrindrainy, R., Raminosoa, T.M., Schütt-Gerowitt, H., Sampo, E., Soura, A.B., Tall, A., Warren, M., Wierzba, T.F., May, J. and Marks, F. (2016). A Multicountry Molecular analysis of *Salmonella enterica* serovar Typhi with reduced susceptibility to ciprofloxacin in Sub-Saharan Africa. *Clinical Infectious Diseases*. 15 (16): 42-46.

Alvarado, I.R. (2011). *Vaccination options to prevent Salmonella Enteritidis: Egg Industry*. Available at: http://www.wattagnet.com/articles/8821-vaccination-options-to-prevent-salmonella-enteritidis [Accessed: 16 May 2015].

Andino, A. and Hanning, I. (2015). *Salmonella enterica*: survival, colonization, and virulence differences among serovars. *The Scientific World Journal*. doi: 10.1155/2015/520179.

Ao, T.T., Feasey, N.A., Gordon, M.A., Keddy, K.H., Angulo, F.J. and Crump, J.A. (2015). Global burden of invasive nontyphoidal *Salmonella* disease, 2010. *Emerging Infectious Diseases*. 21 (6): 941-949.

Asghar, U., Saba, N.V., Samad, A. and Qazilbash, A.A. (2002). Identification, characterization and antibiotic susceptibility of *Salmonella* and *Shigella* species isolated from blood and stool samples of patients visiting N. I. H, Islamabad. *Journal of Medical Science*. 2 (2): 85-88.

Avitia, M., Escalante, A.E., Rebollar, E.A., Moreno-Letelier, A., Eguiarte, L.E. and Souza, V. (2014). Population expansions shared among coexisting bacterial lineages are revealed by genetic evidence. *PeerJ*. 2 (696): 1-12.

Barceloux, D.G. (2012). *Medical toxicology of natural sciences*: *Foods, Fungi, Medical herbs, Plants and Venomous animals*. New Jersey: John Wiley and Sons. Chap 19.

Berkley, J.A., Lowe, B.S., Mwangi, I., Williams, T., Bauni, E., Mwarumba, S., Ngetsa, C., Slack, M.P., Njenga, S., Hart, C.A., Maitland, K., English, M., Marsh, K. and Scott, J.A. (2005). Bacteremia among children admitted to a rural hospital in Kenya. *New England Journal of Medicine*. 352 (1): 39-47.

Bern, C., Martines, J., de Zoysa, I. and Glass, R.I. (1992). The magnitude of the global problem of diarrhoeal disease: a ten-year update. *Bulletin World Health Organisation*. 70: 705–714.

Bertels, F., Silander, O.K., Pachkov, M., Rainey, P.B. and van Nimwegen, E. (2014). Automated reconstruction of whole-genome phylogenies from short-sequence reads. *Molecular Biology and Evolution*. 3: 1077–1088.

Bessong, P.O., Odiyo, J.O., Musekene, J.N. and Tessema, A. (2009). Spatial Distribution of Diarrhoea and Microbial Quality of Domestic Water during an Outbreak of Diarrhoea in the Tshikuwi Community in Venda, South Africa. *Journal of Health, Population and Nutrition*. 27 (5): 652–659.

Boerlin, P. and Reid-Smith, R.J. (2008). Antimicrobial resistance: its emergence and transmission. *Animal Health Research Reviews*. 9 (2): 115–126.

Boxrud, D., Pederson-Gulrud, K., Wotton, J., Medus, C., Lyszkowicz, E., Besser, J. and Bartkus, J.M. (2007). Comparison of multiple-locus variable-number tandem repeat analysis, pulsed-field gel electrophoresis and phage typing for subtype analysis of *Salmonella enterica* serotype Enteritidis. *Journal of Clinical Microbiology*. 45 (2): 536-543.

Cabello, F., Hormaeche, C., Mastroeni, P. and Bonina, L. (1993). *Biology of Salmonella*. Ed 245. New York: Plenum press. pp 63-77.

Campioni, F., Davies, M., Medeiros, M.L.C., Falcão, J.P. and Shah, D,H. (2013). MLVA typing reveals higher genetic homogeneity among *Salmonella* Enteritidis strains isolated from food, humans and chickens in Brazil in comparison to the

North American Strains. *International Journal of Food Microbiology*. 162: 174-181.

Carattoli, A. (2009). Resistance plasmid families in *Enterobacteriaceae*. *Antimicrobial Agents and Chemotherapy*. 53 (6): 2227–2238.

Carlet, J., Jarlier, V., Harbarth, S., Voss, A., Goossens, H., Pittet, D. and Participants of the 3rd World Healthcare-Associated Infections Forum. (2012). Ready for a world without antibiotics? The Pensières Antibiotic Resistance Call to Action. *Antimicrobial Resistance and Infection Control*. 1 (11) http://www.aricjournal.com/content/1/1/11.

Centers for Disease Control and Prevention (CDC). (2013). *National antimicrobial resistance monitoring system: Enteric bacteria (Human isolates final report)*. Available at: http://www.cdc.gov/narms/pdf/2013-annual-report-narms-508c.pdf [Accessed 18 May 2016].

Centres for Disease Control and Prevention (CDC). (2011). Vital signs: incidence and trends of infection with pathogens transmitted commonly through food - foodborne diseases active surveillance network, 10 U.S. Sites, 1996-2010. *Morbidity and Mortality Weekly Report* (*MMWR)*. 60 (22): 749-55.

Chai, S.J., White, P.L., Lathrop, S.L., Solghan, S.M., Medus, M., McGlinchey, B.M., Tobin-D'Angelo, M., Marcus, R. and Mahon, B.E. (2012). *Salmonella enterica* serotype Enteritidis: Increasing incidence of domestically acquired infections. *Clinical Infectious Diseases*. 54 (5): 488-497.

Chamberlain, J.S., Gibbs, R.A., Ranier, J.E., Nguyen, P.N. and Caskey, C.T. (1988). Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. *Nucleic Acids Research*.16: 11141-11156.

Chart, H., Cheesbrough, J.S. and Waghorn, D.J. (2000). The serodiagnosis of infection with *Salmonella* Typhi. *Journal of Clinical Pathology*. 11 (53): 851-853.

Chen, P.L., Lee, H.C., Lee, N.Y., Wu, C.J., Lin, S.H., Shih, H.I., Lee, C.C., Ko, W.C. and Chang, C.M. (2012). Non-typhoidal *Salmonella* bacteraemia in elderly patients: an increased risk for endovascular infections, osteomyelitis and mortality. *Epidemiology and Infection*. 140: 2037-2044.

Cheung, T.K.M., Chu, Y.W., Chu, M.Y., Ma, C.H., Yung, R.W.H. and Kam, K.M. (2005). Plasmid-mediated resistance to ciprofloxacin and cefotaxime in clinical isolates of *Salmonella enterica* serotype Enteritidis in Hong Kong. *Journal of Antimicrobial Chemotherapy*. 56: 586–589.

Chiu, C. and Su, L. (2014). *Salmonella*, non-typhoidal species (*Salmonella* Choleraesuis, *Salmonella* Enteritidis, *Salmonella* Hadar, *Salmonella* Typhimurium) - Infectious disease and antimicrobial agents. Available at: http://www.antimicrobe.org/new/b258.asp [Accessed 16 October 2015].

Cho, S., Boxrud, D.J., Bartkus, J.M., Whittam, T.S. and Saeed, M. (2007). Multiple locus variable-number tandem-repeat analysis of *Salmonella* Enteritidis isolates from human and non-human sources using a single multiplex PCR. *FEMS Microbiology Letters*. 275 (1): 16-23.

Cho, S., Whittam, T.S., Boxrud, D.J., Bartkus, J.M. and Saeed, A.M. (2008). Allele distribution and genetic diversity of VNTR loci in *Salmonella enterica* serotype Enteritidis isolates from different sources. *BMC Microbiology*. 8 (146): 1-11.

Cho, S., Whittam, T.S., Boxrud, D.J., Bartkus, J.M., Rankin, S.C., Wilkins, M.J., Somsel, P., Downes, F.P., Musser, K.A., Root, T.P., Warnick, L.D., Wiedmann, M. and Saeed, A.M. (2010). Use of multiple-locus variable number tandem repeat analysis and phage typing for subtyping of *Salmonella* Enteritidis from sporadic human cases in the United States. *Journal of Applied Microbiology*.108 (3): 859-867.

Clinical and Laboratory Standards Institute. (2013). *Performance standards for antimicrobial susceptibility testing; twenty-third informational supplement*. Pennsylvania: CLSI document M100-S23.

Clinical and Laboratory Standards Institute. (2014). *Performance standards for antimicrobial susceptibility testing; twenty-fourth informational supplement*. Pennsylvania: CLSI document M100-S24.

Clinical and Laboratory Standards Institute. (2015). *Performance standards for antimicrobial susceptibility testing; twenty-fifth informational supplement*. Pennsylvania: CLSI document M100-S25.

Cogan, T.A. and Humphrey, T.J. (2003). The rise and fall of *Salmonella* Enteritidis in the UK. *Journal of Applied Microbiology*. 94: 114-119.

Commission of the European Communities. (2000). *White paper on food safety of 12 January 2000 (COM/99/0719).* Available at: http://ec.europa.eu/dgs/health_food-safety/library/pub/pub06_en.pdf [Accessed 06 April 2015].

Davies, R.H. and Wray, C. (1995). Mice as carriers of *Salmonella* Enteritidis on persistently infected poultry units. *The Veterinary Record*. 137 (14): 337-341.

Dawson MN (2012) Parallel phylogeographic structure in ecologically similar sympatric sister taxa. *Molecular Ecology*. 21 (4): 987-1004.

Den Bakker, H.C., Allard, M.W., Bopp, D., Brown, E.W., Fontana, J., Iqbal, Z., Kinney, A., Limberger, R., Musser, K.A., Shudt, M., Strain, E., Wiedmann, M. and Wolfgang, W.J. (2014). Rapid whole-genome sequencing for surveillance of *Salmonella enterica* serovar Enteritidis. *Emerging Infectious Diseases*. 20 (8): 1306-1314.

Department of Agriculture, Forestry and Fisheries (DAFF). and Agricultural research council- Ondersterpoort Veterinary Institute-ARC. (2000). *Poultry*

diseases. Available at: http://www.nda.agric.za/docs/poultry/poultrydisease.htm [Accessed 17 October 2015].

Dropulic, L.K. and Lederman, H.M. (2009). *Overview of infections in the immunocompromised host: diagnostic microbiology of the immunocompromised host*. Washington, DC: ASM Press. pp 3–43.

Duchet-Suchaux, M., Lechopier, P., Marly, J., Bernardet, P., Delaunay, R. and Pardon, P. (1995). Quantification of experimental *Salmonella* Enteritidis carrier state in B13 leghorn chicks. *Avian diseases*. 39 (4): 796-803.

Dutta, T.K., Parija, S.C., Dutta, J.K. (2012). *Emerging and Re-emerging infectious diseases*. New Delhi: JP Medical Ltd. pp 42-43.

Dwarkin, M. and Alkow, S. (2006). *The prokaryotes*. 6 vol. Proteobacteria; Gamma subclass. New York: Springer. pp 811-835.

Eagar, H., Swan, G. and van Vuuren, M. (2012). A survey of antimicrobial usage in animals in South Africa with specific reference to food animals. *Journal of the South African Veterinary Association*. 83: 15-23.

Eaves, D.J., Randall, L., Gray, D.T., Buckley, A., Woodward, M.J., White, A.P. and Piddock, L.J.V. (2004). Prevalence of mutations within the quinolone resistance-determining region of *gyrA*, *gyrB*, *parC*, and *parE* and association with antibiotic resistance in quinolone-resistant *Salmonella enterica*. *Antimicrobial Agents Chemotherapy*. 48 (10): 4012–4015.

Eibach, D., Al-Emran, H.M., Dekker, D.M., Krumkamp, R., Adu-Sarkodie, Y., Espinoza, L.M.C., 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., Marks, F. and May J. (2016). The Emergence of Reduced Ciprofloxacin Susceptibility in *Salmonella enterica* Causing Bloodstream Infections in Rural Ghana. *Clinical Infectious Diseases*. 62 (1): 32-36.

Elzouki, E., Harfi, H.A., Nazer, H.M., Stapleton, F.B., Oh, W. and Whitley, R.J. (2012). *Textbook of clinical paediatrics*. Berlin: Springer. pp 10301.

Enwere, G., Biney, E., Cheung, Y.B., Zaman, S.M., Okoko, B., Oluwalana, C., Vaughn, A., Greenwood, B., Adegbola, R. and Cutts, F.T. (2006). Epidemiologic and clinical characteristics of community-acquired invasive bacterial infections in children aged 2–29 months in the Gambia. *The Paediatric Infectious Disease Journal*. 25: 700–705.

European Food Safety Authority (EFSA). (2007). *The community summary report on trends and sources of zoonoses, zoonotic agents, antimicrobial resistance and foodborne outbreaks in the European Union in 2006*. 130. Parma, Italy: The EFSA Journal, pp 252-253. Available at: http://www.efsa.europa.eu/en/scdocs/scdoc/130r.htm [Accessed 06 October 2015].

European Food Safety Authority. (2009). *The Community summary report on trends and sources of zoonotic agents in the European Union in 2007*. 223. Parma, Italy: The EFSA Journal 2009, pp 23-29. Available at: http://ecdc.europa.eu/en/publications/Publications/EU-summary-report-trends-sources-of-zoonoses-zoonotic-agents-food-borne-uutbreaks-in-2007.pdf [Accessed 16 April 2015].

European Food Safety Authority. (2014). *Cluster of monophasic Salmonella Typhimurium with previously unseen MLVA pattern in the EU/EEA: Rapid outbreak assessment*. Available at: http://onlinelibrary.wiley.com/doi/10.2903/sp.efsa.2014.EN-657/abstract [Accessed 18 April 2016].

Food and Agriculture Organisation (FAO). and World Health Organisation (WHO). (2002). *Improving Efficiency and Transparency in Food Safety Systems - Sharing Experiences: Proceedings of the Forum: Global Forum of Food Safety Regulators, 28-30 January 2002, Marrakesh, Morocco.* Vol 1. Rome: Food & Agriculture Organisation. pp 136-137.

Feasey, N.A., Archer, B.N., Heyderman, R.S., Sooka, A., Dennis, B., Gordon, M.A. and Keddy, K.H. (2010). Typhoid fever and invasive nontyphoid salmonellosis, Malawi and South Africa. *Emerging Infectious Diseases*. 16 (9): 1448–1451.

Feasey, N.A., Dougan, G., Kingsley, R.A., Heyderman, R.S. and Gordon, M.A. (2012). Invasive non-typhoidal *Salmonella* disease: an emerging and neglected tropical disease in Africa. *Lancet*. 379 (9835): 2489–2499.

Feasey, N.A., Hadfield, J., Keddy, K.H., Dallman, T.J., Jacobs, J., Deng, X., Wigley, P., Barquist, L., Langridge, G.C., Feltwell, T., Harris, S.R., Mather, A.E., Fookes, M., Aslet,t M., Msefula, C., Kariuki, S., Maclennan, C.A., Onsare, R.S., Weill, F.X., Le, Hello, S., Smith, A.M., McClelland, M., Desai, P., Parry, C.M., Cheesbrough, J., French, N., Campos, J., Chabalgoity, J.A., Betancor, L., Hopkins, K.L., Nair, S., Humphrey, T.J., Lunguya, O., Cogan, T.A., Tapia, M.D., Sow, S.O., Tennant, S.M., Bornstein, K., Levine, M.M., Lacharme-Lora, L., Everett, D.B., Kingsley, R.A., Parkhill, J., Heyderman, R.S., Dougan, G., Gordon, M.A. and Thomson, N.R. (2016). Distinct *Salmonella* Enteritidis lineages associated with enterocolitis in high-income settings and invasive disease in low-income settings. *Nature Genetics*. 48 (10): 1211-1217.

Flint, J.A., van Duynhoven, Y.T., Angulo, F.J., DeLong, S.M., Braun, P., Kirk, M., Scallan, E., Fitzgerald, M., Adak, G.K., Sockett, P., Ellis, A., Hall, G., Gargouri, N., Walker, H. and Braam, P. (2005). Estimating the burden of acute gastroenteritis, foodborne disease, and pathogens commonly transmitted by food: an international review. *Clinical Infectious Diseases*. 41 (5): 698–704.

Foley, S.L. and Lynne, A.M. (2008). Food animal-associated *Salmonella* challenges: pathogenicity and antimicrobial resistance. *Journal of Animal Science*. 86 (14): 173-187.

Foxman, B. and Riley, L. (2001). Molecular Epidemiology: Focus on infection. *American Journal of Epidemiology*. 153 (12): 1135-1141.

Galanakis, E., Bitsori, M., Maraki, S., Giannakopoulou, C., Samonis, G. and Tselentis, Y. (2007). Invasive non-typhoidal salmonellosis in immunocompetent infants and children. *International Journal of Infectious Diseases*. 11 (1): 36–39.

Galanis, E., Wong, M.A.L.F., Patrick, M.E., Binsztein, N., Cieslik, A., Chalermchaikit, T., Aidara-Kane, A., Ellis, A., Angulo, F.J. and Wegner, H. (2006). Web-based surveillance and global *Salmonella* distribution, 2000–2002. *Emerging Infectious Diseases*. 12 (3): 381-388.

Gal-Mor, O., Boyle, E.C. and Grassl, G.A. (2014). Same species, different diseases, how and why typhoidal and non-typhoidal *Salmonella enterica* serovars differ. *Frontiers in Microbiology*. 5 (391):1-10.

Gantois, I., Ducatelle, R., Pasmans, F., Haesebrouch, F., Gast, R., Humphery, T.J. and van Immerseel, F. (2009). Mechanisms of egg contamination by *Salmonella* Enteritidis. *FEMS Microbiology Reviews*. 33 (4): 718-738.

Garces, L. (2002). *The detrimental impacts of industrial animal agriculture; A case for humane and sustainable agriculture, Compassion in the World Farming Trust*. Available at: http://www.ciwf.org.uk/includes/documentation_docs/2008/d/detrimental_impact_industrial_animal_agriculture_2002.pdf [Accessed 30 July 2015].

Gaynor, M. and Mankin, A.S. (2003). Macrolide Antibiotics: Binding Site, Mechanism of Action, Resistance. *Current Topics in Medicinal Chemistry*. 3: 949-961.

Giannella, R.A. (1996). *Medical Microbiology*. 4th Ed. Texas: University of Texas. Chap 21.

Gillespie, S., Hawkey, P.M. and Jenkins, C. (2006). *Principles and practice of clinical bacteriology*. 2nd Ed. England: John Wiley and Sons. pp 367-372.

Goetez, G. (2012). *Resistance to Important Antibiotic May Develop in Soil, Study Shows*. Available at: http://www.foodsafetynews.com/ [Accessed 03 May 2015].

Gorbach, S.L., Bartlett, J.G. and Blacklow, N.R. (2004). *Infectious diseases*. 3rd Ed. Philadelphia: Lippincott Williams and Wilkins. pp 624-625.

Gordon, M.A., Graham, S.M., Walsh, A.L., Wilson, L., Phiri, A., Molyneux, E., Zijlstra, E.E., Heyderman, R.S., Hart, A. and Molyneux, M.E. (2008). Epidemics of invasive *Salmonella enterica* serovar Enteritidis and *Salmonella enterica* serovar Typhimurium infection associated with multidrug resistance among adults and children in Malawi. *Clinical Infectious Diseases*. 46 (7): 963-969.

Gordon, M.A. (2009). *Salmonella* infections in immnunocompromised adults. *Journal of Infection*. 56: 413-422.

Graham, S.M. (2002). Salmonellosis in children in developing and developed countries and populations. *Current Opinion in Infectious Diseases*. 15 (5): 507-512.

Graham, S.M., Molyneux, E.M., Walsh, A.L., Cheesbrough, J.S., Molyneux, M.E. and Hart, C.A. (2000a). Nontyphoidal *Salmonella* infections of children in tropical Africa. *The Paediatric Infectious Disease Journal*. 19 (12): 1189–96.

Graham, S.M., Walsh, A.L., Molyneux, E.M., Phiri, A.J. and Molyneux, M.E. (2000b). Clinical presentation of non-typhoidal *Salmonella* bacteraemia in Malawian children. *Transactions of the Royal Society of Tropical Medicine and Hygiene*. 94: 310–314.

Gray, J.T., Hungerford, L.L., Fedorka-Cray, P.J. and Headrick, M.L. (2004). Extended-spectrum-cephalosporin resistance in *Salmonella enterica* isolates of animal origin. *Antimicrobial Agents and Chemotherapy*. 48 (8): 3179-3181.

Grimont, A.D. and Weill, F.X. (2007). *Antigenic formulae of the Salmonella serovars*. 9th Ed. WHO Centre for Reference and Research on *Salmonella*.

Institut Pasteur. France: http://www.pasteur.fr/ip/portal/action/WebdriveActionEvent/oid/01s-000036-089. [Accessed 10 April 2015].

Group for Enteric Respiratory and Meningeal disease Surveillance in South Africa 2012. (2012). GERMS - *SA Annual Report 2012*. Johannesburg: 11-14.

Gruner, E., Martinetti, Lucchini, G., Hoop, R.K. and Altwegg, M. (1994). Molecular epidemiology of *Salmonella* Enteritidis. *Europe Journal of Epidemiology*. 10 (1): 85-89.

Guerrant, R.L., van, G.T., Steiner, T.S., Thielman, N.M., Slutsker, L., Tauxe, R.V., Hennessy, T., Griffin, P.M., DuPont, H., Sack, R.B., Tarr, P., Neill, M., Nachamkin, I., Reller, L.B., Osterholm, M.T., Bennish, M.L. and Pickering, L.K. (2001). Practice guidelines for the management of infectious diarrhoea. *Clinical Infectious Diseases*. 32: 331–351.

Haguenoer, E., Baty, G., Pourcel, C., Lartigue, M., Domelier, A., Rosenau, A., Quentin, R., Mereghetti, L. and Lanotte, P. (2011). A multi locus variable number of tandem repeat analysis (MLVA) scheme for *Streptococcus agalactiae* genotyping. *BMC Microbiology*. 11 (171): 1-13.

Hall, G., Kirk, M.D., Becker, N., Gregory, J.E., Unicomb, L., Millard, G., Stafford, R., Lalor, K. and OzFoodNet Working Group. (2005). Estimating foodborne gastroenteritis, Australia. *Emerging Infectious Diseases*. 11 (8): 1257–1264.

Hamer, D.V. and Gill, C.J. (2008). Balancing individual benefit against public health risk: The impact of cotrimoxazole prophylaxis in HIV-infected patients on antimicrobial resistance. *The American Society of Tropical Medicine and Hygiene*. 79 (3): 299–300.

Hasman, H., Saputra, D., Sicheritx-Ponten, T., Svendsen, C.A., Frimodt-Møller, N. and Aarestrup, F.M. (2014). Rapid whole-genome sequencing for detection

and characterization of microorganisms directly from clinical samples. *Journal of Clinical Microbiology*. 52 (1): 139-146.

Helmuth, R., Stephan, R., Bunge, C., Hoog, B., Steinbeck, A. and Bulling, E. (1985). Epidemiology of virulence-associated plasmids and outer membrane protein patterns within seven common *Salmonella* serotypes. *Infection and Immunity*. 48: 175-182.

Henton, M.M., Eagar, H.A., Swan, G.E., van Vuuren, M. (2011). Antibiotic management and resistance in livestock production. *South African Medical Journal*.101 (8): 583-586.

Herschleb, J., Ananiev, G. and Schwartz, D.C. (2007). Pulsed-field gel electrophoresis. *Nature Protocols*. 2: 677-684.

Hickman-Brenner, F.W., Stubbs, A.D. and Farmer, J.J. (1991). Phage typing of *Salmonella* Enteritidis in the United States. *Journal of Clinical Microbiology*. 29: 2817–2823.

Hoelzer, K., Switt, A.I.M., Wiedmann, M. (2011). Animal contact as a source of human non-typhoidal salmonellosis. *Veterinary research*. 42 (34): 1-27.

Hohmann, E.L. (2001). Nontyphoidal salmonellosis. *Clinical Infectious Diseases*. 32: 263–269.

Hooper, D.C. (2001). Emerging mechanisms of fluoroquinolone resistance. *Emerging Infectious Diseases*. 7 (2): 337-341.

Hopkins, K.L., Davies, R.H. and Threlfall, E.J. (2005). Mechanisms of quinolone resistance in *Escherichia coli* and *Salmonella*: recent developments. *International Journal of Antimicrobial Agents*. 25 (5): 358–373.

Hopkins, K.L., Peters, T.M., de Pinna, E. and Wain, J. (2011). Standardization of multilocus variable-number of tandem-repeat analysis (MLVA) for subtyping of *Salmonella enterica* serovar Enteritidis. *Eurosurveillance*. 16 (32): 19942-19952.

Huehn, S., La, Ragione, R.M., Anjum, M., Saunders, M., Woodward, M.J., Bunge, C., Helmuth, R., Hauser, E., Guerra, B., Beutlich, J., Brisabois, A., Peters, T., Svensson, L., Madajczak, G., Litrup, E., Imre, A., Herrera-Leon, S., Mevius, D., Newell, D.G. and Malorny, B. (2010). Virulotyping and antimicrobial resistance typing of *Salmonella enterica* serovars relevant to human health in Europe. *Foodborne Pathogens and Disease*. 7 (5): 523–535.

Hughes, J.M. (2011). Preserving the lifesaving power of antimicrobial agents. *The Journal of the American Medical Association's*. 305 (10): 1027-1028.

Imen, B.S., Ridha, M. and Mahjoub, A. (2012). *Laboratory Typing Methods for Diagnostic of Salmonella Strains, the "Old" Organism That Continued Challenges, Salmonella - A Dangerous Foodborne Pathogen, Dr. Dr. Barakat S M Mahmoud (Ed.)*. Available at: http://cdn.intechopen.com/pdfs-wm/26435.pdf [Accessed 12 December 2014].

Indar-Harrinauth, L., Daniels, N., Prabhakar, P., Brown, C., Baccus-Tylor, G., Comissiong, E. and Hospedales, J. (2001). Emergence of *Salmonella* Enteritidis Phage Type 4 in the Caribbean: Case-control study in Trinidad and Tobago, West Indies. *Clinical Infectious Diseases*. 32 (6): 890-896.

Jiayong, Z., Yukai, Z., Zhiqiang, X., Jingiing, P., Jia, S., Yujiao, M., Xueyong, H., Baifan, Z. and Shengli, X. (2015). Characteristics of drug resistance and molecular typing research for *Salmonella* Enteritidis isolated in Henan Province from 2011 to 2013. *Chinese Journal of Preventative Medicine*. 50 (3): 261-265.

Koninklijke Nederlandse Vereniging voor Microbiologie (KNVM). (2014). *Multiple-Locus variable number tandem repeat Analysis (MLVA)*. Available from: http://www.microtyping.nl [Accessed 13 October 2015].

Kariuki, S., Revathi, G., Kiiru, J., Mengo, D.M., Mwituria, J., Muyodi, J., Munyalo, A., Teo, Y., Holt, K.E., Kingsley, R.A. and Dougan, G. (2010). Typhoid in Kenya is associated with a dominant multidrug-resistant *Salmonella enterica* serovar Typhi haplotype that is also widespread in Southeast Asia. *Journal of Clinical Microbiology*. 48 (6): 2171-2176.

Keddy, K.H., Dwarika, S., Crowther, P., Perovic, O., Wadula, J., Hoosen, A., Sooka, A., Crewe-Brown, H.H. and Smith, A.M. (2009). Genotypic and demographic characterization of invasive isolates of *Salmonella* Typhimurium in HIV co-infected patients in South Africa. *Journal of Infection in Developing Countries*. 3 (8): 585-592.

Khanum, S., Saba, N.V., Qayyum, M.I.B. and Qazilbash, A.A. (2006). Distribution patterns of *Salmonella* infection in Rawalpindi/Islamabad area and the risks factors associated with the disease prevalence. *Journal of Biological Sciences*. 6: 253-260.

Kidanermariam, A., Engelbrecht, M. and Picard, J. (2010). Retrospective study on the incidence of *Salmonell*a isolations in animals in South Africa, 1996 to 2006. *Journal of the South African Veterinary Association*. 8 (1): 37–44.

Kirk, M.D., Pires, S.M., Black, R.E., Caipo, M., Crump, J.A., Devleesschauwer, B., Döpfer, D., Fazil, A., Fischer-Walker, C.L., Hald, T., Hall, A., Keddy, K.H., Lake, R.J., Lanata, C.F., Torgerson, P.R., Havelaar, A.H. and Angulo, F.J. (2015). World Health Organization estimates of the global and regional disease burden of 22 foodborne bacterial, protozoal, and viral diseases, 2010: a data synthesis. *PLOS Medicine*. 12 (12): e1001921. doi:10.1371/ journal.pmed.1001921.

Kosek, M., Bern, C. and Guerrant, R.L. (2003). The global burden of diarrhoeal disease, as estimated from studies published between 1992 and 2000. *Bulletin World Health Organisation*. 81: 197–203.

Kotetishvili, M., Stine, O.C., Kreger, A., Morris, J.G. and Sulakvelidze, A. (2002). Multilocus sequence typing for characterization of clinical and environmental *Salmonella* strains. *Journal of Clinical Microbiology*. 40: 1626-1635.

Kramer, A., Kretzschmer, M. and Krickeberg, K. (2010). *Modern Infectious Disease Epidemiology; Concepts, Methods, Mathematical models and Public health. Germany*: Springer. pp 125-126.

Kumar, K., Gupta, S.C., Chander, Y. and Singh, A.K. (2005). Antibiotic use in agriculture and its impact on the terrestrial environment. *Advances in Agronomy*. 87: 1-54.

Kwong, J.C., McCallum, N., Sintchenko, V. and Howden, B.P. (2015). Whole genome sequencing in clinical and public health microbiology. *Pathology*. 47 (3): 199-210.

Landeras, E., Onzalez-Hevia, M.A., Alzugaray, R. and Mendoza, M.C. (1996). Epidemiological differentiation of pathogenic strains of *Salmonella* Enteritidis by ribotyping. *Journal of Clinical Microbiology*. 34: 2294-2296.

Landers, T.F., Cohen, B., Wittum, T.E. and Larson, E.L. (2012). A review of antibiotic use in food animals: perspective, policy, and potential. *Public Health Reports*. 127 (1): 4–22.

Langridge, G., Wain, J. and Nair, S. (2012). Invasive salmonellosis in humans. *EcoSal Plus*. 5 (1). doi:10.1128.

Larsson. J.T., Torpdahl, M., Petersen, R.F., Sorensen, G., Lindstedt. B.A. and Nielsen, E.M. (2009). Development of a new nomenclature for *Salmonella* Typhimurium multilocus variable-number of tandem- repeats analysis (MLVA). *Eurosurveillance*.14 (15). pii: 19174.

Lee, S.H., Jung, B.Y., Rayamahji, N., Lee, H.S., Jeon, W.J., Choi, K.S., Kweon, C.H. and Yoo, H.S. (2009). A multiplex real-time PCR for differential detection

and quantification of *Salmonella* spp., *Salmonella enterica* serovar Typhimurium and Enteritidis in meats. *Journal of Veterinary Science*.10 (1): 43-51.

Lee, K.M., Runyon, M., Herrman, T.J., Phillips, R. and Hsieh, J. (2015). Review of *Salmonella* detection and identification methods: Aspects of rapid emergency response and food safety. *Food Control*. 47: 264-276.

Liebisch, B. and Schwarz, S. (1996). Molecular typing of *Salmonella enterica* subsp. *enterica* serovar Enteritidis isolates. *Journal of Medical Microbiology*. 44: 52-59.

Lin, A.W., Usera, M.A., Barrett, T.J. and Goldsby, R.A. (1996). Application of random amplified polymorphic DNA analysis to differentiate strains of *Salmonella* Enteritidis. *Journal of Clinical Microbiology*. 34: 870-876.

Linton, A.H. (1977). Antibiotic resistance: the present situation reviewed. *Veterinary Record*. 100 (17): 354–360.

Liu, L., Johnson, H.L., Cousens, S., Perin, J., Scott, S., Lawn, J.E., Rudan, I., Campbell, H., Cibulskis, R., Li, M., Mathers, C., Black, R.E., for the Child Health Epidemiology Reference Group of WHO and UNICEF. (2012). Global, regional, and national causes of child mortality: an updated systematic analysis for 2010 with time trends since 2000. *Lancet*. 379: 2151–61.

Lorian, V. (2005). *Antibiotics in Laboratory Medicine*. 5th Ed. Philadelphia: Lippincott Williams and Wilkins. pp 551-552.

Lund, B.M. and O'Brien, S.J. (2011). The occurrence and prevention of foodborne disease in vulnerable people. *Foodborne Pathogens and Disease*. 8 (9): 961-973.

Maiden, M.C.J., Graves, J.A., Feil, E., Morelli, G., Russell, J.E., Urwin, R., Zhang, Q., Zhou, J., Zurth, K., Caugant, D.A., Feavers, I.M., Achtman, M. and Spratt, B.G. (1998). Multilocus sequence typing: a portable approach to the identification

of clones within populations of pathogenic microorganisms. *Proceedings of the National Academy of Sciences.* 95: 3140- 3145.

Majowicz, S.E., Musto, J., Scallan, E., Angulo, F.J., Kirk, M., O'Brien, S.J., Jones, T.F., Fazil, A. and Hoekstra, R.M. (2010). The global burden of nontyphoidal *Salmonella* gastroenteritis. *Food Safety*. 50 (6): 882-889.

Malorny, B., Bunge, C. and Helmuth, R. (2007). A real-time PCR for the detection of *Salmonella* Enteritidis in poultry meat and consumption eggs. *Journal of microbiological methods*. 70 (2): 245-251.

Malorny B, Junker, E. and Helmuth, R. (2008). Multi-locus variable-number tandem repeat analysis for outbreak studies of *Salmonella enterica* serotype Enteritidis. *BMC Microbiology*. 8 (84): 1-8.

Malorny, B., Paccassoni, E., Fach, P., Bunge, C., Martin, A., Helmuth, R. (2004). Diagnostic real-time PCR for detection of *Salmonella* in food. *Applied and Environmental Microbiology*. 70: 7046-7052.

Mandomando, I., Macete, E., Sigaúque, B., Morais, L., Quintó, L., Sacarlal, J., Espasa, M., Vallés, X., Bassat, Q., Aide, P., Nhampossa, T., Machevo, S., Ruiz, J., Nhacolo, A., Menéndez, C., Kotloff, K.L., Roca, A., Levine, M.M. and Alonso, P.L. (2009). Invasive non-typhoidal *Salmonella* in Mozambican children. *Tropical Medicine and International Health*. 14 (12): 1467–1474.

Mangni, M.V. and Arvntikis, C. (2010). *Detection of bacteria; viruses; parasites and fungi: Bioterrorism prevention*. Netherlands: Springer Science. pp 125-137.

McDermott, P.F., Walker, R.D. and White, D.G. (2003). Antimicrobials: modes of action and mechanisms of resistance. *International Journal of Toxicology*. 22 (2): 135–143.

McEwen, S.A. and Fedorka-Cray, P.J. (2002). Antimicrobial use and resistance in animals. *Clinical Infectious Diseases*. 34 (3): 93–106.

McKeown, P., Garvey, P. and Cormican, M. (2012). *Elucidating the epidemiology of human salmonellosis: The value of systematic laboratory characterisation of isolates, Salmonella - distribution, adaptation, control Measures and molecular technologies*. Available at: http://www.intechopen.com/books/salmonella-distribution-adaptation-control-measures-and-molecular-technologies/elucidating-the-epidemiology-of-human-salmonellosis-the-value-of-systematic-laboratory-characterisat [Accessed: 15 July 2015].

McQuiston, J.R., Waters, R.J., Dinsmore, B.A., Mikoleit, M.L. and Fields, P.I. (2010). Molecular determination of H antigens of *Salmonella* by use of a microsphere-based liquid array. *American Society of Microbiology*. 49 (2): 565-573.

Medalla, F., Sjölund-Karlsson, M., Shin, S., Harvey, E., Joyce, K., Theobald, L., Nygren, B.L., Pecic, G., Gay, K., Austin, J., Stusrt, A., Blanton, E., Mintz, E.D., Whichard, J.M. and Barzilay, E.J. (2011). Ciprofloxacin-resistant *Salmonella enterica* serotype Typhi, United States, 1999–2008. *Emerging Infectious Diseases*. 17 (6): 1095-1098.

Mendoza, M.C., Landeras, E., Saeed, AM., Gast, R.E., Potter, M.E. and Wall, P.G. (1999). *Molecular epidemiological methods for differentiation of Salmonella enterica serovar Enteritidis strains in humans and animals: epidemiology, pathogenesis, and control*. Iowa: Iowa University Press, Ames. pp 125-140.

Meštrović, T. (2015). *Salmonella History*. Accessed at: http://www.news-medical.net/health/Salmonella-History.aspx [Accessed 23 October 2015].

Millemann, Y., Lesage, M.C., Chaslus-Dancla, E. and Lafont, J.P. (1995). Value of plasmid profiling, ribotyping and detection of IS200 for tracing avian isolates of *Salmonella* Typhimurium and *Salmonella* Enteritidis. *Journal of Clinical Microbiology.* 33: 173-179.

Ministry of Health Singapore. (1996). An outbreak of food poisoning caused by *Salmonella* Enteritidis. *Epidemiological News Bulletin*. 22: 51–53.

Mølbak. K., Baggesen, D.L., Aarestrup, F.M., Ebbesen, J.M., Engberg, J., Frydendahl, K., Gerner-Smidt, P., Petersen, A.M. and Wegener, H.C. (1999). An outbreak of multidrug-resistant, quinolone-resistant *Salmonella enterica* serotype Typhimurium DT104. *New England Journal Medicine*. 341 (19): 1420-1425.

Morpeth, S.C., Ramadhani, H.O. and Crump, J.A. (2009). Invasive non-typhi *Salmonella* disease in Africa. *Clinical Infectious Diseases.* 49 (4): 606-611.

Morris, J.G., Dwyer, Jr., Hoge, D.M., Stubbs, C.W., Tilghman, A.D., Groves, D., Israel, C.E. and Libonati, J.P. (1992). Changing clonal patterns of *Salmonella* Enteritidis in Maryland: evaluation of strains isolated between 1985 and 1990. *Journal of Clinical Microbiology*. 30: 1301-1303.

Moyane, J.N., Jideani, A.I.O. and Aiyegoro, O.A. (2013). Antibiotics usage in food-producing animals in South Africa and impact on human: Antibiotic resistance. *Academic Journals*. 74 (24): 2990-2997.

Murphy, M., Minihan, D., Buckley, J.F., O'Mohony, M., Whyte, P. and Fanning, S. (2008). Multiple-locus variable-number of tandem-repeat analysis (MLVA) of Irish verocytoxigenic *Escherichia* coli O157 from feedlot cattle uncovering strain dissemination routes. *BMC Veterinary Research*. 4 (2): 1-9.

Murray, P.R., Baron, E., Jorgensen, J.H., Landry, M.L., and Pfaller, M.A. (2007). *Manual of clinical microbiology*. 9th Ed. Washington: ASM press. pp 681.

Nadon, C.A., Trees, E., Ng, L.K., Møller, Nielsen, E., Reimer, A., Maxwell, N., Kubota, K.A., Gerner-Smidt, P. and the MLVA Harmonization Working Group. (2013). Development and application of MLVA methods as a tool for inter-laboratory surveillance. *Eurosurveillance*.18 (35). pii: 20565.

National Department of Health. (2015). *Antimicrobial resistance national strategy framework 2014-2024*. Pretoria: Department of Health. pp 1-17. Available at: http://www.mm3admin.co.za/documents/docmanager/3C53E82B-24F2-49E1-B997-5A35803BE10A/00090160.pdf [Accessed 6 April 2016].

National Department of Health South Africa. (2015). *National consolidated guidelines: for the prevention of mother-to-child transmission of HIV (PMTCT) and the management of HIV in children, adolescents and adults.* Available at: http://www.sahivsoc.org/Files/ART%20Guidelines%2015052015.pdf. [Accessed 21 April 2017].

National Institute for Communicable Diseases. (2016). *GERMS-SA*. Available at: http://www.nicd.ac.za/?page=germs-sa&id=97 [Accessed July 2016].

Nesbitt, A., Ravel, A., Murray, R., Mccormick, R., Savelli, C., Finely, R., Parmely, J, Agunos, A., Majowicz, S.E. and Gilmour, M. (2012). Integrated surveillance and potential sources of *Salmonella* Enteritidis in human cases in Canada from 2003 to 2009. *Epidemiology Infection*. 140 (10): 1757–1772.

Ng, D.P., Goh, K.T., Yeo, M.G.C. and Poh, C.L. (1997). An institutional outbreak of *Salmonella* Enteritidis in Singapore. *The Southeast Asian Journal of Tropical Medicine and Public Health*. 28: 85–90.

Noller, A.C., McEllistrem, M.C., Shutt, K.A. and Harrison, L.H. (2006). Locus-specific mutational events in a Multilocus variable-number of tandem-repeat analysis of *Escherichia coli* O157:H7. *Journal of Clinical Microbiology*. 44 (2): 374-377.

Olive, D.M. and Bean, P. (1999). Principles and applications of methods for DNA-based typing of microbial organisms. *Journal of Clinical Microbiology*. 37 (6): 1661-1669.

O'Regan, E., McCabe, E., Burgess, C., McGuinness, S., Barry, T., Duffy, G., Whyte, P. and Fanning, S. (2008). Development of a real-time multiplex PCR assay for the detection of multiple *Salmonella* serotypes in chicken samples. *BMC Microbiology*. 8:156.

Park, H.J., Kim, H.J., Park, S.H., Shin, E.G., Kim, J.H. and Kim, H.Y. (2008). Direct and quantitative analysis of *Salmonella enterica* Serovar Typhimurium

using real-time PCR from artificially contaminated chicken meat. *Journal of Microbiology and Biotechnology*.18:1453-1458.

Parnham, M.J., Bruinvels, J., Schönfeld, W. and Kirst, H.A. (2002). *Macrolide antibiotics: milestones in drug therapy*. Birkhäuser: Springer Basel. pp 1-8.

Parry, C.M. and Threlfall, E.J. (2008). Antimicrobial resistance in typhoidal and nontyphoidal salmonellae. *Current Opinion in Infectious Diseases*. 21 (5): 531–538.

Peters, T., Bertrand, S., Björkman, J. T., Brandal, L. T., Brown, D. J., Erdõsi, T., Heck, M., Ibrahem, S., Johanssan, K., Kornschober, C., Kotila, S.M., Le Hello, S., Lienemann, T., Mathheus, W., Nielsen, E.M., Ragimbeu, C., Rumore, J., Sabol, A., Torpdahl, M., Tress, E., Tuohy, A. and de Pinna, E. (2017). Multi-laboratory validation study of multilocus variable-number tandem repeat analysis (MLVA) for Salmonella enterica serovar Enteritidis, 2015. Eurosurveillance. 22 (9), 30477. http://doi.org/10.2807/1560-7917.ES.2017.22.9.30477.

Peters, T.M., Berghold, C., Brown, D., Coia, J., Dionisi, A.M., Echeita, A., Fisher, I.S., Gatto, A.J., Gill, N., Green, J., Gerner-Smidt, P., Heck, M., Lederer, I., Lukinmaa, S., Luzzi, I., Maguire, C., Prager, R., Usera, M., Siitonen, A., Threlfall, E.J., Torpdahl, M., Tschäpe, H., Wannet, W. and Zwaluw, W.K. (2007). Relationship of pulsed-field profiles with key phage types of *Salmonella enterica* serotype Enteritidis in Europe: results of an international multi-centre study. *Epidemiology & Infection*.135 (8): 1274-81.

Pezzella, C., Ricci, A., DiGiannatale, E., Luzzi, I. and Carattoli, A. (2004). Tetracycline and streptomycin resistance genes, transposons, and plasmids in *Salmonella enterica* isolates from animals in Italy. *Antimicrobial agents and chemotherapy*. 48 (3): 903–908.

Pignato, S., Coniglio, M.A., Faro, G., Lefevre, M., Weill, F.X. and Giammanco, G. (2010). Molecular epidemiology of ampicillin resistance in *Salmonella* spp. and

Escherichia coli from wastewater and clinical specimens. *Foodborne Pathogens and Disease*. 7 (8): 945-951.

Poppe, C., Demczuk, W., McFadden, K. and Johnson, R.P. (1993). Virulence of *Salmonella* enteritidis phage types 4, 8 and 13 and other *Salmonella* species for day-old chicks, hens and mice. *Canadian Journal of Veterinary Research*. 57 (4): 281–287.

Porwollik, S., Boyd, E.F., Choy, C., Cheng, P., Florea, L., Proctor, E. and McClelland, M. (2004). Characterization of *Salmonella enterica* subspecies *I* genovars by use of microarrays. *Journal of Bacteriology*.186: 5883–5898.

Porwollik, S., Santiviago, C.A., Cheng, P., Florea, L. and McClelland, M. (2005). Differences in gene content between *Salmonella enterica* serovar Enteritidis isolates and comparison to closely related serovars Gallinarum and Dublin. *Journal of Bacteriology.*187: 6545– 6555.

Rahman, B.A., Wasfy, M.O., Maksoud, M.A., Hanna, N., Dueger, E. and House, B. (2014). Multi-drug resistance and reduced susceptibility to ciprofloxacin among *Salmonella enterica* serovar Typhi isolates from the Middle East and Central Asia. *New Microbes and New Infections*. 2 (4): 88–92.

Ranjbar, R., Karami, A., Farshad, S., Giammanco, G.M. and Mammina, C. (2014). Typing methods used in the molecular epidemiology of microbial pathogens: a how-to guide. *The New Microbiologica*. 37 (1): 1-15.

Richard, E.M. (2008). *Global Effects of the transcriptional regulators ArcA and FNR in anaerobically grown Salmonella enterica serovar Typhimurium 14028s*. North Carolina: ProQuest. pp 2.

Rodrigue, D.C., Tauxe, R.H. and Rowe, B. (1990). International increase in *Salmonella* enteritidis: a new pandemic. *Epidemiology and Infection*. 105: 21–27.

Ridley, A.M., Threlfall, E.J. and Rowe, B. (1998). Genotypic characterization of *Salmonella* Enteritidis phage types by plasmid analysis, ribotyping and pulsed-field gel electrophoresis. *Journal of Clinical Microbiology*. 36: 2314-2321.

Ribot, E.M., Fair, M.A., Gautom, R., Cameron, D.N., Hunter, S.B., Swaminathan, B. and Barrett, T.J. (2006). Standardization of pulsed-field gel electrophoresis protocols for the subtyping of *Escherichia coli* O157:H7, *Salmonella*, and *Shigella* for PulseNet. *Foodborne Pathogens and Disease*. 3 (1):59-67.

Root, R.K., Waldvogel, F., Corey, L. and Stamm, W.E. (1999). *Clinical infectious diseases: A practical approach*. New York: Oxford University press. pp 252.

Ross, I.L. and Heuzenroeder, M.W. (2009). A comparison of two PCR-based typing methods with pulsed-field gel electrophoresis in *Salmonella enterica* serovar Enteritidis. *International Journal of Medical Microbiology*. 299 (6): 410-420.

Rychlik, I., Gregorova, D. and Hradecka, H. (2006). Distribution and function of plasmids in *Salmonella enterica*. *Veterinary Microbiology*. 112: 1-10.

Scallan, E., Majowicz, S.E., Hall, G., Banerjee, A., Bowman, C.L., Daly, L., Jones, T., Kirk, M.D., Fitzgerald, M. and Angulo, F.J. (2005). Prevalence of diarrhoea in the community in Australia, Canada, Ireland, and the United States. *International Journal of Epidemiology*. 34 (2): 454–460.

Senior, K. (2009). Estimating the global burden of foodborne disease. *Lancet Infectious Diseases*. 9 (2): 80–81.

Shaw, A.V., Reddy, E.A. and Crump, J.A. (2008). *Etiology of community-acquired bloodstream infections in Africa*. L-620. Washington, DC: Infectious Diseases Society of America.

Sibanda, E.L., Weller, I.V.D., Hakim, J.G. and Cowan, F.M. (2011). Does trimethoprim-sulfamethoxazole prophylaxis for HIV induce bacterial resistance to

other antibiotic classes?: Results of a systematic review. *Clinical Infectious Diseases*. 52(9): 1184–1194.

Siegrist, J. (2009). *Differentiation and identification media for Salmonella: Microbiology focus*. Sigma Aldrich Available at: http://www.sigmaaldrich.com/technical-documents/articles/microbiology-focus/salmonella-selective-media.html [Accessed 5 October 2015].

Sigauque, B., Roca, A., Mandomando, I., Morais, L., Quintó, L., Sacarial, J., Macete, E., Nhamposa, T., Machevo, S., Aide, P., Bassat, Q., Bardji, A., Nhalungo, D., Soriano-Gabarró, M., Flannery, B., Menendez, C., Levine, M.M. and Alonso, P.L. (2009). Community-acquired bacteremia among children admitted to a rural hospital in Mozambique. *The Paediatric Infectious Disease Journal*. 28 (2): 108-113.

Simpson, E.H. (1949). Measurement of diversity. *Nature*. 163 (4148): 688.

Siu, L.K., Lu, P.L., Chen, J.Y., Lin, F.M. and Chang, S.C. (2003). High-level expression of *ampC* beta-lactamase due to insertion of nucleotides between -10 and -35 promoter sequences in *Escherichia coli* clinical isolates: cases not responsive to extended-spectrum-cephalosporin treatment. *Antimicrobial Agents and Chemotherapy*. 47 (7): 2138–2144.

Slinko, V.G., McCall, B.J., Stafford, R.J., Bell, R.J., Hiley, L.A., Sanderg, S.M., White, S.A. and Bell, K.M. (2009). Outbreaks of *Salmonella* Typhimurium page type 197 of multiple genotypes linked to an egg producer. *Communicable Diseases Intelligence Quarterly Report*. 33 (4): 419-425.

Smith, A.M., Ismail, H., Henton, M.M., Keddy, K.H. and GERMS-SA Surveillance Network. (2014). Similarities between *Salmonella* Enteritidis isolates from humans and captive wild animals in South Africa. *Journal of Infection in Developing Countries*. 8 (12): 1616-1619.

South African National Veterinary Surveillance and Monitoring Programme for Resistance to Antimicrobial Drugs (SANVAD), University of Pretoria and ARC-

Onderstepoort Veterinary Institute. (2007). Pretoria. http://www.fidssa.co.za/Content/Documents/AMR_Background_document_FINAL_March15.pdf [Accessed 06 April 2015]

Statistics South Africa. (2015). *Mid-year population estimates*. Available at: https://www.statssa.gov.za/publications/P0302/P03022015.pdf [Accessed 06 April 2016].

Su, L., Chiu, C., Chu, C. and Ou, J.T. (2004). Antimicrobial Resistance in Nontyphoid *Salmonella* Serotypes: A Global Challenge. *Clinical Infectious Diseases*. 39 (4): 546-551.

Swaminathan, B., Barrett, T.J., Hunter, S.B. and Tauxe, R.V. (2001). PulseNet: the molecular subtyping network for foodborne bacterial disease surveillance, United States. *Emerging Infectious Diseases*. 7: 382-389.

Swartz, M.N. (2002). Human Diseases Caused by Foodborne Pathogens of Animal Origin. *Clinical Infectious Diseases*. 34 (3): 111-122.

Tadesse, G. (2015). A meta-analysis of the proportion of animal *Salmonella* isolates resistant to drugs used against human salmonellosis in Ethiopia. *BMC Infectious Diseases*. 15 (84): 1-13.

Takem, E.N., Roca, A. and Cunnington, A. (2014). The association between malaria and non- typhoid *Salmonella* bacteraemia in children in Sub-Saharan Africa: a literature review. *Malaria Journal*. 14 (400). http://www.malariajournal.com/content/13/1/400 [Accessed 6 April 2016].

Tennant, S.M., MacLennan, C.A., Simon, R., Martin, L.B. and Khan, M.I. (2016). Nontyphoidal *Salmonella* disease: Current status of vaccine research and development. *Vaccine*. 34 (26): 2907-2910.

Teuber, M. (2001). Veterinary use and antibiotic resistance. *Current Laboratory of Food Microbiology*. 4 (5): 493-499.

Threlfall, E.J. and Frost, J.A. (1990). The identification, typing and fingerprinting of *Salmonella*: laboratory aspects and epidemiological applications. *Journal of Applied Bacteriology*. 68: 5-16.

Threlfall, E.J. (2000). Epidemic *Salmonella* Typhimurium DT 104-a truly international multi-resistant clone. *Journal of Antimicrobial Chemotherapy*. 46: 7-10.

Threlfall, E.J. (2002). Antimicrobial drug resistance in *Salmonella*: problems and perspectives in food-and water-borne infections. *FEMS Microbiology Reviews*. 26: 141-148.

Uzzau, S., Brown, D.J., Wallis, T., Rubino, S., Leori, G., Bernard, S., Casadesús, J., Platt, D.J. and Olsen, J.E. (2000). Host adapted serotypes of *Salmonella enterica*. *Epidemiology and Infection*. 125 (2): 229-255.

Varma, J.K., Molbak, K., Barrett, T.J., Beebe, J.L., Jones, T.F., Rabatsky-Her, T., Smith, K.E., Vugia, D.J., Chang, H.H. and Angulo, F. (2005). Antimicrobial-resistant nontyphoidal *Salmonella* is associated with excess bloodstream infections and hospitalizations. *Journal of Infectious Diseases*. 191: 554 – 561

Vugia, D.J., Samuel, M., Farley, M.M., Marcus, R., Shiferaw, B., Shallow, S., Smith, K., Angulo, F.J., and for the Emerging Infections Program FoodNet Working Group. (2004). Invasive *Salmonella* infections in the United States, FoodNet, 1996–1999: incidence, serotype distribution, and outcome. *Clinical Infectious diseases*. 38 (3): 149-156.

Walsh, C. (2003). *Antibiotics: Actions, Origins, Resistance*. Washington, DC: ASM Press.

Weisblum, B. (1995). Erythromycin resistance by ribosome modification. *Antimicrobial Agents Chemotherapy*. 39 (3): 577-585.

Wheeler, J.G., Sethi, D., Cowden, J.M., Wall, P.G., Rodrigues, L.C., Tompkins, D.S., Hudson, M.J. and Roderick, P. (1999). Study of infectious intestinal disease in England: rates in the community, presenting to general practice, and reported to national surveillance. *British Medical Journal*. 318 (7190): 1046–1050.

World Health Organisation. (2011). *Reduce use of antimicrobials in food producing*. Available at: http://www.who.int/world-health-day/2011/presskit/whd2011_fs4d_subanimal.pdf [Accessed 13 November 2015].

Wiedmann, M. (2002). Subtyping of bacterial foodborne pathogens. *Nutrition Reviews*. 60 (7): 201-208.

Wiedmann, M. and Zhang, W. (2011). *Genomics of foodborne bacterial pathogens*. New York: Springer. pp 403-406.

World Health Organization and Food and Agriculture Organization of the United Nations. (2002). *Microbiological Risk Assessment Series 2: Risk Assessments of Salmonella in Eggs and Broiler Chickens – 2*. Rome: WHO Library Cataloguing-in-Publication Data. Chap 3.

World poultry. (2016). *Case Study: South Africa's growing poultry consumption*. Available at: http://www.worldpoultry.net/Meat/Articles/2015/12/Case-Study-South-Africas-growing-poultry-consumption-2735530W/ [Accessed May 2016].

Yan, S.S., Pendrak, M.L., Abela-Ridder, B., Punderson, J.W., Fedorko, D.P. and Foley, S.L. (2003). An overview of *Salmonella* typing public health concern. *Clinical and Applied Immunology Reviews*. 4: 189-204.

Yim, L., Betancor, L., Martinez, A., Giossa, G., Bryant, C., Maskell, D. and Chabalgoity, J.A. (2010). Differential phenotypic diversity among epidemic-spanning *Salmonella enterica* serovar enteritidis isolates from humans or animals. *Applied and Environmental Microbiology*. 76 (20): 6812–6820.

Younus, M. (2008). *Risk factor for sporadic non-typhoidal Salmonella infections by Michigan children: A population-based case control study*. USA: ProQuest LLC. pp 3-4.

APPENDICES

Appendix A: Site of *Salmonella* Enteritidis isolation

Abscess aspirate
Blood
Bone marrow
Cerebral spinal fluid
Stool
Pleural fluid
Pus
Pus swab
Sputum
Superficial swab
Tissue
Urine

Appendix B: Preparation of TE buffer

TE buffer (10mM Tris, 1mM EDTA pH 8.0)

10 ml of 1M Tris* (pH 8.0)

2 ml of 0.5M EDTA* (pH 8.0)

Dilute to 1000 ml with sterile water and autoclave.

Reagent manufacturer: *Tris (Merck, Darmstadt, Germany); *EDTA (Sigma Aldrich, St Louis, USA)

Appendix C: Real-time PCR for *Salmonella*

Table. Primer and probe sequences used to amplify the target genes for *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis (Malorny *et al*., 2004; O'Regan *et al*., 2008; Park *et al*., 2008).

Target organism	Target gene	PCR primer/probe	Primer/probe sequence	*PCR product size	Manufacturer
Salmonella species	*ttrRSBCA*	ttr-6 (forward)	CTCACCAGGAGATTACAACATGG	95 bp	Inqaba (Pretoria, SA)
		ttr-4 (reverse)	AGCTCAGACCAAAAGTGACCATC		Inqaba (Pretoria, SA)
		ttr-5 probe (*Salmonella*)	**6FAM** - CACCGACGGCGAGACCGACTTT - **BHQ1**		Roche (Johannesburg, SA)
Salmonella Typhimurium	*STM4497*	STM-F1	GCGCACCTCAACATCTTTC	62 bp	Inqaba (Pretoria, SA)
		STM-R1	CGGTCAAATAACCCACGTTCA		Inqaba (Pretoria, SA)
		STM-probe1	**NED** - ATCATCGTCGACATGC - **MGBNFQ**		Life Technologies (Johannesburg, SA)
Salmonella Enteritidis	*sdf*	SES-F1	AAATGTGTTTTATCTGATGCAAGAGG	299 bp	Inqaba (Pretoria, SA)
		SES-R1	GTTCGTTCTTCTGGTACTTACGATGAC		Inqaba (Pretoria, SA)
		SES-probe2	**Cy5** - CGAATGGTGAGCAGACAACAGGCTGATTTA - **BBQ**		Roche (Johannesburg, SA)

*PCR product size: bp- base pair.

Primers and probe re-suspension

Primers and probes are received as lyophilized products and are re-suspended with TE buffer at pH 8.0, according to the manufacturer's instructions to make a 100μM stock solution. The primer/probe mix for *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis real-time PCR reaction was prepared by adding the stock solutions into a 1.5 ml tube as outlined in the Table below. The mix is then stored at -20°C.

Table. Primer/probe mix for *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis real-time PCR reaction.

PCR targets	**Contents**	**Volume**
	PCR/molecular grade water	45 µl
Salmonella genus	ttr-6 (forward) primer	21 µl of 100 µM
	ttr-4 (reverse) primer	21 µl of 100 µM
	ttr-5 probe	4 µl of 100 µM
Salmonella Typhimurium	STM-F1 primer	48 µl of 100 µM
	STM-R1 primer	48 µl of 100 µM
	STM-probe1	9 µl of 100 µM
Salmonella Enteritidis	SES-F1 primer	93 µl of 100 µM
	SES-R1 primer	93 µl of 100 µM
	SES-probe2	18 µl of 100 µM

Preparation of *Salmonella* species, *Salmonella* Typhimurium and *Salmonella* Enteritidis real-time PCR reaction

Preparation of the real-time PCR involves the adding of all reagents outlined on the below table, in each reaction well. Since DNA is not added in the non-template control (NTC) well, autoclaved deionized water is added in substitution.

Table. Reagents and reagent volumes included in the *Salmonella* real-time PCR reaction.

Reagents	**Volume (µl)**	**Manufacturers**
Primer/probe mix	3	-
Invitrogen Express real-time PCR supermix universal	25	Life technologies (Carlsbad, USA)
Autoclaved deionized water	20	-
Crude DNA	2	-
Total	50	

Appendix D: MLVA

Table. Primer sequences used to amplify the five VNTR loci for *Salmonella* Enteritidis (Hopkins *et al*., 2011)

Target gene locus	PCR primer	Primer sequence (5' to 3')	Expected fragment sizes (base pair)	VNTR repeat length (base pair)	Manufacturer
SENTR7	SENTR7-F	**6FAM**-ACGATCACCACGGTCACTTC	117-135	9	Life Technologies (Johannesburg, SA)
	SENTR7-R	CGGATAACAACAGGACGCTTC			Inqaba (Pretoria, SA)
SENTR5	SENTR5-F	**6FAM**-CACCGCACAATCAGTGGAAC	235-301	6	Life Technologies (Johannesburg, SA)
	SENTR5-R	GCGTTGAATATCGGCAGCATG			Inqaba (Pretoria, SA)
SENTR6	SENTR6-F	**NED**-ATGGACGGAGGCGATAGAC	173-236	7	Life Technologies (Johannesburg, SA)
	SENTR6-R	AGCTTCACAATTTGCGTATTCG			Inqaba (Pretoria, SA)
SENTR4	SENTR4-F	**VIC**-GACCAACACTCTATGAACCAATG	112-147	7	Life Technologies (Johannesburg, SA)
	SENTR4-R	ACCAGGCAACTATTCGCTATC			Inqaba (Pretoria, SA)
SE-3	SE-3-F	**VIC**-CAACAAAACAACAGCAGCAT	308-320	12	Life Technologies (Johannesburg, SA)
	SE-3-R	GGGAAACGGTAATCAGAAAGT			Inqaba (Pretoria, SA)

Primers and probe re-suspension

Primers and probes are received as lyophilized products and are re-suspended with TE buffer at pH 8.0, according to the manufacturer's instructions to make a 100µM stock solution. The forward primer and reverse primer mix for the *Salmonella* MLVA PCR reaction was prepared by adding the forward and reverse stock solutions into separate 1.5 ml tubes as outlined below. The mix is then stored at -20°C.

Primer mix - forward primers (primer mix made on amber-coloured tubes)

65 µl of deionized autoclaved water
7 µl of SENTR7-F primer (10 µM)
7 µl of SENTR5-F primer (10 µM)
7 µl of SENTR6-F primer (10 µM)
7 µl of SENTR4-F primer (10 µM)
7 µl of SE-3-F primer (10 µM)

Primer mix - reverse primers (primer mix made on clear tubes)

65 µl of deionized autoclaved water
7 µl of SENTR7-R primer (10 µM)
7 µl of SENTR5-R primer (10 µM)
7 µl of SENTR6-R primer (10 µM)
7 µl of SENTR4-R primer (10 µM)
7 µl of SE-3-R primer (10 µM)

Preparation of *Salmonella* Enteritidis MLVA PCR

Table. Reagents and reagent volumes included in the *Salmonella* Enteritidis MLVA PCR.

Reagents	**Volume (µl)**	**Manufacturers**
Autoclaved deionized water	7.5	-
Qiagen master mix	12.5	Qiagen (Hilden, Germany)
Qiagen Q-solution	2.5	Qiagen (Hilden, Germany)
Forward primer	1	-
Reverse primer	1	-
Crude DNA	1	-
Total	25	

Table: Summary of the 86 MLVA profiles along with their VNTR allele size numbers

MLVA Profile number	SENTR7	SENTR5	SENTR6	SENTR4	SE-3
1	132	268	177	112	318
2	132	268	219	112	318
3	132	280	184	112	318
4	132	280	177	119	318
5	132	280	184	112	306
6	132	280	177	112	306
7	132	280	177	119	306
8	132	280	184	119	306
9	132	244	170	112	306
10	123	262	177	126	306
11	123	250	212	140	306
12	123	226	212	126	306
13	123	226	205	133	0
14	123	226	205	133	318
15	123	226	191	0	306
16	123	274	184	126	318
17	123	226	184	0	318
18	123	262	191	112	318
19	123	274	0	112	318
20	123	262	0	112	318
21	123	274	184	112	318
22	123	262	184	112	318
23	123	280	184	112	318
24	123	244	184	112	318
25	123	286	184	112	318
26	123	268	191	112	318
27	123	268	0	112	318
28	123	268	184	112	318
29	123	268	177	112	306
30	123	268	184	119	306
31	123	268	184	112	306
32	123	226	212	126	0
33	123	262	191	119	306
34	123	262	177	119	306
35	123	208	219	119	306
36	123	280	177	119	306
37	123	274	184	119	306
38	132	268	0	119	306
39	132	274	0	119	306
40	132	274	177	119	306
41	132	286	177	119	306

42	132	268	177	119	306
43	132	256	177	119	306
44	132	208	177	119	306
45	132	292	177	119	306
46	132	238	184	119	306
47	123	250	219	140	306
48	123	268	177	119	306
49	123	280	191	112	318
50	123	268	219	140	306
51	132	268	184	112	318
52	123	280	177	112	318
53	123	274	177	112	318
54	123	268	177	112	318
55	123	268	184	119	318
56	123	262	205	119	306
57	123	262	184	119	306
58	123	280	177	119	318
59	132	268	184	119	306
60	114	226	212	126	0
61	114	268	184	112	318
62	114	280	177	112	306
63	123	274	219	119	306
64	123	244	170	119	306
65	132	286	177	112	306
66	123	286	177	119	306
67	123	286	177	112	306
68	132	286	177	119	318
69	123	226	191	133	306
70	123	262	191	133	306
71	123	280	177	126	306
72	132	268	184	119	318
73	132	262	184	119	306
74	123	292	177	112	306
75	123	256	198	119	306
76	123	256	177	112	318
77	132	274	184	112	318
78	123	262	177	112	318
79	123	268	212	140	306
80	123	244	212	140	306
81	123	268	226	140	306
82	132	280	177	126	306
83	123	268	163	133	306
84	123	250	212	133	306
85	132	262	177	119	306
86	123	268	219	126	306

Appendix E: Information of *Salmonella* Enteritidis outbreaks

Outbreak 1:

Outbreak 1 occurred in the KwaZulu- Natal Province during May 2013. Two people were affected, due to the consumption of liver of a dead goat. The goat was reported to have had diarrhoea prior to its death. Three isolates were received (two human isolates and one goat meat isolate). All isolates belonged to MLVA profile 22 (123_262_184_112_318).

Outbreak 2:

Outbreak 2 occurred in the Mpumalanga Province in November 2013. The outbreak was associated with food poisoning. However, no further details were provided about the outbreak. Three human isolates were received from the outbreak. All isolates belonged to MLVA profile 22 (123_262_184_112_318).

Outbreak 3:

Outbreak 3 occurred in the Limpopo Province in January 2014. This foodborne outbreak occurred in a lodge. Sixty-five people were affected, eight of whom were admitted to hospital in critical condition. Further investigation of the food showed *Salmonella* contamination. Three human isolates were received from the outbreak. All isolates belonged to MLVA profile 28 (123_268_184_112_318).

Outbreak 4:

Outbreak 4 occurred in the Mpumalanga Province in July 2014. The outbreak was associated with food prepared for a funeral. Forty-six people were affected, six of whom were children who were admitted to hospital in critical condition. Fourteen human isolates were received from the outbreak. All isolates belonged to MLVA profile 28 (123_268_184_112_318).

Outbreak 5:

Outbreak 5 occurred in the Free State Province in November 2014. The outbreak was associated with food prepared for a function in a mine. Eighty people were affected, six of whom were hospitalized. Three human isolates were received from the outbreak. All isolates belonged to MLVA profile 28(123_268_184_112_318).

Outbreak 6:

Outbreak 6 occurred in the Eastern Cape Province in December 2014. The outbreak occurred in a TB hospital. However, no further details were provided about the outbreak. Ten human isolates were received from the outbreak. All isolates belonged to MLVA profile 28 (123_268_184_112_318).

Outbreak 7:

Outbreak 7 occurred in the Gauteng Province in October 2015. The outbreak was in a private residence, where chicken feet were cooked by a mother for dinner. Four children were affected (age 4, 7, 8 and 11). Three human isolates were received from the outbreak. All isolates belonged to MLVA profile 21 (123_274_184_112_318).

Appendix F: Ethics clearance

M140740

HUMAN RESEARCH ETHICS COMMITTEE (MEDICAL)

CLEARANCE CERTIFICATE NO. M140740

NAME: (Principal Investigator)	Ms Munyadziwa Muvhali
DEPARTMENT:	School of Pathology Medical School
PROJECT TITLE:	Application of Molecular Epidemiology Methods to Investigate Stairs of Salmonella enterica serovar Enteritidis in South Africa
DATE CONSIDERED:	25/07/2014
DECISION:	Approved unconditionally
CONDITIONS:	
SUPERVISOR:	Dr Anthony Marius

APPROVED BY: _____
Professor PE Cleaton-Jones, Chairperson, HREC (Medical)

DATE OF APPROVAL: 25/07/2014

This clearance certificate is valid for 5 years from date of approval. Extension may be applied for.

DECLARATION OF INVESTIGATORS

To be completed in duplicate and **ONE COPY** returned to the Secretary in Room 10004, 10th floor, Senate House, University.

I/we fully understand the conditions under which I am/we are authorized to carry out the above-mentioned research and I/we undertake to ensure compliance with these conditions. Should any departure be contemplated, from the research protocol as approved, I/we undertake to resubmit the application to the Committee. **I agree to submit a yearly progress report.**

_____ _____
Principal Investigator Signature M140740Date 25/07/2014

PLEASE QUOTE THE PROTOCOL NUMBER IN ALL ENQUIRIES

Appendix G: Plagiarism declaration with Turnitin report

UNIVERSITY OF THE WITWATERSRAND JOHANNESBURG

FACULTY OF HEALTH SCIENCES

PLAGIARISM DECLARATION TO BE SIGNED BY ALL HIGHER DEGREE STUDENTS

SENATE PLAGIARISM POLICY: APPENDIX ONE

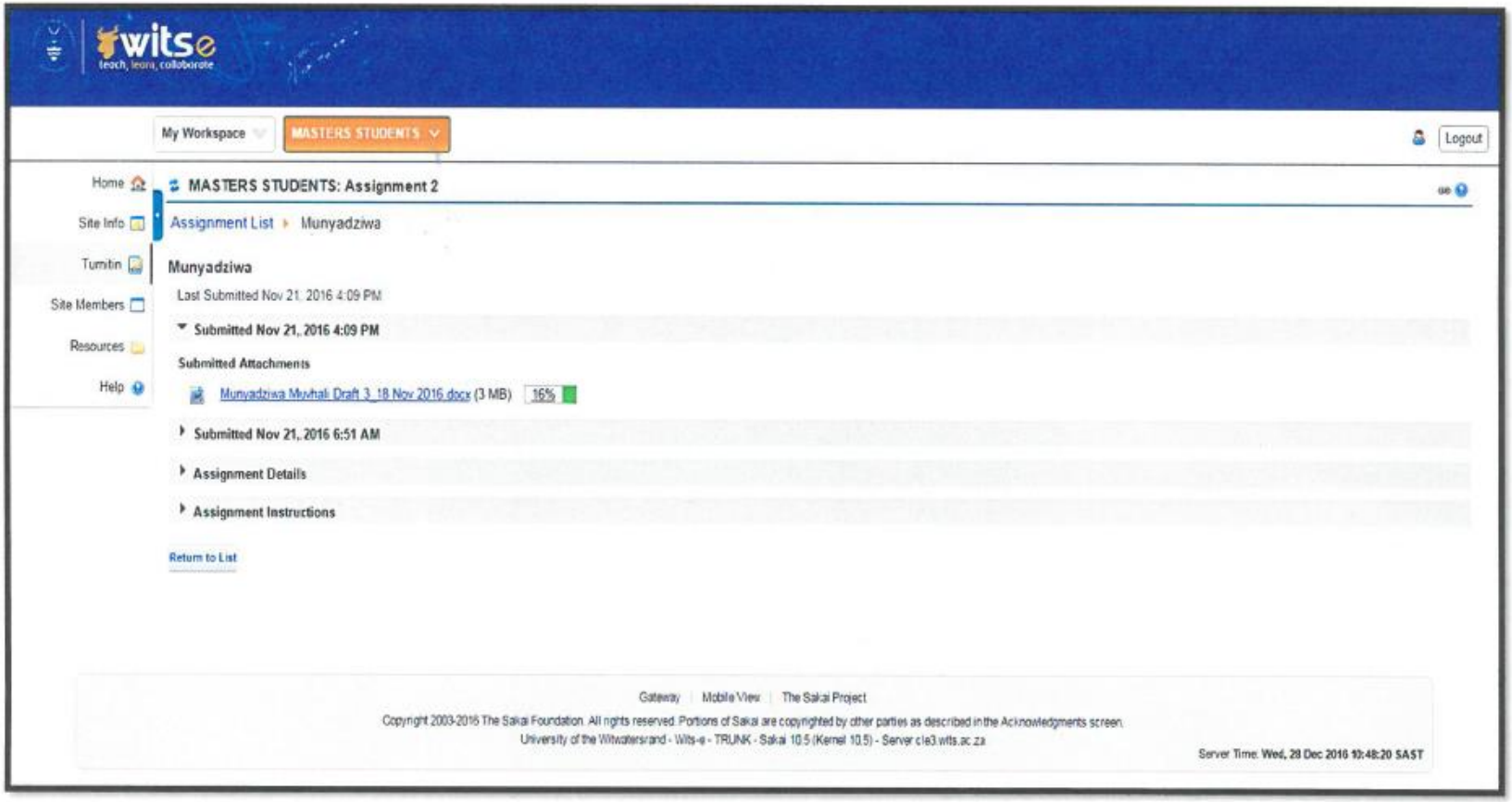
I Munyadziwa Muvhali (Student number: 385400) am a student registered for the degree of Master of Science in Medicine in the academic year 2014.

I hereby declare the following:

- I am aware that plagiarism (the use of someone else's work without their permission and/or without acknowledging the original source) is wrong.
- I confirm that the work submitted for assessment for the above degree is my own unaided work except where I have explicitly indicated otherwise.
- I have followed the required conventions in referencing the thoughts and ideas of others.
- I understand that the University of the Witwatersrand may take disciplinary action against me if there is a belief that this is not my own unaided work or that I have failed to acknowledge the source of the ideas or words in my writing.
- I have included as an appendix a report from "Turnitin" (or other approved plagiarism detection) software indicating the level of plagiarism in my research document.

Signature: Muvhali Date: 05 January 2017

1



DR. ANTHONY M SMITH
BSc (Hons). PhD

5/1/2017